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 monooxygenase. For denitrification, the reduction of nitrite to nitric oxide can be catalyzed by two forms of nitrite reductases, and N₂O 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.

Aerobic 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 (NH₃) 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 (Klemmedtsson 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 kg N ha⁻¹ d⁻¹) to very large (85.6 kg N ha⁻¹ 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 (NO₃⁻) or nitrite (NO₂⁻) to, in the case of complete denitrification, dinitrogen gas (N₂) (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 NO₃ to nitric oxide (NO) is catalyzed by two forms of nitrite reductases (Nir). One gene, nirS, encodes a cytochrome-containing enzyme (cd,Nir). The other gene, nirK, encodes a copper-containing enzyme (Cu,Nir). If denitrification proceeds to completion, N₂O is reduced by nitrous oxide reductase, which is encoded by the gene nosZ (Knowles, 1982). A pool of available NO₂ for denitrification results from the biological oxidation of NH₃ during nitrification (Russow et al., 2009) performed by organisms that produce the enzyme ammonia monoxygenase (AMO). For the ammonia-

---

Copyright © 2011 by the American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America. All rights reserved. No part of this periodical may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopying, recording, or any information storage and retrieval system, without permission in writing from the publisher.

doi:10.2134/jeq2010.0387
Posted online 3 Feb. 2011.
Received 7 Sept. 2010.
*Corresponding author (thomas.ducey@ars.usda.gov).
© ASA, CSSA, SSSA
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 Gary 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; NO₂, nitrite + nitrate; ORP, oxidative reductive potential; qPCR, quantitative polymerase chain reaction; TN, total nitrogen; TSS, total suspended solids; VSS, volatile suspended solids.
oxidizing bacteria, AMO is a multisubunit enzyme that is encoded by the genes \(\text{amoA}, \text{amoB},\) and \(\text{amoC}\). The \(\text{amoA}\) 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 \(\text{amoA}, \text{nirK}, \text{nirS},\) and \(\text{nosZ}\) 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-finish 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 at three depths, for a total of 12 samplings per lagoon.

**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 (\(\text{NH}_4^+\)-N), nitrite and nitrate (\(\text{NO}_2^-\)-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 (°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: \(E = 10^{−1.5\cdot \text{slope}}\) (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 \(\text{nosZ}\) clones corresponding to each of the eight anaerobic lagoons was obtained by performing PCR using the \(\text{nosZ}\) 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™ 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 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* (Kandel 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 ordinations were performed using SAS version 9.2 (SAS Inst., Cary, NC). Canonical correspondence ordination analyses were performed using PCORD version 5 (MJM Software, Gleneden 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.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’ to 3’)</th>
<th>Target</th>
<th>Tm†</th>
<th>Product length‡</th>
<th>Reaction Tm</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>amoA</em>-1F</td>
<td>GGGGTTCCTACCTGGTGGT</td>
<td><em>amoA</em></td>
<td>54.1°C</td>
<td>491 bp</td>
<td>54°C</td>
<td>Rotthauwe et al., 1997</td>
</tr>
<tr>
<td><em>amoA</em>-NEW</td>
<td>CCCCTCBGSAACVCTTCTC</td>
<td><em>amoA</em></td>
<td>58.8°C</td>
<td>491 bp</td>
<td>54°C</td>
<td>Hornek et al., 2006</td>
</tr>
<tr>
<td>1F_nirK</td>
<td>GGGMATGKCCGCTGGCA</td>
<td><em>nirK</em></td>
<td>58.0°C</td>
<td>516 bp</td>
<td>53°C</td>
<td>Braker et al., 1998</td>
</tr>
<tr>
<td>nirK-2R</td>
<td>GCCTCGATCAGRTTRTGG</td>
<td><em>nirK</em></td>
<td>52.8°C</td>
<td>425 bp</td>
<td>55°C</td>
<td>Throback et al., 2004</td>
</tr>
<tr>
<td>cd3aF_nirS</td>
<td>GTAAGCTAGAAGARACSGG</td>
<td><em>nirS</em></td>
<td>57.1°C</td>
<td>453 bp</td>
<td>55°C</td>
<td>Throback et al., 2004</td>
</tr>
<tr>
<td>R3cd_nirS</td>
<td>GASTCGGRTGCGTTCTGA</td>
<td><em>nirS</em></td>
<td>55.8°C</td>
<td>453 bp</td>
<td>55°C</td>
<td>Throback et al., 2004</td>
</tr>
<tr>
<td>nosZ</td>
<td>CCGCGACGCAGCAGCGC</td>
<td><em>nosZ</em></td>
<td>58.6°C</td>
<td>453 bp</td>
<td>55°C</td>
<td>Scala and Kerkhof, 1998</td>
</tr>
<tr>
<td>nosZ-1622R</td>
<td>CGGACCTTCTGGCGCC</td>
<td><em>nosZ</em></td>
<td>63.1°C</td>
<td>453 bp</td>
<td>55°C</td>
<td>Throback et al., 2004</td>
</tr>
<tr>
<td>515F</td>
<td>TGGCCGACGGCGCGGAA</td>
<td>16S v4-v5 region</td>
<td>63.3°C</td>
<td>412 bp</td>
<td>55°C</td>
<td>Weisburg et al., 1989</td>
</tr>
<tr>
<td>927R</td>
<td>CTGGGCCGGGCCCCGGCA</td>
<td>16S v4-v5 region</td>
<td>65.1°C</td>
<td>412 bp</td>
<td>55°C</td>
<td>Rudi et al., 1997</td>
</tr>
</tbody>
</table>

