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Co-Occurring Anammox, Denitrification, and Codenitrification in Agricultural Soils

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Anammox and denitrification mediated by bacteria are known to be the major microbial processes converting fixed N to N_2 gas in various ecosystems. Codenitrification and denitrification by fungi are additional pathways producing N_2 in soils. However, fungal codenitrification and denitrification have not been well investigated in agricultural soils. To evaluate bacterial and fungal processes contributing to N_2 production, molecular and ^{15}N isotope analyses were conducted with soil samples collected at six different agricultural fields in the United States. Denitrifying and anammox bacterial abundances were measured based on quantitative PCR (qPCR) of nitrous oxide reductase (*nosZ*) and hydrazine oxidase (*hzo*) genes, respectively, while the internal transcribed spacer (ITS) of *Fusarium oxysporum* was quantified to estimate the abundance of codenitrifying and denitrifying fungi. ^{15}N tracer incubation experiments with $^{15}NO_3^-$ or $^{15}NH_4^+$ addition were conducted to measure the N_2 production rates from anammox, denitrification, and codenitrification. Soil incubation experiments with antibiotic treatments were also used to differentiate between fungal and bacterial N_2 production rates in soil samples. Denitrifying bacteria were found to be the most abundant, followed by *F. oxysporum* based on the qPCR assays. The potential denitrification rates by bacteria and fungi ranged from 4.118 to 42.121 nmol N_2-N g^{-1} day^{-1}, while the combined potential rates of anammox and codenitrification ranged from 2.796 to 147.711 nmol N_2-N g^{-1} day^{-1}. Soil incubation experiments with antibiotics indicated that fungal codenitrification was the primary process contributing to N_2 production in the North Carolina soil. This study clearly demonstrates the importance of fungal processes in the agricultural N cycle.

The application of inorganic nitrogen fertilizers in agricultural fields causes various environmental problems, including eutrophication and habitat degradation. Accurate quantification of the processes removing fixed nitrogen is increasingly important to gain a better understanding of the fate of nitrogen in agricultural soils. Three microbial processes, denitrification, codenitrification, and anaerobic ammonium oxidation (anammox), are involved in the removal of nitrogen from soils through the production of nitrous oxide (N_2O) or dinitrogen gas (N_2) (1, 2). Denitrification is a microbial process in which nitrate (NO_3^-) and nitrite (NO_2^-) are converted to N_2O and N_2 in aerobic and anaerobic soils. It is a well-studied process in bacteria and has recently been found to occur in archaea and fungi (3). Codenitrification produces N_2O and N_2 through the reduction of nitrite (NO_2^-) by other nitrogen compounds, including azide, ammonium (NH_4^+), salicylhydroxamic acid, and hydroxylamine (4, 5). Codenitrification can occur in both fungi (e.g., *Fusarium oxysporum*) and bacteria (e.g., *Streptomyces antibioticus*) (5, 6) and has been measured in grassland and agricultural soils (4, 7). Fungal codenitrification has been estimated to contribute up to 92% of the N_2 produced in grassland soils (7).

Anammox produces N_2 by oxidizing NH_4^+ with NO_2^- reduction (8, 9). Anammox has been detected in a number of aquatic ecosystems, including marine sediments (10, 11, 12), oxygen-minimum zones (13, 14, 15, 16), freshwater marshes (3, 17), rivers (18), meromictic lakes (19), and river estuaries (20, 21, 22). Anammox bacteria have also been found in various soil types, including permafrost soils (3), reductisol, agricultural soils (17), peat soils (23), and rice paddy soils (24). However, the importance of anammox in soil N cycling has not been fully explored.

The potential rates of both anammox and denitrification can be calculated using ^{15}N isotope-pairing techniques (25). Under anoxic incubation conditions utilizing $^{15}NO_3^-$ and ample $^{14}NH_4^+$, anammox and denitrification produce $^{29}N_2$ and $^{30}N_2$, respectively. The relative importance of anammox can be calculated as the percentage of the total N_2 gas produced by anammox (%anammox). The %anammox has been shown to vary across aquatic environments, from being essentially absent to being the dominant pathway, with up to 79% of the N removed by anammox in various marine sediments (26). However, estimating %anammox in soils becomes difficult because codenitrification can also generate $^{29}N_2$ by reducing $^{47}N_2O$ that is produced from the utilization of $^{14}NH_4^+$ and $^{15}NO_3^-/^{15}NO_2^-$ in ^{15}N isotope-pairing experiments. The contribution of anammox and codenitrification to N_2 production can be presented as the percentage of N_2 produced as $^{29}N_2$ ($\%^{29}N_2$). In addition, both bacteria and fungi can generate $^{30}N_2$ as an end product of anaerobic denitrification. In order to gain a better understanding of soil microbial N cycling,

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TABLE 1 Physical and chemical characteristics of soil samples

Sample site	Soil series	USDA[a] texture	pH	Organic matter (%)	Content (mg/kg)													
					NH_4^+	NO_3^-	P	S	Ca	Mg	K	Na	B	Fe	Mn	Cu	Zn	Al
Beaufort, NC	Portsmouth	Sandy loam	5.6	3.06	8.9	2.5	104	14	1,080	139	69	23	0.27	192	3	0.89	1.32	1,625
Pasquotank, NC	Chapanoke	Loam	6.3	2.24	10.2	3.4	204	16	1,095	276	299	29	0.6	331	50	3.82	6.11	1,007
Currituck, NC	Roanoke	Silt loam	5.9	2.31	8.9	5.9	93	13	1,063	274	131	29	0.32	350	13	1.6	3.39	632
Boone, IA	Nicollette	Loam	5.9	2.91	5.4	3.2	7	9	3,315	530	119	44	0.65	173	21	2.18	0.75	773
Tippecanoe, IN	Chalmers	Silty clay loam	7.2	3.53	5.2	5.5	20	14	3,112	975	192	43	0.76	114	25	3.51	1.92	821
Jewell, KS	Gibbon	Loam	7.1	1.74	8.1	4.1	7	17	2,340	414	275	75	0.67	126	79	1.93	1.19	611

[a] USDA, U.S. Department of Agriculture.

it is important to determine the biological source of N_2 production in soils. The fungal and bacterial contributions to N_2 production may be differentiated using antibiotic inhibition experiments (7). Using antibiotic inhibition coupled with ^{15}N isotope-pairing techniques, the contributions of anammox, codenitrification, and denitrification to total N_2 production in soil samples may be determined.

Molecular methods can be used to determine the genetic potential of organisms involved in N_2 production in soils. Anammox bacteria in the environment can be detected and quantified based on 16S rRNA genes, as well as the functional genes encoding dissimilatory nitrite reductase (*nirS*), hydrazine oxidase (*hzo*), and hydrazine synthase (*hzs*) (19, 27, 28, 29, 30, 31). Nitrous oxide reductase genes (*nosZ*) have been used to quantify N_2-producing denitrifying bacteria in soils and sediments (32, 33, 34). However, genetic markers for codenitrifying and denitrifying fungi have yet to be developed. Alternatively, rRNA genes in selected fungal species can be targeted to estimate, as a proxy, the abundance of fungi capable of codenitrification and denitrification in environmental samples. *F. oxysporum* is the best-characterized fungus capable of codenitrification and denitrification (5). The detection and quantification of *F. oxysporum* have been demonstrated in inoculated soil samples by targeting the internal transcribed spacer (ITS) region of *F. oxysporum* rRNA genes (35).

