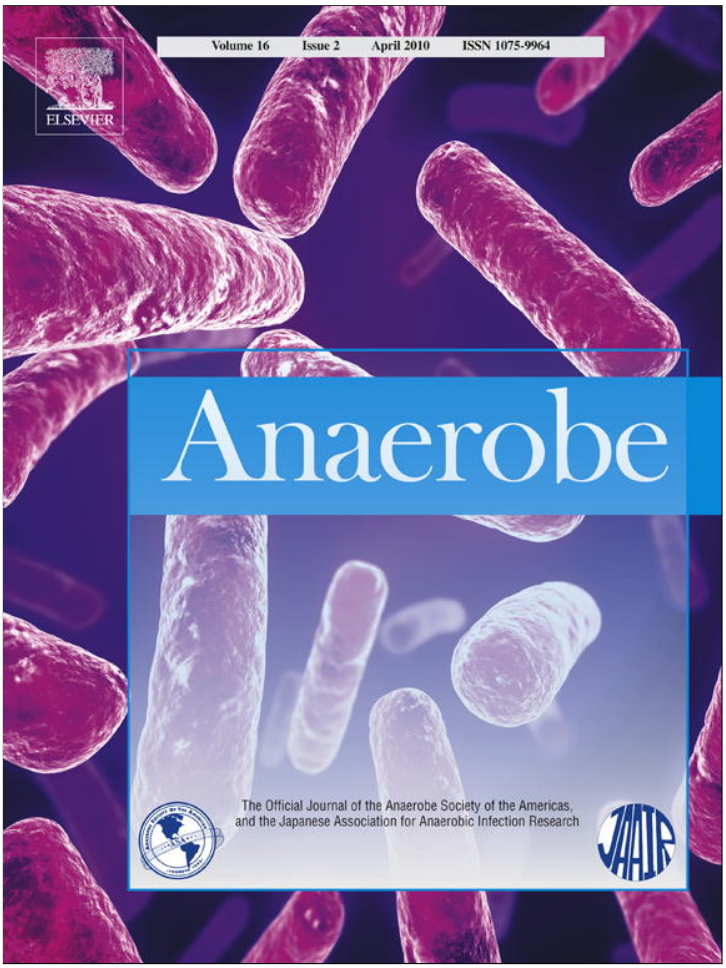
Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.



This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

http://www.elsevier.com/copyright

Author's personal copy

Anaerobe 16 (2010) 74–82

Contents lists available at ScienceDirect

Anaerobe

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

Ecology/environmental microbiology

Spatial and temporal changes in the microbial community in an anaerobic swine waste treatment lagoon

Kimberly L. Cook [a,*], Michael J. Rothrock Jr. [a,1], Nanh Lovanh [a], John K. Sorrell [b], John H. Loughrin [a]

[a] USDA-ARS, AWMRU, Bowling Green, KY 42104, USA
[b] Western Kentucky University, Bowling Green, KY 42104, USA

ARTICLE INFO

Article history:
Received 3 December 2008
Received in revised form
3 June 2009
Accepted 9 June 2009
Available online 16 June 2009

Keywords:
Waste treatment
Denaturing gradient gel electrophoresis (DGGE)
Lagoon
Odor
Swine

ABSTRACT

Microorganisms are central to both the beneficial (organic degradation, nutrient removal, biogas production) and detrimental (odor production, pathogen contamination) effects of swine waste storage systems. In this study, both quantitative (real-time polymerase chain reaction) and qualitative (denaturing gradient gel electrophoresis, cloning, sequence analysis) molecular analyses were used to track spatial and temporal changes in the microbial community of swine slurry from a 0.4 ha anaerobic lagoon. The lagoon, located in a region of western Kentucky which has a humid, subtropical environment, was sampled on a monthly basis ($n = 10$) over a period of one year at four different depths (top, 51 cm from the top, 152 cm from the top, and bottom >198 cm). The concentration and diversity of *Bacteroides* sp. was seasonal (up to 90% decrease between March and June). *Hespellia* sp. and other clostridial species, on the other hand, were endemic in the slurry (concentrations up to 1.0×10^7 cells mL^{-1} slurry) regardless of time of the year or lagoon depth. Results suggest that there were seasonal effects on the microbial community in the swine lagoon, while the effect of depth was not as pronounced. Seasonal changes in the microbial community in stored wastes may be (directly or indirectly) correlated with changes in malodor emissions from lagoons.

Published by Elsevier Ltd.

1. Introduction

Over the last 30 years animal production has become increasingly intensive with fewer operations producing larger numbers of animals. As a consequence, the animal waste is produced and maintained in fewer facilities and in greater concentrations [1]. In swine production systems, this mixture of feces, urine, and wash water is stored in pits beneath the facility or in lagoons located adjacent to confinement areas. The manure is a valuable resource for crop fertilization and soil conditioning. However, there are also significant concerns associated with the handling of increasing volumes of swine wastes including malodor emissions and pathogen contamination. Land application of waste products may pose an additional risk to environmental resources through release of excessive nutrients, salts and organic materials, among others [2,3]. Additionally, long term or excessive application of manure may lead to eutrophication of water sources or soil deterioration.

With the national swine inventory exceeding 65 million [4] and steadily increasing, there is greater need to improve swine waste storage and handling to maximize the benefits while mitigating any negative consequences of animal waste utilization. Microorganisms are central to both the beneficial (organic degradation, nutrient removal, biogas production) and detrimental (odor production, pathogen contamination) effects of swine waste storage systems. Therefore, understanding more about how the microbial community functions in stored swine manure should aid in developing better means for management and usage of waste materials.

The microbial community in swine slurry has been characterized by traditional culture methods [5], 16S rDNA clone sequence analysis [6,7] or other molecular microbial community analysis techniques [8–10]. These studies showed that the slurry community is dominated by low G + C gram positive bacteria (predominantly *Clostridium–Eubacterium* species) and *Bacteroides* from the gram-negative bacteria. This is similar to the population in swine feces in which 81% of clones belonged to the low G + C gram positive bacteria and 11% to *Bacteroides* [11]. As in most

Abbreviations: DGGE, Denaturing gradient gel electrophoresis; QRT-PCR, Quantitative, real-time PCR; CE, *Clostridia/Eubacteria*; BPP, *Bacteroides/Prevotella/Porphryomonas*; PCA, Principle component analysis; UPGMA, Unweighted pair group method with arithmetic mean.

* Correspondence to: Kimberly L. Cook, USDA-ARS, AWMRU, 230 Bennett Lane, Bowling Green, KY 42104, USA. Tel.: +1 270 781 2579x232; fax: +1 270 781 7994.
E-mail addresses: kim.cook@ars.usda.gov (K.L. Cook), michael.rothrock@ars.usda.gov (M.J. Rothrock Jr.), nanh.lovanh@ars.usda.gov (N. Lovanh), john.sorrell@wku.edu (J.K. Sorrell), john.loughrin@ars.usda.gov (J.H. Loughrin).
[1] Present address: Coastal Plains Soil, Water and Plant Research Center, 2611 W. Lucas Street, Florence, SC 29501-1242, USA.

1075-9964/$ – see front matter Published by Elsevier Ltd.
doi:10.1016/j.anaerobe.2009.06.003

Author's personal copy

other environments, culturability is very low in swine slurry (20% or less) [5]. Most of the culturable organisms can be identified (95–97% 16S rDNA sequence similarity to known organisms) while less than 50% of clones from 16S rRNA gene libraries are closely related to those of known organisms [6,7,10]. Others have found pathogenic organisms including *Salmonella* sp. and *Campylobacter* sp. in slurries [12,13]. These recent studies have provided insight into the dominant species in swine slurry and data on the occurrence of pathogens, but more information is needed to understand how the microbial community responds to chemical, physical and environmental fluctuations in these waste storage systems.

