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Comparison of bacterial populations and chemical composition of dairy wastewater held in circulated and stagnant lagoons

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ABSTRACT

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Aims: This study compared the chemical, physical and bacterial composition of circulated and stagnant dairy wastewaters.

Methods and Results: Samples taken from circulated and stagnant wastewater lagoons, over a 1-year period, were analysed for 10 chemical (total N, NH_3, NO_3, NO_2, Na, Ca, HCO_3, Fe, P and K) and six physical (biological oxygen demand, chemical oxygen demand, dissolved solids, electrical conductivity, pH and sodium absorption ratio) parameters and were found to be similar. The 16S rDNA genes from the samples were amplified, cloned and BLAST analysed. In total, 996 stagnant and 1052 circulated wastewater derived sequences were obtained, comprising 294 and 362 operational taxonomic units (OTUs) from the circulated and stagnant wastewaters respectively. Coverage estimates of the OTUs identified were 72·1% for the stagnant, and 63·6% for the circulated wastewater libraries. The greatest difference between the two wastewaters was a *c*. sixfold greater number of sequences representative of the family Chromatiaceae in the circulated wastewater derived library and a *c*. fivefold greater number of sequences representative of the phylum Chloroflexi in the stagnant wastewater derived library.

Conclusions: Circulation of dairy wastewater does not affect any of the chemical or physical parameters tested; however, circulation does alter the bacterial community structure.

Significance and Impact of the Study: This study provides evidence that circulation of dairy wastewater promotes the growth of bacteria within the family Chromatiaceae and that stagnant systems promote the growth of the phylum Chloroflexi.

Keywords: 16S rDNA sequences, circulated wastewater, dairy waste, microbial ecology, wastewater lagoon.

INTRODUCTION

Modern dairy farms generate large amounts of waste, and an effective waste management strategy is an important component of farm management that ensures good animal, as well as human health. In California, the average dairy farm houses 700 milking cows, but some mega-dairies may possess 15 000 cows at a single location. To efficiently house these large herds, the cows spend the majority of their productive life (i.e. lactating periods) in free stall barns where they defecate and urinate on cement floors. The average 650-kg dairy cow produces *c*. 50 kg of waste a day (Miner *et al.* 2000); thus, a 700-cow farm produces *c*. 35 000 kg of waste a day. To remove the waste from the free stall barn floor, it is common to use a hydraulic flush

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system that pumps *c.* 375 l of water per cow across the floor twice a day (Fulhage 1994). The flush water and waste flow into a solid/liquid separation system where the solids are removed, dried, and used as soil amendment or as animal bedding. The liquid portion is pumped into holding lagoons with a hydraulic retention time of *c.* 180 days; thus, for a 700-cow farm the holding lagoon would be *c.* 95 million l. The water from the lagoons is reused to flush the free stall floors and thus re-enters the system. Three to four times a year approximately half of the wastewater from the lagoons is pumped onto surrounding fields as a fertilizer for crop plants destined for animal and/or human consumption. This practice is of concern because pathogenic bacteria, such as *Escherichia coli* O157:H7 (Hancock *et al.* 1998), *Salmonella* (Warnick *et al.* 2001), *Campylobacter* (Wesley *et al.* 2000), etc. have been associated with cow manure and may contaminate the crops (Cieslak *et al.* 1993; Pell 1997: Natvig *et al.* 2002). There is also concern that pathogenic bacteria and nutrients such as sodium chloride, phosphate and nitrate can build up in the soils and leach into surface and ground waters rendering them unsuitable for human and/or animal consumption.

The treatment of dairy wastewater, so that it is microbiologically and chemically acceptable for use in flush and irrigation applications, is of great importance. Ibekwe *et al.* (2003) described the use of constructed wetlands to enhance the quality of dairy wastewater and showed that these systems reduced the chemical oxygen demand, the biological oxygen demand, the concentration of several key chemical species including total nitrogen, ammonia, phosphate, as well as suspended solids and bacterial numbers. However, the cost of constructing and maintaining these systems has so far been prohibitive for widespread use. Another, less costly method of treating wastewater is to hold it in circulated treatment lagoons. A recent study (McGarvey *et al.* 2004) has shown that circulated lagoons also reduce the levels of ammonia, total nitrogen, sulfate and bacteria. However, the efficiency of nutrient removal in circulated wastewater lagoons has never been compared with stagnant lagoon systems and thus it remains unknown if circulated systems are more efficient at nutrient reduction. Several vendors sell wastewater circulators and claim that they alter the nitrogen to phosphate ratio, reduce odours, pathogen levels, ammonia, hydrogen sulfide, sulfuric acid, etc. more efficiently than stagnant systems. However, if any few of these claims have been scientifically proven and thus remain anecdotal.

In this study, we examined the effect of circulation on the microbiological and chemical composition of wastewater at four time points over the course of 1 year (corresponding to spring, summer, fall and winter).