By combining molecular quantification and ^{15}N isotope-pairing techniques with antibiotic inhibition, the contributions of anammox, anaerobic denitrification, and codenitrification to total N_2 production might be assessed in soils. We aimed to (i) identify the abundances of microbes capable of anammox, codenitrification, and denitrification and (ii) quantify the contributions of fungi and bacteria to total N_2 production from agricultural soils.

MATERIALS AND METHODS

Sample collection. In 2009, surface soil samples (30-cm depth increments) were collected in triplicate with a core sampler (10.16-cm diameter) in six different agricultural fields in the United States: Pasquotank County, NC (36°07′30.249″N, 76°10′10.776″W); Beaufort County, NC (35°27.681′N, 076°55.0926′W), Currituck County, NC (36°23′09.77″N, 76°07′18.82″W); Tippecanoe County, IN (40°29′20.027″N, 87°00′07.256″W); Jewell County, KS (39°56.1008′N, 098°2.1027′W); and Boone County, IA (41°55.186′N, 93°44.891′W). All the fields had a history (>5 years) of maize, soybean, and/or wheat production following typical regional management practices. The selected sites had been treated with inorganic fertilizers only in at least the 5 years preceding the study. Soil samples from each core were homogenized separately for subsampling. Two grams of each homogenized soil sample was transferred to 2-ml microcentrifuge tubes and stored in a −80°C freezer for DNA analysis, while the rest was stored in a 4°C cold room in sealed mason jars for rate measurements.

Physical and chemical analyses of soil properties. Additional bulk samples from the same depths and locations were used for soil characterization (Table 1). The soil texture was measured using a hydrometer (36). A 1:1 ratio of soil to water slurry was used to measure the pH (37). Organic matter was measured from the loss on ignition at 360°C (38). The inorganic N (NH_4^+ and NO_3^-) was measured using the methods of Dahnke (39). Phosphorus was measured through the use of the Mehlich III soil test (40). The soil texture and nutrient profiles are reported in Table 1.

DNA extraction from soil samples. Soil DNA extraction was performed with a ZR Soil Microbe DNA kit (Zymo Research Corporation, Orange, CA) using the manufacturer's instructions. The soil DNA concentration was measured with a Quant-It PicoGreen double-stranded DNA (dsDNA) assay kit according to the manufacturer's protocol (Invitrogen, Carlsbad, CA).

qPCR of *hzo* and *nosZ* genes and *F. oxysporum*. The DNA samples extracted from the soil samples were utilized for quantitative-PCR (qPCR) analysis. The abundances of anammox and denitrifying bacteria were quantified using primers targeting the *hzo* gene and *nosZ* gene, respectively. The qPCR primers (HZOQPCR1F, 5′-AAGACNTGYCAYTG GGGWAAA-3′, and HZOQPCR1R, 5′-GACATACCCATACTKGTRTA NACNGT-3′) of *hzo* genes were designed to target highly conserved regions of *hzo* gene cluster 1 after comparing the *hzo* gene cluster 1 sequences available in the NCBI database. qPCR of *hzo* genes generated 224-bp amplicons, which were confirmed to be *hzo* genes belonging to cluster 1 based on cloning and sequence analysis (data not shown). The detection limit of *hzo* gene qPCR was determined to be 10 copies per sample based on a serial dilution of plasmid standards carrying an *hzo* gene (see Fig. S1 in the supplemental material). qPCR of the *nosZ* gene was conducted with nosZ2F and nosZ2R primers as described previously (33). The abundance of the codenitrifying and denitrifying fungus *F. oxysporum* was measured by targeting the ITS region using the primers FOF1 and FOR1 designed by Mishra et al. (41). The qPCR standards were generated by serial dilution of the plasmids carrying the respective gene targets. All qPCR utilized GoTaq qPCR Master Mix Green (Promega, Madison, WI) and a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). The PCR cycling for *hzo* genes included an initial denaturation step for 10 min at 95°C, followed by 50 cycles of 95°C for 45 s, 53°C for 45 s, and 72°C for 35 s and a measurement step for 35 s at 75°C. The PCR cycling for *nosZ* genes started with an initial denaturation step for 10 min at 95°C, followed by 50 cycles of 95°C for 45 s, 55°C for 45 s, and 72°C for 35 s and a measurement step for 35 s at 80°C. The PCR cycling for the *F. oxysporum* ITS region began with an initial denaturation step for 2 min at 95°C, followed by 40 cycles of 95°C for 1 min, 65°C for 30 s, and 72°C for 30 s and a measurement step for 10 s at 79°C. PCR specificity and primer dimer

formation were monitored by analysis of dissociation curves. All qPCRs were performed in triplicate. The R^2 values for the standard curves were 0.998, 0.996, and 0.997 for the *hzo* qPCR, *nosZ* pPCR, and *F. oxysporum* ITS qPCR, respectively. The efficiency and detection limit of the *hzo* qPCR are shown in Fig. S1 in the supplemental material.

Examination of anammox community structures in soils. PCR of *hzo* gene cluster 1 was conducted to examine community structures of soil anammox bacteria using the primers hzocl1F1 and hzocl1R2, following the method of Schmid et al. (42) with some modifications. The PCR mixture was a 25-μl-volume reaction mixture containing 12.5 μl GoTaq Green Master Mix (Promega, Madison, WI), 1 μl of each primer (10 μM), and 1 μl of DNA as the template (10 to 100 ng). The PCR cycle began with a 5-min, 95°C denaturation step, followed by 40 cycles of denaturation at 94°C for 45 s and a primer-annealing step for 1 min at 50°C, and concluding with a 1-min extension step at 72°C. Gel electrophoresis on a 1.0% agarose gel was used to examine the PCR products, which were subsequently purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) according to the manufacturer's instructions. The purified amplicons were cloned using the Perfect PCR Cloning Kit (5Prime, Gaithersburg, MD). Clone libraries for the following sites were constructed: Beaufort, NC (BS), Currituck, NC (CS), Pasquotank, NC (PS), Jewell, KS (JS), Tippecanoe, IN (IS), and Boone, IA (OS) (see Table S1 in the supplemental material). The clones were sequenced using BigDye Terminator (Applied Biosystems, Foster City, CA) and an ABI 3130xl automated genetic analyzer (Applied Biosystems, Foster City, CA). NCBI BLAST (http:/www.ncbi.nih.gov) was used to find closely related sequences. The sequences, along with closely related reference sequences, were aligned using ClustalW (http:/www.ebi.ac.uk/clustalw/). MEGA version 4.0 was utilized to create neighbor-joining trees with bootstrapping of 16S rRNA gene sequences (43). Protein sequences were deduced from *hzo* sequences, and MEGA was utilized to create a Dayhoff model tree with bootstrapping. Similarities were calculated using EBI EMBOSS (http://www.ebi.ac.uk). DOTUR was used to compare the diversity of *hzo* genes detected in six sites based on the Shannon and chao1 indices (44).