More than 150 chemical compounds can be emitted from waste storage systems including small-chain volatile fatty acids, phenols, indoles, ammonia and hydrogen sulfide [9,14,15]. Many of these compounds are problematic due to their low threshold for detection and difficulties involved in identifying any direct relationship between odor production from livestock facilities and concentrations in air emissions. For example, hydrogen sulfide and other sulfur-containing compounds are responsible for as much as half of the odorants in swine waste [15,16]. However, the form and concentration of the emissions depends on a complex interaction of factors including transport efficiency, lagoon physical parameters (temperature and pH) and differences in microbiological populations and activities [3,15].

Many of these malodorous compounds are generated as a consequence of metabolism of waste components by microorganisms [3,14,17]. However, much less is known about the microbial community dynamics in waste storage systems than is known about the physical or chemical parameters within those systems [9,18]. Merrill and Halverson [9] evaluated variations in microbial communities associated with stored swine manure in lagoons. They found that there were seasonal changes in community profiles that may be linked with malodor emissions. However, the method they used for community analysis (fatty acid methyl ester (FAME)) did not permit identification of specific organisms that were associated with shifts in the community. Peu et al. [10] used PCR-single-strand conformation polymorphism (SSCP) to show that the microbial community in anaerobic systems stabilized within 2–3 weeks of storage in anaerobic tanks or lagoons, but shifts significantly on movement from one location to another (i.e., from pit storage to lagoon storage). This kind of information regarding the response of microbial populations to physical and environmental shifts within stored swine slurries is essential for development of systems that will function effectively to reduce odors and pathogens. In this study, molecular analyses (denaturing gradient gel electrophoresis (DGGE), quantitative, real-time PCR (QRT-PCR), cloning and sequence analysis) were used to evaluate temporal (one year of sampling) and spatial (four depths) changes in microbial communities in a swine manure storage lagoon.

2. Materials and methods

2.1. Sample collection and chemical analyses of swine lagoon samples

Swine slurry samples were taken from an anaerobic swine lagoon (0.4 ha) from a farrowing operation (~2000 sows). The lagoon is located in a region of western Kentucky which has a humid, subtropical environment with an average temperature of 19 °C. The area receives an average rainfall of 131 cm per year. The slurry, a mixture of feces, urine and water used to clean stalls, was stored for about 2 weeks in pits located under the house before being evacuated by a flushing procedure to the lagoon. Slurry samples from the lagoon were taken at four different depths (top, 51 cm from the top, 152 cm from the top, and bottom >198 cm) with a pulley system designed so samples can be retrieved at specific depths. The bottom of the lagoon ranged from 250 cm to 300 cm, except at drawdown. Slurry from the lagoon was land-applied during September 2006 and April 2007 resulting in a drawdown of the lagoon depth to 124 cm and 170 cm, respectively. Samples were taken from all depths approximately every month ($n = 10$) from June 2006 to May 2007. Lagoon temperature was monitored with two HOBO water temperature probes (Onset Computer Corporation, Bourne, MA). One was floated on the surface of the lagoon while the other was weighted with lead sinkers and sunk to the bottom of the lagoon. Both probes were moored to an anchor at the edge of the lagoon for retrieval.

A combination electrode (Fisher Scientific, Hampton, NH) was used to determine pH. Chemical oxygen demand (COD) was determined using dichromate reactor digestion kit for high strength wastewater (Chemetrics Inc., Calverton, VA). One mL samples were digested for 2 h at 150 °C and after the samples cooled, absorbance was read at 620 nm. Total dissolved solids (TDS) were determined according to standard method [19]. TDS were filterable-solids fraction which consists of both organic and inorganic molecules and ions that are present in true solution in water. Total organic carbon (TOC) was determined by combustion [20] of the lagoon water using a Vario Max CN analyzer (Elementar Americas, Inc. Mt. Laurel, NJ).

2.2. DNA extraction and PCR amplification

DNA was extracted from swine lagoon slurry samples (0.5 mL) in duplicate for each stratum on each sampling day using the FastDNA® Spin kit for soils (MP Biomedical, Solon, OH, USA) according to manufacturer's specifications. Specific PCR primer sets were used to target the 16S rRNA gene of the total microbial population, the *Clostridia/Eubacteria* (CE) group, or the *Bacteroides/Prevotella/Porphryomonas* (BPP) group in the swine lagoon (Table 1). One to 10 ng of DNA extract was used as PCR template with the appropriate primer set (with 800 nM of each primer).

2.3. DGGE

Total bacterial community 16S rDNA was amplified from the bulk DNA extracts (using 1–10 ng of DNA per PCR) with the general bacterial PCR primer set 341F-GC and 907R, using a previously described PCR protocol [21] in a PTC-200 DNA thermal cycler (MJ Research, Las Vegas, NV). To evaluate the community structure of two of the dominant swine lagoon slurry bacterial groups the *Clostridia/Eubacteria* [22] or the *Bacteroides/Prevotella/Porphryomonas* [23], group-specific PCR primer sets were used according to published protocols (Table 1). The GC designation on the 341F, BPP-F-GC, and W109-R-GC primers represents a 40 bp GC rich region on the 5′ end of the primer necessary to prevent complete denaturation of the DNA strands during electrophoresis. Sequences were amplified using HotStarTaq® MasterMix Kit (Qiagen Inc, Valencia, CA), with 800 nM each primer. The above PCR protocols were modified, adding an additional heating phase (95 °C for 15 min) at the beginning of the reaction, as suggested by the manufacturer of the HotStarTaq® polymerase.

DGGE was used to separate and characterize 16S rRNA gene sequences by using a gradient of denaturants (100% denaturant solution consisting of a combination of 40% [vol/vol] formamide and 7 M urea) in a polyacrylamide gel (37.5:1) to separate DNA fragments according to melting behavior (i.e. sequence, melting domains). GelBond PAG Film (Cambrex BioSciences Rockland, MA) was used during pouring of the DGGE gels to allow for easier manipulation of the polyacrylamide gel after electrophoresis.

Author's personal copy

Table 1
Sequences, target size and Tm of primers used in this study.

Target	Oligo Name	Sequence (5'-3')[a,b]	Tm (°C)	Insert size (Abp)	Application	Reference
All Bacteria	341-F	CCT ACG GGA GGC AGC AG	64.7	566	DGGE	[21]
	341-F-GC	cgc ccg ccg cgc ccc gcg ccc gtc ccg ccg ccc ccg ccc ggC CTA CGG GAG GCA GCA G	87.8			
	907-R	CCG TCA ATT CCT TTG AGT TT	60.5			
All Bacteria	1055-F	ATG GCT GTC GTC AGC T	54.0	337	QRT-PCR	[28]
	B16s-Taq115-F	(FAM)CAA CGA GCG CAA CCC(BHQ-1)				
	1392-R	ACG GGC GGT GTG TAC	59.0			
Bacteroides–Prevotella–Porphyromonas	BPP-F	GAA GGT CCC CCA CAT TG	61.4	418	DGGE	[23]
	BPP-F-GC	cgc ccg ccg cgc ccc gcg ccc gtc ccg ccg ccc ccg ccc gGA AGG TCC CCC ACA TTG	86.4			
	BPP-R	CAA TCG GCG TTC TTC GTG	59.3			
Clostridiaceae[c]/ *Eubacterium*	W18-F	GAG TTT GAT CMT GGC TCA G	57.4	552	DGGE & QRT-PCR	[10]
	W109-R	CCC TTT ACA CCC AGT AA	53.0			[22]
	W109-R-GC	cgc ccg ggg cgc gcc ccg ggc ggg gcg ggg gca cgg ggg gCC CTT TAC ACC CAG TAA	84.9			
Bacteroides spp.	AllBac296-F	GAGAGGAAGGTCCCCCAC	63.6	106	QRT-PCR	[29]
	AllBac412-R	CGCTACTTGGCTGGTTCAG	63.5			
	AllBac375-Bhq-R	(FAM)CCATTGACCAATATTCCTCACTGCTGCCT(BHQ-1)	74.7			

[a] Degeneracy codes: R = A + G, Y = C + T, K = G + T, M = A + C, S = G + C, W = A + T, H = A + T + C, and D = G + A + T.
[b] Lowercase sequence represents the GC clamped portion of the primer.
[c] Includes Clusters I, III, IV, XIVa, XIVb and rumen *Clostridia* spp.