MATERIALS AND METHODS

Sample collection and preparation

The wastewaters examined were from two lagoons chosen because of their similar characteristics. For example, both lagoons are located within 1 km of each other in the San Joaquin Valley of California, and thus are exposed to similar environmental conditions (i.e. amount of rain, sunshine, temperature, etc). In addition, both lagoons are fed by approximately the same number of Holstein milking cows (*c.* 800) and have approximately the same holding capacity (*c.* 95 million l), ensuring similar loading rates. Furthermore, both lagoons receive waste from cows fed similar diets formulated by an animal nutritionist. The only obvious difference between these two lagoons is that one uses three circulators (Circul8 Systems^{TM}; Natural Aeration, Rearden, WA, USA) and the other is a stagnant system. Wastewater samples were taken on 4 August 2003, 12 December 2003, 20 April 2004 and 25 June 2004. Samples were taken as 5 l volumes from the hydraulic flush system that draws wastewater from an inlet located *c.* 1·5 m from the bottom of the lagoon by starting the flush cycle and allowing the water to run for *c.* 2 min to clear the line. A sterile 10 l carboy was placed in the path of the flush and half-filled. The temperatures of the water samples taken were between 11 and 19°C.

Viable counts of bacteria in wastewater samples

Wastewater samples were enumerated for viable bacteria by performing serial dilutions in phosphate-buffered saline which was vortex agitated for 1 min prior to being plated onto brain–heart infusion agar plates and incubated at 25°C for 2 days under normal atmospheric conditions or in an anaerobic chamber. To determine the number of coliform bacteria, samples were diluted as above but plated onto MacConkey agar plates and incubated at 37°C for 18 h. All media were purchased from Difco (Detroit, MI, USA) as dehydrated powders.

Chemical analysis of wastewater samples

Water samples were packed into foam coolers with blue ice packs and sent via next day delivery to A&L Western Agricultural Labs, Modesto CA, a State of California accredited agricultural and environmental testing laboratory for chemical analysis. All analyses were performed using protocols from Standard Methods of the American Public Health Association (1995).

DNA extraction from wastewaters

Micro-organisms from wastewater samples collected on 4 August 2003 and 12 December 2003 were harvested by

centrifugation at 10 000 *g* for 10 min. DNA was extracted from the resulting pellets using a modification of the MoBio UltraClean Fecal DNA Isolation Kit (MoBio, Solano Beach, CA, USA) as described previously (McGarvey *et al.* 2004).

PCR amplification of 16S rDNA sequences and library construction

The PCR amplification of 16S rDNA sequences was carried out using the eubacterial specific-primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1392r (5′-GACGGGCGGTGTGTAC-3′) (Brofft *et al.* 2002). PCRs were performed as recommended by Polz and Cavanaugh (1998) to reduce bias in amplification. Briefly, 50-μl reaction volumes contained 200 μmol l^{-1} deoxynucleotide triphosphates, 100 ng genomic DNA, two units Expand High Fidelity Enzyme mix, (Roche, Nutley, NJ, USA), in Expand High Fidelity buffer with 1·5 mmol l^{-1} $MgCl_2$ and 1 μmol l^{-1} of each primer. PCRs were performed in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) 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, 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, USA) as per the manufacturer's instructions and transformed into *E. coli* TOP10F′ cells (Invitrogen, Carlsbad, CA, USA) by heat shock (42°C for 30 s). Clones were plated on LB agar plates containing kanamycin (Km) (50 μg ml^{-1}), isopropyl-β-D-thiogalactopyranoside (20 mmol l^{-1}) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (80 μg ml^{-1}). White colonies were selected and grown in 96-well plates in LB Km broth. A total of seven PCRs 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 in 96-well plates using the TempliPhi 100 Amplification Kit (Amersham Biosciences, Sunnyvale, CA, USA) as per the manufacturer's instructions. Sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) as described by the manufacturer using the primer 1392r. Sequencing reactions were purified using the DyeEx 96 Kit (Qiagen); electrophoresis and readouts were performed using an Applied Biosystems 3100 Genetic Analyzer (Applied Biosystems). A total of 996 stagnant and 1052 circulated wastewater derived 16 rDNA sequences were obtained and analysed.

DNA sequence analysis, dendrogram construction, rarefaction analysis and statistical methods

DNA sequences were edited manually to correct falsely called bases and trimmed at both the 5′ and 3′ ends using the Chromas software (Technelysium Pty. Ltd, Helensvale, Australia). Only sequences with unambiguous reads longer than 500 bp were used, and each read averaged *c.* 600 bp. The predicted 16S rDNA sequences from this study were compared with 16S rDNA sequences in a BLASTable database constructed previously (McGarvey *et al.* 2004). This database contains sequences downloaded from the Ribosomal Database Project II (http://rdp8.cme.msu.edu/download/SSU_rRNA/unaligned/); Release 8.1. Comparisons were made using the program BLASTALL (ftp://ftp.ncbi/nih.gov/blast/executables/LATEST) and a FASTA-formatted file containing the predicted 16S rDNA sequences. Operational taxonomic units (OTUs) were defined as clones with >97% sequence identity. For dendrogram construction, 40 partial 16S rDNA sequences representing the 20 most prevalent OTUs from each environment (circulated or stagnant) were aligned using CLUSTALX. Also included in this alignment were the most similar 16S rDNA sequences to each OTU from the NCBI nr or env_nr databases. These 16S rDNA sequences were 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 (Kumar *et al.* 2001). The dendrogram was constructed using the neighbour-joining algorithm and the Jukes–Cantor distance estimation method.

