Nitrification Rates Are Affected by Biogenic Nitrate and Volatile Organic Compounds in Agricultural Soils

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The processes regulating nitrification in soils are not entirely understood. Here we provide evidence that nitrification rates in soil may be affected by complexed nitrate molecules and microbial volatile organic compounds (mVOCs) produced during nitrification. Experiments were carried out to elucidate the overall nature of mVOCs and biogenic nitrates produced by nitrifiers, and their effects on nitrification and redox metabolism. Soils were incubated at three levels of biogenic nitrate. Soils containing biogenic nitrate were compared with soils containing inorganic fertilizer nitrate (KNO$_3$) in terms of redox metabolism potential. Repeated NH$_4^+$ addition increased nitrification rates (mM NO$_3^-$ produced g$^{-1}$ soil d$^{-1}$) from 0.49 to 0.65. Soils with higher nitrification rates stimulated ($p < 0.01$) abundances of 16S rRNA genes by about eight times, amoA genes of nitrifying bacteria by about 25 times, and amoA genes of nitrifying archaea by about 15 times. Soils with biogenic nitrate and KNO$_3$ were incubated under anoxic conditions to undergo anaerobic respiration. The maximum rates of different redox metabolisms (mM electron acceptors reduced g$^{-1}$ soil d$^{-1}$) in soil containing biogenic nitrate followed as: NO$_3^-$ reduction 4.01 ± 0.22, Fe$_3^+$ reduction 5.37 ± 0.12, SO$_4^{2-}$ reduction 9.56 ± 0.16, and CH$_4$ production (µg g$^{-1}$ soil) 0.46 ± 0.05. Biogenic nitrate inhibited denitrification 1.4 times more strongly compared to mineral KNO$_3$. Raman spectra indicated that aliphatic hydrocarbons increased in soil during nitrification, and these compounds probably bind to NO$_3$ to form biogenic nitrate. The mVOCs produced by nitrifiers enhanced ($p < 0.05$) nitrification rates and abundances of nitrifying bacteria. Experiments suggest that biogenic nitrate and mVOCs affect nitrification and redox metabolism in soil.

Keywords: nitrification, biogenic nitrate, redox metabolism, mVOCs, 16S rRNA, amoA

INTRODUCTION

Nitrification is a key biogeochemical process for the global nitrogen cycle (Nelson et al., 2016). Therefore, in-depth knowledge on nitrification is essential for agricultural, environmental, and economic reasons. Nitrification of ammonia to nitrate is a two-step process usually performed by two distinct groups of chemolitho-autotrophic microbes (Alfreider et al., 2017), one step oxidizes NH$_4^+$ to NO$_2^-$, while the other oxidizes NO$_2^-$ to NO$_3^-$ (Li Y. et al., 2018). In the first step, most of the NH$_4^+$ is converted to NO$_2^-$, but a small portion of the N is emitted as N$_2$O (Liiimatainen et al., 2018). This is produced as a byproduct when the intermediate HNO is produced during the oxidation of NH$_3$OH to NO$_2^-$.

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NO$_3^{−}$ (Weber et al., 2015). Complete ammonia oxidation (comammox) is energetically feasible and bacteria (Nitrosapira sp.) capable of performing both steps have been identified (Daims et al., 2015). These bacteria encode all enzymes necessary for ammonia oxidation via nitrite to nitrate in their genomes (van Kessel et al., 2015).

Most ammonia oxidizing bacteria (AOB) belong to the Betaproteobacteria (β-AOB) (Pan et al., 2018). There are two distinct phylogenetic clusters within the β-AOB, the Nitrosomonas cluster and the Nitrosospira cluster (Zhao et al., 2015). The Nitrosomonas cluster comprises members of the genus Nitrosomonas. The Nitrosospira cluster comprises the genera Nitrosospira, Nitrosolobus, and Nitrosovibrio. Nitrite (NO$_2^{−}$) oxidizing bacteria have been described in four genera; Nitrobacter, Nitrococcus, Nitrosina, and Nitrospira (Han et al., 2017). Our understanding of the nitrogen cycle has been revised in the past few years by the discovery of ammonia oxidizing archaean (AOA) (Leininger et al., 2006). AOA are members of the proposed archael phylum Thaumarchaea (Gribaldo et al., 2010). However, AOA are difficult to cultivate, so some aspects of their physiology and contribution to biogeochemical pathways are still speculative. AOA are found in almost all environments. Crenarchaeotal 16S rRNA gene sequences have been recovered from different environments including Pacific and Atlantic oceans (Flood et al., 2015), lake sediments (Llirós et al., 2014), the guts of animals (Radax et al., 2012), agricultural soils (Tournel et al., 2011), and forest soils (Isobe et al., 2012). Typically AOA greatly outnumber AOB. In soil samples, the copy number of crenarchaeotal amoA is one to three orders of magnitude higher than bacterial amoA (Wuchter et al., 2006).

Nitrification is carried out by the microbial membrane-bound enzymes. The ammonia monoxygenase (AMO) is responsible for the conversion of NH$_3$ to hydroxylamine (Bock and Wagner, 2013). The end product of nitrification, NO$_3^{−}$, may binds to cationic molecules present in soil or extracellular microbial molecules. Thus, the NO$_3^{−}$ produced by nitrifiers can be different in nature than inorganic NO$_3^{−}$–. The nitrates produced from nitrification may bind to extracellular complex organic compounds to form “biogenic nitrate.” Contrastingly, inorganic forms of NO$_3$ (NaNO$	extsubscript{3}$, KNO$	extsubscript{3}$, NH$_4$NO$	extsubscript{3}$, etc.) are in the form of salts. The bonding between NO$_3^{−}$ and cations (Na, K, NH$_4$ , etc) in inorganic NO$_3$ fertilizer is stronger than the bonding between NO$_3^{−}$ and cellular organic cations in biogenic nitrate. Therefore, nitrate in the inorganic nitrate fertilizer preferably does not bind to cellular organic cations unlike the nitrate produced through nitrification. It is also reported that nitrifiers produce soluble microbial products (SMPs) which serve as supplementary organic substrates for heterotrophic bacteria (Dolinszék et al., 2013). The SMPs are mainly constituted of proteins and humics (Li J. et al., 2018). There is a possibility that after nitrification the product (NO$_3^{−}$) binds to SMPs forming “biogenic nitrate.” Like inorganic nitrate, the biogenic nitrate has two main biological functions. Either it is assimilated by plants and microbes (under aerobic condition (Rubio-Asensio et al., 2014) or it is denitrified when anoxic conditions prevail. Nitrate reduction or denitrification is carried out by dissimilatory nitrate reducing bacteria (Castro-Barros et al., 2017). However, due to its complexation with SMPs, the availability and fate of biogenic nitrate can be different from inorganic fertilizer nitrate (KNO$	extsubscript{3}$).

