Denitrification in Anaerobic Lagoons Used to Treat Swine Wastewater

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Anaerobic lagoons are commonly used for the treatment of swine wastewater. Although these lagoons were once thought to be relatively simple, their physical, chemical, and biological processes are very complex. This study of anaerobic lagoons had two objectives: (i) to quantify denitrification enzyme activity (DEA) and (ii) to evaluate the influence of lagoon characteristics on the DEA. The DEA was measured by the acetylene inhibition method. Wastewater samples and physical and chemical measurements were taken from the wastewater column of nine anaerobic swine lagoons from May 2006 to May 2009. These lagoons were typical for anaerobic swine lagoons in the Carolinas relative to their size, operation, and chemical and physical characteristics. Their mean value for DEA was 87 mg N_2O–N m^{-3} d^{-1}. In a lagoon with 2-m depth, this rate of DEA would be compatible with 1.74 kg N ha^{-1} d^{-1} loss. When nonlimiting nitrate was added, the highest DEA was compatible with 4.38 kg N ha^{-1} d^{-1} loss. Using stepwise regression for this treatment, the lagoon characteristics (i.e., soluble organic carbon, total nitrogen, temperature, and NO_3–N) provided a final step model R^2 of 0.69. Nitrous oxide from incomplete denitrification was not a significant part of the system nitrogen balance. Although alternate pathways of denitrification may exist within or beneath the wastewater column, this paper documents the lack of sufficient denitrification enzyme activity within the wastewater column of these anaerobic lagoons to support large N_2 gas losses via classical nitrification and denitrification.

Anaerobic lagoons are commonly used for the treatment of swine wastewater. Although these anaerobic lagoons were once thought to be relatively simple in their physical, chemical, and biological processes, they are very complex. In the case of the nitrogen cycling microbial community, lagoons and swine wastewater treatments systems have produced communities of cold-tolerant nitrifying bacteria as well as anaerobic ammonia oxidation bacteria (ANAMMOX) that are functional and unique (Ducey et al., 2010; Vanotti et al., 2006). Moreover, one of the most fascinating indications of lagoon biogeochemical complexity is a somewhat enigmatic finding: High levels of N_2 gas were present in anaerobic swine lagoon bubbles (Harper et al., 2004). For a finish-to-farrow swine production operation, Harper et al. (2004) reported 84,358 kg N_2 yr^{-1} from lagoon denitrification. Farrow-to-finish operators handle the pigs from birth to market, including breeding and farrowing the sows and raising the pigs to a market weight of approximately 240 pounds. For a farrow-to-wean operation, they reported 12,483 kg N_2 yr^{-1} from lagoon denitrification. A farrow-to-wean farm raises the piglets to a weaning age, usually 15 to 17 d of age. The piglets are sold to a feeder-to-finish operation. In previous research, Harper et al. (2000) reported significant but lower amounts of N_2 production from multiple anaerobic lagoons. In these lagoons, the N_2 emissions ranged from 11 to 23 kg N_2 ha d^{-1}. As an explanation for these N_2 differences, they discussed the possibility of biological and chemo-denitrification.

In the case of biological denitrification, the amount of oxygen necessary for this conversion via classical nitrification and denitrification is known (Ro et al., 2006; Stevenson and Cole, 1999). The possibility of obtaining the required amount of oxygen for this process via surficial oxygen transfer was assessed by Ro et al. (2006), who used a newly derived equation to predict surficial oxygen transfer at varying wind speeds (Ro and Hunt, 2006; Ro and Hunt, 2007; Ro et al., 2007). They concluded that sufficient oxygen could have been delivered to the lagoons at the prevailing wind speeds of the experiment to produce the 23 kg ha^{-1} d^{-1} of N_2 emissions estimated by Harper et al. (2000), and they showed that

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Abbreviations: ANAMMOX, anaerobic ammonia oxidation bacteria; BOD, biological oxygen demand; CL, chloride-CL; COD, chemical oxygen demand; DEA, denitrification enzyme activity; EC, electrical conductivity; NO_x, nitrite + nitrate-nitrogen; ORP, oxidative reductive potential; SOC, soluble organic carbon; TKN, total Kjeldahl nitrogen; TOC, total organic carbon; TP, total phosphorus; TSS, total suspended solids; VSS, volatile suspended solids.

considerably more N_2 could be obtained via the alternate denitrification process of ANAMMOX (Jetten, 2008; Kartal et al., 2007). However, none of these biological pathways would have had sufficient oxygen to support the very high level of N_2 gas of the farrow-to-finish farm reported by Harper et al. (2004).