Rarefaction analysis was performed using the approximation algorithm of Hurlbert (1971) with 95% confidence intervals estimated as described by Heck *et al.* (1975) using the freeware program aRarefactWin by Holland (1998). The per cent of the total OTUs identified in each sample was calculated using the equation $C = 1 - (n/N) \times 100$, where C is the per cent coverage, n is the number of OTUs and N is the number of clones examined. Several statistical techniques available in the SAS STAT package were employed to test for differences between 16S rDNA libraries as well as the gross microbiological, chemical and physical parameters measured in the circulated and stagnant lagoons. The Student's *t*-test was chosen as the most appropriate method, but with an approximation based on unequal variances between the two lagoons in several instances where tests indicated variances differed significantly (TTEST procedure). When four samples of data were available, probability levels for a group of variables were corrected to yield a family-wise error rate using a step-down bootstrap method in the MULTTEST procedure. Statistical tests were considered significant at the 0·05 probability level.

Comparisons of the 16S rDNA libraries were also analysed using the LIBSHUFF software (Singleton *et al.* 2001).

Spectroscopic analysis of wastewater

To demonstrate the differences in photosynthetic purple sulfur bacteria, we compared the amount of carotenoid pigments contained within the wastewater types. Two millilitre of stagnant or circulated wastewater was centrifuged at 14 000 *g* for 2 min, the supernatant was removed and the pellet was washed three times with distilled deionized water. The resulting pellet was extracted with 1 ml of methanol and centrifuged as above to pellet the cellular debris. The methanol supernatant was discarded and the pellet extracted with 1 ml of ethyl acetate. The resulting solution was centrifuged as above to remove the cellular debris and the supernatant was analysed using a Cary 300 Bio UV/Vis Spectrophotometer (Varian, Walnut Creek, CA, USA).

PCR detection of pathogens

Pathogen-specific PCRs were performed on DNA extracted from wastewaters for *Salmonella*, pathogenic *E. coli*, *Campylobacter* spp., *Yersinia* spp., *Listeria* spp., toxigenic staphylococci and *Clostridium perfringens* as described previously (Lu *et al.* 2003). Controls for PCR experiments were performed by spiking DNA samples of community DNA with low levels of DNA from the appropriate bacteria to ensure that the reactions were working properly.

Nucleotide sequence accession numbers

DNA sequences representative of the 20 most numerous OTUs from each library were deposited into GenBank under the accession numbers AY762906–AY762937.

RESULTS

Cultural, chemical and physical analysis of circulated and stagnant wastewaters

Wastewater samples were taken at four time points over the course of 1 year (roughly corresponding to spring, summer, fall and winter) and analysed for gross microbiological characteristics (aerobic, anaerobic and coliform plate counts), 10 chemical parameters (total N, NH_3, NO_3, NO_2, Na, Ca, HCO_3, Fe, P and K) and six physical characteristics (biological oxygen demand, chemical oxygen demand, dissolved solids, electrical conductivity, pH and sodium absorption ratio) (Table 1). The average aerobic, anaerobic and coliform bacterial counts in the circulated and stagnant wastewaters were similar, and not significantly

Table 1 Cultural, chemical and physical analysis of circulated and stagnant dairy wastewaters

Parameter	Circulated wastewater [average (range)]	Stagnant wastewater [average (range)]	Fold-difference
APC (CFU ml^{-1})	$1{\cdot}5 \times 10^6$ ($3{\cdot}2 \times 10^5$–$2{\cdot}9 \times 10^6$)	$5{\cdot}7 \times 10^5$ ($3{\cdot}1 \times 10^5$–$1{\cdot}0 \times 10^6$)	2·6
AnPC (CFU ml^{-1})	$7{\cdot}1 \times 10^5$ ($2{\cdot}1 \times 10^5$–$2{\cdot}0 \times 10^6$)	$8{\cdot}3 \times 10^5$ ($1{\cdot}1 \times 10^5$–$2{\cdot}1 \times 10^6$)	1·2
CPC (CFU ml^{-1})	$7{\cdot}3 \times 10^3$ ($1{\cdot}7 \times 10^3$–$1{\cdot}1 \times 10^4$)	$3{\cdot}7 \times 10^3$ ($6{\cdot}8 \times 10^2$–$7{\cdot}8 \times 10^3$)	1·9
Total N (mmol l^{-1})	8·6 (6·3–15·6)	14·4 (6·4–22·5)	1·3
NH_3 (mmol l^{-1})	10·5 (9·5–12·1)	11·3 (4·5–19·1)	1·1
NO_3	ND	ND	–
NO_2	ND	ND	–
SO_4 (mmol l^{-1})	0·65 (0·41–0·82)	0·67 (0·13–1·39)	1·0
Na (mmol l^{-1})	6·3 (5·7–7·0)	6·1 (2·0–7·7)	1·0
Ca (mmol l^{-1})	4·6 (2·8–6·5)	5·3 (1·7–7·2)	1·2
HCO_3 (mmol l^{-1})	29 (27·0–32·0)	35 (15·0–61·9)	1·2
Fe (mmol l^{-1})	0·04 (0·006–0·09)	0·02 (0·01–0·13)	2·0
P (mmol l^{-1})	1·3 (1·1–1·5)	1·6 (0·7–2·0)	1·2
K (mmol l^{-1})	1·4 (0·6–2·4)	1·2 (0·3–1·7)	1·2
BOD (mg l^{-1})	483 (168–773)	274 (152–361)	1·8
COD (mg l^{-1})	2210 (1600–2800)	1700 (800–3200)	1·3
DS (mg l^{-1})	2710 (2530–2890)	3244 (1217–4038)	1·2
EC (dS m^{-1})	3·9 (3·6–4·4)	4·3 (1·8–7·4)	1·1
pH	7·5 (7·3–7·6)	7·4 (7·3–7·5)	1·0
SAR	3·2 (3·1–3·3)	3·1 (1·5–4·9)	1·0

