A Novel Screening Tool Using Microarray and PCR to Detect Pathogens in Agriculturally Impacted Waters

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***Abstract*-Microbial populations are present in high concentrations in dairy wastewater treatment lagoons and thus pose a potential public health risk. A broad and robust detection assay is needed to monitor the presence of human pathogens in such systems. The objective of this study was to develop and corroborate a microarray-based assay for screening and monitoring of agriculturally-impacted waters for immediate detection of > 1,500 specific viruses and bacteria. Water samples from two dairy lagoons (Lagoons I and II) were screened using molecular assays (such as ElectroChemical Detection (ECD) 12K microarray) in conjunction with traditional analytical chemical methods. Bacterial signature DNA sequences and viral signature DNA/RNA sequences that are associated with human diseases were detected in higher numbers in both lagoons. The presence of some of these microbial signatures in the lagoons was confirmed using PCR and qPCR techniques. Based on microscopic analyses, most probable number (MPN) counts, total viable counts (CFU), 16S rRNA gene analysis, microarray signal intensity analyses, and qPCR assays, the microbial community density was found to trend higher in Lagoon I than Lagoon II; however, microbial diversity in Lagoon I trended lower. Results from this research confirm the usefulness of ECD-based microarray technology as a pathogen screening method for agriculturally-impacted waters.**

Keywords- ElectroChemical Detection Microarray (ECD); Pathogen Detection; Monitoring; Dairy Lagoons

I. INTRODUCTION

Water is a common pathogen reservoir and thus plays a significant role in the transmission of disease. According to the World Health Organization (WHO), 75% of emerging pathogens are zoonotic and can be transported in the environment through groundwater, aerosols emitted from sewage plants, animal manure, and irrigation water systems (Ford 1999; Ravva et al. 2006). The environmental transmission of water borne pathogens poses serious human health risks, with many diseases acquired after consumption of contaminated water and food that were exposed to water intensive operations, including but not limited to dairy operations (Van Donkersgoed et al. 2001; LeJeune, Besser, and Hancock 2001; Tauxe 2002; Fong and Lipp 2005).

To minimize water borne diseases, regulatory agencies such as the United States Environmental Protection Agency (EPA) are still relying on traditional bacterial indicators (e.g., fecal and total coliforms) to assess water quality; however, there are several pathogens present in water other than bacteria, such as viruses and protozoa (U.S.EPA 2006; Haas et al. 1993). Furthermore, studies have shown that bacterial indicators die rapidly compared to viruses and protozoa (Bordalo, Onrassami, and Dechsakulwatana 2002). Also, bacterial indicators are unable to predict the presence of pathogenic viruses (Griffin et al. 1999; Noble 2001), and recent studies suggest that using viruses that are associated with feces should be considered as a pollution indicator and a replacement or supplement to bacterial indicators because viruses are more resilient and survive longer than bacteria in the environment (Cole, Long, and Sobsey 2003; Wetz et al. 2004; Hejkal et al. 1981; Symonds, Griffin, and Breitbart 2009).

To simultaneously monitor for the presence of bacterial and viral pathogens novel sensitive and rapid methodologies are required. Herein, we describe such an approach to screen simultaneously for thousands of pathogens in a sample in a quick and effective way. This approach if applied widely could provide for improved water quality monitoring worldwide.

For development of this method we employed as our model system dairy wastewater lagoon samples. The lagoon system consisted of two lagoons operated in series (primary Lagoon I which empties into secondary Lagoon II). Both lagoons are evaporative systems, though water is sometimes removed from the lagoons to irrigate nearby pasture. Physiochemical analyses were first performed on water samples from both lagoons. This was followed by immediate microscopic analysis, anaerobic

bacteria culture, and Most Probable Number (MPN) fecal coliform counts. The culture-based techniques are not a reliable assessment for the presence of many potentially pathogenic viruses and bacteria nor can they be used to predict with assurance any health threats caused by the presence of pathogens. Thus, we also utilized several molecular assays including 16S rRNA gene sequence analyses, microarray-based analyses, PCR/qPCR analyses and sequencing to better detect the presence of pathogenic bacteria and/or viral contamination quickly and efficiently.

New molecular approaches to monitor, screen for, and detect potential waterborne pathogens are being explored worldwide. One of the cutting-edge molecular biology-based approaches is DNA microarray technology, which allows scientists to conduct large scale, data-intensive experiments simultaneously on many environmental samples. Scientists have employed this technology to detect the presence or absence of pathogens in environmental samples (Call, Borucki, and Loge 2003; DeSantis et al. 2007). One of the newest types of microarray technology is the ElectroChemical Detection (ECD) 12K microarray (CombiMatrix Corp., Mukilteo, WA). The ECD technology utilizes a semiconductor matrix that contains > 12,000 nucleic acid probes individually synthesized on a single chip. Semiconductor matrix circuitry controls the microelectrodes digitally to allow synthesis of custom probes of specific desired sequences. Each probe "microelectrode" is individually synthesized and activated under control of a computer software program (Montgomery and Undem 2002). Thus, for this investigation a specific microarray procedure was developed using ECD for specific and sensitive pathogen detection. Two arrays were employed: a PanVira™ array that contains signature sequences of >1,000 human viruses, and our own newly designed microarray, a Phage/Host array, that contains signature sequences of 500 bacteriophages and their respective bacterial hosts. Numerous microarray experiments were performed to optimize the assay and make it suitable for detecting pathogens in environmental samples; however, this assay can be customized for many other sequences of interest (Lodes et al. 2006).