Twenty-five μL of PCR product was electrophoresed through a 30–60% (total bacteria and CE group) or 30–50% (BPP group) denaturing gradient according to Nübel et al. [24] for 4 h at 200 V in a Bio-Rad DCode universal mutation detection (Bio-Rad Laboratories, Hercules, CA). The DGGE gels were stained with the Bio-Rad Silver Stain kit according to the manufacturer's specifications, and the images were captured using an Epson Perfection 4990 Photo Scanner (Epson, Long Beach, CA).

DGGE fingerprint analysis was performed using the Fingerprint II software program (Bio-Rad Laboratories, Hercules, CA) using the Basic, Clustering Analysis, Comparative Quantification and Polymorphism Analysis and Dimensioning Techniques modules. The gel images were imported into the software and analyzed according to manufacturer's specifications. The software package performed band matching between the fingerprints, allowing for the principle component analysis (PCA).

2.4. DNA sequencing and phylogenetic analysis

For DGGE band sequencing, each band was excised using a sterile scalpel and forceps and placed into 150 μL of 10 mM Tris buffer. 0.1 mm Zirconia/Silica beads (BioSpec Products, Inc., Bartlesville, OK) were added to each tube, and the samples were placed in a Fast Prep FP120 (Q-BIOgene, Irvine, CA) for 1 min at a speed of 5.5 ms^{-1} followed by overnight incubation at 4 °C. 2 μL of the solution was PCR amplified using the appropriate PCR primer sets (substituting an identical primer without the 40 bp GC-clamp; Table 1), reaction mixture and thermocycling conditions discussed above. The resultant PCR product was cloned into the pCR2.1®-TOPO® plasmid using a TA TOPO Cloning Kit (Invitrogen, Carlsbad, CA) according to manufacturer's specifications and sent to USDA-ARS MSA Genomics Laboratory (Stoneville, MS) for sequencing. DGGE band sequences were submitted to the BLASTn 2.2 search engine [25] to obtain putative phylogenetic assignments for each band. The DGGE band sequences, combined with appropriate known sequences (depending on DGGE bands analyzed) from the GenBank database, were aligned using *MEGA* version 3.1 [26]. The alignment files were used to create bootstrapped ($n = 1000$) Neighbor-joining trees, using the Kimura 2-parameter model in the *MEGA* version 3.1 software package. The classifier function of the Ribosomal Database Project II (RDP) [27] was used to assign 16S rRNA gene sequences to the RDP taxonomical hierarchy at the phylum, order and family levels of resolution. A total of 48 sequences were submitted to the GenBank database, and were assigned the accession numbers of EU834071–EU834119.

2.5. QRT-PCR

QRT-PCR analysis was carried out as previously described for total cells (16S rRNA gene copies) [28] and for all *Bacteroides* sp. [29] using the Qiagen HotStarTaq® MasterMix (Qiagen, Valencia, CA) and primers and probes shown in Table 1. The amplification mixture contained 3.0 mM $MgCl_2$, 600 nM each primer, 200 nM of probe and sample DNA or standard (from 10^2 to 10^8 copies). For 16S rRNA gene copies, the QRT-PCR program was 15 min at 95 °C, 39 cycles at 95 °C for 15 s, 58 °C for 45 s and 72 °C for 45 s. For *Bacteroides* sp., the QRT-PCR program was 15 min at 95 °C, 49 cycles at 95 °C for 30 s, and 60 °C for 45 s. Cell concentrations were calculated by dividing the copy number per mL of slurry by 4.0 or 6.0, the average copy number of 16S rRNA genes in all cells or in *Bacteroides* cells, respectively [30].

QRT-PCR analysis of the CE group was carried out using the primers used for DGGE analysis, but without the GC clamp (Table 1) in a QuantiTect™ SYBR® Green (Qiagen, Valencia, CA) PCR reaction. The amplification mixture contained 800 nM each primer, sample DNA or standard (10^1–10^8 copies). The QRT-PCR program was 15 min at 95 °C, followed by 40 cycles of 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 60 s. Since the SYBR Green dye binds to all double stranded DNA, a melting curve was performed after the reaction to ensure proper amplification had occurred. The melting curve parameters were temperatures ranging from 50 °C to 90 °C, with reads every 0.2 °C after a 1 s hold.

QRT-PCR assays were run on the DNA Engine Opticon 2 (MJ Research, Inc., Waltham, MA) in a total volume of 25 μL. Baseline values were set as the lowest fluorescence signal measured in the well over all cycles. The baseline was subtracted from all values and the threshold was set to one standard deviation of the mean. All PCR runs included duplicates of standards and control reactions without template. Standard DNA consisted of plasmid PCR 2.1 vector (Invitrogen, Carlsbad, CA) carrying the appropriate insert for

Author's personal copy

the given assay. DNA concentrations in each extraction were determined using the Hoechst 33258 nucleic acid stain (Invitrogen, Carlsbad, CA) and measured with a Hoefer DyNA Quant 200 fluorometer (Amersham Biosciences, San Francisco, CA) according to manufacturer's instructions.

3. Results and discussion

3.1. Spatial and temporal changes in total cell numbers

The distribution of cells over the depth of the lagoon was fairly homogenous (Fig. 1A). Total cell numbers averaged $1.4 \pm 0.7 \times 10^8$, $1.1 \pm 0.6 \times 10^8$, $2.7 \pm 1.3 \times 10^8$, and $1.7 \pm 1.3 \times 10^8$ cells mL^{-1} slurry at the top, 51 cm, 152 cm and at the bottom (>198 cm) of the lagoon. The similarity in cell concentrations at the top and bottom of the lagoon occurred despite the fact that all chemical parameters (except pH) were higher at the bottom of the lagoon than at the other depths (Table 2). Although, total cell numbers from this study were lower than those obtained by microscopic cell counts of slurry ($0.6–1.0 \times 10^{10}$ cells mL^{-1} slurry), they were the same or higher than culturable counts ($1 \times 10^6–2 \times 10^9$ cells mL^{-1} slurry) [5,8,31].