APC, aerobic plate counts; AnPC, anaerobic plate counts; CPC, coliform plate counts; ND, none detected; total N, total Kjedahl nitrogen; BOD, biological oxygen demand; COD, chemical oxygen demand; DS, dissolved solids; EC, electrical conductivity; SAR, sodium absorption ratio.

different at the $P = 0{\cdot}05$ level. The 11 chemical parameters measured in the circulated and stagnant wastewaters were similar and not significantly different from each other at the $P = 0{\cdot}05$ level. Lastly, the six physical characteristics measured were also found to be similar and not significantly different.

Analysis of 16S rDNA libraries derived from circulated and stagnant wastewaters

We constructed 16S rDNA libraries from the circulated and stagnant wastewaters containing *c.* 500 sequences each, at two time-points corresponding to summer and winter. The sequences of these clones were BLAST analysed using a 16S rDNA database containing over 33 000 sequences (Cole *et al.* 2003). Both libraries contained sequences derived from bacteria that have not been cultured and thus little information about their physiology can be derived. The sequences representative of these uncultured bacteria represented 32·3% of the circulated, and 31·1% of the stagnant wastewater derived libraries (Table 2). Of the remaining sequences, for which there is information, the majority of OTUs from the circulated wastewater derived library were associated with the phylum Proteobacteria (30·4%) followed by the Firmicutes (26·8%), the Bacteroidetes (2·6%), the Actinobacteria (2·1%), the Verrucomicrobia (1·5%), the Chloroflexi (1·1%), and the Spirochaetes (0·3%); all other phyla represented 1·9% (Table 2a). In the stagnant wastewater derived library the most populous phylum represented was the Firmicutes (25·4%) followed by the Proteobacteria (18·9%), the Bacteroidetes (6·9%) the Chloroflexi (5·9%), the Actinobacteria (3·8%), the Verrucomicrobia (2·6%), and the Spirochaetes (1·6%); all other phyla represented 4·1% (Table 2a). The percentages of each phylum in the two libraries were similar, except for the phyla Chloroflexi and Spirochaetes which were 5·4- and 5·3-fold more numerous in the stagnant wastewater derived library (Fig. 1a), and were found to be significantly different from the circulated wastewater derived library. Although the phylum Proteobacteria was only 1·6-fold more numerous in the circulated wastewater derived library, the class γ-Proteobacteria was 4·6-fold more numerous in the circulated wastewater derived library (Table 2b), and was found to be significantly different from the stagnant wastewater derived library. This difference was predominantly because of the increased abundance of the family Chromatiaceae (purple sulfur bacteria) which were greater than sixfold more abundant in the circulated wastewater derived library (data not shown). The Chromatiaceae impart a reddish colour to the circulated wastewater, because of their high levels of carotenoid pigments. To confirm the difference observed in the 16S rDNA libraries, we extracted the carotenoid pigments from both wastewater types and found >10-fold higher concentration of these pigments in the circulated wastewater (Fig. 1).

Table 2 Bacterial phyla represented in 16S rDNA sequence libraries derived from circulated and stagnant dairy wastewaters. (a) Bacterial phyla represented in libraries derived from circulated and stagnant dairy waste lagoons; (b) classes within the phylum Proteobacteria represented in libraries derived from circulated and stagnant dairy waste lagoons

	% Abundance in libraries derived from		
	Circulated lagoons [average (range)]	Stagnant lagoons [average (range)]	Fold-difference
(a) Phylum			
Unknown	32·3 (28·3–36·2)	31·1 (30·6–31·5)	1·0
Firmicutes	26·8 (23·2–30·3)	25·4 (18·8–32·0)	1·1
Proteobacteria	30·4 (27·2–33·6)	18·9 (20·6–37·1)	1·6
Bacteroidetes	2·6 (2·3–2·8)	6·9 (5·4–8·4)	2·6
Actinobacteria	2·1 (1·6–2·5)	3·8 (1·8–7·8)	1·8
Chloroflexi	1·1 (0·7–1·6)	5·9 (5·4–6·3)	5·4
Verrucomicrobia	1·5 (1·2–1·6)	2·6 (2·2–3·0)	1·7
Spirochaetes	0·3 (0·2–0·4)	1·6 (1·4–1·8)	5·3
All others	1·9 (1·4–2·4)	4·1 (2·8–5·4)	2·2
(b) Class			
α-Proteobacteria	0·5 (0·2–0·7)	0·8 (0·4–1·2)	1·6
β-Proteobacteria	2·9 (3·4–2·3)	3·1 (0·3–3·2)	1·1
γ-Proteobacteria	23·2 (22·2–24·1)	5·1 (4·2–6·0)	4·6
δ-Proteobacteria	3·8 (3·1–4·4)	9·0 (6·8–11·2)	2·3
ε-Proteobacteria	0·8 (0·4–1·2)	0·3 (0·2–0·4)	2·7
Unknown	0·2 (0·1–0·1)	0·1 (0·0–0·2)	2·0