Quantitative Real-Time Polymerase Chain Reaction (qPCR) and quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) assays also are useful to detect small amounts of a specific nucleic acid and are widely used for detection and diagnoses of infectious diseases (Fuhrman, Liang, and Noble 2005; Freeman, Walker, and Vrana 1999). These assays also were utilized in our study to confirm and quantify positive hybridizations that were observed on the microarrays. Furthermore, conventional PCR, and sequencing were used to confirm certain of the positive hybridizations on the microarrays.

II. MATERIAL AND METHODS

A. Site Description and Sample Collection

Wastewater samples were collected from the University of Idaho (UI) dairy lagoons that are located at the campus of University of Idaho, Moscow, ID USA (Figures 1). A primary treatment lagoon (Lagoon I) receives waste manure supernatants where Lagoon II (secondary treatment) receives the effluent of Lagoon I. The lagoons are unmixed, and effluent discharge occurs mainly via evaporation. Approximately 50 ml of lagoon water was collected into sterile falcon tubes (BD, Franklin Lakes, NJ) and immediately delivered on ice to the laboratory for processing or stored at -20°C until analyzed.

Figure 1 Aerial photo of University of Idaho dairy lagoons
Lagoon I is the primary treatment system for settling of solids from the heavily-contaminated wastewater.
Lagoon II is the secondary stage for additional microbiological treatment. Sample locations are indicated by arrows.

B. Light Microscopy

Upon arrival of lagoon water samples, 1 ml of each sample was filtered using 0.45 µm pore-size filters (Millipore Filter Corporation, Billerica, MA). Using a clean wet mount method, 1:100 diluted water samples were visualized for bacterial populations at 1,000x magnification.

C. Bacterial Culture and Fecal Coliform Counts

Serial dilutions were prepared to determine the number of viable organisms under aerobic culture conditions. Aliquots were plated onto Nutrient Agar (NA) and Plate Count Agar (PCA), incubated at 30°C for 48 hrs, and the numbers of colonies were counted. Fecal coliform quantification was performed as most probable number (MPN) using the multiple tube fermentation protocol in accordance with Standard Methods 9221E (Clesceri LS 1998).

D. 16S rRNA Gene Analysis

Total genomic DNA was isolated using the PureLink genomic DNA Mini kit (Invitrogen, Carlsbad, CA) and subjected to PCR amplification using the universal bacterial 16S rRNA gene primer set (8F-AGAGTTTGATCCTTGGCTCAG and 1492R-GCYTACCTTGTTACGACTT) and archaea specific 16S rRNA gene primers (A8F-TCCGGTTGATCCTGCCGG and A1041R-GGCCATGCACCWCCTCTC) (Kolganova et al., 2002). The amplicons were cloned into pCR2.1 (Invitrogen, Carlsbad, CA). Plasmid DNA of 48 potential clones from each library was subjected to restriction analysis with EcoRI. BigDye terminator v3.1 Cycle sequencing half reactions were performed using the 3.2 pmole M13 forward or reverse primers and 50 to 100 ng templates and ran on a ABI 377 sequencer. 16S rRNA gene sequences were BLAST searched in the GenBank database using BLAST software http://ncbi.nlm.nih.gov. Multiple alignments and phylogenetic analyses were performed using Lasergene suite (DNASTAR, Inc., Madison, WI).

E. Water Chemical Analysis

Lagoon water samples were collected to measure the following parameters: soluble reactive phosphate (P), nitrate-N (NO_3-N), ammonia-N (NH_3-N), total suspended solids (TSS), soluble chemical oxygen demand (sCOD), dissolved oxygen (DO), conductivity, and turbidity. For soluble constituents, samples were filtered through a 0.22 µm syringe filter (Millipore Corp., Billerica, MA, USA) prior to testing. P was determined in accordance with Hach (Hach Company, Loveland, CO, USA) method 8048 (equivalent to Standard Methods 4500-PE; (Clesceri LS 1998)). NO_3-N was determined in accordance with Hach method 10020. sCOD tests were performed in accordance with Standard Methods 5220-D (Clesceri LS 1998) using Hach high-range ampoules and a Hach COD reactor. A Spectronic® 20 Genesys™ spectrophotometer (ThermoFisher Scientific Corp, Waltham, MA, USA) was utilized to measure the absorbance of the reacted sample at a wavelength of 890 nm for P, 410 nm for NO_3-N, and 620 nm for sCOD. P, NO_3-N, and sCOD concentrations were determined utilizing a standard curve (R^2>0.99). TSS was measured in accordance with Standard Methods 2540 D (Clesceri LS 1998). DO measurements were collected using a Hach HQ30d Meter with a LDO101 DO Probe. NH_3-N was measured in accordance with Standard Methods 4500-NH_3-D and using an Accumet XL60 meter with a Thermo 9512 Ammonia probe (ThermoFisher Scientific Corp, Waltham, MA, USA). Conductivity measurements were performed using an Accumet AP85 meter (ThermoFisher Scientific Corp, Waltham, MA, USA) fitted with a conductivity probe. Turbidity measurements were performed using a Hach model 2100P turbidimeter.

