2013

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Anaerobe 21 (2013) 50–57

Contents lists available at SciVerse ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Molecular biology, genetics and biotechnology

Microbial community analysis of swine wastewater anaerobic lagoons by next-generation DNA sequencing

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ARTICLE INFO

Article history:
Received 28 September 2012
Received in revised form
1 March 2013
Accepted 10 March 2013
Available online 26 March 2013

Keywords:
Anaerobic lagoon
Swine wastewater
Pyrosequencing
Microbial communities

ABSTRACT

Anaerobic lagoons are a standard practice for the treatment of swine wastewater. This practice relies heavily on microbiological processes to reduce concentrated organic material and nutrients. Despite this reliance on microbiological processes, research has only recently begun to identify and enumerate the myriad and complex interactions that occur in this microbial ecosystem. To further this line of study, we utilized a next-generation sequencing (NGS) technology to gain a deeper insight into the microbial communities along the water column of four anaerobic swine wastewater lagoons. Analysis of roughly one million 16S rDNA sequences revealed a predominance of operational taxonomic units (OTUs) classified as belonging to the phyla Firmicutes (54.1%) and Proteobacteria (15.8%). At the family level, 33 bacterial families were found in all 12 lagoon sites and accounted for between 30% and 50% of each lagoon's OTUs. Analysis by nonmetric multidimensional scaling (NMS) revealed that TKN, COD, ORP, TSS, and DO were the major environmental variables in affecting microbial community structure. Overall, 839 individual genera were classified, with 223 found in all four lagoons. An additional 321 genera were identified in sole lagoons. The top 25 genera accounted for approximately 20% of the OTUs identified in the study, and the low abundances of most of the genera suggests that most OTUs are present at low levels. Overall, these results demonstrate that anaerobic lagoons have distinct microbial communities which are strongly controlled by the environmental conditions present in each individual lagoon.

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1. Introduction

The use of anaerobic lagoons for the passive treatment of swine wastewater remains a steadfast practice of a majority of confined animal feeding operations (CAFOs). These systems rely primarily on microbial activity to reduce organic material and nutrients. While the understanding of the microbial processes occurring in these lagoons has improved over time, there still remains much to be understood about the microbial communities – and their interactions – in these ecosystems. This understanding becomes even more critical when considering that these treatment systems continue to be heavily relied upon by the industry. Over the course of the past two decades, the number of swine operations has been reduced by more than 60%, while the average number of swine per operations has more than quadrupled. Taken together, these two factors have resulted in an extreme concentration of swine wastewater in a constrained space that must be effectively treated and removed from these operations.

Research towards understanding microbiologically related aspects of anaerobic lagoon treatment has focused primarily on the following areas: (1) pathogens [1–4]; (2) nutrient cycling [5–9]; and (3) malodorous compounds [10–14]. The techniques used to address these issues have varied from study to study but have employed both direct culturing methods to isolate, identify, and enumerate bacteria [3,11], and a number of non-culturing molecular methods. The non-culturing molecular techniques include the following: (1) quantitative Real-Time PCR (qPCR) [7,15]; (2) denaturing gradient gel electrophoresis (DGGE) [5]; (3) fluorescent *in situ* hybridization (FISH) [9]; and (4) cloning and sequencing of 16S rDNA or process-specific genes [12,15]. To date however, no studies examining the microbial communities of anaerobic wastewater lagoons have utilized next-generation sequencing (NGS) technologies.

Next-generation sequencing technologies allow – via cost effective, extremely high-throughput sequencing – for deeper taxonomic resolution of microbial communities [16]. These technologies have been utilized to characterize a number of ecosystems [17,18], and have been used to address scientific issues spanning from agriculture [19], the environment [20], to medicine [21]. Bringing these technologies to focus on the microbial communities

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1075-9964/$ – see front matter Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.anaerobe.2013.03.005

of anaerobic wastewater lagoons would provide the most thorough look, to date, at these populations.

To this aim, we collected samples from four North Carolina anaerobic wastewater lagoons with varying chemical compositions. These samples were collected at three depths (surface 25 cm, midway between surface and bottom, and 25 cm from the bottom) along the water column of these lagoons. We then utilized pyrosequencing of variable regions one through three (V1–V3) of the bacterial 16S rDNA to measure each lagoons microbial community composition and diversity.

2. Materials and methods

2.1. Site description and sample collection

Four commercial swine lagoons (labeled L1 through L4) located in North Carolina were chosen for this study. Lagoons were divided into quadrants, and 1 L samples were collected from each of three points within the water column as follows: (a) 15 cm below the surface; (b) midway between surface and bottom (depth range: 70–101 cm); and (c) 15 cm off the lagoon bottom (depth range: 122–191 cm). Samples were collected using a telescopic jar sampler (Lab Safety Supply, Janesville, WI), and stored on ice and transported to the laboratory for analysis. These lagoons were either finish (L1), farrow (L4), or farrow to finish (L2 and L3) operations. Additionally, the lagoons varied in area as follows: 1.58 ha (L1); 2.68 ha (L2); 0.54 ha (L3); and 0.58 ha (L4). For the purposes of this study, lagoon sample naming conventions were based on sampling depth (T, top; M, middle; B, bottom).