Although specific lagoon characteristics vary with design, geographic location, times of year, and loading rates, conditions are generally thought to be favorable for some type and rate of denitrification. If high rates of classical nitrification and denitrification were occurring in these lagoons, the denitrification enzyme activity (DEA) rates should be correspondingly high. Moreover, the DEA treatments provide estimates of both the complete and incomplete (nitrous oxide [N_2O] rather than N_2 end product) denitrification (Hunt et al., 2007; Tiedje, 1994). Although atmospheric N_2O concentrations are far lower than CO_2, it has 298 times that of CO_2 over a 100-yr time period (Forster et al., 2007). Thus, N_2O is now understood to be an important greenhouse gas (Birgand et al., 2007; Goldberg and Gebauer, 2009; IPCC, 2006; Makris et al., 2009; Oehmen et al., 2007; Piña-Ochoa and Álvarez-Cobelas, 2006; Richardson et al., 2009; Zeng et al., 2004). Among the factors affecting N_2O production during denitrification are the polyphosphate-acquiring organisms (Zeng et al., 2004). Moreover, incomplete denitrification is a common pathway when the carbon/nitrogen ratios are low (Hwang et al., 2006; Klemedtsson et al., 2005).

Our objectives of this study were twofold: (i) to quantify DEA, to include an assessment of incomplete denitrification, in samples from the wastewater column of several anaerobic-swine lagoons, and (ii) to evaluate the influence of lagoon characteristics on the rate of DEA.

Materials and Methods

Denitrification enzyme activity was measured on nine commercial swine wastewater lagoons from May 2006 to May 2009. The lagoons were located in the Coastal Plain region of North and South Carolina, USA. They were located on farms with swine production from finishing or farrow-to-finishing operations (Table 1). Swine populations in the farms ranged from 1000 to 9200. Lagoon surface areas ranged from 0.54 to 2.68 ha. Lagoon depths ranged from 0.78 to 2.17 m.

Wastewater samples were taken in four quadrants of each lagoon. The samples were obtained from three depth regions within the wastewater column: (i) surface 25 cm, (ii) midway to the bottom, and (iii) the bottom 25 cm of the lagoon. This constituted 12 wastewater samples for each lagoon. Samples (1000 mL) were collected with a 7300 Series Telescopic Jar Sampler (Ben Meadows, Janesville, WI), which has a chemical-resistant polypropylene sampler head connected to an aluminum telescoping pole along with a sampling jar that opened and closed via a plunger on the telescoping pole. Upon collection, samples were stored on ice and transported to the laboratory. At each depth, the dissolved oxygen, oxidative reductive potential (ORP), pH, electrical conductivity (EC), and temperature were measured with a multiparameter pH/ORP meter (YSI Incorporated, Yellow Springs, OH).

Denitrification enzyme activity was measured by the acetylene inhibition method (Ambus and Lowrance, 1991; Hunt et al., 2003; Tiedje, 1994). For this analysis, wastewater subsamples (20 mL) from each sampling location were placed in 60-mL serum bottles (four bottles per sample per replication). The treatments were (i) 5 mL of a solution containing chloramphenicol (1 g L^{-1}) to inhibit protein synthesis and to measure incomplete denitrification, (ii) 5 mL of a solution containing chloramphenicol (1 g L^{-1}) and 15×10^{-3} L of acetylene (produced from calcium carbide) to block denitrification at the N_2O phase for measuring complete (complete and incomplete) denitrification and DEA, (iii) 5 mL of a solution containing chloramphenicol (1 g L^{-1}) and nitrates (200 mg L^{-1} NO_3–N) to measure non-nitrate–limiting incomplete denitrification, and (iv) 5 mL of a solution containing chloramphenicol (1 g L^{-1}) and nitrates (200 mg L^{-1} NO_3–N) and 15×10^{-3} L of acetylene to block denitrification at the N_2O phase for measuring non-nitrate–limiting DEA.

