

Nitrification and Denitrification Gene Abundances in Swine Wastewater Anaerobic Lagoons

Thomas F. Ducey,* Anthony D. Shriner, and Patrick G. Hunt

Although anaerobic lagoons are used globally for livestock waste treatment, their detailed microbial cycling of N is only beginning to become understood. Within this cycling, nitrification can be performed by organisms that produce the enzyme ammonia monoxygenase. For denitrification, the reduction of nitrite to nitric oxide can be catalyzed by two forms of nitrite reductases, and N_2O can be reduced by nitrous oxide reductase encoded by the gene *nosZ*. The objectives of this investigation were to (i) quantify the abundance of the *amoA*, *nirK*, *nirS*, and *nosZ* genes; (ii) evaluate the influence of environmental conditions on their abundances; and (iii) evaluate their abundance relative to denitrification enzyme activity (DEA). Samples were analyzed via real-time quantitative polymerase chain reaction and collected from eight typical, commercial anaerobic, swine wastewater lagoons located in the Carolinas. The four genes assayed in this study were present in all eight lagoons. Their abundances relative to total bacterial populations were 0.04% (*amoA*), 1.33% (*nirS*), 5.29% (*nirK*), and 0.27% (*nosZ*). When compared with lagoon chemical characteristics, *amoA* and *nirK* correlated with several measured variables. Neither *nirS* nor *nosZ* correlated with any measured environmental variables. Although no gene measured in this study correlated with actual or potential DEA, *nosZ* copy numbers did correlate with the disparity between actual and potential DEA. Phylogenetic analysis of *nosZ* did not reveal any correlations to DEA rates. As with other investigations, analyses of these genes provide useful insight while revealing the underlying greater complexity of N cycling within swine waste lagoons.

ANAEROBIC LAGOONS are the most common method for the storage and passive treatment of liquid manure generated from confined swine production operations. However, their intensive use and concentration of feces and urine in small geographic areas have the potential for significant environmental impacts. For instance, acidification and eutrophication of ecosystems adjacent to anaerobic lagoons can result from ammonia volatilization and subsequent deposition. Reports by Szögi and Vanotti (2007) and Ro et al. (2008) estimated that significant portions of anaerobic swine lagoon N were lost to ammonia (NH_3) volatilization. Additionally, a recent report by the USEPA implicated swine wastewater anaerobic lagoons in the climate change discussion as a source of the potent greenhouse gas nitrous oxide (USEPA, 2010). Nitrous oxide is an intermediary product in the biological process of denitrification (Delwiche and Bryan, 1976) and is often the primary end product when carbon-to-nitrogen (C/N) ratios are low (Klemedtsson et al., 2005; Hwang et al., 2006; Hunt et al., 2007; Ernfors et al., 2008). Classical nitrification and denitrification have long been reported to be involved in the N cycling of swine wastewater lagoons. Recent studies have demonstrated modest ($1.74 \text{ kg N ha}^{-1} \text{ d}^{-1}$) to very large ($85.6 \text{ kg N ha}^{-1} \text{ d}^{-1}$) rates of N loss through denitrification (Harper et al., 2000; Harper et al., 2004; Hunt et al., 2010).

Denitrification is the multistep biological reduction of nitrate (NO_3) or nitrite (NO_2) to, in the case of complete denitrification, dinitrogen gas (N_2) (Fig. 1). This microbial respiratory process is performed under oxygen-depleted conditions, where nitrogen oxides are used as alternative electron acceptors to yield energy (Hayatsu et al., 2008). The reduction of NO_2 to nitric oxide (NO) is catalyzed by two forms of nitrite reductases (Nir). One gene, *nirS*, encodes a cytochrome-containing enzyme (cd_1 -Nir). The other gene, *nirK*, encodes a copper-containing enzyme (Cu-Nir). If denitrification proceeds to completion, N_2O is reduced by nitrous oxide reductase, which is encoded by the gene *nosZ* (Knowles, 1982). A pool of available NO_2 for denitrification results from the biological oxidation of NH_3 during nitrification (Russow et al., 2009) performed by organisms that produce the enzyme ammonia monoxygenase (AMO). For the ammonia-

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*Corresponding author (thomas.ducey@ars.usda.gov).

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5585 Guilford Rd., Madison, WI 53711 USA

USDA–ARS, Coastal Plains Soil, Water and Plant Research Center, 2611 W. Lucas St., Florence, SC 29501. Assigned to Associate Editor Garey Fox. The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

Abbreviations: AMO, ammonia monoxygenase; COD, chemical oxygen demand; DEA, denitrification enzyme activity; FA, free ammonia; NO_2 , nitrite + nitrate; ORP, oxidative reductive potential; qPCR, quantitative polymerase chain reaction; TN, total nitrogen; TSS, total suspended solids; VSS, volatile suspended solids.

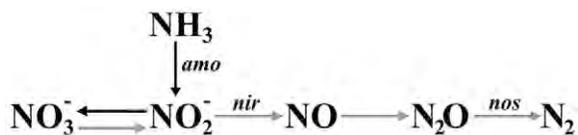


Fig. 1. Schematic of the biological processes of nitrification (black arrows) and denitrification (gray arrows). The genes targeted for analysis in this study are listed at their respective steps in the biological cascade.

oxidizing bacteria, AMO is a multisubunit enzyme that is encoded by the genes *amoA*, *amoB*, and *amoC*. The *amoA* gene encodes the active site of AMO. Its highly conserved nature makes it the target of choice in environmental studies (Bothe et al., 2000; Kowalchuk and Stephen, 2001).

For biological denitrification and nitrification enzymes, the presence of their encoding genes can be measured by real-time quantitative polymerase chain reaction (qPCR) using broad-range primers (Throback et al., 2004). This approach has been used to analyze the abundances of these genes in a number of soil, water, and wastewater treatment environments (Geets et al., 2007; Hallin et al., 2009; Chon et al., 2011). However, few studies have assessed the abundances of nitrification and denitrification genes in anaerobic wastewater lagoons.

Therefore, to better understand the microbial populations and processes in anaerobic lagoons, we examined eight commercial, swine wastewater, anaerobic lagoons from the Carolinas. Based on examination of these eight lagoons, we set out to complete the following objectives: (i) quantify the abundance of the *amoA*, *nirK*, *nirS*, and *nosZ* genes; (ii) evaluate the influence of environmental conditions on their abundances; and (iii) evaluate the abundance of these genes relative to denitrification enzyme activity.

