

Bacterial Population Dynamics in Dairy Waste during Aerobic and Anaerobic Treatment and Subsequent Storage[∇]

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The objective of this study was to model a typical dairy waste stream, monitor the chemical and bacterial population dynamics that occur during aerobic or anaerobic treatment and subsequent storage in a simulated lagoon, and compare them to those of waste held without treatment in a simulated lagoon. Both aerobic and anaerobic treatment methods followed by storage effectively reduced the levels of total solids (59 to 68%), biological oxygen demand (85 to 90%), and sulfate (56 to 65%), as well as aerobic (83 to 95%), anaerobic (80 to 90%), and coliform (>99%) bacteria. However, only aerobic treatment reduced the levels of ammonia, and anaerobic treatment was more effective at reducing total sulfur and sulfate. The bacterial population structure of waste before and after treatment was monitored using 16S rRNA gene sequence libraries. Both treatments had unique effects on the bacterial population structure of waste. Aerobic treatment resulted in the greatest change in the type of bacteria present, with the levels of eight out of nine phyla being significantly altered. The most notable differences were the >16-fold increase in the phylum *Proteobacteria* and the approximately 8-fold decrease in the phylum *Firmicutes*. Anaerobic treatment resulted in fewer alterations, but significant decreases in the phyla *Actinobacteria* and *Bacteroidetes*, and increases in the phyla *Planctomycetes*, *Spirochetes*, and TM7 were observed.

California is the largest dairy-producing state in the United States, housing over 2.5 million dairy cows on approximately 2,300 dairies, with the average farm maintaining 1,000 cows (35). The average 450-kg dairy cow produces approximately 37 kg of waste (manure and urine) per day (27); thus a 1,000 cow dairy produces approximately 37,000 kg of waste per day or 13.5 million kg of waste per year. The waste is usually held in storage lagoons until it can be applied to agricultural fields as a soil amendment/fertilizer for crops destined for animal or human consumption. The average herd size in California has increased by approximately 8% a year for the last 10 years (35), and new challenges associated with the waste stream have emerged. For example, many of the larger dairies produce more waste than they can apply to nearby fields due to excessive nutrient levels (e.g., nitrogen, phosphate, potassium, etc.) and transporting waste to distant agricultural fields is an economic liability. Cow manure has also been associated with pathogenic bacteria such as *Escherichia coli* O157:H7 (14), *Salmonella* sp. (37), *Campylobacter* sp. (38), and *Mycobacterium avium* subsp. *paratuberculosis* (8), and crops fertilized with this material may transmit these pathogens to the consumer. Furthermore, waste lagoons can impair air quality via the release of odorous compounds, leading to nuisance complaints from surrounding residential communities (17). One possible solution to these problems is to treat the waste before it enters the storage lagoons. The most commonly used treat-

ment methodologies for both municipal and agricultural wastes are aerobic and anaerobic digestion (11, 29, 34, 36). Previous studies have shown these techniques to be effective for organic matter, nutrient (19, 33), and pathogen (13) reduction, but little is known about the microbial population dynamics associated with these processes.

Because cultivation methods are estimated to support the growth of only a small fraction of the naturally occurring biodiversity (1), the use of small-subunit rRNA gene (16S) analysis has proven to be a powerful tool to describe the microbial population structure of the human gut (10) and soil (9) and to compare the populations associated with different types of dairy waste storage lagoons (25). Techniques such as terminal fragment length polymorphism (24), denaturing gradient gel electrophoresis (30), and length heterogeneity PCR (32) are popular because they are relatively rapid and inexpensive, but do not provide the detailed information that 16S rRNA gene sequencing does. However, all of these techniques are subject to caveats, including PCR amplification and cloning bias, uneven bacterial cell lysis, and copy number variations of 16S rRNA genes within different species. In this study, 16S rRNA gene sequence analysis was used to determine the bacterial population dynamics of dairy waste treated in aerobic or anaerobic reactors followed by storage in simulated waste storage lagoons and to compare it to the dynamics of untreated waste stored in simulated lagoons. This was accomplished by pumping fresh dairy waste through lab-scale aerobic and anaerobic reactors and holding the effluent in stagnant storage tanks that simulated dairy waste storage lagoons (Fig. 1) or simply holding the waste in simulated storage lagoons. Samples were collected from the fresh waste material, the reactors, and the storage tanks for a period of 6 months and monitored for their

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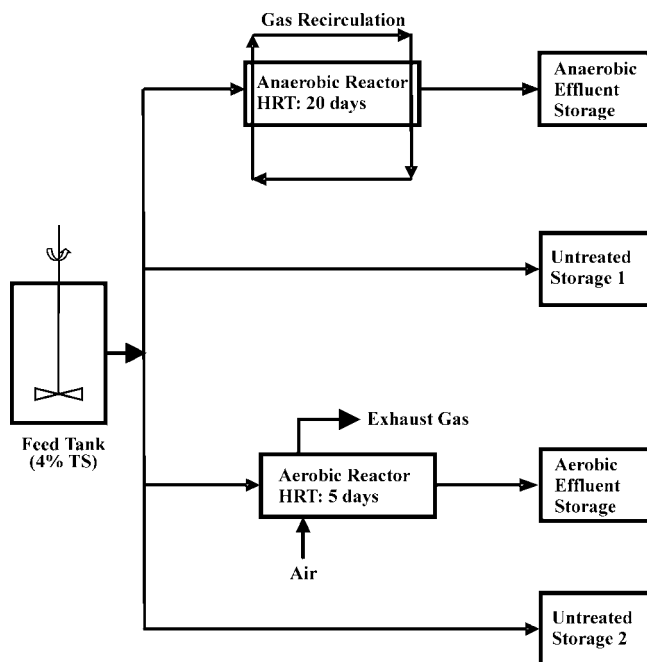


FIG. 1. Diagram of the aerobic and anaerobic reactors. Fresh waste was placed into the feed tank and was pumped into aerobic or anaerobic reactors at an HRT of 5 or 20 days, respectively. Waste was also pumped into untreated storage tanks at the same rates. Treated waste exiting the digesters was collected in holding tanks for the duration of the experiment.

chemical composition and bacterial population structure. Our results confirm that both aerobic and anaerobic treatment are more effective at reducing nutrient levels than storage alone and that each treatment method has a unique effect on the bacterial population structure of dairy waste.