Soil slurry incubation experiments utilizing $^{15}NO_3^-$ or $^{15}NH_4^+$ substrates. The rates of $^{29}N_2$ and $^{30}N_2$ production were measured and calculated using a modification of the method of Dalsgaard and Thamdrup (10). Approximately 2 g of soil was transferred to 12-ml Exetainer tubes (Labco, High Wycombe, United Kingdom) and mixed with 2 ml of Milli-Q water to generate saturated soil slurries. The tubes were sealed with gas-tight septa and flushed with He gas. The tubes with soil slurries were incubated overnight at room temperature to reduce the background concentrations of NO_3^- and NO_2^- (NO_x). The remaining background NO_x levels were measured using reduction by vanadium(III) and chemiluminescent detection with an Antec model 7020 nitric oxide analyzer (Antek Instruments, Houston, TX). After the initial overnight incubation, the tubes were vacuumed and flushed with He gas three times. A final concentration of 1 mM $Na^{15}NO_3$ (99.5 atom%; Cambridge Isotope Laboratory, Andover, MA) was added to each tube. $^{14}NH_4^+$ was not added, as sufficient quantities (>5 mg/kg) were present in the surface soils. Time course incubations were carried out in duplicate (time points 0, 1, 2, 3, and 5 h) at room temperature. A saturated $ZnCl_2$ solution was added at each time point during the incubation in order to stop microbial activity. The N_2 gas in the headspace of each sample was measured on a continuous-flow isotope ratio mass spectrometer (Thermo Finnigan Delta V; Thermo Scientific, Waltham, MA) in line with an automated gas bench interface (Thermo Gas Bench II). All samples from a single site were measured on the same day. $^{29}N_2$ and $^{30}N_2$ production rates were calculated from the samples amended with $^{15}NO_3^-$. The background nitrate levels, based on the nitrate and nitrite reduction measurements, were taken into account in the rates of $^{29}N_2$ and $^{30}N_2$ production, along with tracer dilutio, as described by Dalsgaard and Thamdrup (10).

The presence of anammox and codenitrification were confirmed by conducting additional incubation experiments utilizing $^{15}NH_4^+$ substrate additions. These conditions produce $^{29}N_2$ only if anammox or codenitrification occurs in the soil samples. Approximately 5 g of soil was transferred to 30-ml Wheaton serum bottles (Sigma-Aldrich, St. Louis, MO) and mixed with 5 ml of Milli-Q water to produce saturated soil slurries. The bottles were sealed with gas-tight butyl rubber stoppers and flushed with He gas. After an overnight incubation, the headspace of each serum bottle was vacuumed and flushed with He gas. The serum bottles were injected with He-flushed stock solutions of $(^{15}NH_4)_2SO_4$ (99.2 atom%; Cambridge Isotope Laboratory, Andover, MA) to give a final concentration of 1 mM $^{15}NH_4^+$. The headspace gas in the serum bottles (5 ml) was sampled at the beginning (0 h) and end (24 h) of incubation and transferred to a He-filled 12-ml Exetainer tube (Labco, High Wycombe, United Kingdom) using a gas-tight syringe (Hamilton Company, Reno, NV). The collected gas samples were measured on a continuous-flow isotope ratio mass spectrometer (Thermo Delta V; Thermo Scientific, Waltham, MA). The rate of $^{29}N_2$ production was calculated without considering $^{15}NH_4^+$ tracer dilution.

Soil slurry incubation experiments with antibiotic treatments. Additional soil samples were collected in 2010 from the same agricultural field at Beaufort, NC, following the same sampling procedures, to conduct soil slurry incubation experiments with addition of selective antibiotics of bacteria and fungi. Streptomycin was used to inhibit bacterial activity, while cycloheximide was used to inhibit fungal activity, as described by Laughlin and Stevens (7). The contribution of bacteria and fungi to the production of $^{29}N_2$, $^{30}N_2$, and N_2O was measured using the same incubation conditions described for the $^{15}NO_3^-$ addition experiments with the following modifications. Approximately 2 g of soil was transferred to 12-ml Exetainer tubes (Labco, High Wycombe, United Kingdom) and mixed with 2 ml of Milli-Q water to generate saturated soil slurries. Cycloheximide was added to a series of soil slurries at a final concentration of 15 mg/g, while streptomycin was added to another series of soil slurries at a final concentration of 3 mg/g. A series of soil slurries with no antibiotic additions was used to calculate an activity baseline for $^{29}N_2$, $^{30}N_2$, and N_2O production and to act as a positive control. Another series of soil slurries contained both antibiotics in the concentrations listed above as a negative control. The initial preincubation was extended from overnight to 48 h in order to allow the antibiotics to inhibit microbial activity. After the initial preincubation, the tubes were vacuumed and flushed with He gas three times. A final concentration of 1 mM $Na^{15}NO_3$ (99.5 atom%; Cambridge Isotope Laboratory, Andover, MA) was added to each tube. Time course incubations were carried out in duplicate (time points 0, 3, and 6 h). A saturated $ZnCl_2$ solution was added at each time point during the incubation in order to stop microbial activity. The N_2 gas in the headspace of each sample was measured on a continuous-flow isotope ratio mass spectrometer (Thermo Finnigan Delta V; Thermo Scientific, Waltham, MA) in line with an automated gas bench interface (Thermo Gas Bench II). The fungal and bacterial $^{29}N_2$ and $^{30}N_2$ rates in these incubation experiments were calculated using the methods of Dalsgaard and Thamdrup (10). Separate incubation experiments were set up for N_2O measurements using the same incubation conditions. Gas samples stored in the Exetainer tubes were analyzed for N_2O using a Varian Model 3800 Gas Chromatograph with a Combi-Pal autosampler. In this system, the sample is autoinjected into a 1-ml sample loop and then loaded into columns and routed through a ^{63}Ni electron capture detector (ultrapure 95% argon-5% CH_4 carrier gas). The gas chromatograph was calibrated with commercial blends of N_2O balanced in N_2 (Scott Specialty Gases, Philadelphia, PA) following verification of stated concentrations with standards from the National Institute of Standards and Technology. The precision of analysis, expressed as a coefficient of variation for 10 replicate injections of low and high concentration standards, was consistently <2%. The minimum detectable concentration change was 7 nl N_2O liter^{-1}. The time-linear change in the headspace N_2O molar concentration was used to calculate production rates of N_2O (45, 46). Percent inhibition by antibiotic treatments was calculated by dividing the rates of N_2O, $^{29}N_2$, and $^{30}N_2$ production in three different antibiotic treatments by those measured in the control.

Statistical analysis. The rates calculated from the soil slurry incubation experiments; the abundances of anammox, denitrifying, and codenitrifying organisms; and the physical and chemical characteristics of soil samples were used for principal-component analysis (PCA) and Pearson correlation values using the Canoco program (version 4.5; Microcomputer Power, Ithaca, NY) and Microsoft (Redmond, WA) Excel, respectively. R^2 and P values were calculated from linear regression analyses using Microsoft Excel. Due to the extraordinarily high rate of $^{29}N_2$ production from the Beaufort soils compared to the other sites, the data from the site were excluded from the statistical analysis.

Nucleotide sequence accession numbers. The *hzo* gene sequences were deposited in the NCBI database with accession numbers ranging from JQ314231 to JQ314341.

RESULTS

Physical and chemical properties of soils collected from agricultural fields. The measurements of organic matter, NH_4^+, NO_3^-, and other chemical parameters in soil samples from six agricultural fields are reported in Table 1. High concentrations of NH_4^+ and NO_3^- were characteristic of all soil samples, ranging from 1.1 to 10.2 mg/kg for NH_4^+ and from 1.3 to 5.9 mg/kg for NO_3^-. Soil pH varied considerably from more acidic in North Carolina (ranging from 5.2 to 6.3) to near neutral or basic in Midwestern states (ranging from 5.9 to 8.2). Soil textures ranged from sandy loam in Beaufort to silt loam in Currituck to clay loam in Pasquotank.