2.2. Wastewater analysis

All wastewater analyses, which included biological oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS), volatile suspended solids (VSS), ammonium (NH_4–N), nitrite and nitrate (NO_x-N), total nitrogen (TN), Kjeldahl-N (TKN), orthophosphate–P (PO_4–P), total phosphorus (TP), and chloride (Cl) were performed according to Standard Methods for the Examination for Water and Wastewater [22]. Total organic carbon (TOC) analysis was performed on a Shimadzu TOC-VSCN (Shimadzu Corp., Kyoto, Japan). Dissolved oxygen (DO), electrical conductivity (EC), oxidative reductive potential (ORP), and pH were measured with a multiparameter pH/ORP meter (YSI Inc., Yellow Springs, OH).

2.3. DNA extraction

Prior to DNA extraction a composite sample, consisting of equal volumes from each of four quadrant samples taken at each lagoon depth, was generated as previously described [6]. From this composite sample, a volume of 5 mL was centrifuged at 14,000 × *g* for 5 min, and DNA was extracted from the resultant pellet as described previously [23].

2.4. Pyrosequencing of 16S rDNA gene

The 16S rDNA gene V1–V3 region was amplified by PCR using universal primers 8F and 518R containing the Roche-454 A or B titanium sequencing adapters. While it has been demonstrated that sample bias can be introduced during amplification of the 16S rDNA gene [24], this region is recommended for use in NGS applications [25]. The V1–V3 region provides a dataset with deeper richness compared to other hypervariable regions [26], while at the same time providing a high degree of classification accuracy [27], and a low degree of classification bias towards specific taxonomic groups [28]. Amplification products were quantified using the Quant-iT PicoGreen double-stranded DNA assay (Invitrogen) and quality controlled on an Agilent 2100 BioAnalyzer (Agilent). Pyrosequencing on a Roche Genome Sequencer GS-FLX was then performed as previously described by Martinez et al. [23] by the Center for Applied Genomics and Ecology (CAGE) at the University of Nebraska – Lincoln.

2.5. Pyrosequence processing

Raw data were filtered in order to remove sequences of poor quality [29]. Sequence reads with more than one ambiguous base or with an average quality score of ≥20, as well as reads >200 nucleotide (nt) sequence length were excluded from downstream analysis. Sequence reads were then trimmed to remove adapter and primer sequences.

2.6. Microbial community analysis

Trimmed sequences were assembled and aligned into operational taxonomic units (OTUs), also referred to as phylotypes, in Geneious ver. 5.6.2 (Biomatters Ltd., Auckland, New Zealand) [30]. A minimum of two sequence reads, assembled together at a 97% sequence similarity threshold, were required for designation as an OTU. All sequences that failed to assemble at the 97% sequence similarity threshold were designated as singletons and removed from further phylogenetic analysis. The consensus sequences derived from each OTU were phylogenetically classified using the Ribosomal Database Project's (RDP) pyrosequencing pipeline. Rarefaction curves were calculated using Analytic Rarefaction ver. 1.3 [31]. Simpson's Index of Diversity ($1 - D$ [32],) was calculated where $D = \sum(n_i(n_i - 1)/N(N - 1))$, with n_i the proportion of a given taxon (i), and N representing the total number of sequence reads from an individual lagoon sample. Good's coverage [33] was calculated as $G = 1 - n/N$ where n is the number of singletons based on a 97% sequence similarity threshold, and N is the total number of sequence reads from an individual lagoon sample.

PC-ORD ver. 6 (MJM Software, Gleneden Beach, OR) was used to perform nonmetric multidimensional scaling analysis of bacterial communities found in each of the 12 sites. Comparisons were performed using relative abundances from phylotypes, grouped according to bacterial family, found in at least half of the sites. To avoid redundancy in the environmental data, single variables were selected to represent overlapping environmental data (see Table 3). Distribution of individual bacterial genera across the four lagoons was visualized using Venn diagrams compiled by the software package Venny [34].

2.7. Nucleotide submissions

All sequencing data, in compressed (.zip) format, can be downloaded from the following URL: https://www.ars.usda.gov/Services/docs.htm?docid=23124.

3. Results

From the 12 lagoon sites that were assayed, a total of 987,606 high quality sequence reads were obtained (Table 1). These sequence reads were assigned to a total of 15,682 operational taxonomic units (OTUs). Each lagoon was covered by an average of 246,902 (±54,926) sequence reads and 3921 (±877) OTUs, with an average of 82,300 (±28,962) sequence reads, and 1307 (±371) OTUs per site. Likewise, a total of 34,715 singletons remained, and accounted for between 2.3% and 5.9% of all sequence reads obtained per site. While rarefaction curves (Fig. 1) continued to trend upwards, a Good's coverage estimate of 96.5% (±1.2) suggested that a

Table 1
Sequencing information, based on depth, for each of four swine wastewater lagoons.

Lagoon	Depth	# Reads	OTUs[a]	Singletons	Coverage (%)	1-*D*
Lagoon 1	Top	79,482	1493	4725 (5.9%)	94.06	0.942
	Middle	54,299	1027	2958 (5.4%)	94.55	0.940
	Bottom	93,049	1202	3338 (3.6%)	96.41	0.931
Lagoon 2	Top	131,959	2264	3779 (2.9%)	97.14	0.966
	Middle	85,767	1617	2362 (2.8%)	97.25	0.929
	Bottom	51,113	1264	1416 (2.7%)	97.23	0.881
Lagoon 3	Top	78,360	1231	3778 (4.8%)	95.18	0.934
	Middle	105,995	1264	3733 (3.5%)	96.48	0.947
	Bottom	125,507	1260	4212 (3.4%)	96.64	0.919
Lagoon 4	Top	62,264	1001	1530 (2.5%)	97.54	0.967
	Middle	37,322	782	974 (2.6%)	97.39	0.965
	Bottom	82,489	1277	1910 (2.3%)	97.68	0.964

[a] Based on 97% similarity, excluding singleton sequence reads.

majority of the phylotypes present in the sites had been identified. Additionally, Simpson's reciprocal index of diversity (1/*D*) indicated that bacterial diversity decreased as a function of depth.