Materials and Methods

Site Description and Sample Collection

The study included eight commercial swine wastewater lagoons located in the Coastal Plain region of North and South Carolina. The lagoons were situated on farms with finishing or farrow-to-finishing swine production operations (Hunt et al., 2010). The operations housed approximately 1000 to 9200 head of swine. Lagoons were divided into quadrants, and liquid samples were collected at three depths, for a total of 12 samplings per lagoon. Sampling depths were determined as (i) the top 25 cm, (ii) midway to bottom, and (iii) 25 cm off the lagoon bottom, above the sludge layer. Samples of lagoon liquid (1 L) were collected using a telescopic jar sampler (7300 Series; Lab Safety Supply, Janesville, WI). Upon collection, samples were stored on ice and transported to the laboratory for analysis. Sampling occurred in the 3-mo period between May and July in the years of 2006 and 2007; each site was sampled once during this study.

Sample Analysis

Electrical conductivity, oxidative reductive potential (ORP), and pH were measured with a multiparameter pH/ORP meter (YSI Inc., Yellow Springs, OH). Chemical oxygen demand (COD), total suspended solids (TSS), volatile suspended solids (VSS), ammonium ($\text{NH}_4\text{-N}$), nitrite and nitrate ($\text{NO}_x\text{-N}$), and total nitrogen (TN) were performed according to Standard

Methods for the Examination of Water and Wastewater (APHA, 1998). Denitrification enzyme activity (DEA) was measured by the acetylene blockage method (Tiedje, 1994), as performed by Hunt et al. (2010). Free ammonia (FA) was calculated according to Anthonisen et al. (1976) using measured water temperature ($^{\circ}\text{C}$), pH, and TN.

DNA Extraction

The 12 individual samplings for each lagoon were sorted by depth and combined in equal volumes to obtain three composite samples. From each composite sample, a volume of 2 mL was centrifuged at $14,000 \times g$ for 5 min, and DNA was extracted from the resultant pellet. A SoilMaster DNA Extraction kit (Epicentre, Madison, WI) was used for extraction. This initial DNA preparation was run 5 cm into a 0.5% agarose gel. An additional round of DNA purification was performed using an UltraClean GelSpin DNA extraction kit (MO BIO Laboratories Inc., Carlsbad, CA). The gel was stained with SYBR Safe (Invitrogen, Carlsbad, CA) to visualize DNA and to excise it from the gel. Final DNA quantity was determined via Biophotometer (Eppendorf, Hamburg, Germany), and quality was assessed by electrophoresis on a 1% agarose gel stained with SYBR Safe (Yu and Morrison, 2004).

Quantitative Real-Time Polymerase Chain Reaction Assays

The qPCR assays were run on an iCycler iQ Multi-Color Real Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The primers used in these assays were obtained from Integrated DNA Technologies (Coralville, IA) and are listed in Table 1. Assays were performed using SYBR GreenER qPCR SuperMix (Invitrogen) in a total volume of 25 μL . The final reaction concentrations of the reagents were 1X SYBR GreenER qPCR SuperMix, 200 nM each of forward and reverse primers, and 10 ng of DNA template. The qPCR reaction conditions were as follows: (i) an initial denaturation at 95°C for 5 min; (ii) 50 cycles of denaturation at 95°C for 30 s, the appropriate annealing temperature (Table 1) for 30 s, and elongation at 72°C for 30 s; and (iii) melting curve analysis to confirm amplification product specificity. Fluorescent measurements were taken during the annealing phase of each cycle. Data were collected and processed using the iCycler software package (Bio-Rad Laboratories). All qPCR assays included control reactions without template. Each assay contained appropriate standard DNA reactions with concentrations between 10^1 and 10^9 copies per reaction; these reactions were used to calculate amplification efficiencies according to the equation: $E = 10^{-1/\text{slope}}$ (Pfaffl, 2001). DNA standards consisted of plasmids carrying the appropriate target gene and were sequenced to confirm their identity and primer binding sites (Table 1). Each assay was performed in triplicate. In each assay, composite samples were run in duplicate. This resulted in six measurements per gene per lagoon depth.

Cloning, Sequencing, and Analysis of *nosZ* Sequences

A survey of *nosZ* clones corresponding to each of the eight anaerobic lagoons was obtained by performing PCR using the *nosZ* qPCR primer set. Products were purified and concentrated using the Montage PCR columns (Millipore, Billerica, MA).

Purified products were cloned into pCR2.1-TOPO and transformed into chemically competent *Escherichia coli* Mach1™-T1^R according to manufacturer's specifications (Invitrogen). Colonies were blue/white screened with white colonies passed to fresh LB broth containing ampicillin (100 µg mL⁻¹). DNA from the prospective cultures was extracted by boiling lysis and PCR screened using the M13 primer set. Cultures producing PCR products of appropriate size were transferred and grown in fresh LB broth with ampicillin (100 µg mL⁻¹) for 16 h at 37°C and 250 rpm. Plasmids were then extracted via alkaline lysis (Cloninger et al., 2008) and bidirectionally sequenced on an ABI 3730 using M13 forward and reverse primers.

All DNA sequences were analyzed and edited using Geneious version 4.7.4 (Biomatters Ltd., Auckland, New Zealand) (Drummond et al., 2007). Sequence alignments were performed using the MUSCLE plug-in of Geneious (Edgar, 2004) and were analyzed using MEGA version 4.0 (Tamura et al., 2007). Phylogenetic reconstructions were performed in MEGA using the neighbor-joining algorithm, with bootstrap values calculated from 5000 replicate runs. Evolutionary distances were computed using the Tajima-Nei method (Tajima and Nei, 1984); positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons.

Statistics

Before statistical analysis, all gene abundances were normalized to the amount of DNA collected per sample and log₁₀ transformed. Gene abundances were also corrected for copies per organism, with 1 copy per organism for the denitrification genes *nirK*, *nirS*, and *nosZ* (Kandeler et al., 2006), 2 copies per organism for *amoA* (Chain et al., 2003), and 3.6 copies per organism for the 16S rDNA gene (Klappenbach et al., 2001; Harms et al., 2003). Pearson correlation coefficients, linear regressions, Duncan's multiple range test, and other statistical analyses were performed using SAS version 9.2 (SAS Inst., Cary, NC). Canonical correspondence ordination analy-

ses were performed using PCORD version 5 (MJM Software, Glenden Beach, OR).