Detection and identification of anammox bacteria in agricultural soils. PCR with primers hzocl1F1 and hzocl1R2 generated amplicons with a length of 470 bp from all the soil samples. Cloning and sequencing of the amplicons confirmed the detection of only *hzo* genes associated with *hzo* gene cluster 1 (42). Based on the *hzo* gene detection, anammox bacteria were found to be ubiquitous across the six sample sites in four states. The *hzo* gene sequences were translated into amino acid sequences, which were used to select representative sequences based on sequence identity. Phylogenetic analysis of the representative HZO sequences showed that soil anammox bacteria were closely related to "*Candidatus* Jettenia," sharing 94.1 to 100% sequence similarity (Fig. 1). None of the sequences were closely associated with the HZO sequences found in "*Candidatus* Scalindua spp.," "*Candidatus* Anammoxoglobus spp.," "*Candidatus* Kuenenia spp.," or "*Candidatus* Brocadia spp." There was no clear segregation of anammox bacterial communities associated with the soils sampled from the different locations. However, higher diversity of *hzo* genes was found in the Currituck soil community than in other soil samples (see Table S1 in the supplemental material).

Abundances of *hzo* and *nosZ* genes and the *F. oxysporum* ITS. Quantitative PCR was performed on DNA samples from the top 30 cm of all the sample sites using primers specific for *hzo* and *nosZ* genes for anammox and denitrifying bacteria, respectively. The abundances of *hzo*, *nosZ*, and the *F. oxysporum* ITS are reported in Table 2.

The highest *hzo* gene abundance was found in the Boone soils, while the *nosZ* gene abundance was highest in Beaufort soils. The highest abundance of *F. oxysporum* was recorded in the Currituck soils, but *F. oxysporum* was not detected in soil samples collected from Boone and Jewell.

$^{29}N_2$ and $^{30}N_2$ production from soil slurry incubation experiments. Potential anammox, codenitrification, and denitrification rates in soil samples were measured using two different ^{15}N substrates ($^{15}NH_4^+$ or $^{15}NO_3^-$) (Table 3). The $^{29}N_2$ production from $^{15}NH_4^+$ tracer incubations is considered an indication of the presence of anammox and codenitrification in the soils samples, while the $^{29}N_2$ and $^{30}N_2$ production rates from $^{15}NO_3^-$ tracer incubations were used to calculate the combined potential rates of anammox and codenitrification and the potential rate of denitrification, respectively (Fig. 2).

The $^{29}N_2$ production rates from anammox and codenitrification varied from 2.796 to 147.711 nmol N_2-N g^{-1} day^{-1}, while the denitrification rates ranged from 4.118 to 42.121 nmol N_2-N g^{-1} day^{-1}. The $\%^{29}N_2$ production ranged from 32.1 to 77.9% of total N_2 production. Both of the lowest potential $^{29}N_2$ production and denitrification rates were from the Boone soils, while both of the highest potential $^{29}N_2$ production and denitrification rates were from the Beaufort soils. The highest $\%^{29}N_2$ production was found in Beaufort, while the lowest was in Pasquotank. The overall trend was higher potential $^{29}N_2$ production and denitrification rates in North Carolina than in the other states in the sample set.

The presence of anammox and codenitrification in soil samples was confirmed with the incubation experiments with $^{15}NH_4^+$ addition. The production of $^{29}N_2$ was observed from the incubation conditions, and the potential anammox and codenitrification rates were calculated to be from 0.011 to 0.353 nmol N_2-N g^{-1} day^{-1} based on the $^{29}N_2$ production (Table 3). The lowest potential rate of $^{29}N_2$ production was in the Beaufort soils, and the highest potential rate was in the Tippecanoe soils. Overall, the potential $^{29}N_2$ production rates from North Carolina were lower than those from the other states.

Correlation analysis of rate measurements, gene abundance, and soil properties. Weighted and normalized PCAs were conducted to determine correlations of rate measurements with the soil characteristics and the abundance of anammox and denitrifying bacteria and *F. oxysporum* (Fig. 3). Principal component 1 (PC1) explained 97.1% of the variability, while principal component 2 (PC2) explained 2.9% of the variability. The potential $^{30}N_2$ production rates showed a significant and positive correlation with the abundance of *F. oxysporum* in the soils ($r = 0.748$, $r^2 = 0.560$, and $P = 0.032$). The denitrification rates showed no other statistically significant correlations but exhibited a strong correlation with NO_3^- ($r = 0.895$; $r^2 = 0.801$). The potential $^{29}N_2$ production rates calculated from the $^{15}NO_3^-$ incubation experiments showed strong correlations with the levels of NO_3^- ($r = 0.905$; $r^2 = 0.819$) and the abundance of *F. oxysporum* ($r = 0.799$; $r^2 = 0.638$).

Comparison of bacterial and fungal activities in N_2 and N_2O production. The fungal and bacterial contributions to $^{29}N_2$, $^{30}N_2$, and N_2O production rates were evaluated using the slurry incubation experiments with antibiotic additions. Cycloheximide inhibits both fungal codenitrification and denitrification, while bacterial denitrification and anammox are repressed by streptomycin. Based on the rates calculated from control conditions without antibiotic treatment, the Beaufort soil communities produced larger amounts of N_2O than of N_2. The N_2O production rate was 24.14 nmol N_2O n g^{-1} day^{-1}, while the total N_2 production rate was estimated to be 7.27 nmol N_2-N g^{-1} day^{-1} by combining the $^{29}N_2$ and $^{30}N_2$ production rates (see Table S2 in the supplemental material). Based on the comparison of N_2O and N_2 production rates from the control and antibiotic treatments, cycloheximide was shown to inhibit 65% of the N_2O production and 85% of the total N_2 production (Table 4). Cycloheximide inhibited both $^{29}N_2$ and $^{30}N_2$ production by 85%. Streptomycin inhib-