MATERIALS AND METHODS

Sample collection and preparation. Fresh dairy cow waste (manure and urine <12 h postexcretion) was collected from the research dairy located on the campus of the University of California at Davis weekly from 15 November 2004 until 9 May 2005. The waste was passed through a screen with 2-mm openings to remove large particles that would clog the lines of the reactors. The screened waste was diluted with tap water to yield a slurry of approximately 4% total solids (TS), loaded into a feed tank maintained at 4°C, and used to feed the aerobic and anaerobic reactors. Fresh waste material was added into the feed tank weekly, and any material remaining in the feed tank was discarded when fresh material was added. The aerobic and anaerobic reactors were designed and operated to reduce total solids and biological oxygen demand (BOD_5) by approximately 35 and 80%, respectively, and are described schematically in Fig. 1. Aerobic treatment was performed at room temperature (approximately 25°C) in a 3-liter reactor with a 2-liter working volume and 1-liter headspace with dimensions of 15 cm in diameter and 37 cm in depth, and with a hydraulic retention time (HRT) of 5 days. Atmospheric air was pumped continuously through the reactor to maintain a dissolved oxygen concentration of approximately 2 mg liter⁻¹. Effluent from the aerobic reactor was collected and held in a 100-liter storage tank for the duration of the experiment. Anaerobic treatment was performed in a 5-liter reactor with a 4-liter working volume and a 1-liter headspace with dimensions of 15 cm in diameter and 74 cm in depth. The contents of the anaerobic reactor were maintained at 37°C and mixed for 2 min every hour by recirculating the headspace gas through the liquid. The anaerobic reactor had an HRT of 20 days, and its effluent was collected and stored in a 100-liter tank for the duration of the experiment. Both aerobic and anaerobic reactors were fed once a day from the same feed tank. In addition to the reactors described above, feed material was pumped directly into two storage tanks (100 liters each) to

simulate the storage of untreated waste. One tank was fed at the same rate as the aerobic reactor, and the other tank was fed at the same rate as the anaerobic reactor. The material in these tanks, as in the effluent storage tanks for the aerobic and anaerobic reactors, received no mixing except when samples were taken and was maintained at room temperature for the duration of the experiment. Previous studies in our laboratory have shown that both aerobic and anaerobic treatments of manure reduce microbial diversity and chemical variability (26); thus, a sampling scheme was developed on the hypothesis that the feed material would have the most microbial and chemical variability. Therefore, the feed material was assayed weekly as described below when fresh material was added. It was hypothesized the contents of the aerobic and anaerobic reactors contained the next greatest variability, and they were sampled biweekly. Finally, the aerobic and anaerobic reactor effluent storage tanks and the untreated manure storage tanks were sampled every 4 weeks.

Viable counts of bacteria and chemical analysis of wastewater. Samples were quantified for viable bacteria by performing serial dilutions in phosphate-buffered saline that were vortex agitated for 2 min prior to being plated onto brain heart infusion agar plates (BHI) and incubated at 25°C for 2 days under normal atmospheric conditions or in an anaerobic chamber. To quantify the number of coliform bacteria, samples were diluted as described above, plated onto MacConkey agar plates, and incubated at 37°C for 18 h. All media were purchased from Difco (Detroit, MI) as dehydrated powders. Chemical analysis was performed at A&L Western Agricultural Labs (Modesto, CA), a State of California accredited agricultural and environmental testing laboratory, using standard protocols (2).

DNA extraction from waste samples. Two-milliliter samples of wastewater were centrifuged at 10,000 × *g* for 10 min, and the resultant pellets, or 0.5-g manure samples, were used for DNA extraction. DNA was extracted from the samples using a modification of the MoBio UltraClean fecal DNA isolation kit (MoBio, Solano Beach, CA) as described previously (26).

PCR amplification of 16S rRNA gene sequences and library construction. PCR amplification of 16S rRNA gene sequences was carried out using the primers 27f (5' AGAGTTTGATCCTGGCTCAG 3') and 1392r (5' GACGGG CGGTGTGTAC 3') (21). PCRs were performed as recommended by Polz and Cavanaugh (31) to reduce bias in amplification. Briefly, 50-μl reaction volumes contained 200 μM deoxynucleoside triphosphates, 100 ng genomic DNA, and 2 U Expand high-fidelity enzyme mix (Roche, Nutley, NJ) in Expand high-fidelity buffer with 1.5 mM MgCl₂ and 1 μM of each primer. PCRs were performed in a Tetrad Thermocycler (Bio-Rad, Hercules, CA) under the following conditions: one cycle of 95°C for 5 min; 15 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min; and one cycle of 5 min at 72°C. PCR products were purified by ethanol precipitation, cloned using the QIAGEN PCR cloning kit (QIAGEN, Valencia, CA) as per the manufacturer's instructions, and transformed into *E. coli* TOP10F' cells (Invitrogen, Carlsbad, CA). Clones were plated on LB agar plates containing kanamycin (50 μg ml⁻¹), isopropyl-β-D-thiogalactopyranoside (IPTG) (20 mM), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (80 μg ml⁻¹). White colonies were selected and grown in 96-well plates in LB broth supplemented with kanamycin. Two PCRs and cloning experiments were performed for each sample, and 96 clones were picked from each PCR to minimize potential PCR bias.

DNA template preparation and sequencing. DNA templates were prepared using the TempliPhi 100 amplification kit (Amersham Biosciences, Sunnyvale, CA) as per the manufacturer's instructions. Sequencing reactions were performed in one direction using the primer 1392r and the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequencing reactions were purified using the DyeEx 96 kit (QIAGEN, Valencia, CA); electrophoresis and readout were performed using an Applied Biosystems 3730XL genetic analyzer (Applied Biosystems, Foster City, CA). Two 96-well plates of 16S rRNA gene sequences were analyzed for each sample: a total of 13,824 sequences were analyzed.

DNA sequence analysis and dendrogram construction. DNA sequences were edited manually to correct falsely called bases and trimmed at both the 5' and 3' ends using Chromas (version 2.31; Technelysium Pty. Ltd., Helensvale, Australia). Only sequences with unambiguous reads of >500 bp were used; each read used averaged approximately 600 bp. The predicted 16S rRNA sequences from this study were compared to 16S rRNA sequences in a BLAST database constructed from sequences downloaded from the Ribosomal Database Project (release 8.1; <http://rdp8.cme.msu.edu>). Comparisons were made using the program BLASTALL (<ftp://ftp.ncbi.nih.gov/BLAST/executables/LATEST/>) and a FASTA-formatted file containing the predicted 16S rRNA sequences. Operational taxonomic units (OTUs) were defined as clones with >97% sequence identity. For dendrogram construction, partial 16S rRNA gene sequences representing the 10 most prevalent OTUs from each environment (feed material, aerobic and anaerobic reactor effluents, effluents held in storage tanks, and

TABLE 1. Chemical and cultural analyses of aerobic and anaerobic waste treatment systems