The serum bottles were capped with rubber septa, evacuated, and purged with purified N_2 gas three times. After purging with N_2 gas, the appropriate serum bottles were injected with acetylene. The serum bottles were incubated on a horizontal shaker at 1.5 cycles s^{-1} at 24°C. After 1 and 5 h of incubation, 5 mL of the headspace gases were removed from the serum bottles with a syringe (Plastipak, Franklin Lakes, NJ) and injected into vials (borosilicate glass, crimp top with butyl septum). The time with the maximum value was used. The N_2O–N in the headspace gas was measured with a gas chromatograph (Model 3600 CX; Varian, Palo Alto, CA) equipped with a 15-mCi ^{63}Ni electron capture detector operating at 350°C. Chromatographic separation of the headspace gases was obtained by use of a 1.8-m-long by 2-mm inner diameter stainless steel column packed with 80 to 100 mesh Poropak Q (Alltech Associates, Deerfield, IL). The column and injector temperatures were 70°C, and the carrier gas was purified N_2. Samples were injected into the column by an auto-sampler (Model 8200; Varian). All analyses were performed in triplicate.

The wastewater samples were examined for the following 12 parameters: soluble organic carbon (SOC), chemical oxygen demand (COD), biological oxygen demand (BOD), total suspended solids (TSS), volatile suspended solids (VSS), ammonia (NH_4–N), orthophosphate-P, nitrite + nitrate-N (NO_x), total Kjeldahl-N (TKN), total phosphorus, chloride-CL, and

Table 1. Lagoon and farm characteristics.

Parameter	Lagoon								
	1	2	3	4	5	6	7	8	9
Depth, m	0.78	1.66	1.77	1.10	1.46	2.17	2.06	1.40	2.27
Area, ha	1.89	0.54	0.92	1.25	2.68	0.58	1.58	0.58	1.32
Farm type	F†	FF	F	F	FF	F	F	F	F
Swine, *n*	4500	1000	4360	4900	9200	2900	5280	2200	5880

† F, finishing farm; FF, farrowing-to-finishing farm.

total organic carbon (TOC). The TOC was determined on a Shimadzu TOC-VSCN (Shimadzu Corp., Kyoto, Japan); all the other analyses were performed according to Standard Methods for the Examination of Water and Wastewater (Clesceri et al., 1998).

The data were statistically analyzed using SAS v 9.2 (SAS Institute, 2002). Analysis of variance was done using GLIMIX with lagoon depth and DEA as the treatments and lagoons as the random variable (i.e., replication). The DEA values were also analyzed against wastewater physical and chemical parameters via stepwise regression analysis. Mallow's Cp values were used to stop the addition of parameters at an acceptably low level of collinearity in the stepwise regression model.

Results and Discussion

Lagoon Wastewater Characteristics

As is typical for these types of anaerobic lagoons (Bicudo et al., 1999), the pH was slightly alkaline (pH 7.7 ± 0.23) (Table 2). The ORP values ranged from reduced to moderately reduced with a mean of −228 ± 114 mV. Although the ORP values were consistent with denitrification, they were more reduced than would be expected for a system that was processing major levels of nitrogen loss via nitrification and denitrification. For instance, the ORP of a denitrification tank in a nitrification–denitrification loop of a swine wastewater treatment system was considerably more oxidative (131.7 ± 209.4 mV) (Vanotti et al., 2009). Their tank removed 90% of the nitrogen from the barns of a 5000-head swine farm.

The mean EC for the nine lagoons of the current study was 6.7 ± 2.4 dS m^{-1}. This mean is very similar to the EC for non-purple lagoons reported by Chen et al. (2003). The BOD was 205 ± 63 mg L^{-1}, COD was 1976 ± 549 mg L^{-1}, and TOC was 579 ± 259 mg L^{-1}. The TOC and COD concentrations were similar to the values reported for North Carolina lagoons by Bicudo et al. (1999). The TSS and VSS means were 1199 ± 1465 and 697 ± 687 mg L^{-1}, respectively. The VSS/TSS ratio was 0.58; this ratio was similar to that reported by Bicudo et al. (1999). As would be expected for these lagoons, the nitrate concentrations were <1 mg L^{-1} (Table 3). The NH_4–N was the major component of the total N (mean, 416 ± 157 mg L^{-1}). Similarly, soluble P was the major component of the total P (mean, 64 ± 14 mg L^{-1}).