Like other microorganisms, nitrifiers can produce volatile organic compounds (VOCs). However, information on the VOCs emitted by nitrifiers is scarce. Microbial VOCs (mVOCs) act as signal molecules for different microorganisms (Insam and Seewald, 2010). The mVOCs can modulate activities of the producing species, or of different microbial species. However, it is unclear how the volatiles produced by nitrifiers influence the activity of nitrifiers and denitrifiers. The manuscript aims to define how the NO$_3^{−}$ derived from nitrification is different from that in chemical inorganic nitrate fertilizers.

**MATERIALS AND METHODS**

**Soil Sampling and Characterization**

Experiments were carried out using soil samples collected during September 2016 from the experimental fields of the Indian Institute of Soil Science, Bhopal, Madhya Pradesh, India (23.30 N, 77.40 E, 485 m above mean sea level). The soil is a heavy clayey Vertisol (typic Haplustert, WRB code VR), characterized by: 5.7 g kg$^{-1}$ organic C, 225 mg kg$^{-1}$ available N, 2.6 mg kg$^{-1}$ available P, and 230 mg kg$^{-1}$ available K. The textural composition of soil was: sand 15.2%, silt 30.3%, clay 54.5%, electrical conductivity (EC) 0.43 dS m$^{-1}$, and pH 7.5. The soil had 863.24 µM NO$_3^{−}$, 0.01 µM Fe$^{2+}$, and 101.02 µM SO$_4^{2−}$. Concentration of these ions was estimated by wet chemical method as given below (chemical analysis). After collection, the soil was hand-processed after breaking the clods and removing roots and stones. Soil was then passed through 2-mm mesh sieve and was used within 2 days of collection.

**Nitrification and Biosynthesis of Biogenic Nitrate**

Biogenic nitrate is defined here as the nitrate produced via nitrification. To biosynthesize biogenic nitrate, microcosms were prepared where nitrification was carried out three times (Figure 1). The choice of having three repeated NH$_4$ additions was based on the fact that in agriculture, N fertilizer is often applied in split doses, and for most crops, three split doses of N are recommended (Arregui and Quemada, 2008). Repeated nitrification resulted in different levels of nitrate (biogenic nitrate). Experiments were carried out in six 1000-ml bottles (Figure 1). Three bottles served as controls (AC1–AC3) and the other three were used for biosynthesis of biogenic nitrate and estimation of nitrification (labeled as BC1–BC3). To each bottle 200 g soil was added and sterilized double distilled water was added to maintain soil at 60% moisture holding capacity (MHC). There was no ammonium amendment to “AC” bottles, while 2 ml of 1 M NH$_4$−N (NH$_4$Cl) was added the “BC” bottles, giving a final concentration of 10 mM. Soils were mixed thoroughly using a glass rod and bottles were closed with butyl rubber caps. All the bottles were incubated at 30 ± 2°C. At different times, bottles BC1–BC3 were opened and 1-g soil subsamples were taken out to determine NO$_3^{−}$ concentrations. Control bottles
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FIGURE 1 | Microcosm design for biosynthesis of biogenic nitrate and estimation of nitrification potential of soil under repeated NH$_4^+$-N amendment (setup 1, A). Microcosm setup to evaluate the effect of microbial volatiles (mVOCs) on nitrification and denitrification (setup 2, B). Bottles of 250 ml volume contained 50 g soil and were un-amended (AC1–AC3) or amended with 10 mM NH$_4^+$ (BC1–BC3). After complete nitrification of 10 mM NH$_4^+$ (24 days) a second dose of NH$_4^+$ was added (BC bottles) and after complete nitrification (40 days) a third dose of 10 mM NH$_4^+$ was added (BC bottles). The third nitrification stage was completed in 55 days of incubation. The three complete nitrification phases were designated as nitrification I, nitrification II, and nitrification III. The second setup (B) was designed to evaluate the effect of mVOCs (A1–A6) on nitrification and denitrification. All experimental treatments included three replicates (R1, R2, R3). The six source bottles (A1–A6) were connected to 18 sink bottles (130 ml volume containing 20 g of soil), shown as B1, C1, D1, B2, C2, and D2. The control bottles (130 ml bottle containing 20 g soil) were without exposure to mVOCs (E1, F1, G1 and E2, F2, G2). The connectors fitted with 0.2-µm filters (filled circle) were used to connect source and sink bottles. After each nitrification stage, one sink bottle and one control bottle were further incubated to determine nitrification and denitrification rates as mentioned the methodology.

were also opened for analysis to mimic the treated ones. Nitrate measurement continued till the NO$_3^{-}$ concentrations in BC bottles reached a plateau. Nitrification of the first dose of NH$_4^+$-N (10 mM) was referred as “nitrification I.” After nitrification I, 10 mM of NH$_4^+$-N was again added to BC bottles and the same incubation and measurement protocol applied until the NO$_3^{-}$ was again stabilized. This second nitrification stage was referred as “nitrification II.” Subsequently, the bottles were again opened and amended with a third dose of 10 mM NH$_4^+$-N in BC bottles. The third nitrification stage was referred as “nitrification III.” The three nitrification stages (nitrification I, II, and III) produced three levels of biogenic nitrate. After completion of each nitrification stage, 20-g soil was taken from the bottles (AC and BC) and incubated to evaluate redox metabolism as described below.

The Effect of Biogenic Nitrate on Redox Metabolism
To evaluate the effect of biogenic nitrate on redox metabolism, experiments were carried out as described above (AC1–AC3 and BC1–BC3). In addition, 18 130-ml vials were also used for this analysis. Nine vials were kept for evaluating redox metabolism using soil mixed with equivalent amount of inorganic fertilizer nitrate (KNO$_3$) as observed in nitrification vials (labeled as A). Another nine vials were used for evaluating redox metabolism using the soil in which biogenic nitrate was produced (labeled as B). Each set of nine vials was represented as three nitrification phases and three replicates. Soil (20 g) from AC1–AC3 and BC1–BC3 bottles (collected after nitrification I, II, and III) were placed into 130-ml serum vials. Soil was mixed with 10 mM of CH$_3$COONa, and 50 ml of sterile distilled water. Acetate served as carbon source for anaerobic microbial metabolism. After mixing the contents, bottles were closed with rubber septa and sealed using aluminum crimp seals. Bottles were incubated at 30 ± 2°C with shaking at 100 revolutions per minute (rpm) on an orbital shaker for 30 days. To determine the temporal variation in the reduction of the terminal electron acceptors, 3 ml of slurry from each vial was withdrawn using a syringe (Dispovan, India). Before sampling, the syringes were first flushed with pure N$_2$ to maintain anoxic conditions. Slurry samples
were processed following standard methods to estimate NO$_3^{-}$, Fe$^{3+}$, SO$_4^{2-}$ (see below). Changes in the concentrations of all electron acceptors (NO$_3^{-}$, Fe$^{3+}$, SO$_4^{2-}$) were measured at each sampling time to estimate the rates of redox metabolism. Headspace gas samples of the vials were analyzed via gas chromatography (see below) to quantify CH$_4$ production at the end of the incubation period (30 days).