Treatment and parameter ^a	Avg (range) result for treatment by parameter for:				Untreated storage
	Feed material	Reactor effluent	Effluent storage		
Aerobic					
Total solids (g liter ⁻¹)	44.0 (29.0-58.0)	30.0 (16.0-45.0)	14.0 (7.0-29.0)	28.0 (17.0-54.0)	
BOD ₅ (g liter ⁻¹)	14.9 (13.1-18.0)	3.5 (2.4-4.5)	1.5 (0.9-2.3)	10.6 (8.0-15.3)	
Total Kjeldahl N (g liter ⁻¹)	2.9 (1.5-6.1)	2.2 (1.3-4.7)	1.5 (0.6-4.3)	3.0 (1.2-6.0)	
NH ₄ concn (g liter ⁻¹)	0.8 (0.4-1.6)	0.1 (0.06-0.2)	0.2 (0.1-0.5)	1.1 (0.9-1.5)	
S concn (g liter ⁻¹)	0.3 (0.1-0.6)	0.4 (0.2-0.6)	0.2 (0.1-0.5)	0.2 (0.2-0.4)	
SO ₄ concn (g liter ⁻¹)	0.5 (0.2-1.0)	0.5 (0.3-0.9)	0.2 (0.1-0.3)	0.1 (0.1-0.2)	
K ₂ O concn (g liter ⁻¹)	2.2 (1.3-3.0)	2.2 (1.2-3.2)	2.0 (1.0-3.2)	1.9 (1.2-3.0)	
Na concn (g liter ⁻¹)	0.6 (0.3-1.0)	0.6 (0.4-0.9)	0.6 (0.5-0.8)	0.5 (0.5-0.6)	
APC (CFU ml ⁻¹)	5.4 × 10 ⁷ (0.04 × 10 ⁷ -7.2 × 10 ⁷)	6.1 × 10 ⁷ (0.02 × 10 ⁸ -2.8 × 10 ⁸)	9.1 × 10 ⁶ (0.08 × 10 ⁷ -2.7 × 10 ⁷)	3.0 × 10 ⁶ (0.5 × 10 ⁶ -5.0 × 10 ⁶)	
AnPC (CFU ml ⁻¹)	1.7 × 10 ⁷ (0.1 × 10 ⁷ -5.1 × 10 ⁷)	1.4 × 10 ⁶ (0.01 × 10 ⁶ -4.4 × 10 ⁶)	1.7 × 10 ⁶ (0.02 × 10 ⁶ -8.6 × 10 ⁶)	1.5 × 10 ⁶ (0.04 × 10 ⁶ -5.1 × 10 ⁶)	
CPC (CFU ml ⁻¹)	1.8 × 10 ⁵ (0.02 × 10 ⁶ -1.0 × 10 ⁶)	9.9 × 10 ³ (0.0 × 10 ⁴ -1.7 × 10 ⁵)	1.4 × 10 ³ (0.0 × 10 ³ -6.1 × 10 ³)	7.2 × 10 ³ (0.03 × 10 ⁴ -2.9 × 10 ⁴)	
Anaerobic					
Total solids (g liter ⁻¹)	44.0 (29.0-58.0)	25.0 (16.0-36.0)	18.0 (7.8-22.0)	28.0 (17.0-54.0)	
BOD ₅ (g liter ⁻¹)	14.9 (13.1-18.1)	1.9 (1.4-3.0)	2.2 (0.9-4.7)	10.6 (8.0-15.3)	
Total Kjeldahl N (g liter ⁻¹)	2.9 (1.5-6.1)	2.6 (1.5-4.8)	2.4 (0.7-5.6)	3.0 (1.2-6.0)	
NH ₄ concn (g liter ⁻¹)	0.8 (0.4-1.6)	1.0 (0.7-1.6)	1.0 (1.0-1.4)	1.1 (0.9-1.5)	
S concn (g liter ⁻¹)	0.3 (0.1-0.6)	0.1 (0.1-0.3)	0.1 (0.1-0.2)	0.2 (0.2-0.4)	
SO ₄ concn (g liter ⁻¹)	0.5 (0.2-1.0)	0.3 (0.1-0.7)	0.2 (0.1-0.3)	0.1 (0.1-0.2)	
K ₂ O concn (g liter ⁻¹)	2.2 (1.3-3.0)	1.8 (1.2-2.6)	1.5 (0.8-1.9)	1.9 (1.2-3.0)	
Na concn (g liter ⁻¹)	0.6 (0.3-1.0)	0.5 (0.2-0.8)	0.5 (0.4-0.6)	0.5 (0.5-0.6)	
APC (CFU ml ⁻¹)	5.4 × 10 ⁷ (0.04 × 10 ⁷ -7.2 × 10 ⁷)	2.2 × 10 ⁷ (0.04 × 10 ⁷ -7.3 × 10 ⁷)	2.7 × 10 ⁶ (0.08 × 10 ⁶ -3.9 × 10 ⁶)	3.0 × 10 ⁶ (0.5 × 10 ⁶ -5.0 × 10 ⁶)	
AnPC (CFU ml ⁻¹)	1.7 × 10 ⁷ (0.1 × 10 ⁷ -5.1 × 10 ⁷)	1.9 × 10 ⁵ (0.3 × 10 ⁵ -2.2 × 10 ⁵)	3.3 × 10 ⁶ (0.04 × 10 ⁷ -1.8 × 10 ⁷)	1.5 × 10 ⁶ (0.04 × 10 ⁶ -5.1 × 10 ⁶)	
CPC (CFU ml ⁻¹)	1.8 × 10 ⁵ (0.02 × 10 ⁶ -1.0 × 10 ⁶)	5.4 × 10 ² (0.1 × 10 ³ -1.7 × 10 ³)	1.2 × 10 ² (0.1 × 10 ² -2.2 × 10 ²)	7.2 × 10 ³ (0.03 × 10 ⁴ -2.9 × 10 ⁴)	

^a Abbreviations: APC, aerobic plate count; AnPC, anaerobic plate count; CPC, coliform plate count.