Although these lagoons were typical in terms of size, operation, and chemical and physical characteristics as compared with commercial swine lagoons in the Carolinas, they should have had DEA values that were representative of full-scale swine lagoons in this region. Treatment II measures the DEA occurring under existing conditions. It assesses what would be occurring if the process of denitrification in anaerobic lagoons is one in which NO_3–N is rapidly formed and rapidly denitrified. The mean value for DEA Treatment II was 87 mg N_2O–N m^{-3} d^{-1} (Table 4). The range was 27 to 317 mg N_2O–N m^{-3} d^{-1}. There was no significant difference ($p \geq 0.05$) in DEA with depth. Thus, the mean DEA value was representative of the wastewater column of the entire lagoons.

If the mean DEA rate of 87 mg N_2O–N m^{-3} d^{-1} is assumed for a lagoon of 2-m depth, the rate of DEA would be 1.74 kg

Table 2. Physical and chemical characteristics of lagoon wastewater.

Parameter†‡	Lagoon§										
	1	2	3	4	5	6	7	8	9	Mean	SD
pH	7.6	7.4	7.7	7.8	7.6	7.7	8.0	7.6	8.0	7.7	0.23
ORP, mV	−238	+6	−398	−252	−299	−120	−132	−169	−335	−228	114
DO, mg L^{-1}	0.18	0.71	0.68	0.07	1.13	0.12	0.18	0.21	0.18	0.40	0.37
Conductivity, dS m^{-1}	5.0	2.1	4.8	8.7	10.2	7.5	6.5	6.5	9.1	6.7	2.4
TSS, mg L^{-1}	1541	246	406	3463	4468	384	322	429	442	1199	1465
VSS, mg L^{-1}	1014	193	362	1716	2242	288	258	333	355	697	687
TOC, mg L^{-1}	566	113	342	606	901	910	467	728	–	579	259
COD, mg L^{-1}	2352	920	1803	2832	2452	1883	1354	1888	2259	1976	549
BOD, mg L^{-1}	124	125	276	267	277	185	280	192	187	205	63

† BOD, biological oxygen demand; COD, chemical oxygen demand; DO, dissolved oxygen; ORP, oxidative reductive potential; TOC, total organic carbon; TSS, total suspended solids; VSS, volatile suspended solids.

‡ The parameters were not significantly different for depth via the least significant means procedure with the exception of ORP, which was ~10% higher in the top layer.

§ Mean of four quadrants and three depths.

Table 3. Nutrient characteristics of lagoon wastewater.

Parameter†	Lagoon‡									Mean	SD
	1	2	3	4	5	6	7	8	9		
	mg L^{-1}										
NH_3–N	197	79	506	372	381	285	261	314	516	349	136
PO_4–P	39	74	66	20	29	61	43	51	69	47	21
NO_x–N	0.4	0.1	0.1	0.4	0.4	0.3	0.1	2.0	0	0.3	0.6
Total N	239	109	581	392	499	631	338	528	790	416	157
Total P	84	79	73	62	32	73	60	67	71	64	14

† The parameters were not significantly different for depth via the least significant means procedure.

‡ Mean of four quadrants and three depths.

N_2O–N ha^{-1} d^{-1}. This amount of DEA is very small in contrast to an ammonia emission of 37 kg N ha^{-1} d^{-1} for a North Carolina swine lagoon reported by Szogi and Vanotti (2007). In a 2-m-deep lagoon, a DEA rate of 1500 mg N_2O–N m^{-3} d^{-1} would be required for an N loss of 30 kg ha^{-1} d^{-1}, and this would be less than half of the 84 kg ha^{-1} d^{-1} reported by Harper et al. (2004). Thus, the mean rate of 87 mg N_2O–N m^{-3} d^{-1} DEA is incompatible with a large amount of denitrification proceeding via classical nitrification and denitrification.

It was also important to determine how much the rate of DEA was limited by the availability of nitrate. This limitation was determined with Treatment IV, which had a nonlimited concentration of nitrate added. Treatment IV DEA rates were significantly higher than any of the other DEA treatments at all depths. Although the DEA rate of Treatment IV was more than double that of Treatment II, it was still only 197 mg N_2O–N m^{-3} d^{-1} (range, 27–631 mg N_2O–N m^{-3} d^{-1}). When compared with the surface and middle, the DEA rate was significantly higher (219 mg N_2O–N m^{-3} d^{-1}) at the bottom depth. Even at the DEA rate of this bottom layer with its nonlimiting nitrate, a 2-m-deep lagoon would only produce an N loss of 4.38 kg ha^{-1} d^{-1}. This rate of denitrification removal is substantially lower than the values reported by Harper et al. (2000, 2004). It was also lower than the rates possible by the likely surficial oxygen transfer as estimated by Ro et al. (2006). However, their nitrification estimates based on oxygen transfer assumed that most of the oxygen was available for nitrification.