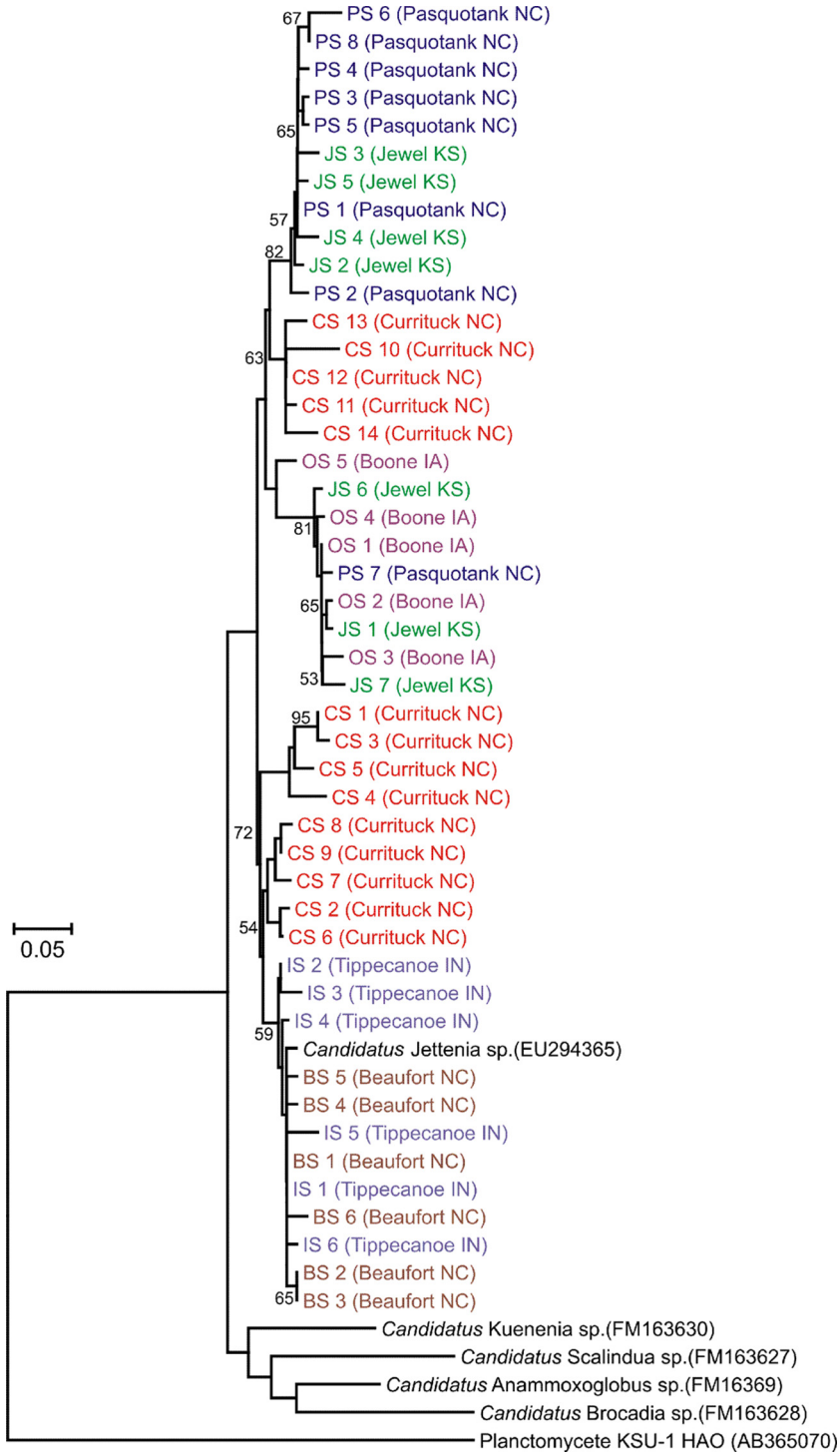

FIG 1 Phylogenetic tree of representative HZO sequences deduced from the *hzo* genes detected from agricultural soils. Neighbor joining with the Dayoff model was used for tree construction. The HAO sequence from the planctomycete KSU-1 was selected as an outgroup. Abbreviations for sample sites are as follows: BS (Beaufort surface layer), CS (Currituck surface layer), IS (Tippecanoe surface layer), JS (Jewell surface layer), OS (Boone surface layer), and PS (Pasquotank surface layer). The bootstrap numbers are percentages of 1,000 iterations.

ited 45% of N_2O production and 62% of total N_2 production. Streptomycin inhibited $^{29}N_2$ and $^{30}N_2$ production by 60% and 68%, respectively. Higher inhibition was observed in both bacterial and fungal N_2 production than N_2O production. The incubations with both antibiotics added nearly completely inhibited N_2 production, while 71% of N_2O production was repressed. Among N_2 production rates measured from the antibiotic treatments, the highest inhibition (62.6%) was found in $^{29}N_2$ production with

TABLE 2 Abundances of denitrifying and anammox bacteria, as well as *F. oxysporum*, in agricultural soils

Sample site	No. of copies g^{-1}		
	hzo	*nosZ*	*F. oxysporum* ITS
Beaufort, NC	$1.24 \times 10^4 \pm 5.30 \times 10^3$	$7.88 \times 10^6 \pm 1.40 \times 10^6$	$1.92 \times 10^5 \pm 2.10 \times 10^4$
Pasquotank, NC	$5.53 \times 10^3 \pm 1.80 \times 10^3$	$3.67 \times 10^6 \pm 7.10 \times 10^5$	$1.19 \times 10^5 \pm 7.50 \times 10^3$
Currituck, NC	$9.04 \times 10^3 \pm 6.10 \times 10^3$	$3.65 \times 10^6 \pm 8.40 \times 10^5$	$3.12 \times 10^5 \pm 4.50 \times 10^4$
Boone, IA	$1.57 \times 10^4 \pm 5.20 \times 10^3$	$3.21 \times 10^6 \pm 7.10 \times 10^5$	ND[a]
Tippecanoe, IN	$4.99 \times 10^3 \pm 8.80 \times 10^2$	$5.15 \times 10^6 \pm 6.50 \times 10^4$	$2.38 \times 10^5 \pm 1.80 \times 10^4$
Jewell, KS	$1.15 \times 10^4 \pm 1.20 \times 10^3$	$3.33 \times 10^6 \pm 4.50 \times 10^5$	ND

[a] ND, not detected.

cycloheximide addition (Table 4). Streptomycin treatment inhibited 44.1% of total N_2 production, which may account for anammox and bacterial codenitrification.

DISCUSSION

Detection and identification of anammox bacteria in agricultural soils. The presence of anammox bacteria in agricultural soils was determined based on the detection of *hzo* genes in this study. This is the first report of finding the *hzo* genes in agricultural soils. Phylogenetic analysis showed that all the detected anammox bacteria are closely associated with "*Ca.* Jettenia spp." with low diversity. This was in contrast to the findings of Humbert et al. (17), which showed high diversity of anammox bacteria across terrestrial ecosystems with "*Ca.* Jettenia," "*Ca.* Brocadia," and "*Ca.* Kuenenia." A number of selective pressures may have contributed to the lack of diversity in anammox bacteria in the agricultural soils. Paramount in these factors is tillage, which introduces air into the soil and disrupts the structural integrity and horizons of the soil profile. Tillage is known to have a detrimental effect on the abundance of denitrifiers in the soil, with up to 60% less denitrifiers in tilled versus untilled soils (47). Another selective pressure in agricultural fields that may have an effect on soil anammox diversity is the long-term application of inorganic nitrogen fertilizers. The application of NH_4^+- and NO_3^--based fertilizers can alter the composition of denitrifier community structures (48). The selective pressures in agricultural soils may have allowed "*Ca.* Jettenia" to be enriched and to predominate in the examined soils. Dominance of "*Candidatus* Jettenia asiatca" was reported in the anammox bacterial enrichment study with peat soils after feeding an increased concentration of NO_2^- and NH_4^+ (23).

Abundance of N_2-producing microorganisms in agricultural soils. Denitrifying bacteria were found to be the most abundant N_2-producing microorganisms in agricultural soils, based on the qPCR assays. The *nosZ* gene abundance was at least 200 times higher than that of the *hzo* genes (Table 2) and fell into the range of *nosZ* gene copy numbers measured in other soils (10^5 to 10^7 gene copies g^{-1}) (33). qPCR of *hzo* genes was used to estimate anammox bacterial abundance in the different soil samples. The *hzo* gene abundance in soils was much lower than those reported in Jiaozhou Bay sediments (27). Lower anammox bacterial abundance in soils might be related to aeration and the physical disruption of soil communities in agricultural fields. Anammox bacteria may be better suited to stratified anoxic sediments than to agricultural soils.

Based on qPCR of the ITS, *F. oxysporum* was detected and quantified in four of the six agricultural soil samples. The abundance of *F. oxysporum* in the soils was greater than in the soil measured by Jimmenez-Fernandez et al. (35), who inoculated soil samples. with *F. oxysporum.* This is the first report to quantify *F. oxysporum* using qPCR in agricultural soils. Sequence analysis of the amplicons showed 100% sequence identity to the ITS region of *F. oxysporum* (data not shown). *F. oxysporum* is just one of the codenitrifying and denitrifying fungi (4). Since the qPCR assay of the *F. oxysporum* ITS region is highly specific for *F. oxysporum* species, the abundance of codenitrifying and denitrifying fungi in soils is underestimated in this study. It is necessary to develop new molecular methods to estimate the abundance of codenitrifying and denitrifying fungi in soil ecosystems.