A total of 22 phyla or candidate divisions were represented amongst the OTUs classified in this study (Fig. 2A), with the vast majority (81.2% ± 3.0%) classified to one of four major phyla: Actinobacteria (7.2%), Bacteroidetes (4.1%), Firmicutes (54.1%), or Proteobacteria (15.8%). The phyla Chloroflexi (1.3%) and Synergistetes (2.5%) accounted for an additional 3.8% (±1.2) of the studies OTUs. Four of the Proteobacterial classes (α, β, δ, and γ) were represented in all 12 sites (Fig. 2B); OTUs classified as ε-proteobacteria were identified in 10 of the 12 sites, the exceptions being L1T and L4M. For Firmicutes, phylotypes belonging to the class Clostridia accounted for the majority of all classified OTUs (83.6% ± 6.3; Fig. 2C).

At the family level, a total of 150 bacterial families were represented from sequences found in at least 1 of the 12 sites (Fig. 3). Only 33 (22%) of these families were represented in all 12 sites, and 84 (56%) were found to be in at least half of the sites examined in this study. The 33 families identified in all twelve sites account for over 50% of all phylotypes in L1, L2, and L4 (53.6% ± 2.3) and 36.8% (±3.1) of the phylotypes in L3. In terms of abundance, the Ruminococcaceae were the best represented amongst the phlyotypes, with 15.2% (±2.8) for L1, 18.4% (±1.8) for L2, 6.2% (±0.9) for L3, and 20.5% (±0.5) for L4. Other well represented families from all twelve sites included the Clostridiaceae (6.8% ± 2.2), Lachnospiraceae

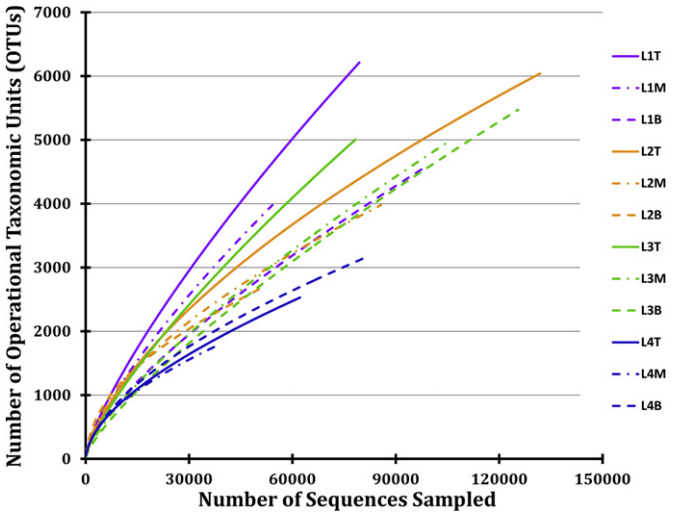

Fig. 1. Rarefaction analysis of bacterial 16S rDNA V1-V3 variable regions from 12 swine wastewater lagoon samples. Rarefaction curves were constructed with a 97% sequence similarity threshold.