One important point of consideration is whether the DEA method would have detected high rates of DEA in swine wastewater. This point was resolved positively. Using the identical method, high rates of DEA were found in the denitrification tank (277 m^3) of a nitrification/denitrification treatment unit in a swine wastewater treatment system (Vanotti et al., 2009). Using Treatment II, the measured DEA was >55,000 mg N_2O–N m^{-3} d^{-1} for this system. This detected rate of DEA in swine wastewater was over 500 times higher than that of the lagoon wastewater. Thus, the method was able to detect very high DEA in swine wastewater, if it existed. The level of DEA has been shown to be a reasonable predictor of nitrogen removal capacity (Hunt et al., 2008), and these tank DEA values were indicative of very high rates of nitrogen removal from the denitrification tanks.

Even if an underestimation factor of 2 were applied to the lagoon DEA estimate (4.38 kg ha^{-1} d^{-1} × 2), the DEA would still only be sufficient for <9 kg N ha^{-1} d^{-1}. Moreover, this rate was obtained only with the addition of a nonlimiting concentration of NO_3–N (Treatment IV). Although these data do not rule out significant denitrification via other biological pathways such as the ANAMMOX (Jetten, 2008; Kartal et al., 2007; Raghoebarsing et al., 2006; Sumino et al., 2006) or chemodenitrification, they clearly show that the lagoons lacked the DEA necessary to have removed major amounts of nitrogen (>30 kg ha^{-1} d^{-1}) via classical nitrification and denitrification.

Relative to incomplete denitrification, Treatment I provided a measurement of N_2O–N production from the lagoon wastewater under anaerobic incubation. Although the mean of 49 N_2O–N mg m^{-3} d^{-1} was low, it was 56% of the DEA (Treatment II), suggesting that the DEA is not proceeding to completion. Even if all of this N_2O was emitted from the lagoon, it would only be equivalent to 1 kg N_2O–N ha^{-1} d^{-1} for a 2-m-deep lagoon. With a 298 equivalent ratio, this rate of N_2O production would be equivalent to 298 kg CO_2 ha^{-1} d^{-1} of CO_2 (IPCC, 2006). Treatment III provided an estimate of N_2O from incomplete denitrification in the presence of nonlimiting nitrate. The rate of N_2O produced after the addition of supplemental nitrate was 79 mg N_2O–N m^{-3} d^{-1}. With the 298 conversion, this would be equivalent to 1.6 kg CO_2 ha^{-1} d^{-1} for a 2-m-deep lagoon. This was 40% of the DEA in the presence of nonlimiting nitrate. As with the nonamended Treatment I, this level of N_2O would not be a large portion of the total lagoon nitrogen balance. However, it would not be an altogether insignificant source of N_2O, which is an important greenhouse gas.

Relationship of Lagoon Characteristics to Denitrification Enzyme Activity

To provide an assessment of the data that went into the stepwise regression without presenting all of the DEA data, the mean and standard deviation are provided for each of the DEA treatments for each of the lagoons (Table 5). In all lagoons, the treatment responded as expected for the method. Treatment II is larger than Treatment I, and Treatment IV is larger than Treatment III. Not one lagoon had a DEA mean above 151 mg N_2O–N m^{-3} d^{-1}. Moreover, not one single lagoon had a mean non-nitrate–limiting DEA as high as 500 mg N_2O–N m^{-3} d^{-1}. The lagoon with the lowest DEA had a mean of 32 mg N_2O–N m^{-3} d^{-1}. It was extremely nitrate limited because it had a non-nitrate–limiting DEA of 144 mg N_2O–N m^{-3} d^{-1}. This extreme nitrate limitation was true for some but not all lagoons. For instance, Lagoon 2 hardly responded to added NO_3–N for complete or incomplete denitrification.

Table 4. Denitrification enzyme activity in swine lagoons by treatment and depth.