**Effects of N$_2$O and Microbial Volatiles on Nitrification**

To test the effect of N$_2$O on nitrification, experiments were carried out by placing 20 g soil into six 130-ml sterilized serum bottles. Soils were moistened with sterilized double distilled water to maintain 60% MHC and NH$_4$–N was added to a final concentration of 10 mM. After mixing the contents, bottles were closed with rubber septa and sealed with aluminum crimp seals. Three bottles were kept as controls and three were injected with pure N$_2$O (Inox Pvt. Ltd., Bhopal, India) to a final mixing ratio of 10 ppmv. Control vials were injected with pure helium (99%) instead of N$_2$O. All bottles were incubated at 30 ± 2°C for 30 days. At different incubation periods, bottles were opened and 1 g amounts of soil taken to measure NO$_3^{-}$. After each NO$_3^{-}$ measurement, bottles were re-incubated with 10 ppmv N$_2$O.

To evaluate the effect of mVOCs on nitrification and denitrification, an experiment was set up as shown in Figure 1; 50 g amounts of soil were placed into 250-ml bottles, and sterile double distilled water added to maintain soil at 60% MHC. To each bottle, 10 mM NH$_4$–N was added. Bottles were closed with rubber stoppers and tightened with screw caps. Three bottles were controls and six “source bottles” were the source of mVOCs originating from nitrification. Another set of 36 “sink bottles” were CO$_2$ and CH$_4$, a Porapak Q column (2 m length, internal diameter 3.175 mm, 80/100 mesh, stainless steel column) was used in combination with the FID. The CO$_2$ was quantified after its conversion to CH$_4$ using a attached methanizer module at 350°C. The injector, column, and detector (FID) were maintained at 120, 60, and 330°C, respectively. N$_2$O was estimated using a stainless steel column (2 ft; diameter, 1/8 in) filled with chromosorb 101 (60–80 mesh) coupled to the ECD. The oven temp was 30°C, the injector and detector (ECD) temp were 120 and 330°C, respectively.

**Raman Spectroscopic Analysis of Soil**

To test the hypothesis that NO$_3^{-}$ derived through nitrification is a complex mixture of NO$_3^{-}$ and cellular derived biomolecules, and to reveal any compositional changes of soil due to nitrification, soil samples were analyzed by Raman spectrophotometry (Guizani et al., 2017). Soil samples of un-nitrified and after third nitrification (nitrification III) were ground using a mortar and pestle and passed through a 0.1-mm sieve. Samples were scanned in a high-resolution Raman spectrometer (RamanStation$^\text{TM}$ 400F, Perkin-Elmer®, Beaconsfield, Buckinghamshire, United Kingdom) fitted with...
Czerny-Turner type achromatic spectrograph. The spectral resolution was 0.4 cm$^{-1}$pixel$^{-1}$ at the spectral range of 200–1050 nm and the source of excitation was a 632.8 nm, air cooled He–Ne laser. Nomenclature of wavelengths and the representing functional groups was based on the earlier publications (Socrates, 2004; Colthup, 2012). Data obtained from the instrument were normalized. Wavelengths representing each functional group were considered for analysis. Intensities of the peaks were added and the average of three replicates was calculated.

**DNA Extraction**
DNA was extracted from 0.5 g field soil samples using the ultraclean DNA extraction kit (MoBio, United States) according to the manufacturer's instructions. The DNA concentrations were determined in a biophotometer (Eppendorf, Germany) by measuring absorbance at 260 nm (A260), assuming that 1 A260 unit corresponds to 50 ng of DNA per µl. DNA extraction was further confirmed by electrophoresis on a 1% agarose gel. The extracted DNA was dissolved in 50 µl TE buffer and stored at −20°C until further analysis.

**Real-Time PCR Quantification of Total Bacteria, Ammonia Oxidizing Bacteria, and Ammonia Oxidizing Archaea**
Microbial abundance was estimated from two experimental setups: first with soil samples of un-incubated control, nitrification I, II, and III soils, and second with soil samples exposed to microbial volatiles (mVOCs) of nitrification III and un-exposed controls. The microbial groups estimated were total bacteria, AOB, and AOA. Real-time PCR was performed on a Step one plus real-time PCR (ABI, United States). Reaction mixtures contained 2 µl of DNA template, 10 µl of 2X SYBR green master mix (Affymetrix, United States), and 200 nM of each primer (GCC Biotech, New Delhi). The final volume of PCR reaction mixture was made to 20 µl with PCR grade water (MP Bio, United States). Primers targeting bacterial 16S rRNA genes, bacterial amoA genes, and archaeal amoA gene were used to quantify the respective microbial abundance. The primers (5’–3’) for bacteria were 1F (CCT ACG GGA GGC AGC AG) and 518R (ATT ACC GCG GCT GG) (Back et al., 2010); nitrifying bacteria 1F (GGG GTT TCT ACT GTG GGT) and amoA 2R1 (CCC CTC TGG AAA GGC TTC TTC) (Okano et al., 2004); nitrifying archaea arc-Amo-F (STA ATG TGC TGT AGA CG; S = G or C); and arc-amoa-R (GGC GCC ATC CAT CTG TAT GT) (Mutlu and Guven, 2011). Thermal cycling was carried out by an initial denaturizing step at 94°C for 4 min, 40 cycles of 94°C for 1 min, the assay-dependent annealing temperature for 30 s, 72°C for 45 s; and a final extension at 72°C for 5 min. The annealing temperature for 16S rRNA genes was 52°C, and for amoA genes of bacteria and archaea were 50 and 52°C, respectively. Fluorescence was measured during the elongation step. Data analysis was carried out with Step one plus software (ABI, United States) as described in user's manual. The cycle at which the fluorescence of target molecule number exceeded the background fluorescence (threshold cycle [C$_T$]) was determined from dilution series of target amplicons with defined target molecule amounts. C$_T$ was proportional to the logarithm of the target molecule number. The quality of PCR amplification products was determined by melting curve analysis with temperature increase of 0.3°C per cycle. Standard for bacteria prepared by using Escherichia coli strain JM 109 (Promega Inc., United States). For preparing standard for amoA genes of nitrifying bacteria and nitrifying archaea, the PCR products of bacterial amoA and archaeal amoA genes were separately cloned to TOPO TA cloning vector (Invitrogen, United States). Constructed plasmids were transformed into competent cells (One Shot Top 10, Invitrogen, United States). Transformed cells (white colonies) were multiplied in LB broth for 24 h at 37°C and their concentration was estimated using a Biophotometer (Eppendorf, Germany). Plasmids from the E. coli or transformed cells were extracted using a plasmid extraction kit (Axygen, United States). Concentration of plasmids was quantified and expressed as ng µL$^{-1}$. Serial dilution for each plasmid was prepared and real-time PCR carried out. Standard curve for each gene was prepared by plotting plasmid concentration (representing cell number or gene copies) versus C$_T$ values (Supplementary Table S1).