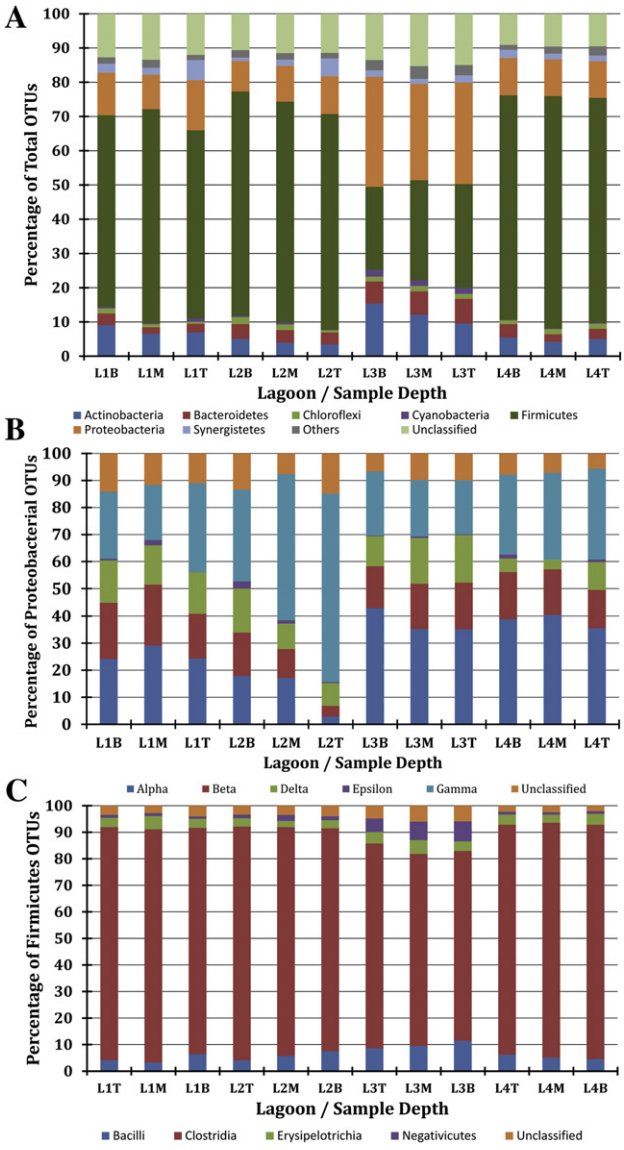

Fig. 2. Relative abundance of bacterial 16S rDNA genes from swine wastewater lagoon samples at the phylum level (A), and breakdown of proteobacteria (B), and Firmicutes (C) OTUs as determined by classification using the Ribosomal Database Project (RDP).

(4.5% ± 1.6), Peptostreptococcaceae (2.2% ± 1.8), Synergistaceae (2.5% ± 1.5), and Chromatiaceae (2.7% ± 1.9). For the L3 sites, well represented families also included Syntrophaceae (1.8% ± 0.5), Methylocystaceae (3.0% ± 0.6), Family IIa (1.6% ± 0.2) of the phylum Cyanobacteria, and the Mycobacteriaceae (1.3% ± 0.4).

Wastewater characteristics were collected for each depth from all four lagoons (Table 2). The characteristics of these lagoons were typical of swine anaerobic lagoons found in the mid-South United States [3,35,36]. All four lagoons presented as anaerobic, reduced environments, with L3 having an ORP indicative of the potential for denitrification, while the others had values typical of sulfate-reduction or methanogenesis. Lagoon pH was slightly alkaline. For L2, TSS and VSS values were elevated, indicating potential overloading, however both TKN and NH_4–N values were within typical ranges for swine anaerobic lagoons, indicating proper lagoon function. These data were used in nonmetric multidimensional scaling (NMS) to examine the relationship between the environmental variables and the microbial community structure of each lagoon (Fig. 5). The microbial community profiles taken from

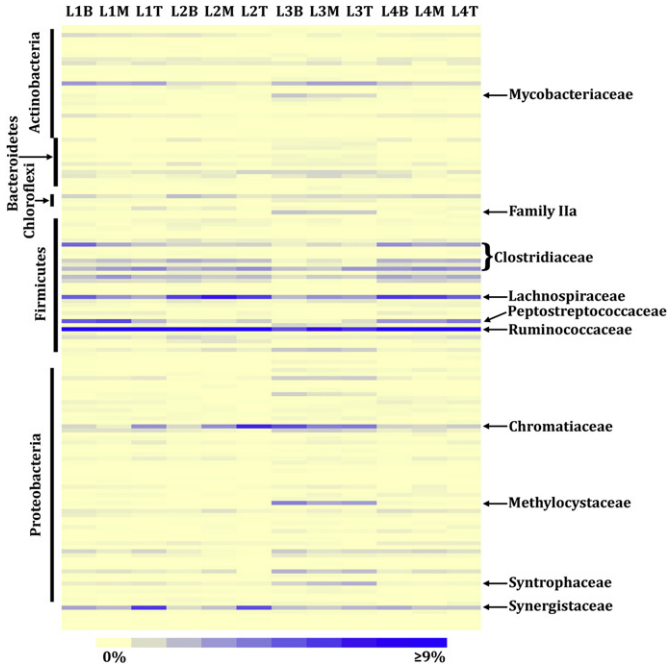

Fig. 3. Heat-map displaying relative abundances of bacterial families. Sites are distinguished by columns, while families are represented as rows. Labeled bars on left of the map indicate different phyla, while specific families are displayed to the right of the map. Color intensity, key provided underneath, indicates the particular families relative abundance in each site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

each site clustered by lagoon, indicative of lagoon-specific phylotypes distributed throughout the water column. Additionally, a number of relationships between microbial communities and environmental variables were identified. Environmental variables that correlated with community structure were TKN ($r^2 = 0.92$), COD ($r^2 = 0.69$), and ORP ($r^2 = 0.68$) along the first axis, and TSS ($r^2 = 0.49$) and DO ($r^2 = 0.48$) along the second axis.

At the genus level, there were 839 genera represented; 223 genera were found in all four lagoons and a total of 321 genera were identified in only one lagoon (Fig. 5). For the four lagoons sampled in this study, the top 25 genera accounted for approximately 20% of all phylotypes (Table 3). The dominate genera, determined by the relative abundance of classified phylotypes, varied from lagoon to lagoon as follows: L1, *Clostridium* cluster XI (3.14%); L2, *Thiolamprovum* (3.81%); L3, *Methylocystis* (2.77%); L4, *Anaerovorax* (2.78%). The low relative abundances of a majority of the genera suggest that most phylotypes are present at low levels.

4. Discussion

Next-generation sequencing (NGS) is rapidly gaining popularity for ecological studies due to its ability to generate data in a cost effective, high-throughput manner. Rather than replace other microbiological and molecular methods, deep 16S rDNA analysis by NGS can provide complementary analysis by identifying members of bacterial communities that were unable to be highlighted by other methods.

For example, studies examining the microbial diversity of anaerobic lagoons [9,37], or alternative swine manure storage systems such as deep pits [11,12], have traditionally utilized either bacterial culture, or the use of clone libraries. These studies however, when compared to the results presented in this study, have identified only a small portion of the organisms inhabiting these

Table 2
Lagoon characteristics.