F. Nucleic Acid Extraction

1) Bacterial Genomic DNA:

Lagoon water samples were filtered using a 5µm pore-size filter (Millipore Filter Corporation, Billerica, MA). Total bacterial DNA was extracted using PureLink™ Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA). Extracted DNA was amplified by Whole Genome Amplification kit (GE Healthcare, Piscataway, NJ) and eluted using 50 ul RNase/DNase-free water.

2) Viral Nucleic Acid (DNA, ssRNA, dsRNA):

Water samples were filtered using 0.22 µm pore-size filters (Millipore Filter Corporation, Billerica, MA). Five ml of filtered water was processed using QIAamp Circulating Nucleic Acid Kit (QIAgen, Valencia, CA) following the manufacturer's instructions and nucleic acids were eluted using 50 µl of RNase-DNase free water. Since carrier RNA is used in the extraction process, 28 ng/µl was subtracted from the final reading. Nucleic acid was split into three tubes; where the first one was processed as viral DNA, the second one as viral ssRNA, and the last one as viral dsRNA. **DNA viruses:** A Whole Genome Amplification Kit was used to amplify single/double stranded DNA (GE healthcare, Piscataway, NJ). To assess proper amplification, negative (water), and positive (lambda bacteriophage DNA) controls were utilized. **ssRNA viruses:** cDNA was made using QuantiTect Rev Transcription Kit (Qiagen, Valencia, CA). The manufacturer's instructions were followed using random hexamer primers. **dsRNA viruses:** dsRNA was denatured by heating at 95°C for 3 minutes then immediately placed on dry ice. Reverse transcription was carried out using random hexamer primers and QuantiTect Rev Transcription kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

3) Nucleic Acid Purity Assessment:

Quality and quantity of eluted DNA/cDNA samples was determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). The ratio of sample absorbance at 260 and 280 nm were obtained to assess the nucleic acid purity (~1.8 for DNA/cDNA and ~2 for RNA). DNA/cDNA was visualized on 1% agarose gels using ethidium bromide staining. To confirm that only viral nucleic acid was extracted, 16S rRNA gene universal primers were used to check for bacterial DNA contamination (Supplemental Table 1). Positive control (E. coli) and negative control (sterile water) were utilized. PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide and no bacterial DNA contaminants were observed.

G. Microarray ElectraSense® Assay

1) Electrochemical Detection (ECD) Microarray Design and Analysis:

Oligonucleotide probes were synthesized on a semiconductor microchip. Each microelectrode harbored a specific DNA probe for a specific organism where each array contains 12,544 electrodes individually addressable with size of 40 µm in diameter. Extensive description of the technical procedures for ElectraSense® Microarray probe synthesis on semi-conductor microarray chip is available in (Lodes et al. 2006) and (Maurer et al. 2006). In this study, two ECD microarray chips were employed. The first array was a commercially available 12K PanVira™ array containing > 1,000 signature sequences of human viruses. Each array contained 10 replicate probes of each virus representing two to three specific genes for each virus. In addition, each array contained factory built-in negative controls that consisted of sequences from the genomes of Arabidopsis thaliana, Agrobacterium tumefaciens and bacteriophage lambda beside standard factory built-in quality controls (no probes) to be used for data normalization and background subtraction (CombiMatrix Corp., Mukilteo, WA). The second array was a custom 12K Phage/Host array of our own design which had probes for 500 bacteriophages and their respective hosts. Multiple probes for each organism were used. Each probe was developed to be species and subspecies specific (constructed from conserved regions of respective genomes) to avoid cross-hybridization. The Phage/Host array contained factory built-in negative controls that consisted of signatures sequences from Arabidopsis thaliana. Standard factory built-in quality controls (no probes) were utilized in each array as well. For both arrays, probe sequences were obtained from the GenBank database, and probe design files were generated with layout designer (CombiMatrix Corp., Mukilteo, WA) and are available upon request.