Nucleotide Sequence Accession Numbers

Sequences for the *amoA*, *nirK*, *nirS*, *nosZ*, and 16S rRNA genes that were cloned in pCR2.1-TOPO and used to generate standard curves were submitted to the GenBank database. They were assigned accession numbers HQ674781 through HQ674785. A total of 67 *nosZ* sequences were also submitted to the GenBank database and were assigned the accession numbers HQ123183 through HQ123249.

Results and Discussion

Lagoon and Wastewater Characteristics and Denitrification Enzyme Activity

Selected physical and chemical properties and denitrification enzyme activity rates of the eight commercial lagoons analyzed in this study are summarized in Table 2. A more in-depth analysis of the physical, chemical, and denitrification enzyme activity characteristics of these eight lagoons was performed by Hunt et al. (2010). Six of the eight commercial lagoons analyzed were on finish-only operations. The exceptions were lagoons D and F, which were farrow-to-finishing operations. Lagoon depths varied from 0.78 to 2.17 m, with total areas of 0.54 to 2.68 ha. Taken as a whole, the wastewater characteristics are concurrent with lagoons typically found in the mid-Atlantic Coastal Plains and mid-South regions of the United States (Bicudo et al., 1999; McLaughlin et al., 2009). The lagoons were all slightly alkaline. With the exception of lagoon F, which was slightly oxidative (+6 mV), all lagoons were reduced; this finding is indicative of an anoxic or anaerobic environment. Four of the lagoons had ORP values in the range of +200 and -200 mV; this range is often considered ideal for the biological process of denitrification (Inniss, 2005). The other four lagoons had ORP values that were indicative of more reduced anaerobic processes

Table 1. Primers and plasmids used in this study.

Primers	Sequence (5' to 3')	Target	Tm†	Product length‡	Reaction Tm	Reference
amoA-1F	GGGGTTTCTACTGGTGGT	<i>amoA</i>	54.1°C	491 bp	54°C	Rotthauwe et al., 1997
amoAr NEW	CCCCTCBGSAAVCCTTCTTC		58.8°C			Hornek et al., 2006
1F_nirK	GGMATGGTKCCSTGGCA	<i>nirK</i>	58.0°C	516 bp	53°C	Braker et al., 1998
nirK5R	GCCTCGATCAGRTRRTGG		52.8°C			Braker et al., 1998
cd3aF_nirS	G TSAACG TSAAGGARACSGG	<i>nirS</i>	57.1°C	425 bp	55°C	Throback et al., 2004
R3cd_nirS	GASTTCGGRTGSGTCTTGA		55.8°C			Throback et al., 2004
nosZF	CGYTGTTCMTCGACAGCCAG	<i>nosZ</i>	58.6°C	453 bp	55°C	Scala and Kerkhof, 1998
nosZ-1622R	CGSACCTTSTTGCCSTYGCG		63.1°C			Throback et al., 2004
515F	TGCCAGCAGCCGCGTAA	16S v4-v5 region	63.3°C	412 bp	55°C	Weisburg et al., 1989
927R	CTTGTGCGGGCCCCGTCAATTC		65.1°C			Rudi et al., 1997
Plasmids	Characteristics	Accession no.				
pCPDamoA1	pCR4.1-TOPO carrying <i>amoA</i> fragment	HQ674785		this study		
pCPDnirS1	pCR4.1-TOPO carrying <i>nirS</i> fragment	HQ674783		this study		
pCPDnirK1	pCR4.1-TOPO carrying <i>nirK</i> fragment	HQ674782		this study		
pCPDnosZ1	pCR4.1-TOPO carrying <i>nosZ</i> fragment	HQ674784		this study		
pCPDv4v5	pCR4.1-TOPO carrying 16S fragment	HQ674781		this study		

† Tm, melting temperature.

‡ Product lengths were calculated based on the primer positions in the *amoA* gene of *Nitrosomonas europaea* ATCC 19718 (NE0944), in the *nirS* gene of *Pseudomonas stutzeri* ZoBell ATCC 14405 (X56813), in the *nirK* gene of *Alcaligenes fecalis* S-6 (D13155), and in the *nosZ* gene of *Pseudomonas aeruginosa* DSM 50071 (X65277).

such as manganese, iron, and sulfur respiration and methane formation (Patrick and Mikkelsen, 1971). None of the eight lagoons had ORP values that have been shown to efficiently remove ammonia via simultaneous nitrification–denitrification (Zhao et al., 1999).

Actual (incomplete) denitrification refers to DEA rates (resulting in N₂O production) occurring under existing lagoon conditions. Potential denitrification is indicative of maximal DEA rates (resulting in N₂O or N₂ production) if NO_x is non-limiting in the lagoons, and the process is halted at the production of N₂O. The mean actual (49.7 ± 25.0 mg N₂O–N m⁻³ d⁻¹) and potential (207.5 ± 135.5 mg N₂O–N m⁻³ d⁻¹) DEA rates for the studied lagoons were low. The higher levels of N₂O–N measured for potential DEA, as compared with the levels for actual DEA, indicate NO_x limitation of the lagoons. Assuming that all the NO_x is converted to N₂O and not N₂, the potential DEA rate still only results in the maximal production of 4.15 kg N₂O–N ha⁻¹ d⁻¹. This number is far short of that previously reported by Harper et al. (2004), who measured average N₂ emissions of 85.6 kg ha⁻¹ d⁻¹ from a series of lagoons in North Carolina. Our data appear to be more closely supported by the work of Szögi et al., which identifies NH₃ volatilization, as opposed to nitrification–denitrification, as the primary cause of N loss from swine anaerobic lagoons (Szögi and Vanotti, 2007).

Abundances of *amoA*, *nirS*, *nirK*, and *nosZ*

Coupled with the low levels of NO_x present in the lagoon wastewater, the low DEA rates led to the hypothesis that the genes responsible for encoding steps in the biological pro-

cesses of nitrification and denitrification would be found in low abundance. To determine relative abundances of each measured gene, we compared the respective gene copy numbers with the total cells per milliliter of lagoon wastewater as determined by quantification of the 16S rRNA gene using a universal primer set (Fig. 2). Whereas gene copies are expressed as copies per milliliter of lagoon wastewater, all relative abundances are expressed as a percentage of the overall number of bacteria determined in the lagoons. Similar to the measured DEA rates, there was no significant difference between the four nitrification and denitrification genes or the total numbers of cells in relation to depth for each lagoon (data not shown). This allowed for the pooling of individual lagoon measurements together for statistical analysis, resulting in a total of 18 measurements per lagoon (six measurements at each of three depths). A similar study looking at anaerobic lagoon microbial abundances at several depths also demonstrated no significant effects based on lagoon depth (Cook et al., 2010).