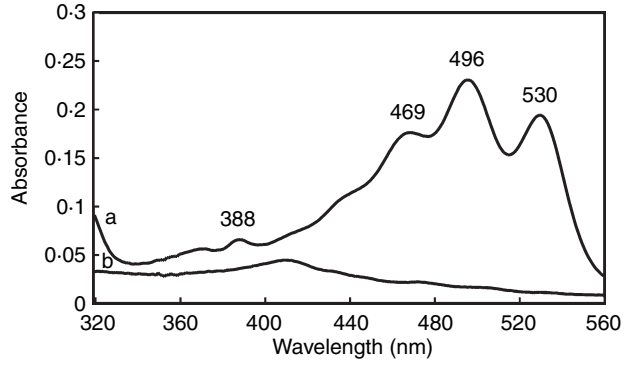

Fig. 1 UV-visible absorption spectra of ethyl acetate extracts of circulated and stagnant wastewater. Spectra for circulated wastewater (trace a) with absorption peaks characteristic of carotenoid pigments found in purple sulfur bacteria and stagnant wastewater (trace b) which is devoid of these absorption peaks

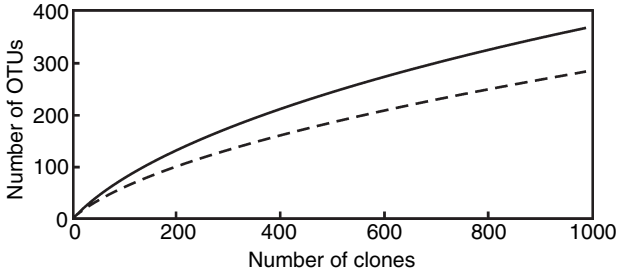

Fig. 2 Rarefaction curves for 16S rDNA clone libraries derived from circulated and stagnant wastewaters using the Rarefaction Calculator (located at http://www.uga.edu/~strata/software/) by Holland. Sequences were grouped into operational taxonomic units based on 97% or greater sequence similarity. Solid lines refer to the stagnant wastewater-derived library, and dotted lines refer to the circulated wastewater-derived library. Average slope for the circulated wastewater derived library in 200 clone increments are 0·65, 0·42, 0·33, 0·26 and 0·24; for the stagnant wastewater derived library are 0·50, 0·33, 0·24, 0·20 and 0·19

Estimates of diversity and the 20 most prevalent OTUs isolated from circulated and stagnant wastewaters

To analyse the levels of diversity within the libraries derived from circulated and stagnant wastewaters we applied rarefaction analysis using the freeware programme of Holland (Holland 1998). The rarefaction curves do not reach saturation (slope = 0/1) suggesting that we did not identify all of the OTUs present (Fig. 2). However, the average slopes of both curves decrease continually to the end points, starting at 0·65 and 0·50 from 0 to 200 clones and ending at 0·24 and 0·19 from 800 to 1000 clones for the stagnant and circulated libraries respectively. This 37–38% decrease in the slopes indicates that the most prevalent species were likely identified. This graph can also be used to elucidate information about the diversity within the libraries. Because the slope of the curve = (number of OTUs)/(total number of clones) it quantifies the diversity within the libraries. The slope of the curve representing the circulated library is consistently less than that of the curve representing the stagnant library, indicating that the stagnant wastewater derived library is more diverse than the circulated wastewater derived library. Coverage analysis supports this conclusion, which estimates the percentage of the total OTUs identified to be 72·1% for the circulated *vs* 63·6% for the stagnant wastewater derived library.

Although the libraries contained significant differences within the family Chromatiaceae and the phyla Chloroflexi, and Spirochaetes the remainder of the libraries were similar. For example, the relative amounts of the other major phyla are very similar (Table 2) and not significantly different at the $P = 0{\cdot}05$ level. To further characterize the libraries we compared the 20 most commonly isolated OTUs from each library (Table 3 and Fig. 3) and determined that eight (40%) are found in both lists. When the 20 most populous OTUs in each library were compared with the entire library of the other sample they showed even greater similarities: 55% for the circulated library and 70% for the stagnant library. These data indicate that although significant differences occur in the ratio of specific phyla and classes between the libraries, most groups of bacteria are common to, and are at similar levels in both wastewater types.