**Statistical Analysis**
All statistical analyses were carried out using the “agricolae” packages of the statistical software R (2.15.1) (Ihaka and Gentleman, 1996). Data obtained were presented as arithmetic mean of three replicated observations. Effect of factors (NH$_4$ amendment) on the parameters (nitrification, denitrification, Fe$^{3+}$ reduction, SO$_4^{2-}$ reduction, CH$_4$ production, and microbial abundance) was tested by analysis of variance (ANOVA). Low P-value and high F statistics indicated significant impact of the factors on the variables. To define the significant difference among the treatments, Tukeys honestly significant difference (HSD) test was performed.

**RESULTS**

**Nitrification Activity of Soil**
Variation of NO$_3^{−}$ concentrations during repeated stages of nitrification is shown in Figure 2. Nitrification increased steadily after 5 days of incubation. Nitrification of the first dose of 10 mM NH$_4$–N occurred within 24 days. Subsequent amendment of NH$_4$–N stimulated nitrification. The second dose of 10 mM NH$_4$–N was nitrified by 40 days while the third dose of 10 mM NH$_4$–N was nitrified by 55 days. The added NH$_4$–N was nitrified by about 84% in nitrification I, 92% in nitrification II, and 87% in nitrification III stages. Potential nitrification rates (PNRs) increased with repeated nitrification (Table 1). PNR of fresh soil was 0.49 mMg$^{-1}$ soil d$^{-1}$ while the PNR of nitrification III soil was highest of 0.65 mMg$^{-1}$ soil d$^{-1}$.

**Effect of Nitrification on Microbial Abundance**
Abundances of total bacteria, nitrifying bacteria, and nitrifying archaea all increased after repeated nitrification (Table 1). The
bacterial population varied from 5 to 43.67 (×10⁶ cells g⁻¹ soil). The nitrifying bacterial population ranged from 4 to 102 (×10⁴ cells g⁻¹ soil) and the nitrifying archaeal population varied from 6 to 94.33 (×10⁴ cells g⁻¹ soil). The lowest abundances were measured in control treatments and the highest in the nitrification III soil samples.

**Effect of Nitrification on Redox Metabolism**

Redox metabolism followed the classical trend of sequential reduction of terminal electron acceptors (Figure 3), starting with NO₃⁻ reduction followed by Fe³⁺ reduction, SO₄²⁻ reduction, and CH₄ production. Soil amended with inorganic KNO₃ exhibited detectable nitrate reduction after 2 days and complete denitrification within 5 days. Iron reduction peaked at 5 days and SO₄²⁻ reduction after 2 weeks. Potential redox metabolic rates are presented in Table 2. Denitrification rates increased with NO₃⁻ concentration originating from either nitrification phases or KNO₃. However, the denitrification rate was lower in the soil that had undergone nitrification than compared to the KNO₃ treated soil. Denitrification may have been inhibited by biogenic nitrate. The reduction rate of Fe³⁺ was also lower in the nitrification soil. Similarly, the reduction of SO₄²⁻ was also low in the nitrification soil. Production of CH₄ was estimated after the end of incubation. CH₄ production was low in nitrification soil compared to non-nitrification soil (Table 2).

**Statistical Analyses**

Analysis of variance indicated that NH₄⁻N addition significantly and positively influenced nitrification (p < 0.0001) (Table 3). It also significantly influenced NO₃⁻ reduction (p < 0.0001), and Fe³⁺ reduction (p < 0.01) compared to or inorganic nitrate amendment. However, SO₄²⁻ reduction and CH₄ production were not significantly affected. Abundances of 16S rRNA genes, amoA genes of nitrifying bacteria, and amoA genes of nitrifying archaea were significantly (p < 0.0001) influenced by the NH₄ amendment.

**Raman Spectra of Soil in Response to Nitrification**

Soil samples were scanned by a Raman spectrometer to examine how soil organic carbon changed due to the metabolism of nitrifiers (Figure 4). Nitrified soil (nitrification III) had high absorbance for the wavelengths (wavenumbers cm⁻¹) between 500–1000 and 1500–2000. Absorbance intensity was
low for the wavelengths ranging from 1200–1600. Raman intensity for the above wavelengths was plotted for both the samples (Figure 4). Nitrification increased the concentration of C–C, C–S, C–O–C molecules and decreased C–NO$_2$ and CH$_2$ molecules.

**Production of N$_2$O and CO$_2$ From Soil During Nitrification**

Headspace N$_2$O and CO$_2$ production were measured from control soil (no added nitrogen) and soil after the three nitrification stages (Table 4). N$_2$O production rates varied from 4.06 to 19.39 µg g$^{-1}$ soil d$^{-1}$. The lowest rate was in control soil and the highest was in nitrification III soil. The amount of headspace CO$_2$ varied from 465 µg g$^{-1}$ soil d$^{-1}$ in control soil to 649 µg g$^{-1}$ soil d$^{-1}$ in nitrification III soil. The values of N$_2$O varied significantly among the treatments.

**Effect of N$_2$O and Nitrifying Microbial Volatiles on Nitrification and Denitrification**

The effect of N$_2$O on the nitrification and denitrification was evaluated by exposing soil to 0 or 10 ppm of N$_2$O. Production of NO$_3^{1-}$ was measured during nitrification, while the decline of NO$_3^{1-}$ was measured during denitrification. Nitrification of 10 mM NH$_4$ was completed in 3 weeks whereas the denitriticaion of NO$_3^{1-}$ (~10 mM) was completed within 8 days. Added N$_2$O had no significant effect on nitrification and denitrification (Figure 5).

The effect of volatiles originating from nitrification was tested on nitrification and denitrification (Figure 5). The composition of nitrifier-derived mVOCs was not evaluated in this study because the primary aim was to reveal the influence of mVOCs on nitrification and denitrification. Soils were exposed to microbial volatiles of three repeated nitrification (nitrification I, II, and III) phases. The mVOCs originating from nitrifiers significantly stimulated nitrification (Figure 5). Time required for complete nitrification of the added NH$_4$ was significantly reduced due to the volatiles. Nitrification rates (mM NO$_3^{1-}$ produced g$^{-1}$ soil d$^{-1}$) varied from 0.425 in control soil to 0.844 in nitrification III soil. Nitrification and denitrification values of the controls remained unchanged over the three nitrification phases. However, mVOCs of nitrifiers did not influence denitrification. Potential denitrification rates (mM NO$_3^{1-}$ reduced g$^{-1}$ soil d$^{-1}$) varied from 1.37 to 1.38 with no statistical difference among the treatments (Table 4).
TABLE 2 | Influence of biogenic NO₃⁻ and inorganic fertilizer KNO₃ on soil denitrification rate, iron reduction rate, sulfate reduction rate, and methane production rate.