Quantification of *amoA*

Examination of *amoA*, the gene responsible for encoding ammonia monooxygenase subunit A, revealed an average of 4.3 × 10⁵ gene copies per milliliter of lagoon wastewater, with a range of 1.9 × 10⁵ to 1.2 × 10⁶ (Fig. 3). These values are an order of magnitude lower than the levels of *amoA* found in previously studied industrial (1.73 × 10⁶ gene copies per milliliter) and municipal (7.5 × 10⁶ and 1.6 × 10⁷ gene copies per milliliter) wastewater treatment systems (Harms et al., 2003; Robinson et al., 2003). Additionally, a study by Geets et al. (2007) looked at several industrial wastewater treatment plants

Table 2. Physical, chemical, and denitrification enzyme activity characteristics of lagoons in study.†

Measurement	Units	Lagoon‡							
		A	B	C	D	E	F	G	H
Physical properties									
Farm type		F§	F	F	FF	F	FF	F	F
Swine	head	4500	5280	4360	9200	4900	1000	2900	2200
Depth	m	0.78	2.06	1.77	1.46	1.1	1.66	2.17	1.4
Area	ha	1.89	1.58	0.92	2.68	1.25	0.54	0.58	0.58
Chemical properties									
pH		7.56	7.98	7.72	7.55	7.78	7.42	7.68	7.61
ORP¶	mV	-238	-132	-398	-299	-252	6	-120	-169
Conductivity	S cm ⁻¹	5020	6513	4825	10,177	8675	2132	7516	6513
TSS	mg L ⁻¹	1541	322	406	4,468	3463	246	384	429
VSS	mg L ⁻¹	1014	258	362	2,242	1716	193	288	333
COD	mg L ⁻¹	2352	1354	1803	2,452	2832	920	1883	1888
NH ₄ -N	mg L ⁻¹	197	261	506	381	372	79	285	314
FA	mg L ⁻¹	7.6	nc#	14.9	17.2	18.9	1.8	15.6	10.7
NO _x -N	mg L ⁻¹	0.4	0.1	0.1	0.4	0.4	0.1	0.3	2
TN	mg L ⁻¹	239	338	581	499	392	109	631	528
DEA									
Actual	mg N ₂ O–N m ⁻³ d ⁻¹	25	30	64	55	24	38	70	93
Potential	mg N ₂ O–N m ⁻³ d ⁻¹	359	179	122	147	144	50	188	470

† Data adapted from Hunt et al. (2010).

‡ Values are the mean of four quadrants and three depths.

§ F, farrow; FF, farrow to finish.

¶ COD, chemical oxygen demand; FA, free ammonia; ORP, oxidative reductive potential; TN, total nitrogen; TSS, total suspended solids; VSS, volatile suspended solids.

nc, not calculated.

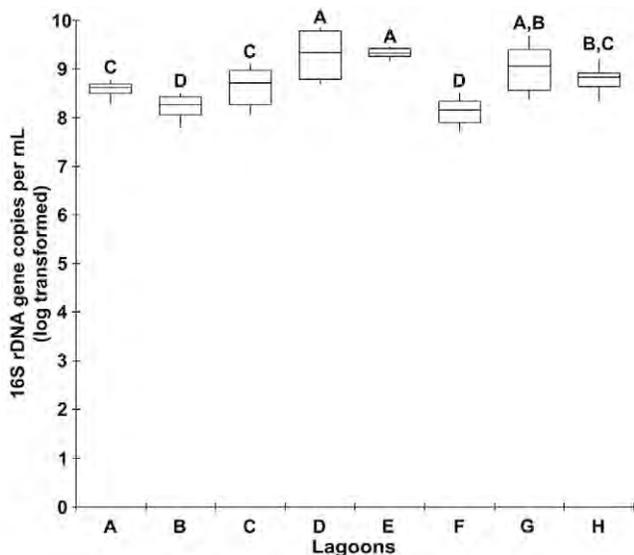


Fig. 2. Gene copy numbers (log transformed) of the 16S rDNA gene per milliliter of lagoon wastewater from eight commercial anaerobic swine wastewater lagoons as measured by quantitative polymerase chain reaction. Statistically different samples ($P < 0.05$) are indicated by letter (A, B, C, D) as determined by Duncan's multiple range test. Values for the quantitative polymerase chain reaction reactions were as follows: slope = -3.526 ; $r^2 = 0.99$; $E = 1.92$.

and revealed *amoA* gene copies in the range of 10^4 to 10^8 copies per milliliter. In the current study, the relative abundances of *amoA*, when compared with 16S rDNA levels, revealed that an average of only 0.04% of the overall lagoon population organisms carried *amoA*. This is also considerably less than the relative abundances measured in a municipal wastewater treatment plant that identified 2.9% of the organisms within the system harboring the *amoA* gene (Harms et al., 2003). These results are similar to a carbohydrate-rich wastewater treatment system studied by Geets et al. (2007), which had 71% ammonium treatment efficiency and *amoA* relative abundance of 0.04%; this demonstrates that low *amoA* relative abundance, in and of itself, is not indicative of poor NH_3 removal. Therefore, low abundances of *amoA* alone, such as those reported in this study, do not necessarily invalidate the large N losses reported by Harper et al. (2004).

Quantification of *nirS* and *nirK*

In the eight examined lagoons, there was an average of 1.8×10^7 gene copies of *nirS* per milliliter (Fig. 4) and 7.3×10^7 gene copies of *nirK* per milliliter (Fig. 5). The range for *nirS* was 1.2 to 2.8×10^7 copies per milliliter. Somewhat similarly, *nirK* had a range of 2.2×10^7 to 1.4×10^8 copies per milliliter. The abundances of *nirS* and *nirK* relative to the overall bacterial population averaged 1.33 and 5.29%, respectively. The relative abundance means for *nirS* were greater than or equal to the relative abundances of *nirS* found in nonagricultural soils, estuarine sediments, and constructed wetlands (Kandeler et al., 2006; Smith et al., 2007; Chon et al., 2011). In contrast, they were generally lower than wastewater treatment systems (Geets et al., 2007).