Total cell numbers in samples taken from 51 cm and from the lagoon bottom increased significantly ($p < 0.05$; over 87% and 79%, respectively) between Mar and Aug (Fig. 1A). Temperature is the most likely cause of the increase in total cell numbers in the slurry rather than a change in livestock management since this is a farrowing operation and as such is highly stable in its routine. Slurry temperatures during summer months (June, July and Aug) averaged 29.3 ± 1.2 °C, while winter month temperatures (Dec thru Feb) averaged 8.1 ± 2.3 °C (Fig. 2). Temperatures in spring (Mar, April and May) and fall (Sept, Oct, and Nov) were transitional and were therefore were more variable over the seasonal span (18.0 ± 4.7 °C and 18.2 ± 5.2 °C, respectively). The change in cell numbers between Mar and Aug tracked with an increase in slurry temperature of 16–18 °C (Fig. 2), but no consistent changes in chemical or physical lagoon characteristics over that time period (data not shown). UPGMA analysis of DGGE banding patterns showed that samples taken on the same date exhibited more similarity among themselves (greater than 70%) than with samples taken on other dates, regardless of depth (data not shown). These results and those from the QRT-PCR suggest that there is greater temporal variability in the general microbial population than spatial variability. Samples taken from the top of the lagoon and from 152 cm did not change significantly ($p > 0.05$). It is uncertain why the mid-levels of the lagoon would be less impacted by the seasonal change than would the bottom.

There was another significant ($p < 0.01$) increase in cell numbers in Sept. This increase may be partially associated with the drawdown of the lagoon which occurred during that month. During this time there was also an increase in COD (25%), TDS (41%) and TOC (29%). However, by Sept there had already been a significant increase in cell numbers over the previous 3–4 months (Fig. 1A). Furthermore, there was also a lagoon drawdown in April and there was no corresponding increase in cell numbers in samples taken following the event in May (Fig. 1A). The change in chemical parameters, however, was also more modest (5–10%) following the drawdown in April. Based on the data obtained in this study, it is difficult to determine if there was an association between the increases in cell numbers observed in Sept and changes in the physical or chemical structure that resulted from the drawdown of the lagoon. However, the data do suggest that there was

Table 2
Yearly average for chemical characteristics of swine lagoon slurry.

Depth (cm)	COD (mg L^{-1})	TDS (mg L^{-1})	TOC (mg L^{-1})	pH
Top	3809 ± 737	3796 ± 878	1968 ± 768	7.59 ± 0.39
51	3516 ± 440	4277 ± 999	1976 ± 538	7.62 ± 0.36
152	3946 ± 365	4450 ± 1006	4474 ± 5562	7.76 ± 0.36
Bottom	50796 ± 13742	11119 ± 5156	15905 ± 8397	7.52 ± 0.27

Values represent yearly average ± standard deviation.
COD, Chemical oxygen demand; TDS, Total dissolved solids; and TOC, Total organic carbon.

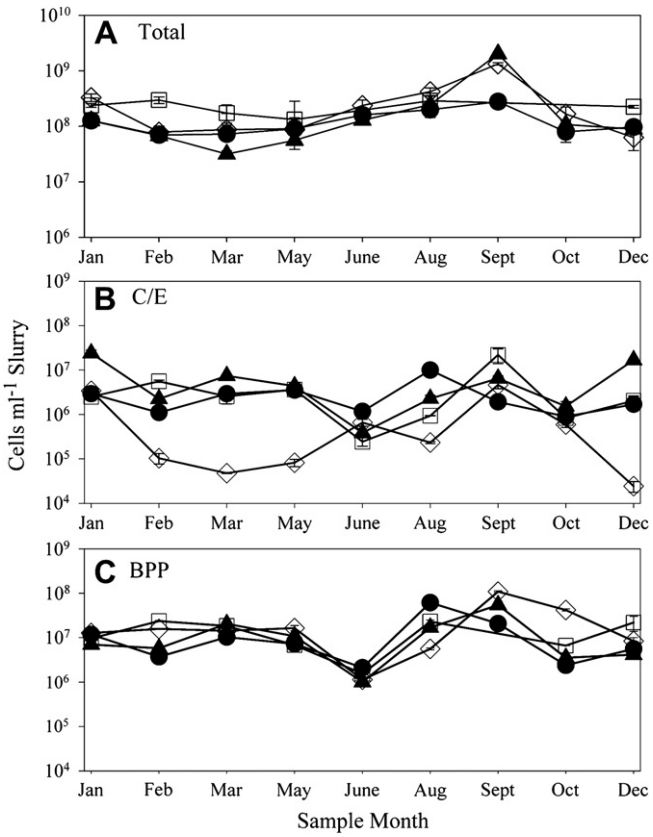


Fig. 1. Log_{10} of the concentration of bacterial cell numbers as determined by quantitative, real-time PCR (QRT-PCR) targeting (A) total cells (16S rRNA gene), (B) *Clostridia–Eubacteria* (CE), and (C) *Bacteroides–Porphyromonas–Prevotella* (BPP) groups. Values are in cells per mL of slurry at the top (●), 51 cm (▲), 152 cm (■) or bottom (◆) of the lagoon. It was assumed that the 16S rRNA gene was present in 4 copies, the CE targeted region was present in 11 copies, and the BPP targeted region was present in 6 copies [30]. Error bars represent the standard deviation of duplicate samples each also run in duplicate (four replicates).

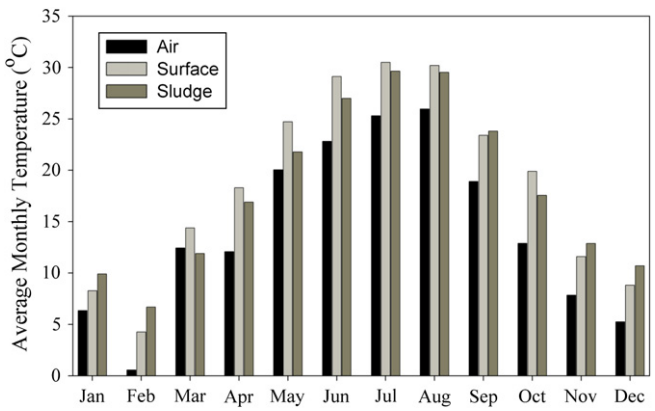


Fig. 2. Average monthly air (■), surface (□) and sludge (■) temperature (°C).

Author's personal copy

a significant increase in microbial cell numbers over the course of the spring and summer seasons.

DGGE analysis was utilized to evaluate changes in the community profile of the bacterial population by depth and over time. PCA of the DGGE banding pattern served to reduce the dimensionality of the data and permit graphic visualization of spatial and temporal changes in the community profiles (Fig. 3A). PCA of the DGGE pattern for the 16S community analysis showed that the community profiles were divided (Factor 1, explaining 22% of the difference among samples) between samples taken in spring and summer and those taken during cold months (Oct through Mar) (Fig. 3A). The second factor (16% the difference among samples) separated Sept (all samples), Oct (top) and Feb (152 cm and bottom) samples. Other than these transition periods, there were no differences based on sampling depth (Fig. 3B). There was a corresponding difference in DGGE band number observed for the warm months (mean bands = 12) versus the cold months (mean bands = 19). The impact of temperature transitions has also been found in other studies, for example, Yu and Mohn [32] found that changes in temperature in an aerated pulp and paper lagoon correlated with the most abrupt changes in the community structure. Merrill and Halverson (2002) found that the seasonal change in FAME profiles of swine slurry in concrete storage systems occurred in summer during the period of peak system temperature. Similarly, the transitional DGGE patterns for the fall (separated on PC2) corresponded to the months that showed the largest shifts in temperature during the course of the year (Fig. 2). Therefore, temperature appeared to have a significant impact on community dynamics based on both QRT-PCR analysis of total cell numbers and on the DGGE community profiling.