2) DNA Labeling and Hybridization:

DNA/cDNA samples from each lagoon were pooled in equal amounts to a final concentration of 3 µg/µl then labeled using a biotin Label IT^{R} µArray Biotin Labeling Kit (Mirus Bio Corp., Madison, WI) following the manufacturer's instructions. Hybridization processes were performed following instructions within the ElectraSense™ Hybridization Manual (CombiMatrix Corp., Mukilteo, WA). Post-hybridization steps were processed following the methods described in the ElectraSense™ Detection Kit manual (CombiMatrix Corp., Mukilteo, WA). Detection of hybridization signals was performed using an ElectraSense™ microarray reader. Data were extracted immediately using ElectraSense™ Application Software followed by the use of CombiMatrix Blist Software for data analysis. Each experiment was repeated in three replicates including positive and negative controls. Positive organisms (candidates) were identified in all three replicates, and then the average signal intensity was calculated. The ElectraSense™ Manual and software are available at http://cores.montana.edu/uploads/Genomics%20Core/PTL006_00_12K_Hyb_Imaging.pdf

3) Microarray Stripping (Re-Use):

Microarray stripping procedure was performed at the end of each experiment for chip re-use using the CombiMatrix CustomArray™ stripping kit according to the manufacturer's instructions (CombiMatrix Corp., Mukilteo, WA). Each stripped chip was scanned using ElectraSense™ reader to verify complete stripping. The stripped chip is kept in 1x PBS solution at 4 °C ready for next experiment. CombiMatrix CustomArray™ stripping protocol can be found at: http://cores.montana.edu/uploads/Genomics%20Core/PTL001_12K_StripReHyb.pdf

H. PCR and Amplicon Sequencing

The PCR reaction for each target gene was carried out using previously published primers and/or newly designed primers (Ley, Higgins, and Fayer 2002; Asakura et al. 2007; Nilsson and Haggard-Ljungquist 2001; Smith et al. 2009; Giovannoni 1991) (supplemental Table 1). Primer Express® Software v3.0 (Applied Biosystems, Foster City, CA) was used for primer design. All PCR mixtures had a total volume of 25 µl which contained 0.5 to 2 µl of DNA/cDNA, 12.5 µl of Fermentas PCR master mix (2X) (Maryland, USA), 1 to 3 µm of forward and reverse primers, and sterile nuclease-free water to final volume. For each primer set, appropriate positive and negative controls were employed. PCR amplicons were visualized on a 1.5% agarose gel stained with ethidium bromide. The positive (correct size) amplicons were processed further for PCR sequencing procedure as follows: PCR products were purified using a QIAquick PCR purification kit (Qiagen, Velencia, CA). Each sequencing PCR tube contained 1 µl PCR product (80 ng), 1 µl primer (3.2 pmol/µl), 4 µl big dye mix (Applied Biosystems, Foster City, CA), and nuclease free water up to 10 µl. Sequencing PCR conditions as follows: 95°C for 10 s, 50°C for 15 s, 60°C for 4 min, 24 cycles. The PCR products were cleaned using gel columns and sequencing was done using an ABI3730XL sequencer (Applied Biosystems, Foster City, CA) (Macrogen, Rockville, MD). Nucleotide sequences were blasted against NCBI database (http://www.ncbi.nl-m.nih.gov/blast) to determine sequence identity (>97% sequence identity).

I. qPCR/ qRT-PCR

qPCR experiments were carried out to quantify and corroborate some of the positive hybridization observed on the microarray chips. In order to establish a standard curve, positive controls were selected based on the availability of organisms in our laboratory (specifically E coli, Salmonella, Adenovirus C, and Enterobacteria phage (MS2)). To determine the mass of genomic DNA that corresponds to the gene copy numbers of target nucleic acid sequences, Applied Biosystems' calculation sheet was used. Five points were created to generate the standard curve, starting from 3×10^5 and moving to 3×10^1 gene copy number/µl. Serial dilutions (1:10) using UltraPure™ DNase/RNase-Free water were used to create the standard curve. For the qPCR assay we employed previously published primers and/or newly designed primers (Supplemental Table 1) (Hoorfar, Ahrens, and Radstrom 2000; Ebner, Pinsker, and Lion 2005). Each 25 µl reaction included: 12.5 µl of Power SYBR® Green

PCR Master Mix (Applied Biosystems, Foster City, CA), 100 nm to 250 nm of each primer, 2 µl DNA/cDNA template, and remaining volume displaced with UltraPure™ DNase/RNase-Free water. Tubes were briefly centrifuged then placed into a StepOnePlus qPCR machine (Applied Biosystems, Foster City, CA). qPCR conditions were as follows: 10 min an initial setup at 95°C, followed by 40 cycles of 15 sec denaturing step at 95°C, 1 min of annealing step at 60°C, and 1 min of extending step at 72°C. All samples were processed in triplicate, including the standard positive and negative controls. Melt curve and standard analysis were performed to determine the specificity and efficiency of each qPCR reaction. PCR amplicons were visualized on a 1.5% agarose gel stained with ethidium bromide. Data and statistical analysis were computed with StepOnePlus qPCR software to calculate the efficiency %, slope, coefficient of correlation (R^2), gene copy number and threshold cycle (C_t). Student's T-test with P value of$\leq$ 0.05 was used to evaluate the significant differences between samples obtained from different lagoons (Lagoon I and Lagoon II).