Detection of 16S rDNA sequences similar to those of pathogenic bacteria

We identified 16S rDNA sequences with similarity to known pathogenic bacteria. From the library derived from the circulated wastewater we identified seven clones with 16S rDNA sequences 98–99% similar to *Arcobacter cryaerophilus*, and three clones with 98% similarity to *Clostridium septicum*. From the stagnant wastewater derived library we identified one clone with 99% similarity to *Rhodococcus equi*. Three sequences from the circulated and 17 sequences from the stagnant wastewater derived library have low sequence similarity to *Treponema* spp. associated with human periodontal disease, and may be associated with papillomatous digital dermatitis (PDD) also known as hairy foot wart disease. Pathogen-specific PCR to the common food borne pathogens: *E. coli* O157 : H7, *Salmonella*, *Campylobacter*, *Listeria*, *Salmonella*, *Staphylococcus aureus*, *Yersinia* and *Mycobacterium avium* ssp. *paratuberculosis* was performed, but no DNA fragments were amplified.

DISCUSSION

The object of this study was to determine if circulation alters the microbiological, chemical or physical properties of dairy wastewater. We compared two lagoons that were similar in many ways including: geographical location, wastewater holding capacity, loading rate, etc.; however, one lagoon used three circulators and the other was a stagnant system. Thus, it was not surprising that the wastewaters were similar in many aspects. For example, the gross microbiological, chemical and physical characteristics of the circulated and stagnant wastewaters were not significantly different. The similarities observed in the water chemistry are consistent with the observations of Rumburg *et al.* (2004) who installed the same brand of circulators in a dairy wastewater lagoon and observed no change in ammonia or total nitrogen levels. The COD and BOD values we observed were also not significantly different between the stagnant and circulated wastewaters, indicating that the circulators do not incorporate large amounts of oxygen into the wastewater, but only mix them. Thus, we were unable to identify any physical, chemical or

Table 3 The 20 most commonly isolated operational taxonomic units (OTUs) from each library

OTU	Most similar sequence in GenBank	Number of clones	% Similarity	Phylum
Circulated wastewater clones				
C1	*Rhabdochromatium marinum*	109	91–94	Proteobacteria (γ)
C2	*Clostridium lituseburense*	58	97–99	Firmicutes
C3	*Pseudomonas anguilliseptica*	56	96–99	Proteobacteria (γ)
C4	*Arcobacter cibarius*	53	96–98	Proteobacteria (ε)
C5	AY438769	32	98–100	Unknown
C6	*Thiolamprovum pedioforme*	29	96–97	Proteobacteria (γ)
C7	AY438880	28	95–98	Unknown
C8	U81676	28	95–98	Unknown
C9	U81706	21	97–100	Unknown
C10	*Eubacterium tenue*	20	98–99	Firmicutes
C11	*Turicibacter sanguinis*	20	98–99	Firmicutes
C12	*Achromatium* sp. HK15	20	91–94	Proteobacteria (γ)
C13	*Desulfobulbus* sp. RPf35L17	12	96–98	Proteobacteria (δ)
C14	AY438835	12	95–98	Unknown
C15	AY345500	10	92–94	Unknown
C16	AF050628	10	94–96	Firmicutes
C17	AF276459	10	93–94	Unknown
C18	AY438730	9	98–99	Unknown
C19	*Aquaspirillum metamorphum*	7	98–99	Proteobacteria (β)
C20	*Arcobacter cryaerophilus*	7	98–99	Proteobacteria (ε)
Stagnant wastewater clones				
S1	AY218558	32	92–94	Unknown
S2	AJ009469	26	95–98	Chloroflexi
S3	*Clostridium lituseburense*	23	96–99	Firmicutes
S4	AY438769	22	98–100	Unknown
S5	AY438715	20	97–100	Unknown
S6	*Achromatium* sp. HK15	19	91–94	Proteobacteria (γ)
S7	AF050542	17	96–99	Bacteroidetes
S8	AY345500	17	94–97	Unknown
S9	U81676	17	94–97	Unknown
S10	*Cytophaga* sp.	14	95–97	Bacteroidetes
S11	AF050534	14	96–99	Proteobacteria (δ)
S12	AF050570	13	97–100	Chloroflexi
S13	AJ278162	13	94–97	Proteobacteria (δ)
S14	U81706	12	97–100	Unknown
S15	AY438835	11	92–94	Unknown
S16	AF050628	10	95–97	Firmicutes
S17	*Clostridium thermocellum*	10	92–95	Firmicutes
S18	*Lachnospiraceae* 19gly4	9	95–96	Firmicutes
S19	AY160830	9	92–95	Unknown
S20	*Smithella propionica*	9	95–97	Proteobacteria (δ)

gross microbiological differences in the wastewaters because of circulation. However, based on rarefaction analysis, coverage analysis and the percentage of the total sequences represented by the 20 most prevalent OTUs the stagnant wastewater derived library was found to have greater diversity than the circulated wastewater derived library. It is possible that circulation eliminates chemical and thermal stratification and creates a more homogenous environment that selects for fewer types of bacteria than the stagnant system which would be expected to be divided into thermo- and chemo-clines creating multiple environmental conditions that can be exploited by different types of bacteria.