The *nirK* patterns of relative abundances were similar to published values in natural and treatment system environments. Specifically, the abundances of *nirK* in the measured lagoons

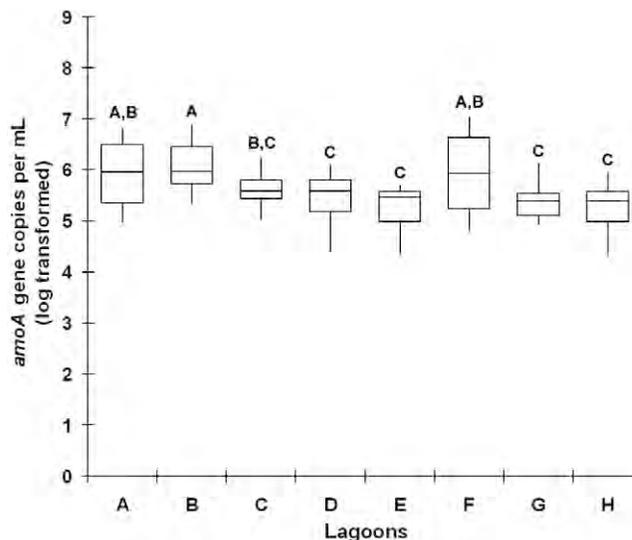


Fig. 3. Gene copy numbers (log transformed) of *amoA* per milliliter of lagoon wastewater from eight commercial anaerobic swine wastewater lagoons as measured by quantitative polymerase chain reaction. Statistically different samples ($P < 0.05$) are indicated by letter (A, B, C) as determined by Duncan's multiple range test. Values for the quantitative polymerase chain reaction reactions were as follows: slope = -3.647 ; $r^2 = 0.98$; $E = 1.88$.

were greater than or equal to the abundances of *nirK* found in soil (Henry et al., 2006; Kandeler et al., 2006). Likewise, they were greater than or equal to the relative abundances found in most of the studied wastewater treatment systems (Geets et al., 2007). Taken together, the relative abundances of *nirS* and *nirK* appear to indicate that the potential for conversion of NO_x to NO would be high given the proper environmental conditions and presence of electron acceptors. However, actual rates of NO production and the associated denitrification in these lagoons may well be impeded by the lack of NO_x and by certain environmental conditions, such as ORP.

Quantification of *nosZ*

The mean lagoon copy numbers of the *nosZ* gene was 4.0×10^6 gene copies per milliliter. The lagoons were rather similar, with a range of only 2.4 to 9.2×10^6 copies per milliliter (Fig. 6). The mean relative abundance of *nosZ* was 0.27% of the overall bacterial lagoon population. Although low, these results are similar to *nosZ* abundances in constructed and estuarine wetlands reported by Chon et al. (2011) and an agricultural soil examined by Henry et al. (2006). Both studies demonstrated relative abundances of *nosZ* with values typically $<1.0\%$ of the overall populations. Additionally, like this report, these studies demonstrated *nosZ* levels in lower abundance than the two nitrite reductases, *nirS* and *nirK*. Nonetheless, when compared with the study of Geets et al. (2007), the relative abundances of *nosZ* in the lagoons of this study were drastically lower than a majority (8 out of 9) of the wastewater treatment systems, some of which saw *nosZ* relative abundances as high as 98%. These results suggest that if NO_x were to become readily available and environmental conditions were to shift to allow for denitrification, NO and N_2O could be a large portion of the final N product. Additionally, nitrite reductases (Averill, 1996) typically demonstrate higher rates of enzymatic activity when compared with nitrous oxide reductases (Kristjansson and Hollocher,

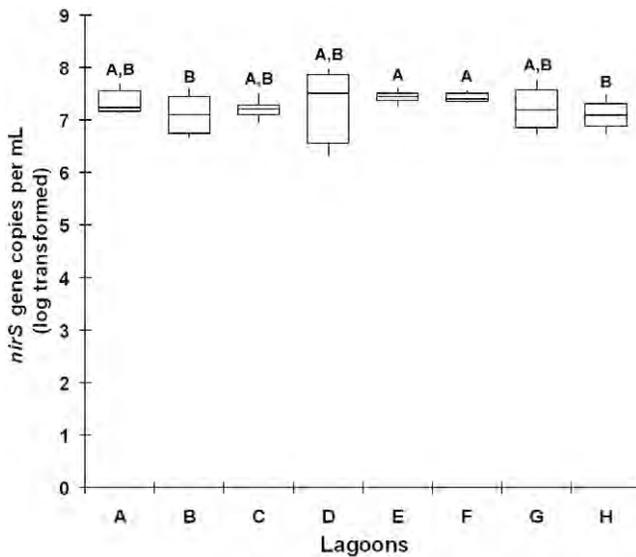


Fig. 4. Gene copy numbers (log transformed) of *nirS* per milliliter of lagoon wastewater from eight commercial anaerobic swine wastewater lagoons as measured by quantitative polymerase chain reaction. Statistically different samples ($P < 0.05$) are indicated by letter (A, B) as determined by Duncan's multiple range test. Values for the quantitative polymerase chain reaction reactions were as follows: slope = -3.351 ; $r^2 = 0.95$; $E = 1.97$.

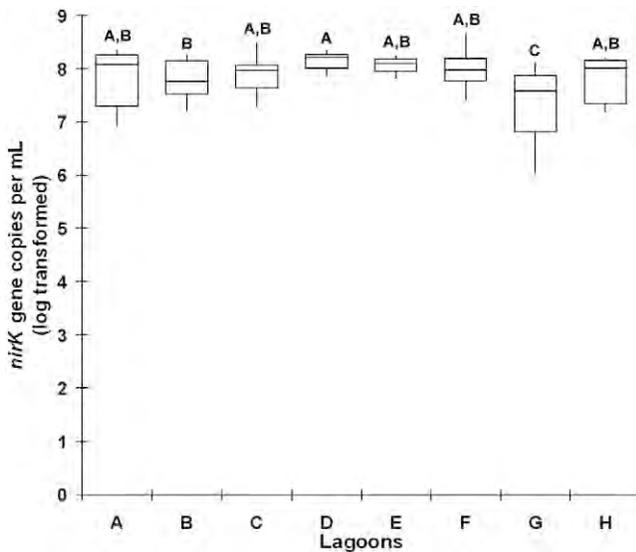


Fig. 5. Gene copy numbers (log transformed) of *nirK* per milliliter of lagoon wastewater from eight commercial anaerobic swine wastewater lagoons as measured by quantitative polymerase chain reaction. Statistically different samples ($P < 0.05$) are indicated by letter (A, B, C) as determined by Duncan's multiple range test. Values for the quantitative polymerase chain reaction reactions were as follows: slope = -3.581 ; $r^2 = 0.93$; $E = 1.90$.