TABLE 2. Percentage of rRNA gene clones assigned to phyla before and after treatment and storage

Treatment and phylum assignment	Avg (range) % of clones assigned to phylum			
	Feed material	Reactor effluent	Effluent storage	Untreated storage
Aerobic				
<i>Actinobacteria</i>	5.3 (2.1–10.1)	10.9 (6.1–34.9)	5.1 (1.9–9.3)	3.0 (1.7–4.7)
<i>Bacteroidetes</i>	16.1 (9.0–24.3)	15.4 (4.7–22.0)	12.8 (9.8–17.8)	8.9 (6.1–10.5)
<i>Deinococcus-Thermus</i>	ND ^a	1.2 (0.0–1.7)	0.8 (0.7–1.3)	ND
<i>Firmicutes</i>	74.8 (61.8–88.0)	9.5 (4.0–17.4)	21.7 (13.4–27.4)	78.0 (72.2–80.1)
<i>Planctomycetes</i>	ND	1.1 (0.6–2.3)	5.7 (1.3–7.5)	ND
<i>Proteobacteria</i>	3.4 (0.0–5.1)	55.1 (46.0–59.9)	48.8 (37.5–58.2)	3.3 (2.1–3.8)
<i>Spirochetes</i>	0.2 (0.0–1.6)	ND	0.4 (0.0–1.2)	1.5 (0.6–3.2)
TM7	ND	4.5 (0.6–11.0)	1.1 (0.6–1.6)	ND
<i>Verrucomicrobia</i>	ND	1.1 (0.0–1.7)	0.3 (0.0–0.7)	ND
Unknown	0.7 (0.0–1.9)	1.1 (0.6–1.8)	3.4 (0.7–7.4)	4.9 (2.3–6.1)
Anaerobic				
<i>Actinobacteria</i>	5.3 (2.1–10.1)	2.0 (1.2–3.7)	1.8 (1.2–2.9)	2.2 (1.3–3.9)
<i>Bacteroidetes</i>	16.1 (9.0–24.3)	9.9 (5.6–15.9)	10.8 (8.4–14.5)	11.5 (6.5–13.8)
<i>Deinococcus-Thermus</i>	ND	ND	4.6 (0.0–13.4)	ND
<i>Firmicutes</i>	74.8 (61.8–88.0)	74.3 (64.0–79.3)	64.2 (74.5–56.2)	76.5 (73.2–81.7)
<i>Planctomycetes</i>	ND	0.5 (0.0–0.7)	0.5 (0.0–1.4)	ND
<i>Proteobacteria</i>	3.4 (0.0–5.1)	4.9 (2.2–11.2)	9.3 (2.3–17.3)	4.5 (0.8–7.8)
<i>Spirochetes</i>	0.2 (0.0–1.6)	0.8 (0.5–1.4)	0.5 (0.0–1.9)	0.8 (0.6–1.3)
TM7	ND	0.2 (0.0–0.7)	0.2 (0.0–0.6)	ND
<i>Verrucomicrobia</i>	ND	ND	ND	ND
Unknown	0.7 (0.0–1.9)	7.2 (3.4–10.8)	8.0 (4.2–9.8)	4.1 (1.4–6.7)

^a ND, not determined.

control untreated material in storage) and the most similar 16S rRNA gene sequences to each OTU from the NCBI nonredundant (nr) database were aligned using CLUSTALX. The 16S rRNA gene sequences from the nr database were first reverse complemented and trimmed to approximate the start point and length of the OTU sequences. Phylogenetic and molecular evolutionary analyses were performed using MEGA version 2.1 (20); the dendrogram was constructed using the neighbor-joining algorithm and the Kimura two-parameter distance estimation method.

Rarefaction analysis and statistical methods. Rarefaction analysis was performed using the approximation algorithm of Hurlbert (18) with 95% confidence intervals estimated as described by Heck (16) using the freeware program aRarefactWin by S. Holland (University of Georgia, Athens; <http://www.uga.edu/~strata/AnRareReadme.html>). The percent coverage of the total OTUs identified in each sample was calculated using the equation $C = [1 - (n/N)] \times 100$, where C is the percent coverage, n is the number of OTUs, and N is the number of clones examined. Student's t test, available in the SAS STAT package, was employed to test for differences between 16S rRNA gene libraries as well as the cultural and chemical parameters measured. Each OTU was assigned to a phylum using the Classifier software (7), which assigns an OTU sequence to a phylum using a naïve Bayesian rRNA classifier trained on the known type strain 16S sequences. Once the OTUs of each library were assigned to a phylum, pairwise comparisons of the phyla within the libraries were performed using Student's t test. In addition, comparisons of the 16S rRNA libraries were analyzed using the Library Compare software (7), which estimates the likelihood that the frequency of membership in a given taxon is the same for the two libraries using the equation:

$$p(y/x) = \binom{N_2}{N_1} \frac{(x+y)!}{x!y! \binom{N_2}{1+N_1}^{(x+y+1)}}$$

where N_1 and N_2 are the total number of sequences for libraries 1 and 2, respectively, and x and y are the number of sequences assigned to an OTU from libraries 1 and 2, respectively. The percentage of a phylum in one library was considered significantly different from that in another library if both statistical methods (Student's t test and Compare) were in agreement. The diversity within the libraries was measured with the Shannon-Wiener index (H), species richness (S), and evenness (E) using the equations $H = -\sum p_i \ln(p_i)$, where p_i is the proportion of the total number of OTUs made up to the i^{th} OTUs; and $E = H/\log(S)$, where S = total number of OTUs in the community.

Nucleotide sequence accession numbers. DNA sequences representative of the 10 most prevalent OTUs from each library were deposited into GenBank under accession no. DQ673153 to DQ673212.

RESULTS

Cultural and chemical analyses of waste, reactor effluents, and stored material. Changes in aerobic, anaerobic, and coliform plate counts, TS, BOD₅, total Kjeldahl N (TKN), and NH₄, S, SO₄, K₂O, and Na concentrations were measured before and after treatment and are presented in Table 1. After aerobic treatment, significant reductions in BOD₅ (77%), NH₄ (87%), anaerobic (92%), and coliform (95%) plate counts were observed as compared to those in the feed material at the $P < 0.05$ level. Analysis of the aerobic reactor effluent stored in stagnant holding tanks showed significant reductions in TS (68%), BOD₅ (83%), TKN (48%), NH₄ (69%), SO₄ (56%), and aerobic (90%) and coliform (99%) plate counts as compared to those in the feed material ($P < 0.05$). After anaerobic digestion, significant reductions in TS (43%), BOD₅ (87%), S (58%), SO₄ (43%), and anaerobic (99.9%) and coliform (99.7%) plate counts were observed as compared to those in the feed material. Anaerobic reactor effluent stored in stagnant holding tanks had significant reductions in TS (59%), BOD₅ (85%), S (61%), SO₄ (65%), and anaerobic (81%) and coliform (99.9%) plate counts compared to the feed material. The feed material held in an untreated control tank showed significant reductions in BOD₅ (29%), SO₄ (70%), and anaerobic (91%) and coliform (96%) plate counts compared to the feed material at the $P < 0.05$ level.