	Units	L1T	L1M	L1B	L2T	L2M	L2B	L3T	L3M	L3B	L4T	L4M	L4B
Depth[a]	cm	15	101	191	15	73	131	15	81	147	15	70	122
Operation		Finish			Farrow to finish			Farrow to finish			Farrow		
Area	ha	1.582			2.68			0.54			0.58		
Swine	*n*	5280			9200			1,000			2,900		
TOC	mg L^{-1}	439 ± 39	474 ± 42	488 ± 89	909 ± 22	881 ± 65	913 ± 79	79 ± 8	90 ± 15	83 ± 6	930 ± 47	913 ± 33	888 ± 48
COD[b]	mg L^{-1}	1430 ± 65	1310 ± 94	1323 ± 46	2663 ± 848	881 ± 65	913 ± 79	79 ± 8	90 ± 15	83 ± 6	702 ± 73	719 ± 70	761 ± 30
BOD	mg L^{-1}	272 ± 39	287 ± 25	280 ± 38	285 ± 43	2235 ± 21	2458 ± 67	648 ± 56	643 ± 59	645 ± 34	1874 ± 193	1905 ± 218	1885 ± 201
DO[b]	mg L^{-1}	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	1.1 ± 0.7	271 ± 55	276 ± 51	54 ± 6	55 ± 5	55 ± 19	191 ± 84	175 ± 10	210 ± 106
ORP/eH[b]	mV	−86 ± 25	−141 ± 15	−170 ± 11	−253 ± 16	1.0 ± 0.5	1.2 ± 0.6	1.3 ± 0.3	1.1 ± 0.3	1.1 ± 0.4	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
pH[b]		8.0 ± 0.0	8.0 ± 0.0	7.9 ± 0.1	7.7 ± 0.0	−314 ± 45	−330 ± 33	44 ± 9	24 ± 20	−4 ± 40	−151 ± 24	−170 ± 19	−187 ± 17
Conduct[b]	S m^{-1}	6440 ± 70	6478 ± 70	6623 ± 66	10680 ± 236	7.6 ± 0.0	7.3 ± 0.1	7.5 ± 0.0	7.5 ± 0.0	7.5 ± 0.0	7.6 ± 0.1	7.7 ± 0.0	7.6 ± 0.1
TSS[b]	mg L^{-1}	314 ± 60	322 ± 44	337 ± 35	4373 ± 58	10390 ± 55	9462 ± 501	2188 ± 21	2168 ± 45	2248 ± 126	6626 ± 85	6481 ± 113	6431 ± 20
VSS	mg L^{-1}	259 ± 57	255 ± 44	263 ± 35	2194 ± 29	4429 ± 65	4737 ± 778	187 ± 9	243 ± 86	264 ± 139	410 ± 29	444 ± 16	435 ± 21
NH_4–N	mg L^{-1}	264 ± 16	264 ± 7	254 ± 8	397 ± 34	2223 ± 32	2377 ± 390	142 ± 4	190 ± 84	219 ± 142	316 ± 30	337 ± 7	345 ± 19
PO_4–P	mg L^{-1}	43 ± 2	44 ± 2	42 ± 5	27 ± 2	380 ± 23	366 ± 37	72 ± 9	74 ± 9	73 ± 8	304 ± 56	327 ± 76	315 ± 46
NO_x–N	mg L^{-1}	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.4 ± 0.1	29 ± 3	31 ± 5	74 ± 3	75 ± 7	76 ± 7	51 ± 6	51 ± 1	51 ± 2
TKN[b]	mg L^{-1}	338 ± 16	341 ± 19	335 ± 25	477 ± 21	0.4 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	2.2 ± 0.8	0.9 ± 0.4	2.7 ± 2.6
TP[b]	mg L^{-1}	59 ± 1	60 ± 2	60 ± 3	30 ± 3	537 ± 43	484 ± 21	87 ± 4	88 ± 2	92 ± 6	514 ± 60	547 ± 68	524 ± 77
TN	mg L^{-1}	264 ± 16	264 ± 7	254 ± 8	478 ± 21	35 ± 5	32 ± 1	84 ± 3	84 ± 3	88 ± 2	66 ± 3	68 ± 2	68 ± 7
Cl[b]	mg L^{-1}	139 ± 4	139 ± 3	138 ± 7	189 ± 2	537 ± 43	484 ± 21	87 ± 4	88 ± 2	93 ± 6	516 ± 59	548 ± 68	526 ± 77

[a] Maximum lagoon depths were as follows: L1, 206 cm; L2, 146 cm; L3, 162 cm; L4, 140 cm.
[b] Used in NMS ordination analyses.

Table 3
Relative abundance (%) and number of operational taxonomic units (OTUs) of top 25 genera.