| Source of NO₃⁻ | Nitrification phases | Denitrification rate (mM NO₂⁻ reduced g⁻¹ soil d⁻¹) | Iron reduction rate (µM Fe³⁺ reduced g⁻¹ soil d⁻¹) | Sulfate reduction rate (µM SO₄²⁻ reduced g⁻¹ soil d⁻¹) | CH₄ production (µg CH₄ produced g⁻¹ soil) |
|----------------|---------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|
| Biogenic NO₃⁻ | Nitrification I      | 1.22 ± 0.04                                | 5.29 ± 0.26                                | 9.41 ± 0.14                                | 0.54 ± 0.04                                |
|                | Nitrification II     | 2.03 ± 0.02                                | 2.95 ± 0.10                                | 9.35 ± 0.03                                | 0.41 ± 0.08                                |
|                | Nitrification III    | 2.80 ± 0.04                                | 2.89 ± 0.10                                | 9.19 ± 0.06                                | 0.38 ± 0.04                                |
| Inorganic fertilizer NO₃⁻ | Nitrification I | 1.63 ± 0.17                                | 8.55 ± 0.58                                | 10.89 ± 0.17                               | 0.63 ± 0.11                                |
| (KNO₃)         | Nitrification II     | 2.84 ± 0.18                                | 5.84 ± 0.10                                | 10.45 ± 0.19                               | 0.57 ± 0.10                                |
|                | Nitrification III    | 4.01 ± 0.22                                | 5.37 ± 0.12                                | 9.56 ± 0.16                                | 0.46 ± 0.05                                |

The three nitrification stages were referred as nitrification I, nitrification II, and nitrification III. Soil sub-samples collected at the end of the three nitrification phases were incubated for redox metabolism. Values represent arithmetic means and standard deviations of three replicates.

Microbial Abundance in Response to Microbial Volatiles

The effect of nitrifying mVOCs on the soil microbial abundance was estimated by quantifying the 16S rRNA genes of eubacteria, amoA genes of nitrifying bacteria, and amoA genes of nitrifying archaea. Exposure of soils to mVOCs of nitrification (nitrification III) did not increase microbial abundance in soils (Table 5). This indicated that the mVOCs were not a substantial substrate for growth of soil microorganisms. However, prior exposure of soils to mVOCs and subsequent incubation for nitrification resulted in a significant increase in the growth of nitrifying bacteria. Probably, the mVOCs may have activated the nitrifiers in some way resulting high microbial abundance.

Raman Spectra of Soil Exposed to Nitrifying Microbial Volatiles

Soils after exposure to the nitrification III and control (unexposed) treatments were analyzed by Raman spectra (Figure 5). The Raman intensity across the total wavelengths of the two samples was mostly equivalent with no apparent change.

DISCUSSION

NO₃⁻ influences (mostly negatively) reduction of Fe³⁺ (Ionescu et al., 2015), SO₄²⁻ (Ontiveros-Valencia et al., 2013), and methanogenesis (Rissanen et al., 2017). Denitrification is thermodynamically more favorable than the reduction of other electron acceptors (Fe³⁺, SO₄²⁻, CO₂). This influence of inorganic nitrate on redox metabolism is well understood. However, the role of biogenic NO₃⁻ on redox metabolism is not known. Therefore, the interaction between nitrification (which produces biogenic nitrate) and redox metabolism was explored.

Soils were amended with 10 mM NH₄-N and the progress of nitrification was monitored. The PNRs measured were similar to those observed in other soils (Fierer et al., 2001). The nitrification was repeated three times to generate three levels of NO₃⁻ (biogenic nitrate). Nitrification rates increased over repeated NH₄-N amendments, as did the abundance of both nitrifying bacteria and archaea. After each nitrification stage, the soils were evaluated for redox metabolism. Soil samples were incubated under flooded moisture regime to test the effect of the biogenic nitrate versus inorganic nitrate (control) on redox metabolism. Biogenic nitrate inhibited reduction of electron acceptors compared to inorganic NO₃⁻. This is reasonable as any compound or processes that inhibited denitrification will ultimately affect the reduction of subsequent terminal electron acceptors (Fe³⁺, SO₄²⁻, CO₂).

The production of biogenic nitrate via nitrification significantly (p < 0.05) inhibited redox metabolism compared to the addition of inorganic NO₃⁻. Several soil factors may have been affected by the nitrification phase. One possibility is that nitrifiers produced biomolecules which inhibited the redox metabolism. To identify those biomolecules, soils of non-nitrified control soil and soil from the nitrification III stage were analyzed by Raman spectrometer. Soils of nitrification III were selected for Raman spectra analysis because these soils had undergone maximum nitrification. Raman spectra differentiated soil of control (with an equivalent amount of KNO₃) from soils of nitrification III. Nitrification increased the abundance of functional groups including C–C, C–S, C–O–C. Spectra also indicated that nitrification decreased the amount of functional groups including C–NO₂, CH₂/CH₃, C–NO₂, C–N, esters, and alkynes. Therefore, the
inhibition of redox metabolism by nitrification may have been due to the presence and/or absence of these functional groups. Under anaerobic conditions, denitrifiers oxidize aliphatic bonds (C–C and C–O–C) to CO$_2$ through NO$_3^{−}$-dependent oxidation (Zedelius et al., 2011). Theoretically, the occurrence of aliphatics would stimulate the redox metabolism by acting as substrates for the anaerobic microorganisms. However, in the current experiment, they were correlated with reduced redox metabolism. Probably, the biogenic nitrate was less reactive (denitrifying) than the inorganic NO$_3^{−}$ as mentioned above. This could be due to the complex interaction or bonding between NO$_3^{−}$ and the extracellular aliphatics. In control (non-nitrified soil), the C–NO$_2$ functional groups were high. Spectral data indicated occurrence of biogenic nitrate in soil that has undergone nitrification. Biogenic nitrate is a complex form of nitrate containing organic molecules. The organic molecules can be short- or long-chain aliphatics. The complex structure and bonding between aliphatics and NO$_3^{−}$/NO$_2^{−}$ makes it less reactive to undergo denitrification (Figure 6). It has been found that organic compounds may inhibit denitrification (Gilbert et al., 1997). Probably, the biogenic NO$_3^{−}$ was denitrified after separation of NO$_3^{−}$ and aliphatics, which might have been carried out by anaerobic microorganisms (Rabus et al., 2016). The processes of decomposition of the biogenic nitrate by microorganisms probably delayed the availability of NO$_3^{−}$ for denitrification. Thus, due to the delayed denitrification, there was delay in the reduction of subsequent electron acceptors comprising Fe$^{3+}$, SO$_4^{2−}$, and CH$_3$COO$^{1−}$ (CH$_4$ production).