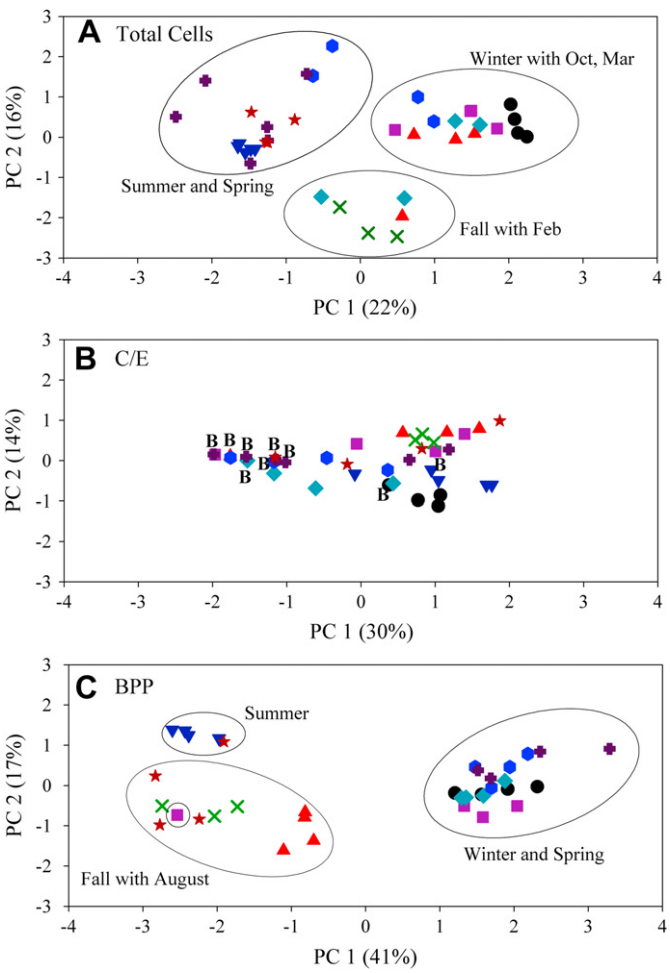


Fig. 3. Principal component analysis (PCA) of DGGE banding patterns for (A) total cells (16S rRNA gene), (B) *Clostridia–Eubacteria* (CE), and (C) *Bacteroides–Porphyromonas–Prevotella* (BPP) groups. Values in parentheses indicate the contribution of PC1 or PC2 to the total variation in the DGGE profile. Jan (●), Feb (◆), Mar (⬢), May (✕), June (▼), Aug (★), Sept (✚), Oct (▲), Dec (■). For CE samples, lagoon bottom samples are in bold and marked with "B".

3.2. Phylogenetic analysis of DGGE band sequences

Twenty-nine of the dominant DGGE bands were cloned and sequenced (Fig. 4). Nineteen (65%) of the cloned 16S rRNA gene sequences were similar (90% or greater) to database sequences from Firmicutes; thirteen (45%) of which grouped with the *Clostridiales*. Not surprisingly, the clostridial clone sequences matched most closely to other uncultured clones from swine pits, biogas reactors, sludge treatment or other anaerobic waste treatment systems [33–36](Fig. 4). Six clones grouped with the *Bacillus–Lactobacillus–Streptococcus* (21%) sub-group and matched other clones from studies of the human gut or swine manure compost [33,37]. These *Bacillales*-related sequences were 90% similar to *Lysinibacillus* sp. or *Urebacillus* sp. RDP classified these sequences as *Erysipelothrix* sp., which causes erysipelas an acute or chronic septicemia in swine [38]. However, the DGGE band sequences were less than 80% similar to those of *Erysipelothrix* sp. sequences from GenBank. Eight other clone sequences (28%) aligned with those from the gram-negative BPP group. These sequences matched uncultured clones from swine lagoons, anaerobic digester or sediment microcosms [39]. One cloned band sequence grouped with the *Spirochaetales* and one with *Burkholderiales* (Fig. 4). The grouping and distribution of clones predominantly between the *Clostridiales*, *Bacillales*, and the BPP groups was similar to that found in the swine GI tract [11] and in swine slurries [5–8]. Some species common to the swine gastrointestinal tract (i.e., *Lactobacillus*) were absent from the 16S DGGE gel band sequences [11]. However, the microbial community in swine slurry would not be expected to mimic that of the swine intestine given the fact that both physical conditions (temperature, frequency of emptying, retention time) and chemical makeup (urine, feces, barn debris, wash water) are quite different in the lagoon [5–8].

The seven most common and distinct DGGE bands (16S Bands 18, 8, 6, 26, 15, 9 and 17) contained sequences that were similar to *Clostridium butryicum*, *Hespellia stercorisuis*, *Aminobacterium* sp., *Acidaminococcus* sp., and *Peptinophilus* sp. *Clostridia* are commonly found in swine slurry and are involved in fermentation of lipids, sugars and amino acids with concomitant production of malodorous volatile fatty acids (VFA) [8,40]. *Hespellia* sp. are a new group of non-spore-forming, strictly anaerobic organisms that form a cluster within the *Clostridium coccoides* group [36]. Those organisms were originally isolated from swine slurry pits. The band (B6, Fig. 4) corresponding to a sequence similar to *H. stercorisuis* (98% similarity) was present in all slurry samples that were taken. Three other clones containing common band sequences were similar to those from genera containing amino acid degraders (*Aminobacterium* sp., *Acidaminococcus* sp., and *Peptinophilus* sp.). Proteins are one of the main organic substrates in anaerobic slurries, therefore, it is not surprising that three of the dominant species are related to organisms that degrade amino acids in the rumen and in anaerobic wastewaters [41,42]. Amino acid degraders are important in anaerobic slurry as they are responsible for production of some of the most offensive compounds associated with swine odor [5]. Data from this study suggest that these species are dominant and common in the swine slurry regardless of lagoon depth or season.