When the bacterial population structure of circulated and stagnant wastewaters was examined we observed that the relative percentages of 16s rDNA sequences, representing 14 of 16 bacterial phyla present in both systems, were not significantly different. These data, taken together with the gross microbiological, chemical and physical data are consistent with our initial assumption that the two lagoons are very similar except that one lagoon is circulated and the

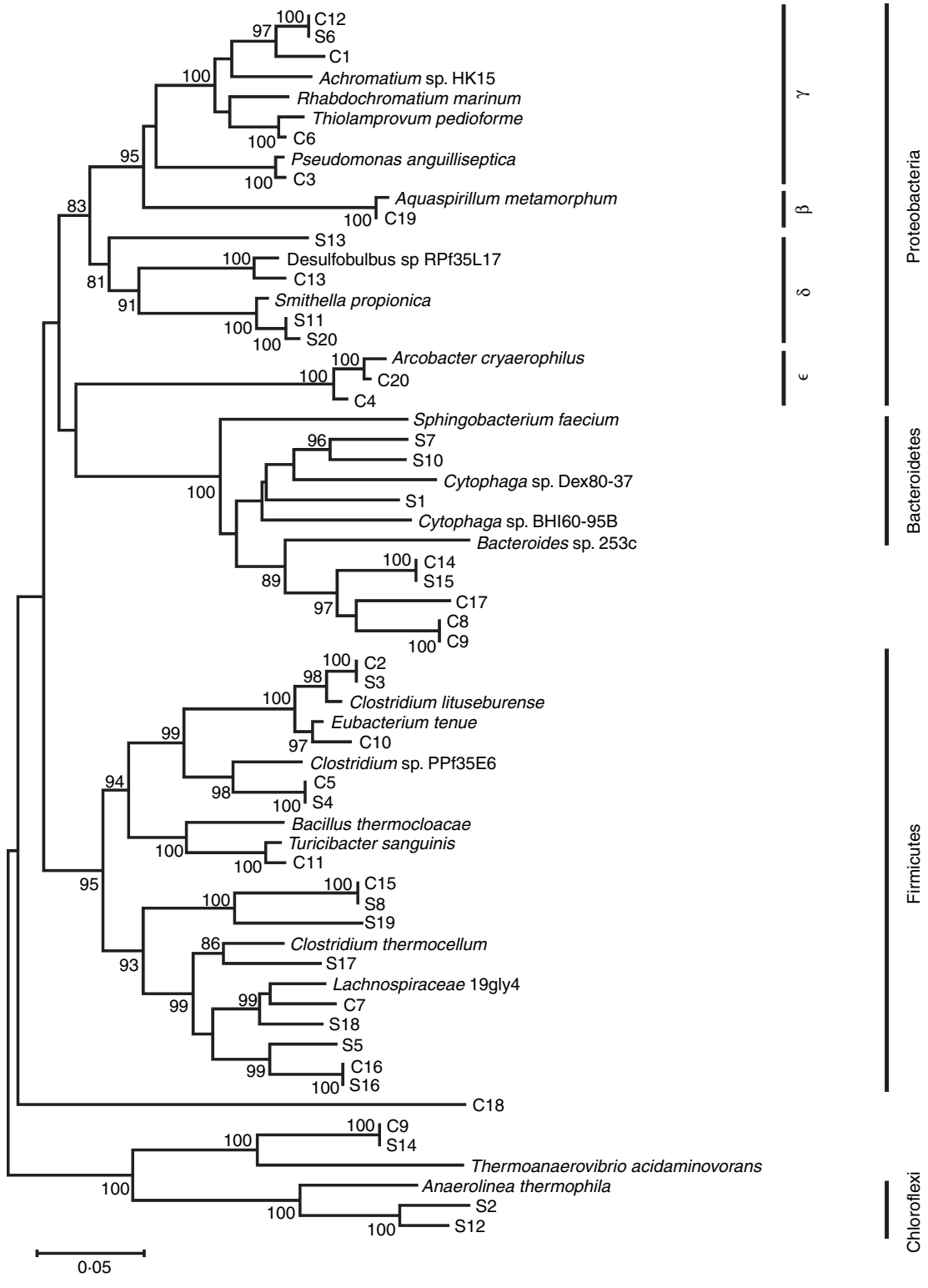

Fig. 3 Phylogenetic relationship of the operational taxonomic units isolated from circulated (C) and stagnant (S) wastewater. The dendrogram was constructed using the neighbour-joining algorithm and the Jukes–Cantor distance estimation method. Bootstrap values (>70%) generated from 1000 replicates are shown at the nodes. The scale bar represents substitutions per site. Phylum and class designations are indicated on the right. Labels were added in Corel Draw 11 (Corel, Ottawa, ON, Canada).

other is stagnant. However, statistical analysis of the entire 16S rDNA libraries using the freeware program LIBSHUFF (Singleton *et al.* 2001) determined that the libraries were statistically different at the $P = 0{\cdot}05$ level. These differences are most notable in the family Chromatiaceae and the phyla Chloroflexi and Spirochaetes.