Analysis of 16S rRNA libraries derived from dairy waste, reactor effluents, and stored material. To determine the effect of aerobic and anaerobic digestion on the bacterial population

TABLE 3. The 10 most commonly isolated OTUs from each library

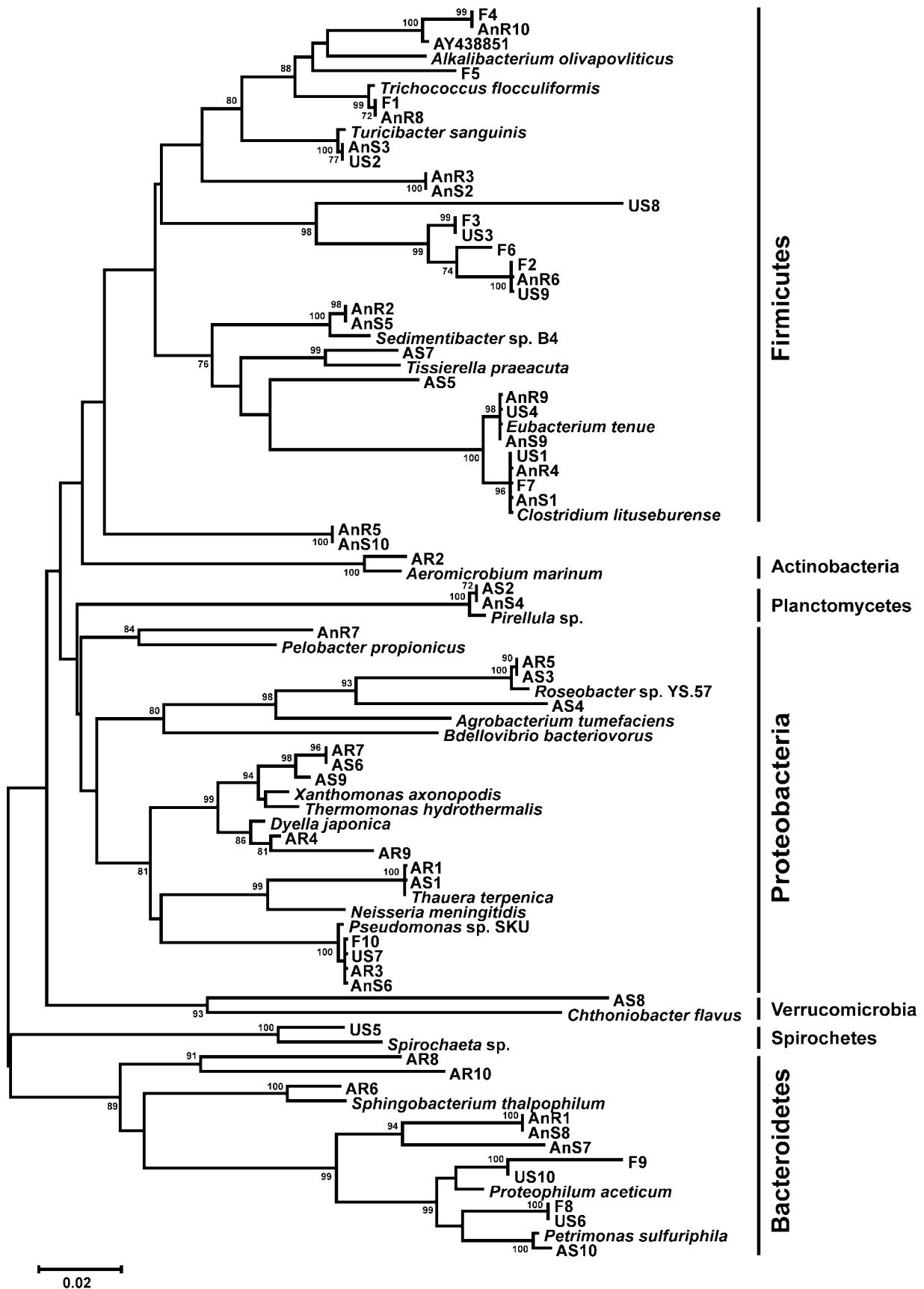
OTU ^a	No. of clones	% of total	Phylum (% confidence threshold) ^b	Best match in GenBank	% Similarity
F1	204	6.0	<i>Firmicutes</i> (100)	<i>Trichococcus flocculiformis</i>	97–98
F2	151	4.4	<i>Firmicutes</i> (100)	AY438851	98–99
F3	142	4.2	<i>Firmicutes</i> (100)	AF371787	99
F4	96	2.8	<i>Firmicutes</i> (100)	AY100573	98–99
F5	86	2.5	<i>Firmicutes</i> (100)	AY438899	98
F6	83	2.4	<i>Firmicutes</i> (100)	AY438880	97–98
F7	77	2.3	<i>Firmicutes</i> (100)	<i>Clostridium lituseburense</i>	98–100
F8	60	1.8	<i>Bacteroidetes</i> (100)	AY438832	98–99
F9	58	1.7	<i>Bacteroidetes</i> (100)	AB219992	92–94
F10	58	1.7	<i>Proteobacteria</i> (100)	<i>Pseudomonas</i> sp. strain SKU	98–99
AR1	154	9.6	<i>Proteobacteria</i> (100)	<i>Thauera terpenica</i>	99–100
AR2	57	3.5	<i>Actinobacteria</i> (100)	<i>Aeromicrobium marinum</i>	97–98
AR3	54	3.4	<i>Proteobacteria</i> (100)	<i>Pseudomonas</i> sp. strain SKU	98–100
AR4	48	3.0	<i>Proteobacteria</i> (100)	<i>Dyella japonica</i>	96–98
AR5	40	2.5	<i>Proteobacteria</i> (100)	<i>Roseobacter</i> sp. strain YS-57	99
AR6	35	2.2	<i>Bacteroidetes</i> (100)	<i>Sphingobacterium thalophilum</i>	97–98
AR7	34	2.1	<i>Proteobacteria</i> (100)	<i>Xanthomonas axonopodis</i>	95–97
AR8	34	2.1	<i>Bacteroidetes</i> (96)	UBA318142	95–96
AR9	33	2.1	<i>Proteobacteria</i> (100)	<i>Dyella korensis</i>	97–98
AR10	26	1.6	<i>Bacteroidetes</i> (99)	AF507866	96–97
AnR1	144	8.6	<i>Bacteroidetes</i> (100)	CR933150	97–99
AnR2	139	8.3	<i>Firmicutes</i> (100)	<i>Sedimentibacter</i> sp. B4	96–97
AnR3	130	7.8	<i>Firmicutes</i> (88)	DQ191708	96–97
AnR4	125	7.5	<i>Firmicutes</i> (100)	<i>Clostridium lituseburense</i>	98–100
AnR5	58	3.5	<i>Actinobacteria</i> (62)	AB092855	99–100
AnR6	43	2.7	<i>Firmicutes</i> (100)	AY438851	98
AnR7	32	1.9	<i>Proteobacteria</i> (62)	AB232562	96–97
AnR8	31	1.8	<i>Firmicutes</i> (100)	<i>Trichococcus flocculiformis</i>	97–98
AnR9	31	1.8	<i>Firmicutes</i> (100)	<i>Eubacterium tenue</i>	99–100
AnR10	30	1.8	<i>Firmicutes</i> (100)	AY100573	98–99
AS1	194	17.6	<i>Proteobacteria</i> (100)	<i>Thauera terpenica</i>	99–100
AS2	58	5.3	<i>Planctomycetes</i> (100)	<i>Pirellula</i> sp.	98–99
AS3	31	2.8	<i>Proteobacteria</i> (100)	<i>Roseobacter</i> sp. YS-57	97–99
AS4	20	1.8	<i>Proteobacteria</i> (100)	<i>Rhodobacter gluconicum</i>	99–100
AS5	19	1.7	<i>Firmicutes</i> (100)	AY570630	98–99
AS6	18	1.6	<i>Proteobacteria</i> (100)	<i>Xanthomonas axonopodis</i>	95–96
AS7	16	1.5	<i>Firmicutes</i> (100)	<i>Tissierella praeacuta</i>	93–95
AS8	16	1.5	<i>Proteobacteria</i> (78)	AY438740	99
AS9	16	1.5	<i>Proteobacteria</i> (100)	<i>Thermomonas hydrothermalis</i>	95–97
AS10	15	1.4	<i>Bacteroidetes</i> (100)	<i>Petrimonas sulfuriphila</i>	98–99
AnS1	80	7.3	<i>Firmicutes</i> (100)	<i>Clostridium lituseburense</i>	98–100
AnS2	64	5.9	<i>Firmicutes</i> (100)	DQ191708	95–96
AnS3	53	4.9	<i>Firmicutes</i> (100)	<i>Turicibacter sanguinis</i>	99–100
AnS4	48	4.4	<i>Planctomycetes</i> (100)	<i>Pirellula</i> sp.	98–100
AnS5	47	4.3	<i>Firmicutes</i> (100)	<i>Sedimentibacter</i> sp. strain B4	96–97
AnS6	46	4.2	<i>Proteobacteria</i> (100)	<i>Pseudomonas</i> sp. strain SKU	99–100
AnS7	46	4.1	<i>Bacteroidetes</i> (100)	AY953168	97–98
AnS8	33	3.0	<i>Bacteroidetes</i> (100)	CR933150	98–99
AnS9	30	2.7	<i>Firmicutes</i> (100)	<i>Eubacterium tenue</i>	99–100
AnS10	28	2.6	<i>Actinobacteria</i> (55)	AB092855	99–100
US1	166	14.5	<i>Firmicutes</i> (100)	<i>Clostridium lituseburense</i>	98–100
US2	48	4.2	<i>Firmicutes</i> (100)	<i>Turicibacter sanguinis</i>	99–100
US3	36	3.2	<i>Firmicutes</i> (100)	AF371787	99
US4	35	3.1	<i>Firmicutes</i> (100)	<i>Eubacterium tenue</i>	99–100
US5	30	2.6	<i>Spirochaetes</i> (64)	AY228699	97–98
US6	24	2.1	<i>Bacteroidetes</i> (100)	AY438832	98–99
US7	20	1.8	<i>Proteobacteria</i> (100)	<i>Pseudomonas</i> sp. strain SKU	99–100
US8	19	1.7	<i>Firmicutes</i> (100)	AY622268	94–95
US9	19	1.7	<i>Firmicutes</i> (100)	AY438851	98–99
US10	17	1.5	<i>Bacteroidetes</i> (100)	AY953229	96–98