Author's personal copy

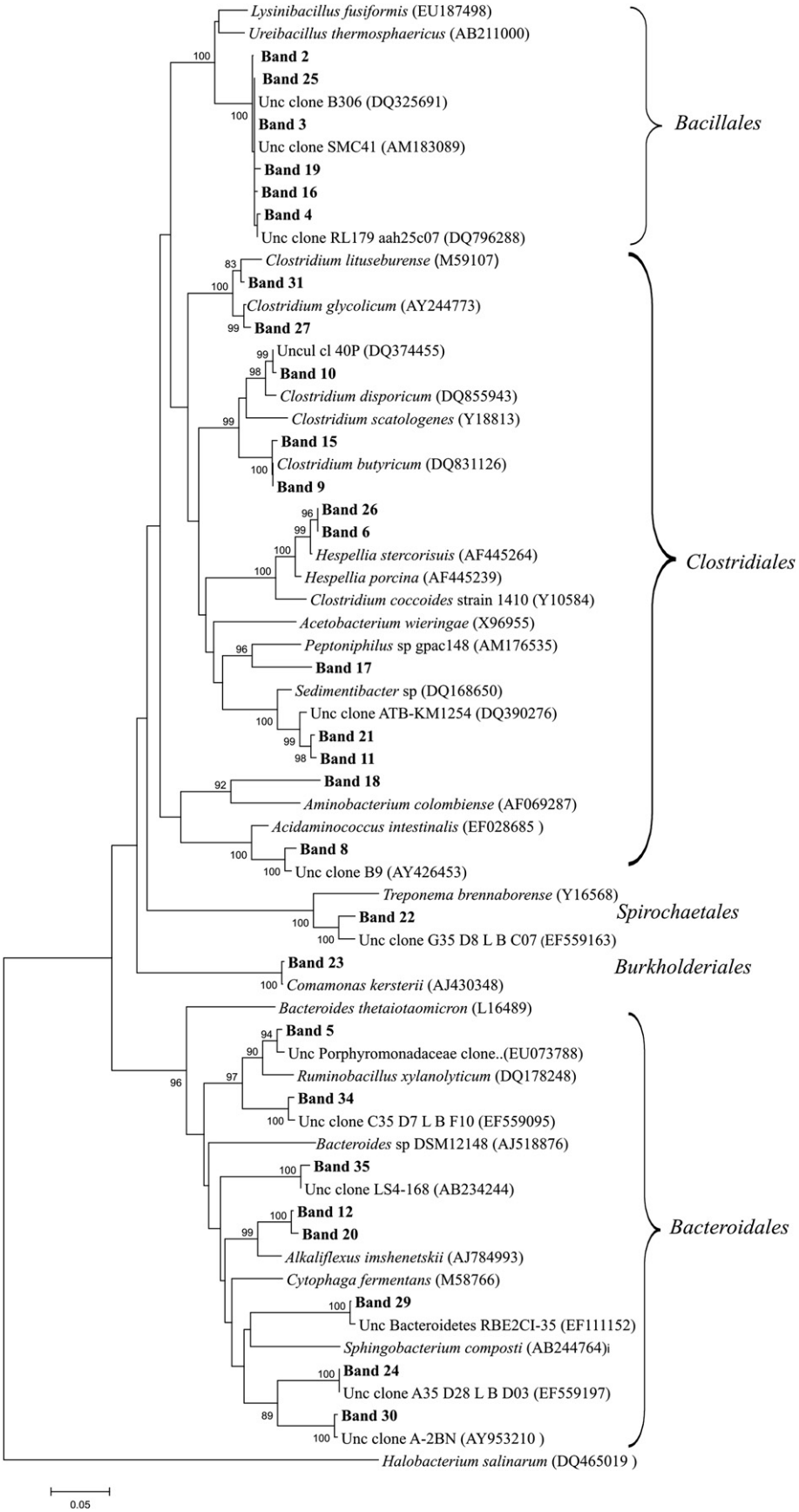


Fig. 4. Neighbor-joining tree showing the phylogenetic relationship between DGGE band sequences (bold text) and 16S rRNA gene sequences retrieved from the GenBank database (accession numbers in parentheses). The tree represents the alignment of an approximately 600-bp region of sequences from DGGE bands and from GenBank. The scale bar represents 5% estimated change. The major phylogenetic groups found in this study are indicated along the right side of the figure. An archaea was used as an outgroup [*Halobacterium salinarinum* (DQ465019)]. Numbers at each node indicate percentage of occurrences in 500 bootstrap iterations for values over 75%.

Author's personal copy

Based on the 16S rRNA gene sequence analysis of data from the cloning of DGGE gel bands, *Clostridia* sp. and *Bacteroides* sp. were the two largest groups in the slurry. Furthermore, correlation of the DGGE profile with the band sequences suggests that the difference in DGGE banding patterns between the warm months and the cool months in the 16S DGGE profile correlated with the absence of band sequences from several *Bacteroidales* and one band that matched *Sedimentibacter* sp. Others have found that using primers for specific microbial groups permits visualization of subdominant populations that may not be visible in the total bacterial profile [10,23]. Therefore, we focused more specifically on the CE and BPP groups by using group-specific primers (Table 1) to obtain PCR products from the swine slurry extract to be used for DGGE analysis.

3.3. Spatial and temporal dynamics of Clostridiales populations

Amplification of swine lagoon DNA extract with primers targeting the CE group resulted in DGGE profiles in which the bands were concentrated at the top or bottom of the gel. Peu et al. [10] also found, using SSCP which separates DNA fragments based on size and secondary structure, that the CE sub-group represented a distinct cluster within the total SSCP profile. In this study, PCA of the DGGE banding profile for the CE group showed that the profile for the CE group was not as significantly influenced by time of year as was the general 16S rDNA DGGE pattern (Fig. 3B). The first axis (explaining 30% of variability in the data) separated all bottom samples (Fig. 3B) along with samples from Feb, Mar and May. This was due to fewer bands in the bottom samples (mean bands = 3) than in samples from the other depths (mean bands = 11). QRT-PCR analysis targeting the *C. coccoides*/*Hespellia* sp. group of CE sequences showed that cell concentrations corroborated the results of DGGE analysis. The concentration of the CE group was lower in the bottom samples, especially in Dec, Feb, Mar and May (Fig. 2B). This decrease did not correlate to changes in physical or chemical parameters during those times (i.e., COD, TOC, TDS or pH).

Ten bands from the CE group DGGE gel were excised, cloned and sequenced. Five of the ten cloned band sequences fell into groups that were similar to the *Clostridiales* sequences obtained from the 16S DGGE band sequences (Table 3). Four CE group band sequences were over 97% similar to *C. coccoides* and *Hespellia* sp. and two of the 16S rDNA band sequences (B6, B26) aligned with the same group (Fig. 4). Two CE group band sequences and two 16S rDNA band sequences matched over 98% to those from *Sedimentibacter* sp. and two other band sequences were similar to a *Clostridium disporicum* (DQ855943) strain that has been found in the swine hindgut, pork manure biosolids and in swine slurry [8,43]. Two sequences matched an uncultured clone from swine lagoon slurry (AY953242), but were less than 90% similar to any known sequences in the GenBank database. A clone that was 99% similar to *Hespellia porcina* strain PPC80 (AF445239) was present in all of the samples and was one of the few bands present in the bottom samples. Two other clones matched those from swine slurry, and although not close to any cultured species they were most closely linked with the *Hespellia* group. Those two clones coupled with the four other clones from this library and two from the 16S library, as well as the QRT-PCR data which suggest that this group is ubiquitous within the slurry suggests that *Hespellia* species are important in swine slurry and the group should be studied further.

3.4. Spatial and temporal dynamics of Bacteroides sp. populations

The PCA of the DGGE profile produced by products from PCR amplification of swine slurry with BPP group-specific primers was much more distinctive than that of the CE group (Fig. 3C). Fall samples (with Aug) separated from the spring and winter months (Dec thru May), accounting for 41% of the variability in the data. On the second axis (17% of variability), June (and Aug Top) samples separated from the other warm and cold months (Fig. 3C). Ten bands from the BPP group DGGE profile were excised, cloned and sequenced. In contrast to the clone sequences from the analysis of the CE group, the BPP group sequences were distinct from the 16S rDNA sequences that aligned with the BPP group (Table 3). The eight cloned 16S DGGE band sequences matched most closely with the Porphyromonadaceae and Rikenallaceae families or the Sphingobacteria, while the clone sequences obtained from the BPP group DGGE gel aligned more closely with those from the Bacteroideaceae or Prevotellaceae families (Table 3). Two cloned band sequences that were present in all samples matched those associated with *Bacteroidetes* clones from fecal pollution [44] or from a study of swine gut microbiota [11]. Two cloned band sequences that were missing in warm month samples matched those from studies of the human and swine gut [11,45].

Evaluation of the DGGE band profile suggests that the difference in DGGE banding patterns correlated with a lower diversity of

Table 3
Phylogenetic grouping of cloned DGGE bands.