In the circulated wastewater derived library, sequences representative of the γ-Proteobacteria were 4·6-fold more abundant than in the stagnant wastewater derived library. This increase was predominantly because of the >6-fold increase in members of the family Chromatiaceae, also known as the purple sulfur bacteria. The increased prevalence of these bacteria can be visually observed by the red/purple colour of the circulated wastewater, which is caused by the carotenoid pigments contained within these bacteria. Chemical extraction of carotenoid pigments from the wastewaters is consistent with the 16S library data, which indicate that the purple photosynthetic bacteria are more numerous in the circulated than in the stagnant wastewater. We have observed blooms of purple sulfur bacteria in other circulated wastewater lagoons in the area, but have only rarely observed them in stagnant wastewater lagoons. Photosynthetic purple bacteria have been associated with sewage treatment systems (Holm and Vennes 1970; Gieseke *et al.* 2003; van der Steen *et al.* 2003) and have been shown to utilize a wide variety of volatile organic acids (VOAs) and other waste related compounds as electron and carbon sources for photoheterotrophic growth (Holm and Vennes 1970; Do *et al.* 2003; van der Steen *et al.* 2003). Indeed, the rate of VOA emissions from waste lagoons has been shown to be inversely proportional to the number of these bacteria (Do *et al.* 2003). We have consistently noted that the circulated lagoons are less malodorous than stagnant lagoons and speculate that the purple sulfur bacterial blooms are responsible for the reduced odour and believe this to be the major benefit of circulation.

The 16S rDNA sequences representative of the phylum Chloroflexi (green nonsulfur bacteria) were significantly more abundant in the stagnant, than in the circulated wastewater derived library. The Chloroflexi have been shown to be abundant in some types of wastewater treatment systems (Bjornsson *et al.* 2002). The two most prominent Chloroflexi observed in our study, OTUs S2 and S12, which are 95–98% and 97–100% similar to AJ009469 and AF050570, respectively, have been associated with trichlorobenzene and other hydrocarbon contaminated ecosystems (Dojka *et al.* 1998; von Wintzingerode *et al.* 1999). Indeed, the vast majority of the Chloroflexi sequences identified from the stagnant wastewater (91%) belong to subdivision 1A, which has been associated with activated sludge and hydrocarbon degrading consortia (Bjornsson *et al.* 2002). It is likely that these organisms are able to utilize the complex organic material in wastewater; however, the specific-cultural parameters that result in their selective advantage in stagnant wastewater remain unclear.

The 16S rDNA sequences representative of the phylum Spirochaetes were also significantly more abundant in the stagnant than in the circulated wastewater derived library. The greater abundance of the Spirochaetes-like sequences in the stagnant wastewater derived library may be related to the higher incidence of PDD, also called hairy foot warts, on the farm with the stagnant system. This disease is speculated to be caused by several different species of strictly anaerobic *Treponema* spp. that have 16S rDNA sequences similar to those of *Treponema* spp. associated with human periodontal disease (Choi *et al.* 1997; Trott *et al.* 2003). Interestingly, the majority (69%) of the 16S sequences isolated from the stagnant wastewater derived library are similar to those of *Treponema* spp. isolated from humans with periodontal disease, suggesting a link between these isolates and PDD. It should be noted that the number of these sequences were low in both wastewater types, with only three sequences detected in the circulated and 16 sequences detected in the stagnant wastewater derived library; however, this difference is statistically significant at the $P = 0{\cdot}05$ level. Lastly, it remains unclear if the increased abundance of *Treponema* spp. in the stagnant lagoon is because of their ability to survive better in this system or if the higher incidence of PDD on this farm introduces more of these bacteria into the lagoons.

Bacteria with 16S rDNA sequences similar to those of pathogenic bacteria were identified in both wastewater types. Most important were the seven clones with sequences 98–99% similar to *Arcobacter cryaerophilus* in the circulated wastewater. *Arcobacter cryaerophilus* has been isolated from humans with abdominal illness and septicaemia (Vandamme *et al.* 1992) and have been observed in dairy cattle waste (Wesley *et al.* 2000). *Arcobacter* are similar to *Campylobacter*, but grow at temperatures from 15 to 37°C (Neill *et al.* 1985). We also isolated three sequences with 98% similarity to *Clostridium septicum* from the circulated lagoons. *Clostridium septicum* is the aetiological agent of pseudoblackleg in cattle and sheep, an infection of skeletal and heart muscle that results in the inflammation of these tissues and is often fatal (Nervig *et al.* 1981). From the stagnant wastewater derived library we identified one sequence with 99% similarity to *R. equi*, an important cause of necrotizing bronchopneumonia in grazing animals (Meijer and Prescott 2004). Lastly, we identified 16S sequences similar to those from organisms believed to cause PDD from both systems. These data indicate that circulation does not categorically reduce pathogen levels in wastewater, but likely reduces the levels of some while enhancing the ability of others to persist.

Our findings suggest that circulation does not enhance nutrient reduction of wastewater or alter any of the gross microbiological, chemical or physical parameters that we measured when compared with stagnant systems. The most dramatic benefit associated with circulation we observed was the increase in purple sulfur bacteria that may be related to the reduction of malodorous compound emission. These compounds, which include VOAs and other volatile organic compounds (VOCs), are known greenhouse gasses. The emission of these compounds from wastewater lagoons is becoming more of a concern to regulatory agencies, which up to now have not regulated their discharge. However, with poor air quality in agricultural communities becoming an important issue, VOA and VOC emissions from waste lagoons may become regulated in the near future. We are now investigating the role of circulation in the reduction of VOC and VOA emission.

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