^a Abbreviations are as follows: F, feed material; AR, aerobic reactor effluent; AnR, anaerobic reactor effluent; AS, aerobic reactor effluent storage tank; AnS, anaerobic reactor effluent storage tank; US, untreated storage tank.

^b The confidence threshold is an estimation of the classification reliability using bootstrapping.

structure of dairy waste, we constructed 16S rRNA libraries from DNA extracted from waste, the effluent of the aerobic and anaerobic reactors, the effluent held in storage tanks, as well as control untreated material held in storage tanks over a

6-month period (Table 2). These sequences were analyzed using the Classifier software to determine the type of bacteria from which the sequences were most likely derived. At the phylum level, the majority of the 16S rRNA sequences derived



from the feed material were assigned to the *Firmicutes*, followed by the *Bacteroidetes*, the *Actinobacteria*, the *Proteobacteria*, and the *Spirochetes* (Table 2). The library derived from the aerobic reactor effluent showed the greatest difference from the feed material, with the levels of eight out of nine phyla being significantly different. The most notable differences were the >16-fold increase in the phylum *Proteobacteria* and the approximately 8-fold decrease in the phylum *Firmicutes*. Other significant differences included the phyla *Actinobacteria*, *Deinococcus-Thermus*, *Planctomycetes*, *Spirochetes*, TM7, and *Verrucomicrobia*. The sequences derived from the aerobic reactor effluent held in storage vessels showed significant increases in the phyla *Firmicutes*, *Planctomycetes*, and *Spirochetes* and a decrease in the phylum TM7 as compared to the aerobic reactor-derived library. After anaerobic digestion, the bacterial population structure showed statistically significant decreases in the phyla *Actinobacteria* and *Bacteroidetes* and a statistically significant increase in the phyla *Planctomycetes*, *Spirochetes*, and TM7. The sequences derived from the anaerobic reactor effluent held in a storage tank showed a significant increase in the phylum *Deinococcus-Thermus*, while all other phyla in the library showed no significant change from those of the anaerobic reactor. Comparisons between the libraries derived from the feed material and the control untreated material held in storage tanks revealed a significant increase in the level of *Spirochetes*, while all other phyla levels remained unchanged. Comparisons of libraries derived from the two untreated control storage tanks, which differed from each other only in the volume of material that was pumped into them each day, showed no significant differences in any of the phyla. Comparisons of the aerobic and anaerobic reactor effluent libraries showed significant differences in all phyla except the *Planctomycetes* and the *Bacteroidetes*.