Microbial interaction of N_2 production in agricultural soils. Microbial N_2 production in soils can be mediated by anammox, denitrification, and codenitrification. Both anammox and codenitrification can generate $^{29}N_2$ gas from ^{15}N isotope-pairing experiments with $^{15}NH_4^+$ or $^{15}NO_3^-$ substrates. The N_2 production rates from $^{15}NH_4^+$ treatments were significantly lower than the rates measured from the $^{15}NO_3^-$ treatments, which may have been due to dilution of the $^{15}NH_4^+$ tracer with unlabeled $^{14}NH_4^+$ continuously generated from remineralization of organic nitrogen during the incubation. Lower $^{29}N_2$ production rates of $^{15}NH_4^+$ treatments observed in the North Carolina soils could be

TABLE 3 N_2 production rates calculated from ^{15}N isotope-pairing experiments

Sample site	$^{15}NH_4^+$ (nmol N_2-N g^{-1} day^{-1}) $^{29}N_2$	$^{15}NO_3^- + {}^{14}NH_4^+$ (nmol N_2-N g^{-1} day^{-1})		%$^{29}N_2$	Residual NO_3^-/NO_2^- (mM)	$^{15}NO_3^-$ enrichment (%)
		$^{29}N_2$	$^{30}N_2$			
Beaufort, NC	0.011 ± 0.006	147.711 ± 1.848	42.121 ± 3.168	77.9	0.251	74.9
Pasquotank, NC	0.024 ± 0.017	19.212 ± 6.371	40.811 ± 4.812	32.0	0.141	85.9
Currituck, NC	0.018 ± 0.024	21.611 ± 3.651	33.611 ± 3.816	39.1	0.361	63.9
Boone, IA	0.181 ± 0.097	2.796 ± 0.408	4.188 ± 0.288	40.0	0.182	81.8
Tippecanoe, IN	0.353 ± 0.266	8.172 ± 0.528	11.291 ± 0.168	42.0	0.051	94.9
Jewell, KS	0.040 ± 0.019	13.212 ± 2.892	12.011 ± 2.052	52.4	0.211	78.9

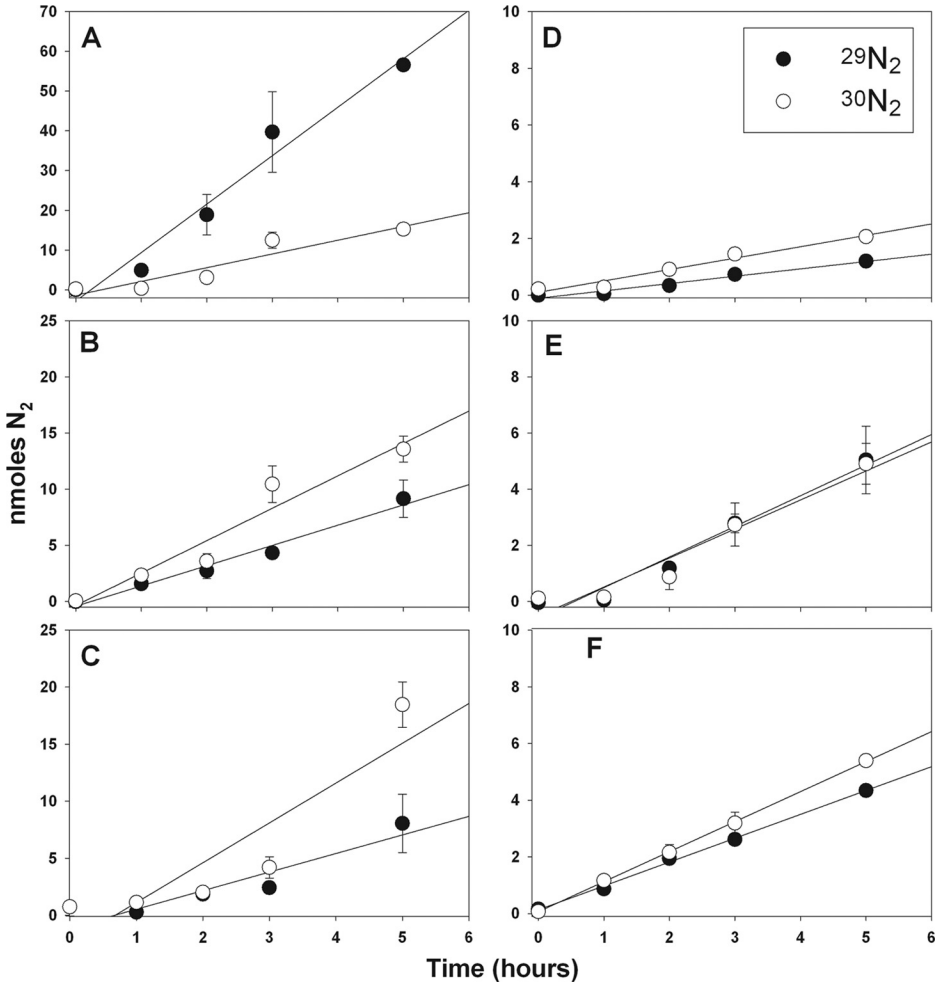

FIG 2 $^{29}N_2$ and $^{30}N_2$ production from $^{15}NO_3^-$ addition incubation experiments. (A) Beaufort. (B) Currituck. (C) Pasquotank. (D) Boone. (E) Jewell. (F) Tippecanoe. The error bars indicate standard deviations.

explained by higher remineralization coupled with denitrification.

The $^{29}N_2$ production from the $^{15}NO_3^-$ treatments ranged from 2.8 to 147.71 nmol N_2-N g^{-1} day^{-1}, and the $\%^{29}N_2$ production ranged from 32.1 to 77.9% of the total N_2 produced. Both $^{29}N_2$ production rates and $\%^{29}N_2$ production were higher than those reported in rice paddy soils (24). The potential denitrification rates based on the $^{30}N_2$ production fall into the lower range of N_2 gas fluxes from denitrification measured in other agricultural soils (49). There was no significant correlation between the abundance of *hzo* and *nosZ* genes and the production rates of $^{29}N_2$ and $^{30}N_2$ gases. This may indicate that anammox and bacterial denitrification are not major N_2-producing pathways in the agricultural soils. Alternatively, there might be a detection limitation of the *nosZ* gene primers used for qPCR analyses. The diversity of N_2O-

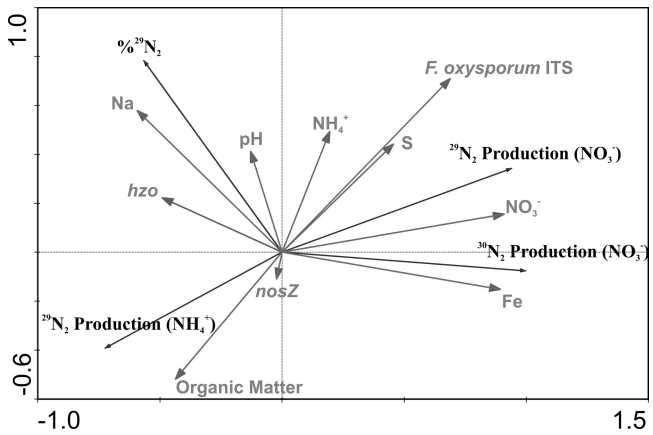

FIG 3 PCA plot comparing $^{29}N_2$ and $^{30}N_2$ production rates, qPCR data on microbial abundance, and soil characteristics.