Phylum	Genus	Lagoon #1		Lagoon #2		Lagoon #3		Lagoon #4	
		Ra	# OTUs	Ra	# OTUs	Ra	# OTUs	Ra	# OTU
Firmicutes	*Anaerovorax*	2.58%	96	2.45%	126	1.94%	73	2.78%	85
Proteobacteria	*Thiolamprovum*	1.59%	59	3.81%	196	1.38%	52	1.14%	35
Firmicutes	*Clostridium* sp. cluster XI	3.14%	117	0.70%	36	0.40%	15	2.16%	66
TM7	TM7	0.89%	33	1.24%	64	1.04%	39	1.34%	41
Firmicutes	*Clostridium* (sensu stricto)	1.53%	57	0.64%	33	0.48%	18	1.63%	50
Firmicutes	*Oscillibacter*	0.43%	16	1.13%	58	0.85%	32	0.98%	30
Actinobacteria	*Leucobacter*	1.67%	62	0.45%	23	0.37%	14	0.78%	24
Proteobacteria	*Methylocystis*	0.11%	4	0.10%	5	2.77%	104	0.00%	0
Firmicutes	*Tissierella*	0.67%	25	0.86%	44	0.05%	2	1.18%	36
Firmicutes	*Acetanaerobacterium*	1.05%	39	0.56%	29	0.16%	6	1.05%	32
Firmicutes	*Turicibacter*	1.42%	53	0.40%	19	0.13%	5	0.85%	26
Firmicutes	*Saccharofermentans*	0.67%	25	0.70%	36	0.67%	25	0.33%	10
Firmicutes	*Lachnospiracea*	0.38%	14	0.80%	41	0.56%	21	0.62%	19
Actinobacteria	*Corynebacterium*	0.73%	27	0.52%	27	0.59%	22	0.56%	17
Firmicutes	*Sporobacter*	0.54%	20	0.76%	39	0.05%	2	0.75%	23
Synergistetes	*Aminobacterium*	0.73%	27	0.51%	26	0.24%	9	0.26%	8
Firmicutes	*Lactobacillus*	0.38%	14	0.45%	23	0.64%	24	0.29%	9
Proteobacteria	*Desulfomonile*	0.38%	14	0.06%	3	1.23%	46	0.20%	6
Proteobacteria	*Acinetobacter*	0.40%	15	0.45%	23	0.19%	7	0.59%	18
Actinobacteria	*Mycobacterium*	0.13%	5	0.08%	4	1.25%	47	0.03%	1
Cyanobacteria	Group IIa	0.00%	0	0.00%	0	1.52%	57	0.00%	0
Actinobacteria	*Actinomyces*	0.70%	26	0.25%	13	0.11%	4	0.39%	12
Proteobacteria	*Thioflavicoccus*	0.00%	0	0.00%	0	1.46%	55	0.00%	0
Bacteroidetes	*Prevotella*	0.16%	6	0.27%	14	0.53%	20	0.42%	13
Actinobacteria	*Klugiella*	0.02%	1	0.00%	0	1.09%	41	0.07%	2
Total		20.30%	755	17.19%	882	19.70%	740	18.40%	563

ecosystems. For example, studies by Whitehead and Cotta [12], Cotta et al. [11], Goh et al. [9], and Cardinali-Rezende [37] identified a total of approximately 200 bacterial phylotypes in 60 genera, many of which overlap. In contrast, this study identified 15,682 identified phylotypes, classified into 839 distinct genera. It should be noted however that all the aforementioned studies characterized populations consisting primarily of anaerobic, Gram-positive, low G + C bacteria involved in odor production, fermentation, and nutrient cycling. These studies provide a very important, initial step, into elucidating bacterial community structure and function within these lagoons. However the caveat that presents itself in studies which employ DNA amplification, with or without cloning, is that they are incapable of differentiating between viable and dead organisms [38]. Likewise, even studies relying on culture techniques cannot differentiate between dormant organisms versus those which are actively growing in the environment. These become particular points of concern when dealing with swine production systems, as the animal management practices utilize a variety of antimicrobial compounds which are eventually excreted into swine anaerobic lagoons [39]. In order to address these issues in future studies, we propose utilizing NGS techniques such as metatranscriptomics, to look at the functional microbial populations of anaerobic lagoons [40].

A sizeable body of work has been performed on anaerobic lagoons to identify and quantify organisms responsible for malodorous compounds, in particular hydrogen sulfide (H_2S). Hydrogen sulfide is produced by sulfate-reducing bacteria (SRB) upon the utilization of sulfate as a terminal electron acceptor during the degradation of organic compounds. Cook et al. [15] utilized qPCR and sequencing of the *dsrA* gene, that encodes for the enzyme dissimilatory sulfite reductase, to determine the abundances and types of SRB in swine manure pits and anaerobic lagoons. Their study utilized primers directed towards SRB from the genera *Desulfobacterium*, *Desulfobulbus*, and *Desulfovibrio* [15]; to date, over 30 known genera of SRB have been characterized [41]. In our study phylotypes were classified to 26 SRB genera, with phylotypes classified to seven SRB genera found in all four anaerobic lagoons. These seven were *Desulfatirhabdium*, *Desulfobulbus*, *Desulfocurvus*, *Desulfomonile*, *Desulfovibrio*, *Desulfovirga*, and *Thermodesulfobium*. Of the remaining 19, five were identified in three lagoons with *Desulfoglaeba*, *Desulfonispora*, and *Desulfosporosinus* in L1,L 2, and L4, and *Desulfatiferula* and *Desulforegula* in L1, L2, and L3. Five additional genera were identified in two lagoons, *Desulfobacca* and *Desulfofaba* in L1 and L3, *Desulfofustis* in L1 and L2, and *Desulfococcus* and *Desulfomicrobium* in L1 and L4. Lastly, *Desulfarculus* (L3), *Desulfonema* (L1), *Desulforhabdus* (L3), *Desulforhopalus* (L3), *Desulfospira* (L3), *Desulfovermiculus* (L3), *Desulfurispora* (L4), *Syntrophobacter* (L2), and *Thermodesulforhabdus* (L3) were all identified in a single lagoon. In addition to being an odorous compound, H_2S is part of the larger S-cycle, and a number of phylotypes associated with other steps in S-cycling were also detected. These phylotypes were classified as genera known to be involved in the reduction of elemental sulfur (*Desulfuromonas*, L4), sulfite (*Desulfitibacter*, L1 and L3), and thiosulfate (*Dethiobacter*, L2; *Dethiosulfatibacter*, L1 and L2; and *Dethiosulfovibrio*, L4).