Identification of the 10 most prevalent OTUs from each library. The 10 most numerous OTUs identified in each library are presented in Table 3 and Fig. 2. These results are consistent with the phylum assignment data. For example, 7 of the 10 most prominent OTUs from the feed material are members of the phylum *Firmicutes* (the most predominant phylum), 2 are from the phylum *Bacteroidetes* (the second largest phylum), and 1 is from the phylum *Proteobacteria* (the fourth most predominant phylum). This trend continues throughout the aerobic effluent-, anaerobic effluent-, and storage-derived libraries. Samples subjected to a similar treatment regimen yielded libraries displaying similar 16S rRNA gene sequence composition. For example, the feed material-derived library shares 50% of the 10 most prevalent OTUs with the untreated storage-derived library, but only 10% with the aerobic reactor effluent-derived library. Likewise, the anaerobic reactor effluent-derived library shares 60% of the OTUs with the anaerobic

effluent storage-derived library but has none in common with the aerobic effluent storage-derived library.

Estimates of diversity, coverage, and rarefaction. The diversity within the libraries, as measured by the Shannon-Wiener diversity index (H), is presented in Table 4. H was greatest for the feed material-derived library (5.28), indicating that this library contained the greatest diversity. H decreased in the aerobic reactor effluent-derived library (5.04) and decreased further in the aerobic effluent storage tank-derived library (4.69), indicating that both aerobic treatment and subsequent storage have negative effects on diversity. H also decreased in the anaerobic reactor-derived library (4.46), but increased slightly in the anaerobic effluent storage tank-derived library (4.60). The index of evenness (E), which is proportional to the number of individuals that belong to each OTU, was 0.82 for the feed material-derived library and declined in the anaerobic reactor effluent-derived library (0.77) but increased in the aerobic reactor-derived library (0.84). Reductions in evenness indices were observed in the aerobic reactor effluent storage material (0.82) but increased in the anaerobic storage tank-derived library (0.81). The libraries derived from untreated waste held in storage tanks had very similar H values (4.79 and 4.81) and the same E values (0.83). Analysis of the libraries revealed that the coverage within the feed material-derived library was the highest (82.0%), followed by those in the anaerobic reactor-derived library (80.8%), the aerobic reactor-derived library (74.8%), the anaerobic effluent storage tank-derived library (72.4%), and the aerobic effluent storage tank-derived library (69.7%). Both of the untreated control material-derived libraries had similar coverage levels (72.2 and 73.6%). Rarefaction analysis of the 16S rRNA libraries indicates that our sampling was not exhaustive, but that most predominant OTUs were likely identified as the slopes of all of the curves decrease greatly towards the end points (Fig. 3). These graphs are in agreement with the Shannon-Wiener index data, indicating diversity is lost after aerobic or anaerobic treatment and continued to decline during the storage of the aerobic reactor effluent while storage of the anaerobic reactor effluent resulted in increased diversity.

DISCUSSION

Modern high-intensity dairies generate copious amounts of waste that is usually stored in holding lagoons until it is applied to agricultural land as a fertilizer. This practice is becoming more problematic due to changes in agricultural demographics that concentrate large confined animal feeding operations in geographically limited regions like the San Joaquin Valley of California. These changes result in greater amounts of waste being deposited on crop fields with the potential to contribute

FIG. 2. Phylogenetic relationship of the operational taxonomic units isolated from feed material (F), aerobic and anaerobic reactor effluent (AR and AnR, respectively), aerobic and anaerobic storage tanks (AS and AnS, respectively), and untreated storage (US). 16S rRNA gene sequences representing the 10 most prevalent OTUs from each environment and the most similar 16S rRNA gene sequences to each OTU from the NCBI nr database were aligned using CLUSTALX. The dendrogram was constructed using the neighbor-joining algorithm and the Kimura two-parameter distance estimation method. Bootstrap values of >70%, generated from 1,000 replicates, are shown at the nodes. The scale bar represents the number of substitutions per site. Phylum designations are indicated on the right.

TABLE 4. Estimation of diversity within rRNA gene libraries

Library	No. of clones	Richness (no. of OTUs)	% Coverage	Evenness index (E)	Shannon index (H)
Feed material	3,434	618	82.0	0.82	5.28
Reactor					
Aerobic	1,593	401	74.8	0.84	5.04
Anaerobic	1,693	325	80.8	0.77	4.46
Storage					
Aerobic	1,017	308	69.7	0.82	4.69
Anaerobic	1,047	289	72.4	0.81	4.60
Untreated					
Storage 1	1,177	327	72.2	0.83	4.79
Storage 2	1,221	323	73.6	0.83	4.81

to food-borne illness (4), surface and groundwater contamination (15), and poor air quality (17). A possible solution to these challenges is the treatment of waste before storage and subsequent land application. The objective of the present study was

to model a typical dairy waste stream, monitor the chemical and bacterial population dynamics that occur during aerobic or anaerobic treatment and subsequent storage, and compare them to those of waste held without treatment in a simulated storage lagoon.

Our results indicate that both aerobic and anaerobic treatments followed by storage were superior to storage alone for the reduction of total solids, BOD₅, and coliform bacteria. In addition to these reductions, each system had unique remediation properties. For example, aerobic treatment significantly reduced both total nitrogen and ammonia levels. These reductions are likely the result of the deamination of proteins and peptides and the hydrolysis of urea to ammonia by ruminant bacteria (12, 28). In the oxygen-rich environment of the aerobic reactor, ammonia likely became nitrified by ammonia-oxidizing bacteria of the genus *Nitrosomonas*, whose 16S rRNA gene sequences were only observed in the aerobic treatment system (data not shown). When the oxidized nitrogen species entered the anoxic conditions of the storage tank, they were