TABLE 4 Percent inhibition of N_2O and N_2 production with antibiotic treatments

Antibiotic treatment	% Inhibition			
	N_2O	N_2	$^{29}N_2$	$^{30}N_2$
Cycloheximide	64.8	85.1	62.6	22.6
Streptomycin	45.6	61.6	44.1	18.2
Streptomycin + cycloheximide	70.6	99.9	72.5	26.4

respiring bacteria in the examined soils might be greater than those reported by Henry et al. (33).

The presence of *F. oxysporum* in the soils suggests that codenitrification and denitrification by fungi may play important roles in $^{29}N_2$ and $^{30}N_2$ production. PCA showed a strong correlation between the abundance of *F. oxysporum* and the production rates of $^{29}N_2$ and $^{30}N_2$ (Fig. 3). The results from soil slurry incubation with antibiotics confirmed that fungal codenitrification and denitrification had a greater contribution to the total N_2 production in the examined soil samples than did anammox and bacterial denitrification (Table 4; see Table S2 in the supplemental material). Greater inhibition of $^{29}N_2$ production by fungus was also observed in the antibiotic inhibition experiments. Interestingly, the sum of percent inhibition by either cycloheximide or streptomycin in N_2 production is larger than the percent inhibition of both antibiotic treatments. This may due to an unknown mechanism of inhibition by either cycloheximide or streptomycin on bacteria or fungus, respectively. The highest inhibition of N_2 production by cycloheximide indicates that fungal codenitrification is a major N_2-producing process in the examined soil communities. This, along with the higher overall $^{29}N_2$ production rates compared to the $^{30}N_2$ production rates, suggests that fungal codenitrification is the dominant pathway of N_2 production in the Beaufort soils. Anammox and bacterial codenitrification were also found to make significant contributions to the total N_2 production compared to that of denitrification.

Future studies must continue to address the fungal contribution to the production of N_2 and N_2O in agricultural soils with the development of new methods. Current denitrification, codenitrification, and anammox calculations based on the production of labeled N_2 cannot fully differentiate the $^{29}N_2$ produced by bacterial codenitrification and anammox (7, 25, 50). Due to the characteristics of anammox bacteria, which, like other planctomycetes, lack cell walls, antibiotics targeting cell wall synthesis, coupled with fungal inhibitors, may inhibit both bacterial and fungal codenitrification, thereby allowing anammox to be measured separately from bacterial codenitrification (51). Characterization and development of molecular probes targeting genes in the codenitrification pathway may prove useful in gaining further understanding of the abundance of codenitrifiers in soils.

REFERENCES

1. **Davidson EA, Seitzinger S.** 2006. The enigma of progress in denitrification research. Ecol. Appl. **16**:2057–2063.
2. **Hayatsu M, Tago K, Saito M.** 2008. Various players in the nitrogen cycle: diversity and functions of the microorganisms involved in nitrification and denitrification. Soil Sci. Plant Nutr. **54**:33–45.
3. **Philipot L, Hallin S, Schloter M.** 2007. Ecology of denitrifying prokaryotes in agricultural soil. Adv. Agron. **96**:249–305.
4. **Spott O, Strange CF.** 2011. Formation of hybrid N_2O in a suspended soil due to co-denitrification of NH_2OH. J. Plant Nutr. Soil Sci. **174**:554–567.
5. **Tanimoto T, Hatano K-I, Kim D-H, Uchiyama H, Shoun H.** 1992. Co-denitrification by the denitrifying system of the fungus *Fusarium oxysporm*. FEMS Microbiol. Lett. **93**:177–180.
6. **Kumon Y, Sasaki Y, Kato I, Takaya N, Shoun H, Beppu T.** 2002. Codenitrification and denitrification are dual metabolic pathways through which dinitrogen evolves from nitrate in *Streptomyces antibioticus*. J. Bacteriol. **184**:2963–2968.
7. **Laughlin RJ, Stevens RJ.** 2002. Evidence for fungal dominance of denitrification and codenitrification in a grassland soil. Soil. Sci. Soc. Am. J. **66**:1540–1548.
8. **Penton CR, Devol AH, Tiedje JM.** 2006. Molecular evidence for the broad distribution of anaerobic ammonium-oxidizing bacteria in freshwater and marine sediments. Appl. Environ. Microbiol. **72**:6829–6832.
9. **van de Graaf AA, Mulder A, de Bruijn P, Jetten MS, Robertson LA, Kuenen JG.** 1995. Anaerobic oxidation of ammonium is a biologically mediated process. Appl. Environ. Microbiol. **61**:1246–1251.
10. **Dalsgaard T, Thamdrup B.** 2002. Factors controlling anaerobic ammonium oxidation with nitrite in marine sediments. Appl. Environ. Microbiol. **68**:3802–3808.
11. **Hietanen S, Kuparinen J.** 2008. Seasonal and short-term variation in denitrification and anammox at a coastal station on the Gulf of Finland, Baltic Sea. Hydrobiologia **596**:67–77.
12. **Rich JJ, Dale OR, Song B, Ward BB.** 2008. Anaerobic ammonium oxidation (anammox) in Chesapeake Bay sediments. Microbiol. Ecol. **55**:311–320.
13. **Dalsgaard T, Canfield DE, Petersen J, Thamdrup B, Acuña-González J.** 2003. N_2 production by the anammox reaction in the anoxic water column of Golfo Dulce, Costa Rica. Nature **422**:606–608.
14. **Kuypers MM, Sliekers AO, Lavik G, Schmid M, Jørgensen BB, Kuenen JG, Sinninghe Damsté JS, Strous M, Jetten MS.** 2003. Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. Nature **422**:608–611.
15. **Kuypers MM, Lavik G, Woebken D, Schmid M, Fuchs BM, Amann R, Jørgensen BB, Jetten MS.** 2005. Massive nitrogen loss from the Benguela upwelling system through anaerobic ammonium oxidation. Proc. Natl. Acad. Sci. U. S. A. **102**:6478–6483.
16. **Stevens H, Ulloa O.** 2008. Bacterial diversity in the oxygen minimum zone of the eastern tropical South Pacific. Environ. Microbiol. **10**:1244–1259.
17. **Humbert S, Tarnawski S, Fromin N, Mallet MP, Aragno M, Zopfi J.** 2010. Molecular detection of anammox bacteria in terrestrial ecosystems: distribution and diversity. ISME J. **4**:450–454.
18. **Zhang Y, Ruan XH, Op den Camp HJ, Smits TJ, Jetten MS, Schmid MC.** 2007. Diversity and abundance of aerobic and anaerobic ammonium-oxidizing bacteria in freshwater sediments of the Xinyi River (China). Environ. Microbiol. **9**:2375–2382.
19. **Schubert CJ, Durisch-Kaiser E, Wehrli B, Thamdrup B, Lam P, Kuypers MM.** 2006. Anaerobic ammonium oxidation in a tropical freshwater system (Lake Tanganyika). Environ. Microbiol. **8**:1857–1863.
20. **Dale OR, Tobias CR, Song B.** 2009. Biogeographical distribution of diverse anaerobic ammonium oxidizing (anammox) bacteria in Cape Fear River Estuary. Environ. Microbiol. **11**:1194–1207.
21. **Risgaard-Petersen N, Meyer RL, Schmid M, Jetten MS, Enrich-Prast A, Pysgaard S, Revsbech NP.** 2004. Anaerobic ammonium oxidation in an estuarine sediment. Aquat. Microbiol. Ecol. **36**:293–304.
22. **Trimmer M, Nicholls JC, Deflandre B.** 2003. Anaerobic ammonium oxidation measured in sediments along the Thames estuary, United Kingdom. Appl. Environ. Microbiol. **69**:6447–6454.
23. **Hu BL, Rush D, van der Biezen E, Zheng P, van Mullekom M, Schouten S, Sinninghe DamstÉ JS, Smolders AJ, Jetten MS, Kartal B.** 2011. New anaerobic, ammonium-oxidizing community enriched from peat soil. Appl. Environ. Microbiol. **77**:966–971.
24. **Zhu G, Wang S, Wang Y, Wang C, Risgaard-Petersen N, Jetten MS, Yin C.** 2011. Anaerobic ammonia oxidation in a fertilized paddy soil. ISME J. **5**:1902–1912.
25. **Thamdrup B, Dalsgaard T.** 2002. Production of N_2 through anaerobic ammonium oxidation coupled to nitrate reduction in marine sediments. Appl. Environ. Microbiol. **68**:1312–1318.
26. **Engstrom P, Dalsgaard T, Hulth S, Aller RC.** 2005. Anaerobic ammonium oxidation by nitrite (anammox): implications for N_2 production in coastal marine sediments. Geochim. Cosmochim. Acta **69**:2057–2065.
27. **Dang H, Chen R, Wang L, Guo L, Chen P, Tang Z, Tian F, Li S, Klotz MG.** 2010. Environmental factors shape sediment anammox bacterial communities in hypernutrified Jiaozhou Bay, China. Appl. Environ. Microbiol. **76**:7036–7047.
28. **Harhangi HR, Le Roy M, van Alen T, Hu BL, Groen J, Kartal B, Tringe SG, Quan ZX, Jetten MS, Op den Camp HJ.** 2012. Hydrazine synthase, a unique phylomarker with which to study the presence and biodiversity of anammox bacteria. Appl. Environ. Microbiol. **78**:752–758.
29. **Hirsch M, Long Z, Song B.** 2011. Anammox bacterial diversity in various aquatic ecosystems based on the detection of hydrazine oxidase genes (*hzoA/hzoB*). Microbiol. Ecol. **61**:264–276.
30. **Lam P, Lavik G, Jensen MM, van de Vossenberg J, Schmid M, Woebken D, Gutiérrez D, Amann R, Jetten MS, Kuypers MM.** 2009. Revising the nitrogen cycle in the Peruvian oxygen minimum zone. Proc. Natl. Acad. Sci. U. S. A. **106**:4752–4757.