III. RESULTS AND DISCUSSION

A. Sample Collection and Light Microscopy

In this study we first established a thorough analysis of the bacterial and viral populations in the dairy lagoons. The lagoons are located on the University of Idaho campus (Moscow, ID, USA) and consist of two lagoons (Lagoon I and Lagoon II) that operate in series. Lagoon I is the primary lagoon, receiving raw dairy manure wastewater, while Lagoon II receives effluent from Lagoon I (Figure 1). The physical appearances of Lagoon I and Lagoon II wastewater were different. Water from Lagoon I was more turbid and purple in color as compared to Lagoon II which was yellowish in color and less turbid (Figure 2). Using wet mount methods, we noted the presence of what we postulated were Thiocapsa species (family Chromatiaceae). The recognition was based on their unique morphology (spherical to slightly ovoid, tetrads) and was confirmed by 16S rRNA gene analysis (Figures 3 and 5). Thiocapsa species were observed in greater numbers in Lagoon I as compared to Lagoon II (Figure 3). We concluded that the purple color of Lagoon I wastewater was due to the presence of dominant Thiocapsa species, photosynthetic purple sulfur bacteria (PSB), that contains carotenoid pigments (Fowler et al. 1984). Thiocapsa is an anaerobic sulfur bacterium that uses reduced sulfur as an electron donor during photosynthesis (Caumette et al. 2004). The presence of PSB in lagoons and wastewater has been reported previously (Holm and Vennes 1970; Siefert, Irgens, and Pfennig 1978). The presence of PSB is considered to be advantageous to the wastewater industry because PSB reduces the rate of production of volatile organic compounds by utilizing hydrogen sulfide as electron donor (Guyoneaud et al. 1998; Imhoff, Suling, and Petri 1998; Do et al. 2003). Recent studies have focused on the induction of the purple sulfur bacteria in dairy wastewater by circulation concluding that PBS reduced H_2S as well as emissions of volatile organic compounds and alcohols leading to an enhanced air quality for residents near agricultural areas (McGarvey et al. 2009; McGarvey et al. 2005).

Figure 2 Physical appearance of water from Lagoon I (left; primary treatment system) and Lagoon II (right; secondary treatment system) Lagoon I water was purple and more turbid as compared to water from Lagoon II (see text) which was less turbid and yellowish in color.

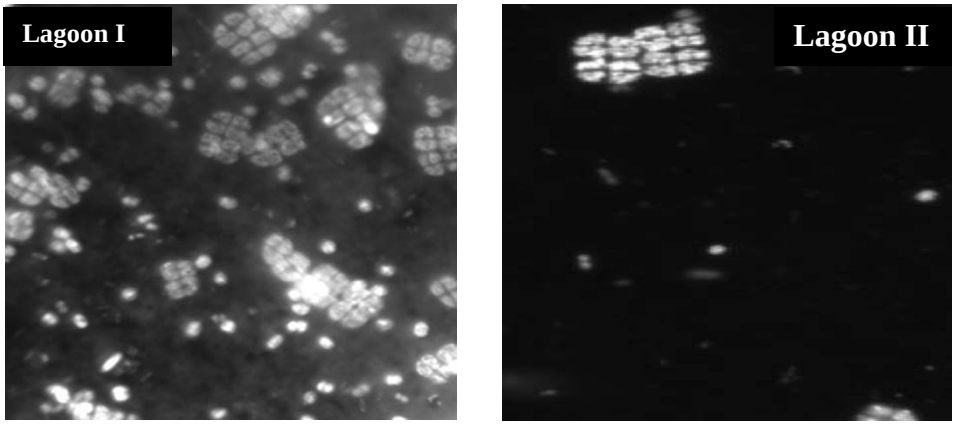


Figure 3 Microscopic analyses of water from Lagoon I (left) and Lagoon II (right)
Water samples were diluted 1:100 and examined using the wet mount method under 1000X magnification.
Thiocapsa spp. were detected in higher numbers in Lagoon I as compared to Lagoon II.

B. Bacterial Culture and Coliform Counts

More than 10-fold higher numbers of culturable bacteria were observed on standard aerobic agar medium (Nutrient Agar (NA) and Plate Count Agar (PCA)) in Lagoon I as compared to Lagoon II water samples. Lagoon I had 2.2 x 10^7 CFU/ml on NA and 4.0 x 10^7 CFU/ml on PCA in contrast to Lagoon II that had 1.0 x 10^6 CFU/ml on NA and 1.1 x 10^6 CFU/ml on PCA. However, bacterial diversity (based on the visual observation of colony morphologies and 16S rRNA gene analyses) in Lagoon I seemed to be lower than Lagoon II, though no attempt was made to quantify this observation. On a most probable number (MPN) basis, the fecal coliform count was substantially higher in Lagoon I ($9x10^4$ MPN/ml) than Lagoon II (80 MPN/ml). Lagoon I exhibited nearly anaerobic conditions (dissolved oxygen level of 0.23 mg/l) while Lagoon II was aerobic (dissolved oxygen of 4.28 mg/l); fecal coliform bacteria are facultative anaerobes and thus would be expected to proliferate better in the Lagoon I environment. To further characterize these coliforms, genomic DNA was isolated from selected colonies then sequenced. We observed that most of the coliforms examined were E. coli derivatives with only two to three nucleotide differences between them. Sequence of two isolates matched the 16s rRNA gene sequence of E. coli O157:H7. To examine the possibility that these were shigatoxin-producing pathogenic E. coli, PCR using stx1, stx2 and uidA specific primers was conducted (Jin et al. 2007). Although the sequencing suggested a match, none of the isolates, including the O157:H7-like strains, produced an amplicon of the correct size when compared to the positive control indicating that we had not isolated shigatoxin-producing E coli.