Depth region	Treatments				
	Control (I)	Control and acetylene (II)	Control and nitrate (III)	Control plus nitrate and acetylene (IV)	Mean
	mg N_2O–N m^{-3} d^{-1}				
Top	44c†	80c	75c	195a	99ab
Middle	48c	88c	75c	178b	97b
Bottom	55c	92c	87c	219a	113a
Mean	49b	87b	79b	197a	

† Means followed by the same letter are not significantly different at the 0.05 level by the least means square procedure.

More insight into the responses of DEA to the lagoon characteristics is presented in the following discussion of stepwise regression. In stepwise regression, the first step is the parameter that provides the best fitting simple linear regression. The parameters used in the stepwise regression were SOC, conductivity, temperature, COD, BOD, DO, Eh, pH, TSS, VSS, NH_4, PO_4, NO_2, NO_x, TKN, TP, TN, CL, COD/TN, BOD/TN, and SOC/TN. With the stepwise analyses, there was a first-step linear regression of the best linear fitted variable. Slopes and intercepts are provided in Tables 5 through 9. Subsequently, the regression was expanded by stepwise regressions to determine if any additional parameters provided significant improvement to the regression. Generally, five to nine variables were significant for the stepwise regression, with a P value of ≤0.05. Additionally, the Cp values for the final step of the stepwise regressions did not exceed the value that corresponded to the number of variables used in the final regression step. Thus, these Mallow's Cp values were consistent with an acceptably low level of collinearity in the stepwise regression model.

For Treatment I, the best fit linear regression was the COD/TN ratio (Table 6). This was a reasonable result based on the fact the C/N ratio is known to control the production of N_2O via incomplete denitrification (Hwang et al., 2006; Klemedtsson et al., 2005). When pH, NO_x, PO_4, and TP were added to the stepwise regression, R^2 improved to 0.31. Moreover, the Cp value of 5 was equivalent to the number of parameters in the stepwise regression. The involvement of these parameters is reasonable (Hunt et al., 2007; Hwang et al., 2006; Meyer et al., 2005; Zeng et al., 2003a,b). For Treatment II, the best linear regression was with the BOD/TN ratio (Table 7). With the addition of temperature, TP, CL, and SOC, R^2 improved to 0.35. The Cp value did not approach the number of variables in the stepwise regression. The parameters controlling DEA in lagoons were somewhat different from those controlling DEA in swine wastewater treatment wetlands or in swine wastewater–affected riparian buffers (Hunt et al., 2003, 2004, 2006, 2009). In those systems, DEA rates were generally well correlated to total N or total C concentrations. This is likely because they were more often limiting factors. The limiting factor in

Table 5. Denitrification enzyme activity values for each lagoon.

Lagoon	Treatments†			
	Control (I)	Control and acetylene (II)	Control and nitrate (III)	Control plus nitrate and acetylene (IV)
	mg N_2O–N m^{-3} d^{-1}			
1	25 ± 12	90 ± 75	54 ± 15	359 ± 182
2‡	38 ± 10	48 ± 11	37 ± 10	50 ± 14
3	64 ± 23	103 ± 58	73 ± 41	122 ± 86
4	24 ± 4	32 ± 2	26 ± 4	144 ± 53
5‡	55 ± 15	87 ± 17	57 ± 15	147 ± 83
6	70 ± 49	121 ± 88	110 ± 67	188 ± 79
7	30 ± 16	55 ± 16	47 ± 23	179 ± 54
8	93 ± 66	151 ± 72	253 ± 58	470 ± 74
9	44 ± 10	93 ± 12	56 ± 41	118 ± 80
Mean	49 ± 23	87 ± 39	79 ± 30	197 ± 78

† Denitrification enzyme activity values are the mean for four quadrants and three depths.

‡ Farrow-to-finish farms.

Table 6. Stepwise regression of the lagoon wastewater control nitrous oxide (Treatment I).

Step	Variable entered†	Parameter estimate‡	Partial R^2	Model R^2	Mallow's Cp	Pr > F
1	COD/TN	−4.80	0.12	0.12	56	<0.0001
2	pH	−17.74	0.07	0.19	36	<0.0001
3	NO_x	10.93	0.05	0.24	23	0.0003
4	PO_4	0.55	0.02	0.26	19	0.0151
5	TP	−0.63	0.05	0.31	5	0.0001

† COD, chemical oxygen demand; NO_x, nitrite + nitrate-nitrogen; TN, total nitrogen; TP, total phosphorus.