1980), which could further exacerbate emissions of NO and N_2O over N_2 . This is a result that would not typically be seen in wastewater treatment systems designed to effectively remove N.

When comparing *amoA*, *nirS*, *nirK*, and *nosZ* gene copy numbers and relative abundances in anaerobic lagoons, natural ecosystems, and wastewater treatment systems, anaerobic lagoons are found to be on par with natural ecosystems in terms of copy numbers and abundances. Anaerobic lagoons routinely have lower gene copy numbers and relative abundances of all four genes studied when compared with wastewa-

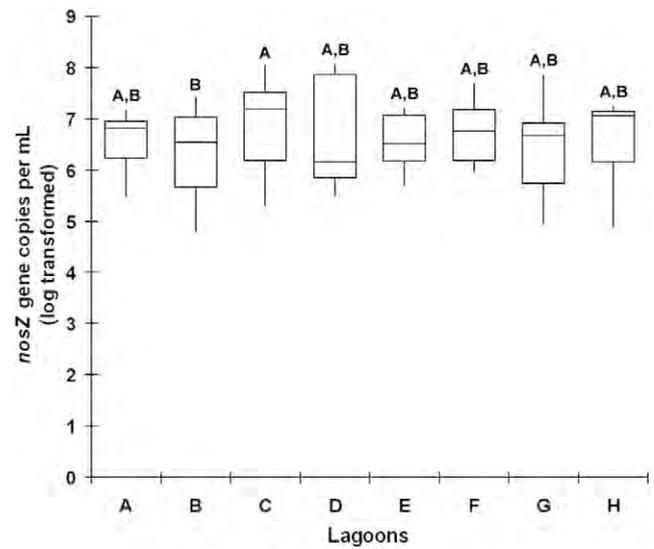


Fig. 6. Gene copy numbers (log transformed) of *nosZ* per milliliter of lagoon wastewater from eight commercial anaerobic swine wastewater lagoons as measured by quantitative polymerase chain reaction. Statistically different samples ($P < 0.05$) are indicated by letter (A, B) as determined by Duncan's multiple range test. Values for the quantitative polymerase chain reaction reactions were as follows: slope = -3.351 ; $r^2 = 0.92$; $E = 1.99$.

ter treatment plants, which are designed specifically to remove N. This observation supports the findings of Szögi and Vanotti (2007) that N loss in anaerobic lagoons is primarily through NH_3 -N volatilization.

Relationship of *amoA*, *nirS*, *nirK*, and *nosZ* Abundances to Environmental Variables

Correlation analysis, which included selected environmental variables as independent factors and *amoA*, *nirS*, *nirK*, and *nosZ* gene copy numbers per milliliter of lagoon sample as dependent factors, was performed to examine the potential corresponding relationships (Table 3). Although *nirS* and *nosZ* failed to correlate ($P > 0.05$) with any of the selected environmental variables, a number of environmental variables had a significant impact on *amoA* and *nirK* (Table 3).

Copy numbers of *amoA* negatively correlated with conductivity, COD, NH_4 -N, FA, and TN. The negative correlation with the three nitrogen variables has been previously documented in wastewater treatment systems and is most likely indicative of ammonia toxicity. Anthonisen et al. (1976) reported that FA levels in the range of 10 to 150 $mg L^{-1}$ —a threshold met by six of the eight lagoons in this study—were sufficient to inhibit nitrifier activity. In this study, the calculated FA levels (range, 1.8–18.9 $mg L^{-1}$) appear to be playing a role in reducing overall nitrifier populations. Additionally, lagoon TN (range, 109–631 $mg L^{-1}$) was predominantly in the form of NH_4 -N (range, 79–506 $mg L^{-1}$), a factor favorable for the formation of FA.

In addition to being adversely affected by lagoon N, copy numbers of *amoA* negatively correlated with lagoon C. A strong negative relationship with COD was observed. This may reflect the inability of nitrifiers to compete with heterotrophic populations (Nogueira et al., 2002). A negative correlation was also seen between *amoA* copy numbers and conductivity. This most likely reflects an inhibition due to the high salt content

of the lagoons (Georgacakis and Sievers, 1979). Even though lagoon ORP values were below levels considered conducive to nitrification, *amoA* copy numbers positively correlated with this measure. It is possible that the nitrifiers remain dormant within the lagoons or operate at extremely low levels of activity in select microenvironments within the lagoons (e.g., at the surface-liquid interface where there may be sufficient oxygen available to drive nitrification) (Ro et al., 2008). Studies have demonstrated restoration of nitrification activity in anaerobic wastewater after extensive aeration (Loynachan et al., 1976), which is a clear indication of the presence of nitrifiers before any changes in treatment regimes.

Because the gene *amoA* is restricted to a total of three bacterial genera and represents a small fraction of the population in these lagoons (0.04%), it is not surprising to see it affected by so many environmental variables. In contrast, the gene *nirK* is found in a wide variety of bacterial genera (Coyne et al., 1989) and was the most well represented gene in this study (5.29%). However, it was positively correlated with lagoon conductivity, TSS, VSS, COD, and FA. The strong correlation between *nirK* gene copy numbers and lagoon TSS and VSS is likely due to the high representation of the *nirK* gene in the microbial populations, and accordingly the overall microbial biomass, of these anaerobic lagoons. Measurements of VSS, which showed a very strong linear relationship with TSS ($y = 0.4754x + 131.57$; $R^2 = 0.99$; $P < 0.0001$), are often used as a rough estimate of microbial biomass (Metcalf and Eddy, 2002). Gene copy numbers of *nirK* have been positively correlated with C in previous studies (Kandeler et al., 2006; Bárta et al., 2010), and a similar correlation was determined with lagoon COD in this study. Positive correlations of *nirK* gene copy numbers with conductivity and FA suggest that organisms carrying *nirK* may have a selective advantage in anaerobic lagoons. Studies have demonstrated that increased conductivity and FA result in inhibition of microbial activity (Anthonisen et al., 1976; Georgacakis and Sievers, 1979). These findings are reflected in the *amoA* gene copy numbers in the lagoons. However, studies have shown that organisms carrying *nirK* inhabit a wide variety of ecosystems (Coyne et al., 1989). It is possible that organisms carrying *nirK*, as opposed to

nirS, are better able to survive harsh, high-ammonia, high-salt environmental conditions. This observation is borne out by the correlation of *nirK/nirS* ratios with FA.