Our counts of fecal contaminants provided us with useful information regarding the presence of coliforms in both lagoons and indicated a large reduction of coliforms in the transition from Lagoon I to Lagoon II. Such observations were expected knowing that Lagoon I receives heavily contaminated water (primary treatment) in contrast to Lagoon II which receives the effluent of Lagoon I (secondary treatment). Traditionally fecal coliforms have been used as indicators of the likely presence of pathogens in water samples. Though they are not pathogenic themselves, their presence in high number is usually indicative of the presence of fecal contamination. As of today fecal coliforms remain the predominant indicators used to assess water quality (Rosen 2000); however, recent studies have shown that the presence of fecal indicators does not always correlate with the presence of pathogens (Lipp, Farrah, and Rose 2001; Kramer et al. 1996). Furthermore, the presence of coliforms does not correlate well with the presence of viruses or protozoa (Bitton 2005; Griffin et al. 1999). These drawbacks of fecal indicators have encouraged us and others to develop indicators that can directly detect specific bacteria, viruses and protozoa to help regulators to better assess water quality.

C. Lagoon I and Lagoon II Physiochemical Composition

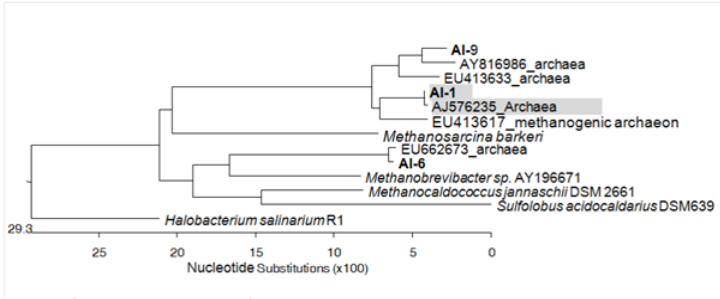
Results of determinations of select physiochemical parameters differed significantly between the two lagoons. Physiochemical analyses of lagoon water were evaluated in terms of measuring P, NH_3-N, NO_3-N, sCOD, TSS, DO, conductivity, temperature and turbidity. Overall, the levels of the physiochemical parameters are as follows: the soluble reactive phosphate (P) was higher in Lagoon I (60.4 mg/l) than Lagoon II (29.9 mg/l) and the ammonia-N measured 109 mg/l in Lagoon I compared to Lagoon II at 29.5 mg/l. P removal would occur principally through microbial growth while ammonia-N can be removed through either biological growth or nitrification (i.e., ammonia-N aerobically oxidized to nitrate-N). The physiochemical data indicated that the DO in Lagoon I was less than in Lagoon II (0.23 mg/l and 4.28 mg/l, respectively) which showed that Lagoon II is largely aerobic as compared to Lagoon I which is anaerobic. The value obtained for turbidity in Lagoon I was greater than Lagoon II (>800 NTU and 156 NTU, respectively) which is expected since Lagoon I receives manure supernatants and primary wastewater. Conductivity measurements of lagoon water showed slight differences in the conductivity between lagoons, with measured at 3.89 µS whereas in Lagoon II the value was 4.12 µS. The TSS values were 860 mg/l in Lagoon I and 160 mg/l in Lagoon II. The concentration of nitrate-N was higher in Lagoon I (42 mg/l) than Lagoon II (22.8 mg/l). The low DO concentration in Lagoon I would not favor significant nitrification, although the presence of nitrate does confirm that sufficient localized aerobic conditions prevailed to induce some ammonia-N oxidation. The sCOD in Lagoon I showed higher value (1007.7 mg/l) compared to Lagoon II (649.9 mg/l). The reduced sCOD in Lagoon II also reflected the enhanced aerobic environment which would select for chemoheterotrophic microbes capable of organic carbon oxidation. Similar to the results of the bacterial density, the physiochemical compositions of the lagoons' waters indicated that a partial stabilization of the wastewater had occurred as treatment proceeded from Lagoon I to Lagoon II. The decrease in macronutrient (carbon, nitrogen, and phosphorus) concentrations in Lagoon II also indicated an improvement in the water quality in the transition between the lagoons. However, while there is an improvement in the water quality, the water is not of suitable quality for discharge to a water body due to high concentrations of pollutants such as sCOD, TSS, and NH_3. It is true that wastewater contains essential nutrients for plant growth; however, the accumulations of high dose of those nutrients may cause adverse affects on irrigated areas and might leach into surface and ground waters (Mohammad 2004; Nunez-Delgado, Lopez-Periago, and Diaz-Fierros Viqueira 2002). Further treatments and tests are needed to improve the lagoons water quality prior to re-use.