† Tm, melting temperature.
‡ Product lengths were calculated based on the primer positions in the *amoA* gene of *Nitratosomas europaea* ATCC 19718 (NE0944), in the *nirS* gene of *Pseudomonas stutzeri* ZoBell ATCC 14405 (X56813), in the *nirK* gene of *Alcaligenes faecalis* 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 N\textsubscript{2}O production) occurring under existing lagoon conditions. Potential denitrification is indicative of maximal DEA rates (resulting in N\textsubscript{2}O or N\textsubscript{2} production) if NO\textsubscript{x} is non-limiting in the lagoons, and the process is halted at the production of N\textsubscript{2}O. The mean actual (49.7 ± 25.0 mg N\textsubscript{2}O–N m\textsuperscript{-3} d\textsuperscript{-1}) and potential (207.5 ± 135.5 mg N\textsubscript{2}O–N m\textsuperscript{-3} d\textsuperscript{-1}) DEA rates for the studied lagoons were low. The higher levels of N\textsubscript{2}O–N measured for potential DEA, as compared with the levels for actual DEA, indicate NO\textsubscript{x} limitation of the lagoons. Assuming that all the NO\textsubscript{x} is converted to N\textsubscript{2}O and not N\textsubscript{2}, the potential DEA rate still only results in the maximal production of 4.15 kg N\textsubscript{2}O–N ha\textsuperscript{-1} d\textsuperscript{-1}. This number is far short of that previously reported by Harper et al. (2004), who measured average N\textsubscript{2} emission of 85.6 kg ha\textsuperscript{-1} d\textsuperscript{-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 NH\textsubscript{3} volatilization, as opposed to nitrification–denitrification, as the primary cause of N loss from swine anaerobic lagoons (Szögi and Vanotti, 2007).