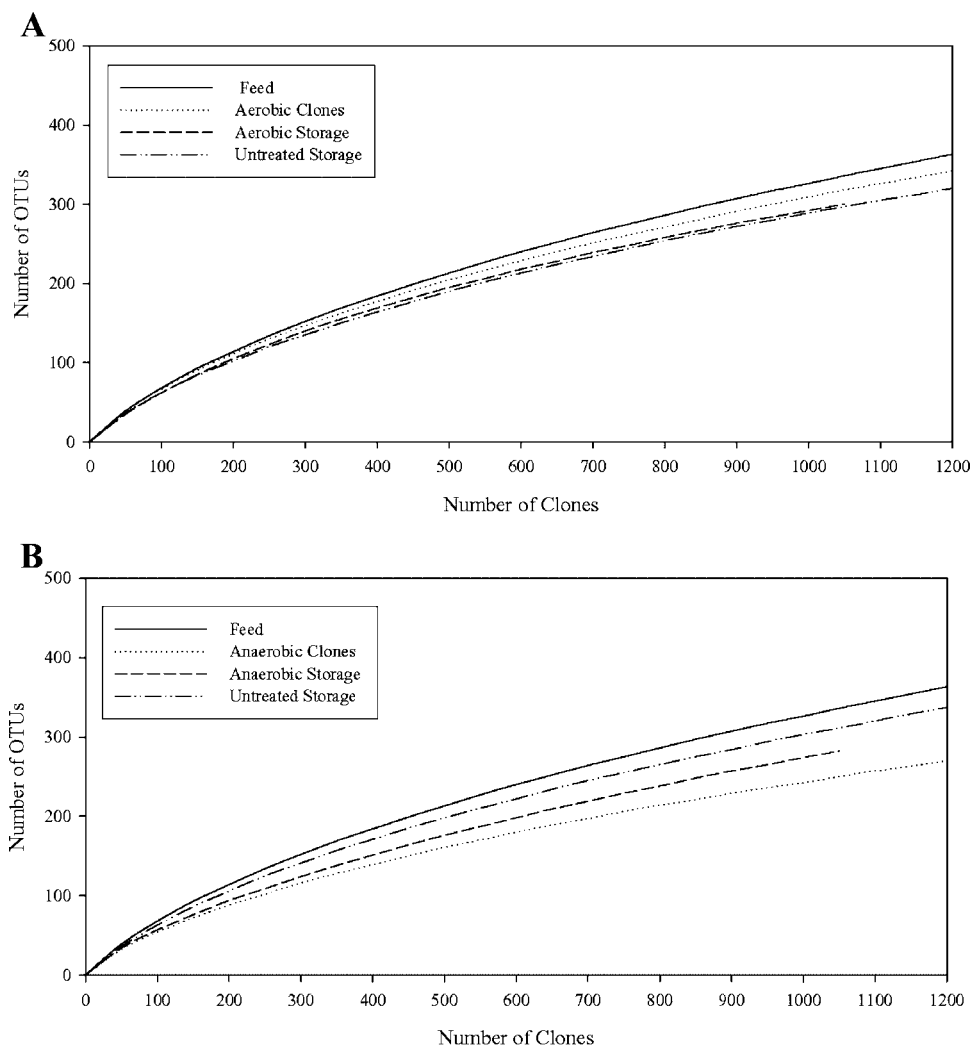


FIG. 3. Rarefaction curves for the (A) aerobic reactor and (B) the anaerobic reactor. Rarefaction analysis was performed using the approximation algorithm of Hurlbert (18) with 95% confidence intervals estimated as described by Heck et al. (16) using the freeware program aRarefactWin by Holland (<http://www.uga.edu/strata/software/Software.html>).

denitrified to volatile nitrogen-containing gases that escaped into the atmosphere. In addition, some ammonia was likely volatilized and assimilated by the bacteria. In the anaerobic system, significant reductions in sulfate and total sulfur were observed. This loss is likely explained by dissimilatory sulfate reduction to form hydrogen sulfide and other volatile sulfur-containing compounds (3, 5, 6) and, to a lesser extent, by assimilation.

At the phylum level, the feed material-derived 16S rRNA gene library was very similar to a library constructed from dairy waste reported previously (26). In both of these libraries, the greatest percentages of sequences were from members of the phylum *Firmicutes* (74% in this study versus 77% in the previous one), followed by the phyla *Bacteroidetes* (16% versus 7%), *Actinobacteria* (11% versus 9%), and *Proteobacteria* (3% versus 5%). The feed material library also possessed similarities to libraries derived from human feces (10), the gastrointestinal tracts of pigs (22), and, to a lesser extent, broiler chicken litter (23). The aerobic reactor effluent library had similarities to a library derived from a circulated dairy waste lagoon. In these libraries, the phylum *Proteobacteria* was most prominent followed by the *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* (25). However, these libraries differed in the abundance of the phylum *Firmicutes*, which represented 26.8% of the circulated waste lagoon-derived library, as compared to only 9.5% in the aerobic reactor effluent-derived library. This difference may be explained by the growth inhibition of many of the obligate anaerobic members of the *Firmicutes* in the aerobic reactor which maintained an oxygen concentration of 2 mg liter⁻¹ compared to the circulated waste lagoon, which was essentially anoxic. The predominance of *Firmicutes* 16S rRNA sequences increased to 21.7% after storage in a simulated waste lagoon, making it more closely resemble the library derived from the circulated dairy waste lagoon reported previously (25). The increased number of *Firmicutes*-like sequences may be explained by the anoxic conditions encountered in the simulated waste lagoon that support the growth of the obligate anaerobic species within this phylum. The library generated from the anaerobic reactor was similar to a library derived from a stagnant dairy waste lagoon (25); however, the relative levels of the *Proteobacteria* and *Bacteroidetes* were inverted. Subsequent storage of the anaerobic reactor effluent did little to change the bacterial community structure at the phylum level, with only a slight increase in the phylum *Deinococcus-Thermus* observed.

Of the 10 most prevalent OTUs in the waste-derived library, most have been recovered previously in dairy waste (F7) (26), wastewater lagoons (F2, -5, -6, and -8) (25), or the gastrointestinal tracts of swine (F3) (22). Storage without treatment does little to change the predominance of these OTUs, with the vast majority resembling those isolated previously in dairy waste (US1) (26), dairy wastewater (US2, -4, -6, -7, and -9) (25), or swine waste (US3 and -10) (22). Aerobic treatment and subsequent effluent storage resulted in the greatest changes in the most commonly observed OTUs, with only 3 of 20 OTUs previously associated with waste (AR3 and -6 and AS7), and the rest were similar to environmental isolates. Anaerobic treatment and subsequent storage resulted in fewer changes in the OTUs identified. Many of the 10 most prevalent OTUs have been recovered previously in manure or stagnant

dairy waste lagoons (AnR4, -6, -8, -9, and -10 and AnS1, -3, -6, -8, and -9) (25, 26).

The results presented here were obtained from bench-scale (3 to 5 liters) reactors and thus may not exactly replicate the much larger systems needed for a 1,000-cow dairy farm. For example, the hydraulic retention times, reactor temperatures, and mixing methods will likely require modifications during scale up. However, the results obtained using this model provide insights as to how the full-scale reactors will perform. Ultimately, the type of waste treatment utilized on dairies and other confined animal feeding operations will depend on multiple factors, including cost, type and amount of nutrient reduction desired, and government-imposed emission regulations. Because nitrogen is usually the limiting nutrient in animal waste-based fertilizers, anaerobic digestion, which tends to conserve nitrogen and is also the least expensive method to employ, will likely be popular. In addition, methane can be collected from anaerobic reactors and used as a fuel to generate heat and electricity or to run farm equipment. Another key factor is volatile chemical emissions, which are becoming a major problem in agricultural regions such as the San Joaquin Valley of California, where dairy farming is intensive. We are currently examining the emissions of various gases, including volatile organic compounds, from these processes.

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