D. 16S rRNA Gene Analysis

Analyses of 16S rRNA gene sequences from environmental DNA samples are a useful tool for investigating the biodiversity of microorganisms in environmental samples. In this study, 16S rRNA gene analyses were conducted to establish a base assessment of the bacterial biodiversity within Lagoons I and II, and we found substantial differences. Archaea-specific

primers for 16s rRNA genes were used to amplify products of about 1000 bp from each lagoon sample. We determined the nucleotide sequence of 13 clones for each lagoon sample (Figure 4). The predominant archaeon in Lagoon I was even more enriched in Lagoon II samples. Furthermore, the results revealed that most of the archaeons that were detected in both lagoons showed similarity to sequences of uncultured organisms recovered in other studies (Kolganova, Kuznetsov, and Turova 2002). Universal primers for the 16s rRNA bacterial genes were tested as well, revealing that Lagoon I had more Thiocapsa spp. and anaerobic bacteria as compared to Lagoon II which had an apparently increased bacterial diversity as shown in our 16s RNA gene sequence data (Figure 5). The 16S rRNA gene sequences in Lagoon I samples showed that 45% of 16S rRNA sequences belong to γ-Proteobacteria with a close relation to Thiocapsa spp., also responsible for the pink coloration of the water. In addition, 25% clones belong to the phylum Firmicutes (low-G+C, gram-positive bacteria), 12.5% clones were β-Proteobacteria and 12.5% clones were Sphingobacteria. In Lagoon II, the most common bacteria observed were classified into the β-Proteobacteria (representing 56.7% of the clones), followed by Sphingobacteria (16.2% clones), γ-Proteobacteria (8.1% clones), Firmicutes (8.1% clones), α-Proteobacteria (8% clones) and finally δ-Proteobacteria (2.7% clones). Gamma-proteobacteria phylum was predominant in Lagoon I which consists of important groups such as Enterobacteriaceae (E coli), Vibrionaceae and Pseudomonadaceae and these results in agreement with other approaches that were used in this study (such as microarray, PCR, and sequencing). However, in contrast to Lagoon I, β-Proteobacteria was the most abundant phylum in Lagoon II which includes groups of aerobic and/or facultative bacteria that contain chemolithotrophic genera (i.e., the ammonia-oxidizing bacteria) and some phototrophs that play a critical role in nitrogen fixation (Emerson et al. 2007). The detection of Firmicutes in both lagoons was expected since many ruminant bacteria such as Bacillus and Clostridium reside within this group. Our results shared similarities with previous studies regarding the phyla that were present in dairy lagoons; however, the abundance of those phyla differed. McGarvey et al identified the bacterial populations in dairy lagoons using 16S rRNA gene analysis revealing that the phylum Firmicutes was predominant followed by Proteobacteria (McGarvey et al. 2004). Such differences might reflect the unique nature of each lagoon system. Also, it is important to note that the microbial community of a dairy lagoon is complex and is always subject to local physiochemical changes as well as metabolic activities of co-inhabitating organisms (von Wintzingerode, Gobel, and Stackebrandt 1997). Admitting the limitations of our limited 16S rRNA gene analyses, we did not observe specific pathogenic bacteria such as E. coli O157:H7 in these dairy lagoons. This is of course a good result since these strains are of very high concern from a public health perspective (Ritter et al. 2002). Although this study provided new insights into the microbiology of the University of Idaho's dairy lagoon system, further evaluations are needed with larger numbers of clones and metagenomic DNA.

Lagoon I	Access #	Best Match
7/13 clones	AJ576235	Uncultured methanogenic archaeon, clone GZK61
5/13 clones	AYU816986	Uncultured archaeon clone P41
1/13 clones	AB662673	Uncultured euryarchaeote



Lagoon II	Access #	Best Match
12/13 clones	AJ576235	Uncultured methanogenic archaeon, clone GZK61
1/13 clones	EU885167	Uncultured archaeon, clone ArchA1

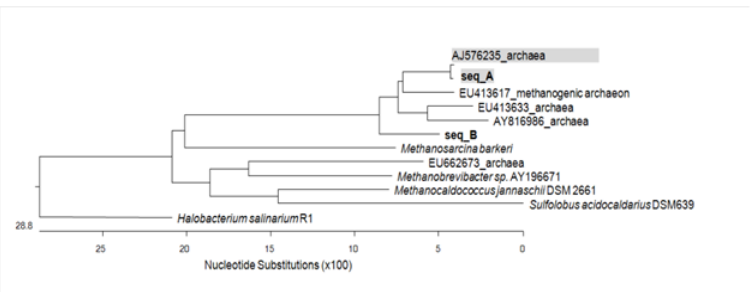


Figure 4 Phylogenetic tree of 16s rRNA clones from lagoon I and II illustrating the diversity of archaea present in the lagoon community. The abundance of previously unknown archaeal sequences was detected. Archaea-specific primers were used to amplify approximately 1000 bp of the 16s rRNA gene of lagoon isolates. The pcr products of 13 isolates from each lagoon were cloned into pcr2.1 and sequenced. The predominant archaeon observed was methanogenic.