‡ The denitrification enzyme activity intercept was 218.

Table 7. Stepwise regression of the lagoon wastewater denitrification enzyme activity (Treatment II).

Step	Variable entered†	Parameter estimate‡	Partial R^2	Model R^2	Mallow's Cp	Pr > F
1	BOD/TN	−11.09	0.15	0.15	103	<0.0001
2	temperature	−8.04	0.07	0.22	79	<0.0001
3	TP	−1.78	0.09	0.31	46	<0.0001
4	Cl	−0.25	0.04	0.34	34	0.0005
5	SOC	0.04	0.01	0.35	33	0.0797

† BOD, biological oxygen demand; SOC, soluble organic carbon; TN, total nitrogen; TP, total phosphorus.

‡ The denitrification enzyme activity intercept was 400.

the lagoons is generally NO_3–N. This is shown by the denitrification results of Treatments III and IV.

When nonlimiting NO_3–N was added to the wastewater in Treatment III, there was a reasonably good fit with simple linear regression (Table 8). The R^2 was 0.45 for DEA vs. NO_x. By adding temperature, SOC, and SOC/TN, the R^2 was improved to 0.67. The Cp value was an acceptable 36. The parameter estimates were DEA = 162 + 47 mg NO_x − 6°C + 0.04 mg SOC − 8.07 SOC/TN. The involvement of SOC is reasonable because the limitation of this energy source would produce incomplete denitrification with the attendant N_2O. As with the N_2O production without the addition of nitrate (Treatment I), these parameters are consistent with those expected to affect N_2O production under anoxic conditions (Hunt et al., 2007; Hwang et al., 2006; Li et al., 2008; Meyer et al., 2005; Tallec et al., 2008).

When NO_3–N was added along with acetylene (Treatment IV), the best simple linear fit was with SOC/TN (Table 9). The R^2 for this regression was 0.24. The stepwise regression determined that eight more parameters added significant additions to the model R^2; it gave a final step model R^2 of 0.69. The Cp value of 14 was acceptable. The parameters were temperature, NO_x, COD, NH_4, BOD, PO_4, TKN, and TN. The model equation was DEA = 543 + 88.51 SOC/TN − 28.67°C + 91.14 mg NO_x + 0.09 mg COD − 0.94 mg NH_4 + 0.97 mg BOD − 0.81 mg PO_4 + 1.64 mg TKN − 1.45 TN. These parameters seem to be reasonably intuitive in light of the literature revolving around C, N, and anaerobic conditions (Hunt et al., 2007; Hunt et al., 2009; Hwang et al., 2006; Meyer et al., 2005; Ro et al., 2006; Zeng et al., 2004; Zeng et al., 2003a,b). However, we did not find a clear and large correlation of DEA to TN or TKN as might be expected from results of swine wastewater treated in treatment wetlands (Hunt et al., 2006; Hunt et al., 2009).

Remaining questions need to be resolved about the rates of DEA in the lagoon sediments and other processes for nitrogen removal as N_2. For instance, if the amount of N_2 gas reported by Harper et al. (2004) is considered reliable, our results demonstrate that processes other than classical nitrification and denitrification must be responsible for the N_2 gas losses. This was an option presented in their paper. While bearing in mind the possibility that the results of Harper et al. (2004) could have contained analytical artifacts, there is now ample evidence for alternate biological N_2 removal pathways. For example, ANAMMOX has been demonstrated as being a useful process for the removal of ammonia from animal wastewater (Vanotti et al., 2006). Accordingly, ANAMMOX has been increasingly described in treatment systems (Furukawa et al., 2009; Qiao et al., 2009; Sumino et al., 2006). The development of new molecular methods for the detection of ANAMMOX species will allow for more rapid identification and classification of these species in lagoons and other wastewater treatment systems.

The coupling of anaerobic methane oxidation to denitrification could play a role in anaerobic lagoons in the production of N_2 gas. In this process, methane is oxidized to carbon dioxide; the nitrite or nitrate that is used as the electron receptor is reduced to nitrogen gas. The occurrence of this process was linked to a bacterial and *archaea* consortium. They were initially believed to exist at the oxic/anoxic interface (Raghoebarsing et al., 2006). More recent evidence suggested that this process is solely bacterial in nature and driven under anoxic conditions (Ettwig et al., 2008). The process seems to be driven by the uncultured phylum NC10. This phylum's organisms have been shown to exist in a number of freshwater environments. However, like ANAMMOX, the full extent of this process in the formation of N_2 gas remains unknown (Ettwig et al., 2009). What is known about nitrogen losses from lagoons

Table 8. Stepwise regression of the nitrous oxide accumulation in lagoon wastewater amended with nitrate (Treatment III).