Examination of the genera identified in this study, and their relative abundances, revealed that the relative abundance of *Desulfomonile* was higher in L3 as compared to the other lagoons (Table 2). This is interesting because examination of the lagoons anaerobic characteristics, in particular ORP values, suggests that this lagoon is more likely to use nitrate − ORP range of +50 mV to −50 mV − to degrade organic compounds as opposed to sulfate [42]. On the other hand, the remaining three lagoons had ORPs in a range ($\leq$−50 mV) conducive to sulfate-reduction, and concomitantly, H_2S production. Earlier studies have demonstrated that anaerobic lagoons undergo mixing of the water column [43], and the abundance of OTUs classified as SRB in L3 may be resultant of this mixing process, as populations from more reduced layers are brought up through the water column. It has also been demonstrated that there is a periodic effect to the chemical makeup of these lagoons [44], with L3 potentially undergoing a slightly less reduced phase. In this case, the SRB may be dormant, only active in

increasingly anaerobic portions of the lagoon (e.g., sludge layer), while the overall conditions favor denitrification. While such conditions could favor amelioration of lagoon odor, the presence of SRB indicates that a return to more reduced conditions may result in renewed odor production.

In order to produce H_2S, SRB oxidize a number of endogenously and exogenously produced fermentative products. A number of phylotypes were classified in this study that identify with genera, such as *Saccharofermentans* [45], that are capable of fermenting numerous carbohydrates. Other organisms, belonging to genera such as *Aminobacterium* [46], are capable of degrading proteins. Together, these processes produce substrates utilized by SRB during growth. For example, a non-comprehensive list of products include: acetate, butyrate, ethanol, lactate, and propionate. Our study revealed a number of phylotypes classified to genera, also not to be considered comprehensive, known to produce these SRB substrates. The following fermentation products along with genera known for its production were identified in all four lagoons: acetate, *Acetanerobacterium*, *Acetivibrio*, and *Acetomicrobium*; butyrate, *Anaerovorax*, *Butyricicoccus* and *Butyrivibrio*. ethanol, *Ethanoligenens*; lactate; *Lactobacillus* and *Lactococcus*; and propionate, *Propionibacterium*.

Additionally, acetate can undergo a dismutation reaction by methanogenic archaea to produce methane (CH_4) and carbon dioxide (CO_2) [47]. While we did not attempt to identify the archaea in this study, the low redox conditions (see Table 3) as indicated by ORP, and the presence of phylotypes classified to several methanotrophic genera, suggest that methanogenesis is occurring to some degree in, if not the water column, then the sludge layer of these anaerobic lagoons. While no phylotypes classified as methanotrophic were found across all four lagoons, *Methylocystis* (L1, L2, and L3) and *Methylohalobius* (L1, L2 and L4) were each found in three.

Although a majority of respiration in anaerobic lagoons involves sulfate-reduction or methanogenesis, it has been demonstrated that anaerobic lagoons are also capable of denitrification [7]. This was confirmed in a report by Ducey et al. [6], that quantified the abundance of nitrification and denitrification genes in the water column of eight anaerobic swine wastewater lagoons. They hypothesized that, though redox conditions tend to favor sulfate-reduction and methanogenesis, nitrification and denitrification could potentially occur in select microenvironments, such as the surface–liquid interface, where sufficient oxygen diffusion exists [48]. The identification of significant numbers of nitrifiers in the Ducey et al. study are supported by the identification of genera known to be involved in the oxidation of ammonia (NH_3) (*Nitrosomonas* and *Nitrosococcus*) and nitrite (NO_2) (*Nitrospira*) in all four lagoons. Two additional genera, involved in the oxidation of NH_3 (*Nitrosospira*) and NO_2 (*Nitrobacter*) were found in L1 and L4. These findings are further supported by the preponderance of ammonia oxidizing archaea (AOA) identified in a Brazilian anaerobic lagoon [37].

A cursory examination of the distribution of organisms discussed above, all involved in a variety of biological processes, demonstrates that a significant portion of the microbial community is conserved across lagoons. There remains however, a portion that is specific for each. This observation is reflected both in the NMS (Fig. 4) and Venn diagrams (Fig. 5). These observations also demonstrate that anaerobic lagoons are more microbially diverse that previously understood, though the impact that some of these rare species have in the functioning of anaerobic lagoons remains to be elucidated. Further study also needs to be conducted on the interplay between a lagoons microbial community, and its chemical and nutrient characteristics. These characteristics in turn are controlled by a number of extraneous factors which are reflected in the management principles for each operation. These factors include, but are not limited to, differences in the following: dietary supplements and feed [49]; use of antibiotics [1,50]; number of animals [51]; lagoon depth and acreage [52]; and the surrounding soil microbial ecology [53].