**TABLE 4 | Production rates of N$_2$O, CO$_2$, potential rates of nitrification and denitrification in soil in response to repeated ammonium additions.**

| Nitrification    | N$_2$O production (µg produced g$^{-1}$ soil d$^{-1}$) | CO$_2$ production (µg produced g$^{-1}$ soil d$^{-1}$) | Potential nitrification rate (mM NO$_3^{−}$ produced g$^{-1}$ soil d$^{-1}$) | Potential denitrification rate (mM NO$_3^{−}$ reduced g$^{-1}$ soil d$^{-1}$) |
|------------------|-----------------------------------------------------|-----------------------------------------------------|-----------------------------------------------------------------|---------------------------------------------------------------|
| Control          | 4.06 ± 0.06                                         | 465 ± 50.16                                         | 0.42 ± 0.01                                                     | 1.37 ± 0.05                                                   |
| Nitrification I  | 11.97 ± 0.84                                        | 575 ± 94.85                                         | 0.47 ± 0.02                                                     | 1.38 ± 0.06                                                   |
| Nitrification II | 15.87 ± 1.80                                        | 605 ± 36.02                                         | 0.57 ± 0.01                                                     | 1.38 ± 0.01                                                   |
| Nitrification III| 19.39 ± 2.61                                        | 649 ± 39.02                                         | 0.84 ± 0.06                                                     | 1.38 ± 0.04                                                   |

The three successive nitrification stages are referred as nitrification I, nitrification II, and nitrification III. Soil without added ammonium served as control. Values represent arithmetic means and standard deviations of three replicates.
FIGURE 5 | Effect of N\textsubscript{2}O and microbial volatile organic compounds (mVOCs) on nitrification and denitrification activity in soil (A,B). The mixing ratios of N\textsubscript{2}O were either 0 or 10 ppm. To evaluate the effect of microbial volatiles (mVOCs) produced during nitrification on nitrification and denitrification activity, soils were exposed to the gas phase of soils during nitrification I, nitrification II, and nitrification III stages. Soil without exposure served as controls. After exposure, soils were incubated to determine nitrification (C) and denitrification (D) rates. The x-axis represents incubation period and the y-axis represents NO\textsubscript{3}\textsuperscript{−} concentration. Data points are arithmetic means and standard deviations of three replicates. Raman spectra of soil under the influence of nitrifying microbial volatiles (E). Soils were exposed to nitrifying microbial volatiles (nitrification III) or not exposed (control). The x-axis represents wavenumber cm\textsuperscript{−1} and the y-axis represents Raman intensity. Data points are arithmetic means of three replicates.

TABLE 5 | Effect of microbial volatiles (mVOCs) produced during nitrification on the abundance of different microbial groups.

| Nitrifying microbial volatiles | Nitrification | Eubacteria (×10\textsuperscript{6} 16S rRNA gene copies g\textsuperscript{−1} soil) | Nitrifying bacteria (×10\textsuperscript{4} bacterial amoA gene copies g\textsuperscript{−1} soil) | Nitrifying archaea (×10\textsuperscript{4} archaean amoA gene copies g\textsuperscript{−1} soil) |
|--------------------------------|---------------|---------------------------------|---------------------------------|---------------------------------|
| Un-exposed                     | Before nitrification | 5.67 ± 0.57                      | 4.33 ± 0.57                      | 6.49 ± 0.65                      |
|                                | After nitrification  | 43.67 ± 4.50                     | 103.33 ± 6.11                    | 93.00 ± 8.54                     |
| Exposed to nitrifying volatiles (mVOCs) | Before nitrification | 6.00 ± 1.00                      | 4.67 ± 1.11                      | 6.67 ± 1.15                      |
|                                | After nitrification  | 64.67 ± 3.51                     | 195.67 ± 12.85                   | 139.33 ± 16.16                   |

Soils were exposed to the volatiles originating from nitrification over three successive ammonium additions. Soils without exposure served as an un-exposed control. After exposure to mVOCs, soils were amended with 10 mM NH\textsubscript{4}–N and incubated. Microbial abundance was estimated before and after nitrification of this added ammonium. Values represent arithmetic means and standard deviations of three replicates.

It was observed that unlike other microbial activities, nitrification progressed steadily in spite of a constant increase in NO\textsubscript{3}\textsuperscript{−} concentration. Therefore, the formation of biogenic NO\textsubscript{3}\textsuperscript{−} may be a mechanism used by nitrifiers to block the feedback inhibition of NO\textsubscript{3}\textsuperscript{−} to nitrification. Production of CO\textsubscript{2} did not significantly vary with nitrification potential. However, N\textsubscript{2}O production varied significantly among the treatments. Nitrous oxide was generally produced from nitrification, because active nitrification (continuous increase in the NO\textsubscript{3}\textsuperscript{−} concentration) was observed over the incubation period. N\textsubscript{2}O production through denitrification cannot be ruled out, because some denitrification might have occurred in the soil microaggregates. However, NO\textsubscript{3}\textsuperscript{−} production from NH\textsubscript{4} was consistent and there was no decline in the NO\textsubscript{3}\textsuperscript{−} level. Therefore, the denitrification mediated N\textsubscript{2}O production could be marginal. A follow-up experiment was carried out to determine the effect of N\textsubscript{2}O on nitrification and denitrification. It was observed that there was no significant effect of N\textsubscript{2}O on nitrification and denitrification.
Apart from CO₂ and N₂O, other gaseous products emitted by soil microorganisms include mVOCs. Soil microbes produce VOCs including alkenes, alcohols, ketones, terpenes, benzenoids, pyrazines, acids, and esters (Lemfack et al., 2013). Microbial volatiles act as signal molecules to other microorganisms, plants, and animals (Insam and Seewald, 2010). The composition of mVOCs originating from nitrification was beyond the scope of this research, which aims only to provide primary information about the influence of mVOCs on nitrification and denitrification. Based on the current study, conceptual models were developed depicting the potential interaction of mVOCs and nitrifiers (Figure 6). This experiment suggested that mVOCs stimulate nitrification, but no effect on denitrification. Probably, the mVOCs acted as signal molecules for the nitrifiers and stimulated their activity (nitrification). Exposure of soil to mVOCs did not increase the abundance of bacteria, nitrifying bacteria, and nitrifying archaea, suggesting that mVOCs stimulated the nitrifiers by increasing cell activity. Many bacteria decompose VOCs in soil (Tyc et al., 2016). The degraded products could have played important role in the activation of microbial population, resulting in high nitrification rates compared to the unexposed control. Soils after exposure to mVOCs were further tested by Raman spectrometer to evaluate if the volatiles altered chemical composition. However, mVOCs did not change the measured soil properties. We propose that the mVOCs stimulate nitrifiers by acting as signal molecules rather than altering the soil properties.