**Abundances of amo\textsubscript{A}, nir\textsubscript{S}, nir\textsubscript{K}, and nos\textsubscript{Z}**

Coupled with the low levels of NO\textsubscript{x} present in the lagoon wastewater, the low DEA rates led to the hypothesis that the genes responsible for encoding steps in the biological processes 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 amo\textsubscript{A}**

Examination of amo\textsubscript{A}, the gene responsible for encoding ammonia monoxygenase subunit A, revealed an average of 4.3 × 10\textsuperscript{5} gene copies per milliliter of lagoon wastewater, with a range of 1.9 × 10\textsuperscript{5} to 1.2 × 10\textsuperscript{6} (Fig. 3). These values are an order of magnitude lower than the levels of amo\textsubscript{A} found in previously studied industrial (1.73 × 10\textsuperscript{6} gene copies per milliliter) and municipal (7.5 × 10\textsuperscript{6} and 1.6 × 10\textsuperscript{7} 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.†**

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

† 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.

Ducey et al.: (De)Nitrification Gene Abundances in Anaerobic Lagoons 613
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 $\times$ $10^7$ gene copies of nirS per milliliter (Fig. 4) and 7.5 $\times$ $10^7$ gene copies of nirK per milliliter (Fig. 5). The range for nirS was 1.2 to 2.8 $\times$ $10^7$ copies per milliliter. Somewhat similarly, nirK had a range of 2.2 $\times$ $10^7$ to 1.4 $\times$ $10^8$ 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 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 $4.0 \times 10^6$ gene copies per milliliter. The lagoons were rather similar, with a range of only 2.4 to 9.2 $\times$ $10^6$ 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$_2$O could be a large portion of the final N product. Additionally, nitrite reductase (Averill, 1996) typically demonstrate higher rates of enzymatic activity when compared with nitrous oxide reductases (Kristjansson and Hollocher, 2006).
(1980), which could further exacerbate emissions of NO and N\textsubscript{2}O over N\textsubscript{2}. This is a result that would not typically be seen in wastewater treatment systems designed to effectively remove N. This observation supports the findings of Szögi and Vanotti (2007) that N loss in anaerobic lagoons is primarily through NH\textsubscript{3}–N volatilization.

Relationship of \textit{amoA}, \textit{nirS}, \textit{nirK}, and \textit{nosZ} Abundances to Environmental Variables

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

Copy numbers of \textit{amoA} negatively correlated with conductivity, COD, NH\textsubscript{3}–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\textsuperscript{−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\textsuperscript{−1}) appear to be playing a role in reducing overall nitrifier populations. Additionally, lagoon TN (range, 109–631 mg L\textsuperscript{−1}) was predominantly in the form of NH\textsubscript{4}–N (range, 79–506 mg L\textsuperscript{−1}), a factor favorable for the formation of FA.

In addition to being adversely affected by lagoon N, copy numbers of \textit{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 \textit{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 (Loyanachan 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 significant negative correlation with nirK gene copy numbers with conductivity and FA, 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 cd−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.

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>amoA</th>
<th>nirK</th>
<th>nirS</th>
<th>nirK/nirS</th>
<th>nosZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>ns†</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>ORP‡</td>
<td>0.421*</td>
<td>0.408*</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Conductivity</td>
<td>−0.640***</td>
<td>0.664***</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>TSS</td>
<td>ns</td>
<td>0.664***</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>VSS</td>
<td>ns</td>
<td>0.665***</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>COD</td>
<td>−0.601**</td>
<td>0.430*</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>NH₄-N</td>
<td>−0.648***</td>
<td>0.432*</td>
<td>ns</td>
<td>0.454*</td>
<td>ns</td>
</tr>
<tr>
<td>FA</td>
<td>−0.690***</td>
<td>0.432*</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>NO₃-N</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>TN</td>
<td>−0.732***</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

* 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 *Rhodoferax 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 unlinked 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 (Klemdestsson 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.

Acknowledgments

We thank William Brigman, Samantha Lemman, and Terry A. Matheny for sample collection; Philip Bauer and Kenneth C. Stone for assistance with statistical analysis; and Michael J. Rothrock, Ariel A. Szögi, and Jeffrey M. Novak for critical evaluation of this manuscript.

References