Step	Variable entered†	Parameter estimate‡	Partial R^2	Model R^2	Mallow's Cp	Pr > F
1	NO_x	47.27	0.45	0.45	199	<0.0001
2	Temperature	−5.58	0.13	0.58	103	<0.0001
3	SOC	0.04	0.07	0.64	53	<0.0001
4	SOC/TN	−8.07	0.02	0.67	36	<0.0001

† NO_x, nitrite + nitrate-nitrogen; SOC, soluble organic carbon; TN, total nitrogen.

‡ The denitrification enzyme activity intercept was 162.

Table 9. Stepwise regression of the non-nitrate–limiting denitrification enzyme activity of lagoon wastewater amended with nitrate (Treatment IV).

Step	Variable entered†	Parameter estimate‡	Partial R^2	Model R^2	Mallow's Cp	Pr > F
1	SOC/TN	88.51	0.24	0.24	314	<0.0001
2	Temperature	−28.67	0.17	0.40	203	<0.0001
3	NO_x	91.14	0.13	0.52	118	<0.0001
4	COD	0.09	0.06	0.58	79	<0.0001
5	NH_4	−0.94	0.03	0.61	62	<0.0001
6	BOD	0.97	0.03	0.64	44	<0.0001
7	PO_4	−0.81	0.02	0.66	329	0.0006
8	TKN	1.64	0.01	0.67	30	0.0597
9	TN	−1.45	0.03	0.69	14	<0.0001

† BOD, biological oxygen demand; COD, chemical oxygen demand; NO_x, nitrite + nitrate-nitrogen; SOC, soluble organic carbon; TKN, total Kjeldahl nitrogen; TN, total nitrogen.

‡ The denitrification enzyme activity intercept was 54.

is that ammonia emission can be significant (Ro et al., 2008; Szogi and Vanotti, 2007).

There remains much to be done to fully understand the biogeochemistry of livestock wastewater treatment via anaerobic lagoons. Nonetheless, this paper documents the lack of sufficient denitrification enzyme activity in the wastewater column of these commercial anaerobic lagoons to support large amounts of classical denitrification. This is in distinct contrast to the high rate of denitrification enzyme activity occurring in swine wastewater treatment systems that have high rates of nitrification and denitrification treatment (Vanotti et al., 2009; Vanotti et al., 2007).

Conclusions

The nine commercial lagoons used in this study were typical of operating commercial swine lagoons located in the Carolinas relative to their size, operation, and chemical and physical characteristics. Their mean DEA was 87 mg N_2O–N m^{-3} d^{-1} (range, 27–317 mg N_2O–N m^{-3} d^{-1}). In a 2-m-deep lagoon, this rate of DEA would be consistent with 1.74 kg N ha^{-1} d^{-1} loss as N_2. This amount of nitrogen would be a small part of the lagoon nitrogen balance. It is also very small relative to a published ammonia emission of 37 kg N ha^{-1} d^{-1} for a North Carolina swine lagoon. When nitrate was added, the DEA mean was 197 mg m^{-3} d^{-1} (range, 27–631 mg N_2O–N m^{-3} d^{-1}). Although this level of DEA is greater, it would only be consistent with 4.38 kg ha^{-1} d^{-1} loss as N_2. Relative to incomplete denitrification and N_2O production, the measured levels would not have been a significant part of the system nitrogen balance. However, this level of N_2O would not be an altogether insignificant source of an important greenhouse gas. The lagoon DEA (Treatment II) was not particularly well predicated by the lagoon biogeochemical characteristics via stepwise regression. However, when nitrate was added (Treatment IV), the final R^2 was 0.69. The major contributing components were SOC/TN, temperature, and NO_x. The low DEA rates do not preclude the possibility of other biological denitrification pathways or ammonia volatilization. Either of these could be involved as a major component of the lagoon nitrogen balance. Nevertheless, this paper documents the absence of sufficient denitrification enzyme activity within the wastewater column of these anaerobic lagoons to support large N_2 gas losses via classical nitrification and denitrification.

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