Group[a]	Universal			BPP			CE		
	Phylum	Order	Family	Phylum	Order	Family	Phylum	Order	Family
	Number of clones in group								
Bacteroidetes	8			10					
Rikenallaceae			3						
Unclassified bacteroidetes			5						
Prevotellaceae						4			
Bacteroidaceae						6			
Firmicutes	19						10		
Bacillales		6							
Clostridiales		13						8	
Acidaminococcaceae			1						
Syntrophomonadaceae			1						
Peptostreptococcaceae			3						2
Clostridiaceae			8						3
Unclassified Clostridiales									3
Unclassified Firmicutes								2	
Burkholderiales		1							
Spirochaetales		1							

BPP, *Bacteroides*, *Prevotella*, *Porphyromonas*; and CE, *Clostridium–Eubacterium*.
[a] Group based on Ribosome Database Project designation.

Author's personal copy

species in warm months (mean bands = 7) versus cold months (mean bands = 10). There was no apparent shift in the species present (i.e., no shift in the dominant bands) in the winter months, rather the differences in the DGGE profiles were due to the decrease in bands in the warm months. The significant shift in *Bacteroides* sp. in slurry was confirmed by QRT-PCR analysis. *Bacteroides* sp. were approximately 10% of the total population (averaging 1.0×10^7 cells mL^{-1} slurry) (Fig. 1C). In June, however, the *Bacteroides* sp. population dropped to 1% or less of the population at all depths relative to total 16S QRT-PCR analysis. By Aug, the concentration of *Bacteroides* sp. had increased by an order of magnitude at the top, 51 cm and 152 cm depths. This increase was delayed at the bottom of the lagoon; however, there was a two order of magnitude increase between June and Sept (Fig. 1C). This data correlates well with PCA of DGGE profiles which suggest that samples from Aug, Sept and Oct were distinct from Dec through May samples and that June separated from all of the other months. There was no effect of depth. Okabe and Shimazu [46], studying the effect of temperature and salinity on the die-off of strains of *Bacteroides*, found that the organism died off more rapidly at higher temperatures (1–3 log decrease within 2 days at 20 °C and 30 °C). In this system, loss of *Bacteroides* sp. in the warm months may be due to competition from species that are more tolerant of warmer temperatures. Perhaps fewer species become more dominant in the warm months and thereby inhibit some *Bacteroides* sp. Other physical factors may also be important. For example, the pH of the lagoon slurry also increased to its highest point (pH 8.1) in June [47]. The influence of pH on the microbial community as a whole and in particular on the *Bacteroides* sp. is unknown. However, the availability of many waste components is influenced by pH [15] as is the activity of many microbial enzymes [14]. The interaction of temperature and pH and their influence on nutrient concentrations and availability may be responsible for the changes in the *Bacteroides* population in this study.

It is interesting that the *Bacteroides* population in the lagoon followed a very seasonal pattern and the odor profiles within swine lagoons have also been shown to be seasonal [48,49]. Loughrin et al. (2008) found that concentrations of malodorous compounds increase over winter months. In this study, *Bacteroides* sp. were found to be more diverse in winter months and present at high concentrations (Fig. 1C). Transitions in the *Bacteroides* populations seen in June, the concomitant increase in pH and the high temperatures during summer months may be associated with changes in the microbial community that correlate with reduced odor production from the lagoon in summer. These results suggest that shifts in the *Bacteroides* population correlate with the seasonal changes observed for odor production in the swine lagoon. These populations may be partially responsible for this effect in the lagoon either directly or working in synergy with other organisms. Although the link or significance cannot be determined from data obtained in this study, it does warrant further investigation. Based on this data, it seems that the *Bacteroides* population has a much more seasonal aspect to their presence in the lagoon than do the *Clostridiales*.

4. Conclusions

Data from this study show that there is a distinct seasonal effect in the microbial community in swine lagoon storage systems. *Hespellia* sp. and other clostridial species appear to be endemic in the slurry, present at all depths and throughout the year. Based on sequence analysis of 16S DGGE bands, PCA analysis of BPP group DGGE profile and on the QRT-PCR analysis, the *Bacteroides* sp. appear to be seasonal in their distribution in the slurry. Therefore, *Bacteroides* may account for the seasonal effect in the 16S DGGE profile and perhaps play an important role (either directly or in synergy with other microbial groups) in malodor emissions from waste storage systems [50]. Future work should evaluate the role of this group in community dynamics and odor production in the lagoon system. Furthermore, trends in these large groups may say little about the changes or physiological ecology of important functional groups within the system (methanogens, acetogens, sulfate reducers). These groups may have a major impact on biomass conversion and odor production within the lagoon.

Acknowledgements

We would like to thank Rohan Parekh, Jason Simmons, Stacy Antle and Joe St. Claire (USDA-ARS AWMRU) for their technical assistance. This research was performed as part of the USDA-ARS National Program 206: Manure and By-product Utilization. Mention of a trademark or product anywhere in this paper is to describe experimental procedures and does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

References

[1] Harkin T. Animal waste pollution in America: an emerging national problem. Washington, D.C.: Report filed by the minority staff of the United States Senate Committee on Agriculture, Nutrition, and Forestry; 1997.
[2] Bradford SA, Segal E, Zheng W, Wang Q, Hutchins SR. Reuse of concentrated animal feeding operation wastewater on agricultural lands. J Environ Qual 2008;37(Suppl. 5):S97–115.
[3] Zahn JA, Hatfield JL, Do YS, Dispirito AA, Laird DA, Pfeiffer RL. Characterization of volatile organic emissions and wastes from a swine production facility. J Environ Qual 1997;26:1687–96.
[4] NASS. U.S. hog inventory by month and year, http://wwwnassusdagov/Charts_and_Maps/Hogs_and_Pigs/qtr_easp; 2007.
[5] Cotta MA, Whitehead TR, Zeltwanger RL. Isolation, characterization and comparison of bacteria from swine faeces and manure storage pits. Environ Microbiol 2003;5:737–45.
[6] Snell-Castro R, Godon J-J, Delgenes J-P, Dabert P. Characterization of the microbial diversity in a pig manure storage pit using small subunit rDNA sequence analysis. FEMS Microbiol Ecol 2005;52:229–42.
[7] Whitehead TR, Cotta MA. Characterization and comparison of microbial populations in swine feces and manure storage pits by 16S DNA gene sequence analyses. Anaerobe 2001;7:181–7.
[8] Leung K, Topp E. Bacterial community dynamics in liquid swine manure during storage: molecular analysis using DGGE/PCR of 16S rDNA. FEMS Microbiol Ecol 2001;38:169–77.
[9] Merrill L, Halverson LJ. Seasonal variation in microbial communities and organic malodor indicator compound concentrations in various types of swine manure storage systems. J Environ Qual 2002;31:2074–85.
[10] Peu P, Brugère H, Pourcher A-M, Kérouredan M, Godon J-J, Delgenès J-P, et al. Dynamics of a pig slurry microbial community during anaerobic storage and management. Appl Environ Microbiol 2006;72:3578–85.
[11] Leser TD, Amenuvor JZ, Jensen TK, Lindecrona RH, Boye M, Moller K. Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. Appl Environ Microbiol 2002;68:673–90.
[12] Chinivasagam HN, Thomas RJ, Casey K, McGahan E, Gardner EA, Rafiee M. Microbiological status of piggery effluent from 13 piggeries in the south east of Queensland region of Australia. J Appl Microbiol 2004;97:883–91.
[13] Watabe M, Rao JR, Stewart TA, Xu J, Millar BC, Xiao L, et al. Prevalence of bacterial faecal pathogens in separated and unseparated stored pig slurry. Lett Appl Microbiol 2003;36:208–12.
[14] Mackie RI, Stroot PG, Varel VH. Biochemical identification and biological origin of key odor components in livestock waste. J Anim Sci 1998;76:1331–42.
[15] Spoelstra SF. Origin of objectionable odorous components in piggery wastes and the possibility of applying indicator components for studying odour development. Agric Environ 1980;5:241–60.
[16] Clark OG, Morin B, Zhang Y, Sauer WC, Feedes JJR. Preliminary investigation of air bubbling and dietary sulfur reduction to mitigate hydrogen sulfide and odor from swine waste. J Environ Qual 2005;34:2018–23.
[17] Miller DN, Varel VH. In vitro study of the biochemical origin and production limits of odorous compounds in cattle feedlots. J Anim Sci 2001;79:2949–56.
[18] Zhu J. A review of microbiology in swine manure odor control. Agric Ecosyst Environ 2000;78:93–106.
[19] Clesceri LS, Greenberg AE, Eaton AD, editors. Standard methods for the examination of water and wastewater. 20th ed. Washington, DC.: American Public Health Association; 1998.