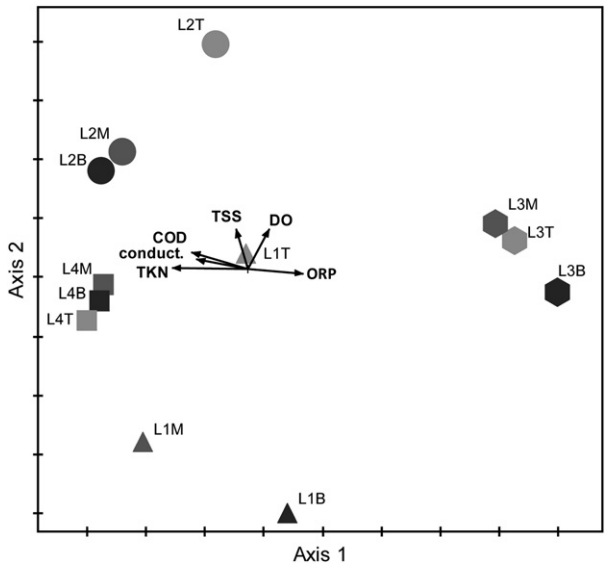

Fig. 4. Nonmetric multidimensional scaling (NMS) plot of microbial communities (based on the relative abundance of bacterial families) identified in the 12 lagoon sites sampled in this study. Only explanatory environmental variables with an $r^2 > 0.5$ are included as vectors. Lagoons are designated by symbol (L1, triangle; L2, circle; L3, hexagon; L4, square), with increasing depth indicated by darkening of the symbol.

Examination of NMS revealed no strong connection between lagoon microbial community structure and operation type. The two lagoons associated with farrow to finish operations (L2 and L3) were separated along the first axis of the NMS ordination, with L2 clustered more closely to lagoons associated with finish (L1) and farrow (L4) operations. The chemical characteristics of these three lagoons were more similar to each other, as compared to L3. It should be noted that L3 was associated with a smaller number of animals and would, as a consequence, receive lower inputs of manure. As mentioned previously, L3, while still anaerobic, was less reduced than the other three lagoons, and also had lower levels of nitrogen (NH4-N, TKN, and TN), suspended solids (TSS and VSS), organic carbon (TOC), oxygen demand (COD and BOD), and EC. Reduced levels of salts, as measured by EC, could be of particular significance. A report by Georgacakis and Sievers reported altered bacterial activity in manure with high concentrations of salt [54],

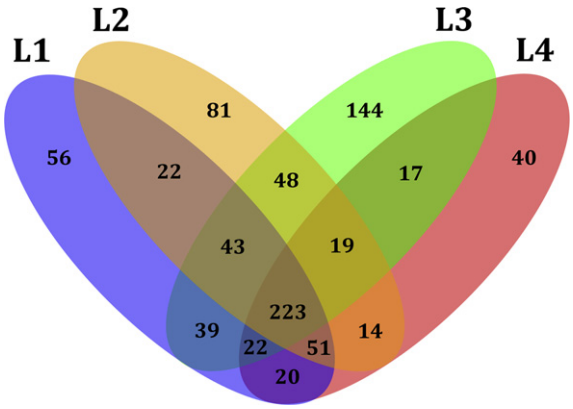

Fig. 5. Venn diagram of all 839 identified genera distributed across the four lagoons examined in this study.

while McLaughlin et al. reported correlations between EC and levels of certain bacterial groups [3].

The NMS results also indicate that depth was a factor in microbial community composition, though the effect varied from lagoon to lagoon. Analysis of NMS revealed significant separation of the microbial communities at each depth in L1. Conversely, while L2T and L3B clustered loosely with the two remaining depths from their respective lagoons, when compared to the other lagoon samples, they remained most closely related to the samples collected from the same lagoon. Taken together, these results indicate a degree of mixing amongst the lagoon layers, a finding that is supported by previous studies [5,6].

Significant portions of the phylotypes identified were found in relatively low abundances. Despite low relative abundances, such organisms should not be disregarded. These organisms may occupy a specialized niche within the lagoon ecosystem, or they may perform similar or overlapping functions with similarly low proportioned organisms, thereby having a meaningful, cumulative effect. Further studies will need to be performed in order to substantiate either of these hypotheses.

5. Conclusions

Similar to other studies examining anaerobic swine wastewater lagoons, our results found a large number of phylotypes that could be classified to organisms that performed functions typical of these ecosystems. These functions include odor production, fermentation, sulfate-reduction, as well as N- and S-cycling. A majority of the phylotypes represented anaerobic, Gram-positive organisms, with low G + C content. A significant portion of the phylotypes identified in this study were found across all four lagoons, but each lagoon did contain its own distinct population.

In 2001, Whitehead and Cotta [12] discussed the potential for a large number of undefined microbial organisms in swine wastewater treatment and storage systems. Since that time however, no studies of a large scope, designed to identify those microbes, have been undertaken. The results of our study constitute a significant and revealing characterization of anaerobic swine wastewater lagoon microbial community structure and diversity.

Acknowledgements

We thank the members of the Core for Applied Genomics and Ecology at the University of Nebraska at Lincoln, in particular Andrew K. Benson and Jaehyoung Kim, to whom we contracted out the pyrosequencing. We would also like to thank Ariel A. Szogi for technical assistance in the revision of this manuscript. 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.

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