**CONCLUSION**

The current experiment addressed four key questions about nitrification. First, how does nitrification progress under repeated N amendment? Second, how does nitrification influence redox metabolism? Third, how does the nitrate produced from nitrification (biogenic nitrate) differ from inorganic nitrate? Fourth, do the nitrifiers communicate by means of VOCs? Nitrification activity was observed under three repeated N amendments. Nitrification increased steadily in respect to the NH₄–N amendment, due to increasing abundance of nitrifying bacteria and nitrifying archaea. After each nitrification stage, soils were incubated to undergo redox metabolism. An initial nitrification phase inhibited redox rates compared to the addition of an equivalent amount of inorganic NO₃⁻ (KNO₃). Raman spectra of the nitrified soils revealed an increased concentration of aliphatics. Based on these observations, it was hypothesized that during nitrification, biogenic nitrates are produced by complex interaction (bonding) between NO₃⁻ and the aliphatics, and that this biogenic nitrate is less reactive toward...
denitrification than is inorganic nitrate. Nitrifiers emitted VOCs which stimulated nitrification. Nitrification was accelerated by both VOCs and biogenic nitrate. The current experiment mostly indicated the formation of biogenic nitrate and mVOCs by nitrifiers which regulate nitrification and redox metabolism. However, there is need of comprehensive studies on the biochemical characteristics of biogenic nitrate and mVOCs to better understand the nitrification. Further studies are also warranted with other soil types as well as under field conditions to verify complex interaction between biogenic nitrate, VOCs, and nitrification.

AUTHOR CONTRIBUTIONS

SRM conceptualized the experiments, and drafted the manuscript. MN executed experiments and performed most of the wet chemical analysis. RP assisted in setting up experiments. UA contributed in analyzing redox moieties of soil samples. AP facilitated experiments. GD performed qPCR reactions to quantify functional genes. BK analyzed data statistically and contributed in drafting and revising the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00772/full#supplementary-material

REFERENCES

Alfreider, A., Baumer, A., Bogensperger, T., Posch, T., Salcher, M. M., and Summerer, M. (2017). “CO2 assimilation strategies in stratified lakes: diversity and distribution patterns of chemolithoautotrophs.” Environ. Microbiol. 19, 2754–2768. doi: 10.1111/1462-2920.13786

Arregui, L. M., and Quemada, M. (2008). Strategies to improve nitrogen use efficiency in winter cereals grown under rainfed conditions. Agron. J. 100, 277–284. doi: 10.2134/agronj2007.0187

Baeck, K. H., Park, C., Oh, H.-M., Yoon, B.-D., and Kim, H.-S. (2010). Diversity and abundance of ammonia-oxidizing bacteria in activated sludge treating different types of wastewater. J. Microbiol. Biotechnol. 20, 1128–1133. doi: 10.4014/jmb.0907.07021

Bock, E., and Wagner, M. (2013). “Oxidation of inorganic nitrogen compounds as an energy source,” in The Prokaryotes, eds E. Osenberg, E. F. DeLong, S. Lory, E. Stackebrandt, and F. Thompson (Berlin: Springer), 83–118.

Castro-Barros, C. M., Jia, M., van Loosdrecht, M. C., Volcke, E. I., and Winkler, M. K. (2017). Evaluating the potential for dissimilatory nitrate reduction by anammox bacteria for municipal wastewater treatment. Bioresour. Technol. 233, 363–372. doi: 10.1016/j.biortech.2017.02.063

Colthup, N. (2012). Introduction to Infrared and Raman Spectroscopy: Amsterdam: Elsevier.

Daims, H., Lebedeva, E. V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., et al. (2015). Complete nitrification by Nitrosipia bacteria. Nature 528:504. doi: 10.1038/nature16461

Doliniek, J., Lagkouvardos, I., Wanek, W., Wagner, M., and Daims, H. (2013). Interactions of nitrifying bacteria and heterotrophs: identification of a Micavibrio-like putative predator of Nitrospira spp. Appl. Environ. Microbiol. 79, 2027–2037. doi: 10.1128/AEM.03408-12

Fierer, N., Schimpel, J. P., Cates, R. G., and Zou, J. (2001). Influence of balsam poplar tannin fractions on carbon and nitrogen dynamics in Alaskan taiga floodplain soils. Soil Biol. Biochem. 33, 1827–1839. doi: 10.1016/S0038-0717(01)00111-0

Flood, M., Frabutt, D., Floyd, D., Powers, A., Ezegwe, U., Devol, A., et al. (2015). Ammonia-oxidizing bacteria and archaea in sediments of the Gulf of Mexico. Environ. Sci. Technol. 36, 124–135. doi: 10.1021/es503330c.2014.942385

Gilbert, F., Stora, G., Bonin, P., LeDéreaux, Y., Mille, G., and Bertrand, J.-C. (1997). Hydrocarbon influence on denitrification in bioturbed Mediterranean coastal sediments. Hydrobiologia 345, 67–77. doi: 10.1023/A:100293142250

Gribaldo, S., Poole, A. M., Daubin, V., Forterre, P., and Brochier-Armanet, C. (2010). The origin of eukaryotes and their relationship with the Archaea: are we at a phyllogenomic impasse? Nat. Rev. Microbiol. 8, 743–752. doi: 10.1038/nrmicro2426

Guizani, C., Haddad, K., Limousy, L., and Jeguirim, M. (2017). New insights on the structural evolution of biomass char upon pyrolysis as revealed by the Raman spectroscopy and elemental analysis. Carbon 119, 519–521. doi: 10.1016/j.carbon.2017.04.078

Han, S., Luo, X., Liao, H., Nie, H., Chen, W., and Huang, Q. (2017). Nitrospira are more sensitive than Nitrobacter to land management in acid, fertilized soils of a rapeseed–rice rotation field trial. Sci. Total Environ. 599, 135–144. doi: 10.1016/j.scitotenv.2017.04.086

Ihaka, R., and Gentleman, R. (1996). R: a language for data analysis and graphics. J. Comput. Graph. Stat. 5, 299–314.

Insam, H., and Seewald, M. S. (2010). Volatile organic compounds (VOCs) in soils. Biol. Fertil. Soils 46, 199–213. doi: 10.1007/s00374-010-0442-3

Ionescu, D., Heim, C., Polerecky, L., Ramette, A., Haeusler, S., Bizic-Ionescu, M., et al. (2015). Diversity of iron oxidizing and reducing bacteria in flow reactors in the Aspo hard rock laboratory. Geomicrobiol. J. 32, 207–220. doi: 10.1080/01490451.2014.884196

Isebo, K., Koba, K., Suwa, Y., Ikutani, J., Fang, Y., Yoh, M., et al. (2012). High abundance of ammonia-oxidizing archaea in acidified subtropical forest soils in southern China after long-term N deposition. FEBS Microbiol. Ecol. 80, 193–203. doi: 10.1111/j.1574-6941.2011.01294.x

Jackson, M. L. (1958). Soil Chemical Analysis. Englewood, NJ: Prentice- Hall, Inc.

Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G. W., et al. (2006). Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature 442, 806–809. doi: 10.1038/nature04983

Lemlack, M. C., Nickel, J., Dunkel, M., Preissner, R., and Piechulla, B. (2013). mVOC: a database of microbial volatiles. Nucleic Acids Res. 42, D744–D748. doi: 10.1093/nar/gkt1250

Li, J., Wei, J., Ngu, H. H., Guo, W., Liu, H., Du, B., et al. (2018). Characterization of soluble microbial products in a partial nitrification sequencing batch biofilm reactor treating high ammonia nitrogen wastewater. Bioresour. Technol. 249, 241–246. doi: 10.1016/j.biortech.2017.10.013

Li, Y., Chapman, S. J., Nicol, G. W., and Yao, H. (2018). Nitrification and nitrifiers in acidic soils. Soil Biol. Biochem. 116, 290–301. doi: 10.1016/j.soilbio.2017.10.023

Limataman, M., Voigt, C., Martikainen, P. J., Hytönen, J., Regina, K., Öskarsson, H., et al. (2018). Factors controlling nitrous oxide emissions from managed northern peat soils with low carbon to nitrogen ratio. Soil Biol. Biochem. 122, 186–195. doi: 10.1016/j.soilbio.2018.04.006

Lliros, M., Ingeoglu, O., Garcia-Armisen, T., Auguet, J. C., Morana, C., Darchambeau, F., et al. (2014). “Ammonia oxidising Archaea in the OMZ of a freshwater African Lake,” in Proceedings of the 46th International Lige
Colloquium, Low Oxygen Environments in Marine, Estuarine and Fresh Waters, (Liège).

Mutlu, M. B., and Guven, K. (2011). Detection of prokaryotic microbial communities of Çamaltı Saltern, Turkey, by fluorescence in situ hybridization and real-time PCR. *Turk. J. Biol.***35,*** 687–695.

Nelson, M. B., Martiny, A. C., and Martiny, J. B. (2016). Global biogeography of microbial nitrogen-cycling traits in soil. *Proc. Natl. Acad. Sci. U.S.A.* **113,*** 8033–8040. doi: 10.1073/pnas.1601070113

Okano, Y., Hristova, K. R., Leutenegger, C. M., Jackson, L. E., Denison, R. F., Gebreyesus, B., et al. (2004). Application of real-time PCR to study effects of ammonium on population size of ammonia-oxidizing bacteria in soil. *Appl. Environ. Microbiol.* **70,** 1008–1016. doi: 10.1128/AEM.70.2.1008-1016.2004

Ontiveros-Valencia, A., Ilhan, Z. E., Kang, D.-W., Rittmann, B., and Krajmalnik-Brown, R. (2013). Phylogenetic analysis of nitrate- and sulfate-reducing bacteria in a hydrogen-fed biofilm. *FEMS Microbiol. Ecol.* **85,** 158–167. doi: 10.1111/1574-6941.12107

Pan, K.-L., Gao, J.-F., Li, H.-Y., Fan, X.-Y., Li, D.-C., and Jiang, H. (2018). Ammonia-oxidizing bacteria dominate ammonia oxidation in a full-scale wastewater treatment plant revealed by DNA-based stable isotope probing. *Bioreour. Technol.* **256,** 5–28. doi: 10.1016/j.biortech.2018.02.012

Rabus, R., Hoffmann, F., Rapp, H. T., Leininger, S., and Schleper, C. (2012). Anaerobic microbial degradation of hydrocarbons: from enzymatic reactions to the environment. *J. Mol. Microbiol. Biotechnol.* **26,** 5–28. doi: 10.1159/000439997

Radax, R., Hofmann, F., Rapp, H. T., Leininger, S., and Schleper, C. (2012). Ammonia-oxidizing archaea as main drivers of nitrification in cold-water sponges. *Environ. Microb.***14,** 909–923. doi: 10.1111/j.1462-2920.2011.02661.x

Rissansen, A. J., Karvinen, A., Nykänen, H., Peura, S., Tiirola, M., Mäki, A., et al. (2017). Effects of alternative electron acceptors on the activity and community structure of methane-producing and -consuming microbes in the sediments of two shallow boreal lakes. *FEMS Microbiol. Ecol.* **93,** 1–16. doi: 10.1093/femsec/ffx078

Rubio-Asensio, J. S., López-Berenguer, C., García-de la Garma, J., Burger, M., and Bloom, A. J. (2014). “Root strategies for nitrate assimilation,” in *Root Engineering*, eds A. Morte and A. Varma (Berlin: Springer), 251–267.

Schmidt, E. L., and Belser, L. W. (1982). *Nitrifying bacteria.*

Searle, P. L. (1979). Measurement of adsorbed sulphate in soils—effects of varying soil: extractant ratios and methods of measurement. *N. Z. J. Agric. Res.* **22,** 287–290. doi: 10.1080/00288233.1979.10430749

Socrates, G. (2004). *Infrared and Raman Characteristic Group Frequencies: Tables and Charts.* Hoboken, NJ: John Wiley & Sons.

Stokey, L. L. (1970). Ferrozine—a new spectrophotometric reagent for iron. *Anal. Chem.* **42,** 779–781. doi: 10.1021/ac60289a016

Tourna, M., Stiegmeier, M., Spang, A., Könneke, M., Schindlmeister, A., Urich, T., et al. (2011). Nitrososphaera viennensis, an ammonia oxidizing archaeon from soil. *Proc. Natl. Acad. Sci. U.S.A.* **108,** 8420–8425. doi: 10.1073/pnas.1013488108

Tyc, O., Song, C., Dickschat, J. S., Vos, M., and Garbeva, P. (2016). The ecological role of volatile and soluble secondary metabolites produced by soil bacteria. *Trends Microbiol.* **25,** 280–292. doi: 10.1016/j.tim.2016.12.002

van Kessel, M. A., Speth, D. R., Albertsen, M., Nielsen, P. H., den Camp, H. J. O., Kartal, B., et al. (2015). Complete nitrification by a single microorganism. *Nature* **528:**555. doi: 10.1038/nature16459

Weber, E. B., Lehtovirta-Morley, L. E., Prosser, J. I., and Gubry-Rangin, C. (2015). Ammonia oxidation is not required for growth of Group 1.1c soil *Thaumarchaeota.* *FEMS Microbiol. Ecol.* **91:**fv001. doi: 10.1093/femsec/fv001

Wuchter, C., Abbas, B., Cooled, M. J., Herfort, L., van Bleijswijk, J., Timmers, P., et al. (2006). Archaeal nitrification in the ocean. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 12317–12322. doi: 10.1073/pnas.0600756103

Zedelius, J., Rabus, R., Grundmann, O., Werner, I., Brodkorb, D., Schreiber, F., et al. (2011). Alkane degradation under anoxic conditions by a nitrate-reducing bacterium with possible involvement of the electron acceptor in substrate activation. *Environ. Microbiol. Rep.* **3,** 125–135. doi: 10.1111/j.1758-2229.2010.00198.x

Zhao, J., Wang, B., and Jia, Z. (2015). Phylogenetically distinct phylotype modulate nitrification in a paddy soil. *Appl. Environ. Microbiol.* **81,** 3218–3227. doi: 10.1128/AEM.00426-15

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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