31. **Li M, Hong YG, Klotz MG, Gu JD.** 2010. A comparison of primer sets for detecting 16S rRNA and hydrazine oxidoreductase genes of anaerobic ammonium-oxidizing bacteria in marine sediments. Appl. Microbiol. Biotechnol. **86**:781–790.
32. **Dandie CE, Burton DL, Zebarth BJ, Henderson SL, Trevors JT, Goyer C.** 2008. Changes in bacterial denitrifier community abundance over time in an agricultural field and their relationship with denitrification activity. Appl. Environ. Microbiol. **74**:5997–6005.
33. **Henry S, Bru D, Stres B, Hallet S, Philippot L.** 2006. Quantitative detection of the *nosZ* gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, *narG*, *nirK*, and *nosZ* genes in soils. Appl. Environ. Microbiol. **72**:5181–5189.
34. **Philippot L, Cuhel J, Saby NP, Chèneby D, Chronáková A, Bru D, Arrouays D, Martin-Laurent F, Simek M.** 2009. Mapping field-scale spatial patterns of size and activity of the denitrifier community. Environ. Microbiol. **11**:1518–1526.
35. **Jimmenez-Fernandez D, Montes-Borrego M, Navas-Cortés JA, Jiménez-Díaz RM, Landa BB.** 2010. Identification and quantification of *Fusarium oxysporum in planta* and soil by means of an improved specific and quantitative PCR assay. Appl. Soil Ecol. **46**:372–382.
36. **Day P.** 1965. Hydrometer method of particle size analysis. Methods Soil Anal. Monogr. **9**:562–566.
37. **Mishra PK, Fox RTV, Culham A.** 2003. Development of a PCR-based assay for rapid and reliable identification of pathogenic fusaria. FEMS Microbiol. Lett. **218**:329–332.
38. **Schulte EE, Hopkins BG.** 1996. Estimation of soil organic matter by weight loss-on-ignition. p 21–32. *In* Magdoff FR, Tabatabai MA, Hanlon EA, Jr (ed), Soil organic matter: analysis and interpretation. Special publication no. 46. Soil Science Society of America, Madison, WI.
39. **Dahnke WC.** 1990. Testing soils for available nitrogen, p 120–140. *In* Westerman RL (ed), Soil testing and plant analysis. Soil Science Society of America book series 3. American Society of Agronomy, Madison, WI.
40. **Mehlich A.** 1984. Mehlich-3 soil test extractant: a modification of Mehlich-2 extractant. Commun. Soil Sci. Plant Anal. **15**:1409–1416.
41. **Mulder A, Vandegraaf AA, Robertson LA, Kuenen JG.** 1995. Anaerobic ammonium oxidation discovered in a denitrifying fluidized-bed reactor. FEMS Microbiol. Ecol. **16**:177–183.
42. **Schmid MC, Hooper AB, Klotz MG, Woebken D, Lam P, Kuypers MM, Pommerening-Roeser A, Op den Camp HJ, Jetten MS.** 2008. Environmental detection of octahaem cytochrome c hydroxylamine/hydrazine oxidoreductase genes of aerobic and anaerobic ammonium-oxidizing bacteria. Environ. Microbiol. **10**:3140–3149.
43. **Tamura K, Dudley J, Nei M, Kumar S.** 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24**:1596–1599.
44. **Schloss PD, Handelsman J.** 2005. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl. Environ. Microbiol. **71**:1501–1506.
45. **Phillips RL, Tanaka DL, Archer DW, Hanson JD.** 2009. Fertilizer application timing influences greenhouse gas fluxes over a growing season. J. Environ. Qual. **38**:1569–1579.
46. **Phillips RL, Wick AF, Liebig M, West M.** 2011. Biogenic emissions of CO_2 and N_2O increase exponentially at multiple depths during a simulated soil thaw for a northern prairie Mollisol. Soil Biol. Biochem. **45**:14–22.
47. **Doran JW.** 1980. Soil microbial and biochemical changes associated with reduced tillage. Soil Sci. Soc. Am. J. **44**:765–771.
48. **Enwall K, Philippot L, Hallin S.** 2005. Activity and composition of the denitrifying bacterial community respond differently to long-term fertilization. Appl. Environ. Microbiol. **71**:8335–8343.
49. **Stevens RJ, Laughlin RJ.** 1998. Measurement of nitrous oxide and dinitrogen emissions from agricultural soils. Nutr. Cycl. Agroecosyst. **52**: 131–139.
50. **Spott O, Strange CF.** 2007. A new mathematical approach for calculating the contribution of anammox, denitrification and atmosphere to an N_2 mixture based on ^{15}N tracer technique. Rapid Commun. Mass Spectrom. **21**:2398–2406.
51. **König E, Schlesner H, Hirsch P.** 1984. Cell wall studies on budding bacteria of the *Planctomyces/Pasteuria* group and on a *Prosthecomicrobium* sp. Arch. Microbiol. **138**:200–205.