A direct comparison of the copy numbers and abundances of the two nitrate reductases in this study revealed *nirK* levels significantly higher than those of *nirS* ($P < 0.01$). These results contrast with other studies that report *nirS* gene copy numbers greater than *nirK* gene copy numbers by a couple of orders of magnitude (Kandeler et al., 2006; Geets et al., 2007). An immunological study performed by Coyne et al. (1989) demonstrated that although microbes carrying *cd₁-Nir* (*nirS*) were more predominant than those carrying Cu-Nir (*nirK*), Cu-Nir was found in more ecological niches. Additional studies have examined the response of *nirK*- and *nirS*-containing denitrifier communities to environmental gradients and have demonstrated that these populations do not respond congruently (Hallin et al., 2006; Oakley et al., 2007; Smith and Ogram, 2008; Jones and Hallin, 2010). Maeda et al. (2010), looking at denitrifier communities in cattle manure composting, demonstrated that organisms carrying *nirK* were selected over organisms carrying *nirS*. Therefore, anaerobic swine wastewater lagoons may present an ecological niche that is more conducive to *nirK*-carrying denitrifiers. This appears to be supported by a study by Hallin et al. (2009), which reported a significant increase in *nirK* gene copy levels over *nirS* in agricultural soils treated with sewage sludge. This treatment regime was most similar to the anaerobic wastewater examined in this study and is the most likely to result in increased FA levels.

Relationship of *amoA*, *nirS*, *nirK*, and *nosZ* Abundances to DEA

Given the NO_x limitation on DEA rates in these lagoons, the relationships between *amoA* copy number and relative abundances, with actual or potential DEA rates, were analyzed. This examination revealed no correlation ($P > 0.05$). None of the denitrification genes *nirS*, *nirK*, or *nosZ* demonstrated a correlative relationship between their copy numbers and relative abundances and DEA rate ($P > 0.05$). However, there was a significant negative correlation between *nosZ* copy number and the ratio between actual and potential DEA values of each

Table 3. Correlation coefficients of selected environmental variables versus *amoA*, *nirK*, *nirS*, and *nosZ* gene copy numbers and *nirK/nirS* ratio.

	Gene				
	<i>amoA</i>	<i>nirK</i>	<i>nirS</i>	<i>nirK/nirS</i>	<i>nosZ</i>
pH	ns†	ns	ns	ns	ns
ORP‡	0.421*	ns	ns	ns	ns
Conductivity	-0.640***	0.408*	ns	ns	ns
TSS	ns	0.664***	ns	ns	ns
VSS	ns	0.665***	ns	ns	ns
COD	-0.601**	0.430*	ns	ns	ns
NH ₄ -N	-0.648***	ns	ns	ns	ns
FA	-0.690***	0.432*	ns	0.454*	ns
NO _x -N	ns	ns	ns	ns	ns
TN	-0.732***	ns	ns	ns	ns

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level.

† NS, not significant.

‡ COD, chemical oxygen demand; FA, free ammonia; ORP, oxidative reductive potential; TN, total nitrogen; TSS, total suspended solids; VSS, volatile suspended solids.

lagoon ($y = -0.0369x + 7.1963$; $r = -0.44$; $P < 0.001$). This is significant because it indicates that as *nosZ* copy numbers increased, the disparity between actual DEA (incomplete denitrification) and potential DEA (complete and incomplete denitrification) decreased. These results coincide with a study by Philippot et al. (2009), which demonstrated a strong correlation between *nosZ* abundances and $N_2O/(N_2 + N_2O)$ ratios in grasslands subjected to cattle grazing. Monitoring of *nosZ* gene copy numbers, in addition to management practices to keep *nosZ* gene copy numbers high, could help push NO reduction processes toward complete denitrification, resulting in a reduction of the greenhouse gas N_2O .

Phylogenetic Analysis of *nosZ*

Our finding that *nosZ* abundances correlated to the disparity between potential and actual DEA rates led us to examine whether specific *nosZ* gene sequences could also be correlated to DEA rates. A total of 67 unique *nosZ* partial sequences from the eight commercial anaerobic lagoons were characterized. Analysis of the phylogenetic tree demonstrated that the sequences formed three broad, but distinct, clusters (Fig. 7). The largest cluster, with representatives from each of the eight lagoons, contained a majority of the identified *nosZ* sequences (48 of 67). These sequences were most similar to *nosZ* sequences from α -proteobacteria, such as *Paracoccus denitrificans* and *Bradyrhizobium japonicum*. A total of 11 sequences from lagoons B, D, and G formed a cluster with other β -proteobacterial *nosZ* sequences. The remaining eight sequences derived from lagoon E and were 83% similar to *Pseudomonas stutzeri* in the γ -proteobacterial cluster. Sixteen lagoon *nosZ* sequences had a high degree of similarity to *nosZ* genes from cultured organisms. Nine sequences, from lagoons A, D, E, and G, had 99.3 to 100% similarity to *P. denitrificans*; another sequence from lagoon G (*nosZ*_G3) had 86.6% similarity to *B. japonicum*; and six sequences from lagoons B and D had 77.3 to 81.7% similarity to *Rhodoferrax ferrireducens*.

Ordination analysis did not reveal a significant pattern in the distribution of lagoon *nosZ* sequences in relation to actual DEA, potential