Author's personal copy

[20] Watson M, Wolf A, Wolfe N. Total nitrogen. In: Peters J, editor. Recommended methods of manure analysis. Madison, WI: University of Wisconsin Extension; 2003. p. 18–24.
[21] Casamayor EO, Schäfer H, Bañeras L, Pedrós-Alió C, Muyzer G. Identification of and spatio-temporal differences between microbial assemblages from two neighboring sulfurous lakes: comparison by microscopy and denaturing gradient gel electrophoresis. Appl Environ Microbiol 2000;66: 499–508.
[22] Van Dyke MI, McCarthy AJ. Molecular biological detection and characterization of *Clostridium* populations in municipal landfill. Appl Environ Microbiol 2002;68:2049–53.
[23] Wise MG, Siragusa GR. Quantitative analysis of the intestinal bacterial community in one- to three-week-old commercially reared broiler chickens fed conventional or antibiotic-free vegetable-based diets. J Appl Microbiol 2007;102:1138–49.
[24] Nübel U, Garcia-Pichel F, Muyzer G. PCR primers to amplify 16S rRNA genes from cyanobacteria. Appl Environ Microbiol 1997;63:3327–32.
[25] Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997;25:3389–402.
[26] Kumar S, Tamura K, Nei M. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinformatics 2004;5:150–63.
[27] Cole JR, Chai B, Farris RJ, Wang Q, Kulam SA, McGarrell DM, et al. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res 2005;33:D294–6.
[28] Harms G, Layton AC, Dionisi HM, Gregory IR, Garrett VM, Hawkins SA, et al. Real-time PCR quantification of nitrifying bacteria in a municipal wastewater treatment plant. Environ Sci Technol 2003;37:343–51.
[29] Layton AC, McKay L, Williams D, Garrett V, Gentry R, Sayler GS. Development of *Bacteroides* 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. Appl Environ Microbiol 2006;72:4214–24.
[30] Klappenbach JA, Dunbar JM, Schmidt TM. rRNA operon copy number reflects ecological strategies of bacteria. Appl Environ Microbiol 2000;66: 1328–33.
[31] Spoelstra SF. Enumeration and isolation of anaerobic microbiota of piggery wastes. Appl Environ Microbiol 1978;35:841–6.
[32] Yu Z, Mohn WW. Bacterial diversity and community structure in an aerated lagoon revealed by ribosomal intergenic space analyses and 16S ribosomal DNA sequencing. Appl Environ Microbiol 2001;67:1565–74.
[33] Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, et al. Metagenomic analysis of the human distal gut microbiome. Science 2006;312: 1355–9.
[34] Klocke M, Mahnert P, Mundt K, Souidi K, Linke B. Microbial community analysis of a biogas-producing completely stirred tank reactor fed continuously with fodder beet silage as mono-substrate. Syst Appl Microbiol 2007;30: 139–51.
[35] Wang X, Hoefel D, Saint CP, Monis PT, Jin B. The isolation and microbial community analysis of hydrogen producing bacteria from activated sludge. J Appl Microbiol 2007;103:1415–23.
[36] Whitehead TR, Cotta MA, Collins MD, Lawson PA. *Hespellia stercorisuis* gen. nov., sp. nov. and *Hespellia porcina* sp. nov., isolated from swine manure storage pits. Int J Syst Bacteriol 2004;54:241–5.
[37] Guo Y, Zhu N, Zhu S, Deng CY. Molecular phylogenetic diversity of bacteria and its spatial distribution in composts. J Appl Microbiol 2007;103:1344–54.
[38] Makino S-I, Okada Y, Maruyama T, Ishikawa K, Takahashi T, Nakamura M, et al. Direct and rapid detection of *Erysipelothrix rhusiopathiae* DNA in animals by PCR. J Clin Microbiol 1994;32:1526–31.
[39] Hiraishi A, Kaiya S, Miyakoda H, Futamata H. Biotransformation of polychlorinated dioxins and microbial community dynamics in sediment microcosms at different contamination levels. Microbes Environ 2005;20:227–42.
[40] Zhu J, Jacobson LD. Correlating microbes to major odorous compounds in swine manure. J Environ Qual 1999;28:737–44.
[41] Baena S, Fardeau M-L, Labat M, Ollivier B, Thomas P, Garcia J, et al. *Aminobacterium colombiense* gen. nov. sp. nov., an amino acid-degrading anaerobe isolated from anaerobic sludge. Anaerobe 1998;4:241–50.
[42] Eschenlauer SCP, McKain N, Walker ND, McEwan NR, Newbold CJ, Wallace RJ. Ammonia production by ruminal microorganisms and enumeration, isolation, and characterization of bacteria capable of growth on peptides and amino acids from the sheep rumen. Appl Environ Microbiol 2002;68:4925–31.
[43] Su Y, Yao W, Perez-Gutierrez ON, Smidt H, Zhu W-Y. 16S ribosomal RNA-based methods to monitor changes in the hindgut bacterial community of piglets after oral administration of *Lactobacillus sobrius* S1. Anaerobe 2008;14:78–86.
[44] Lamendella R, Santo Domingo JW, Oerther DB, Vogel JR, Stoeckel DM. Assessment of fecal pollution sources in a small northern-plains watershed using PCR and phylogenetic analyses of Bacteroidetes 16S rRNA gene. FEMS Microbiol Ecology 2007;59:651–60.
[45] Hayashi H, Sakamoto M, Benno Y. Phylogenetic analysis of the human gut microbiota using 16S rDNA clone libraries and strictly anaerobic culture-based methods. Microbiol Immunol 2002;46:535–48.
[46] Okabe S, Shimazu Y. Persistence of host-specific *Bacteroides–Prevotella* 16S rRNA genetic markers in environmental waters: effects of temperature and salinity. Appl Microbiol Biotechnol 2007;76:935–44.
[47] Lovanh N, Loughrin JH, Cook KL, Rothrock MJ, Sistani KR. The effect of stratification and seasonal variability on the profile of anaerobic swine waste treatment lagoon. Bioresource Techol 2009;100:3706–12.
[48] Do YS, Schmidt TM, Zahn JA, Boyd WL, de la Mora A, Dispirito AA. Role of *Rhodobacter* sp. strain PS9, a purple non-sulfur photosynthetic bacterium isolated from an anaerobic swine waste lagoon, in odor remediation. Appl Environ Microbiol 2003;69:1710–20.
[49] Loughrin JH, Lovanh N, Mahmood R. Equilibrium sampling used to monitor malodors in a swine waste lagoon. J Environ Qual 2008;37:1–6.
[50] Cook KL, Rothrock MJ, Loughrin JH, Doerner KC. Characterization of skatole-producing microbial populations in enriched swine lagoon slurry. FEMS Microbiol Ecol 2007;60:329–40.