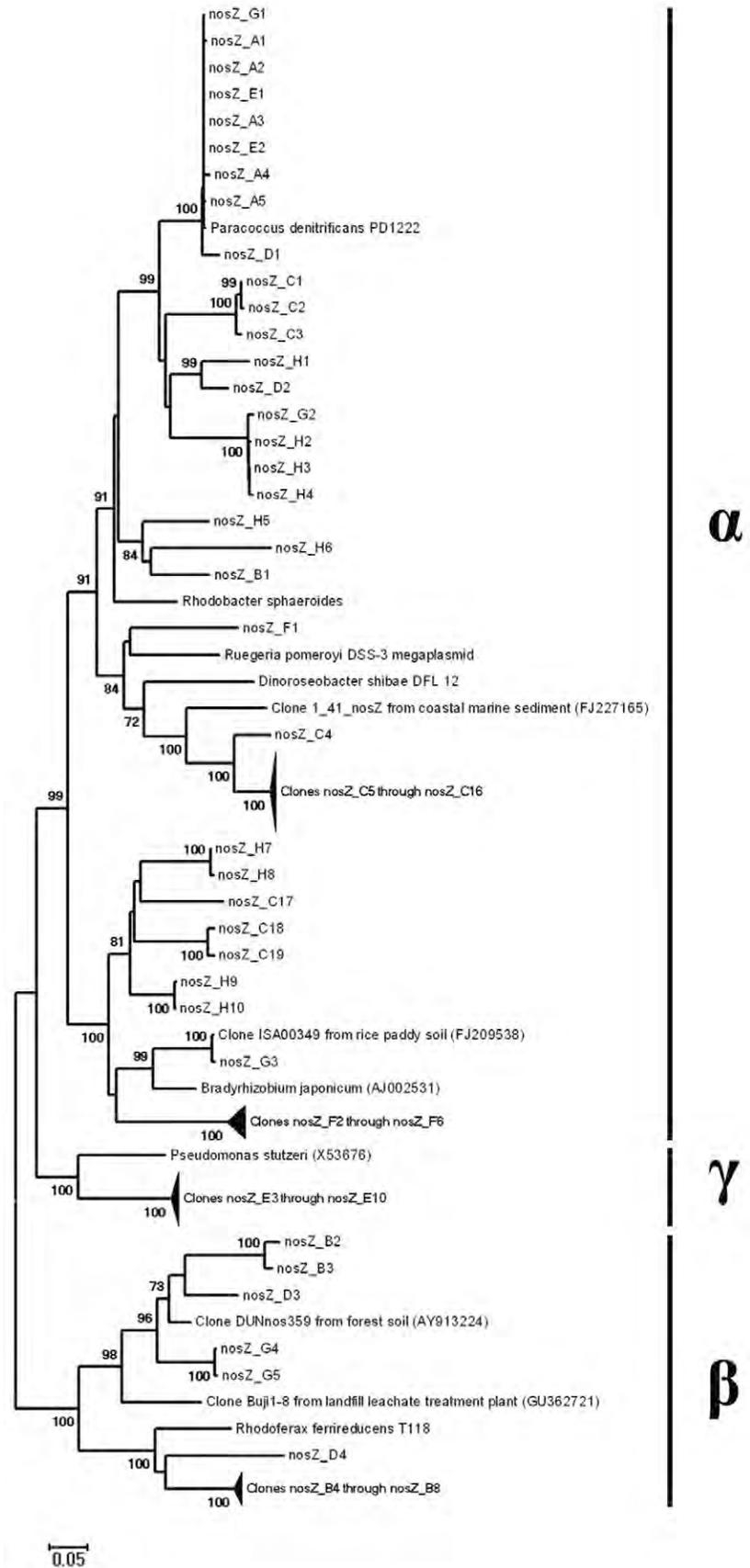


Fig. 7. Neighbor-joining tree showing the phylogenetic relationship between cloned *nosZ* sequences and *nosZ* sequences retrieved from the GenBank database (accession numbers in parentheses). The tree represents an alignment of an approximately 420-bp intergenic region of the *nosZ* gene. The scale bar represents 5% estimated change. Major phylogenetic clades identified in this study are labeled to the right of the tree and correspond to the α -, β -, and γ -subclasses of the proteobacterial phylum. The frequency (%) with which a given branch was recovered in 5000 bootstrap replications is shown by branches recovered in more than 70% of bootstrap replicates.

DEA, or the ratio of the two (data not shown). Although *nosZ* is often the last gene in the denitrification pathway to be activated, leading to a transient accumulation of N₂O in the environment, it is possible that the differences in the intergenic regions of *nosZ* cannot be used to indicate whether a particular microbial species or community has the potential to produce lower or higher amounts of N₂O. It is also possible that *nosZ* abundance and sequence composition are unlinked to the process of denitrification and N₂O emissions. This hypothesis is supported by several reports that demonstrated that N₂O emissions were uncoupled from denitrifier community composition and gene abundance and were instead coupled to environmental factors (Dandie et al., 2008; Ma et al., 2008). Indeed, although gene abundances did not correlate to DEA rates in this study, Hunt et al. (2010) were able to correlate C/N ratios to DEA activity. This finding is supported by a number of reports that have identified environmental C/N ratios as responsible for the accumulation of N₂O in soils and the sludge layer of anaerobic lagoons (Klemmedtsson et al., 2005; Hunt et al., 2007; Ernfors et al., 2008; Hunt et al., 2009).

Henry et al. (2006) demonstrated that, although two different sets of *nosZ* primers gave similar quantitative results, the topologies of the phylogenetic trees produced by sequencing those qPCR products were dissimilar and most likely attributable to the different specificities of the primer sets. Therefore, although this primer set can be used to accurately quantify *nosZ*, it may not be the best candidate for phylogenetic analysis of *nosZ*-carrying microbial communities. In fact, it may be masking microbial species that are playing an important role in the accumulation or reduction of N₂O. Because amplification- and culture-based techniques introduce biases when describing the diversity of a population, a method that avoids these pitfalls is necessary to accurately determine microbial communities in the environment. Advances in sequencing technology have ushered in the era of affordable metagenomics and may provide the answer to measuring microbial populations while avoiding many of the pitfalls of the current technologies used to estimate diversity (Bentley, 2006; Kowalchuk et al., 2007).

Conclusions

The results of this study demonstrate that swine wastewater anaerobic lagoons contain microbial organisms that carry genes responsible for encoding nitrification and denitrification functions. Relative abundances for the four nitrification and denitrification genes analyzed in this study were 0.04% (*amoA*), 1.33% (*nirS*), 5.29% (*nirK*), and 0.27% (*nosZ*) of the overall bacterial populations. Overall, these abundances are similar to those found in natural ecosystems but lower than those found in a majority of studied wastewater treatment systems. Gene copy numbers of *amoA* and *nirK* correlated significantly with several lagoon environmental variables. Although actual and potential DEA rates did not correlate with gene abundances, *nosZ* abundance did correlate significantly with the disparity between each lagoon's actual and potential DEA rates. Phylogenetic analysis of *nosZ* across the eight lagoons did not identify a link between particular sequences and DEA rates. Further studies, such as metagenomics, are needed for a deeper understanding of the

complex variables and communities involved in the N-cycling of anaerobic swine wastewater lagoons.

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