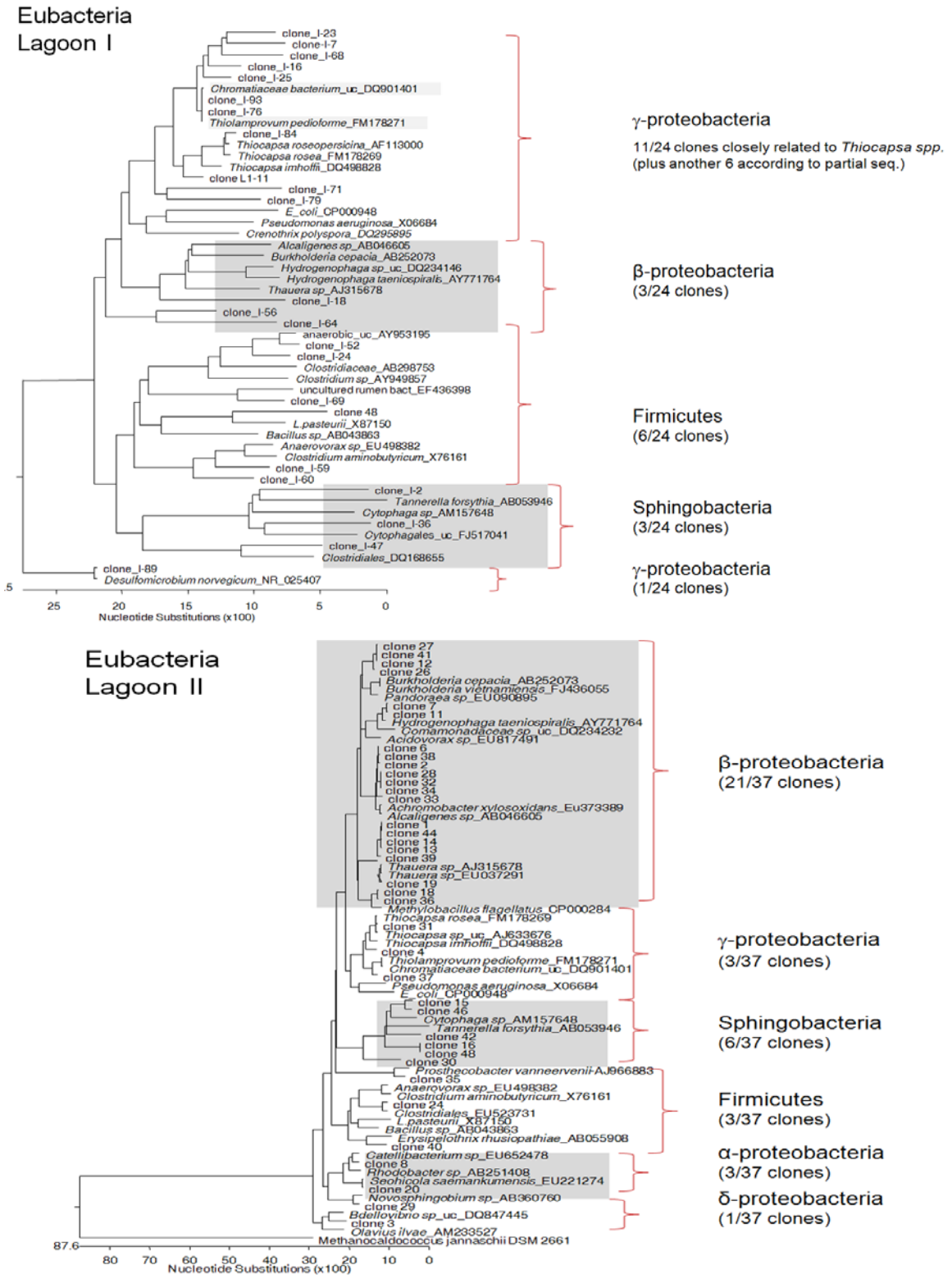


Figure 5 Phylogenetic relationships of the Bacterial 16s rRNA gene sequences of isolates from Lagoons I and II
The dendrogram was constructed using the neighbor-joining algorithm. The Bacterial communities differ substantially between the two lagoons. Lagoon I contained predominantly Thiocapsa spp., which was responsible for the pink coloration of the water. We noticed methane production indicating anoxic conditions. In Lagoon II the most common bacteria observed were classified into the β– proteobacteria.

E. Pathogen screening: Microarray

Microarray data were extracted using ElectraSense™ Application Software followed by the use of CombiMatrix Blist Software for background subtraction and data analysis. Organisms showing 80% positive hybridization (8 out of 10 probes) per array and appearing on all three replicates were compiled into a Microsoft Excel spreadsheet to calculate the average signal intensity ($\geq$ 500). The average signal intensity was the most significant independent variable useful to identify a positive

hybridization and thus was used for analysis. After averaging the positive hybridization values for the three replicates, the indicated phage, host, or human virus was determined. Figure 6 shows a summary of the positive hybridizations for phages, hosts and human viruses in Lagoons I and II.

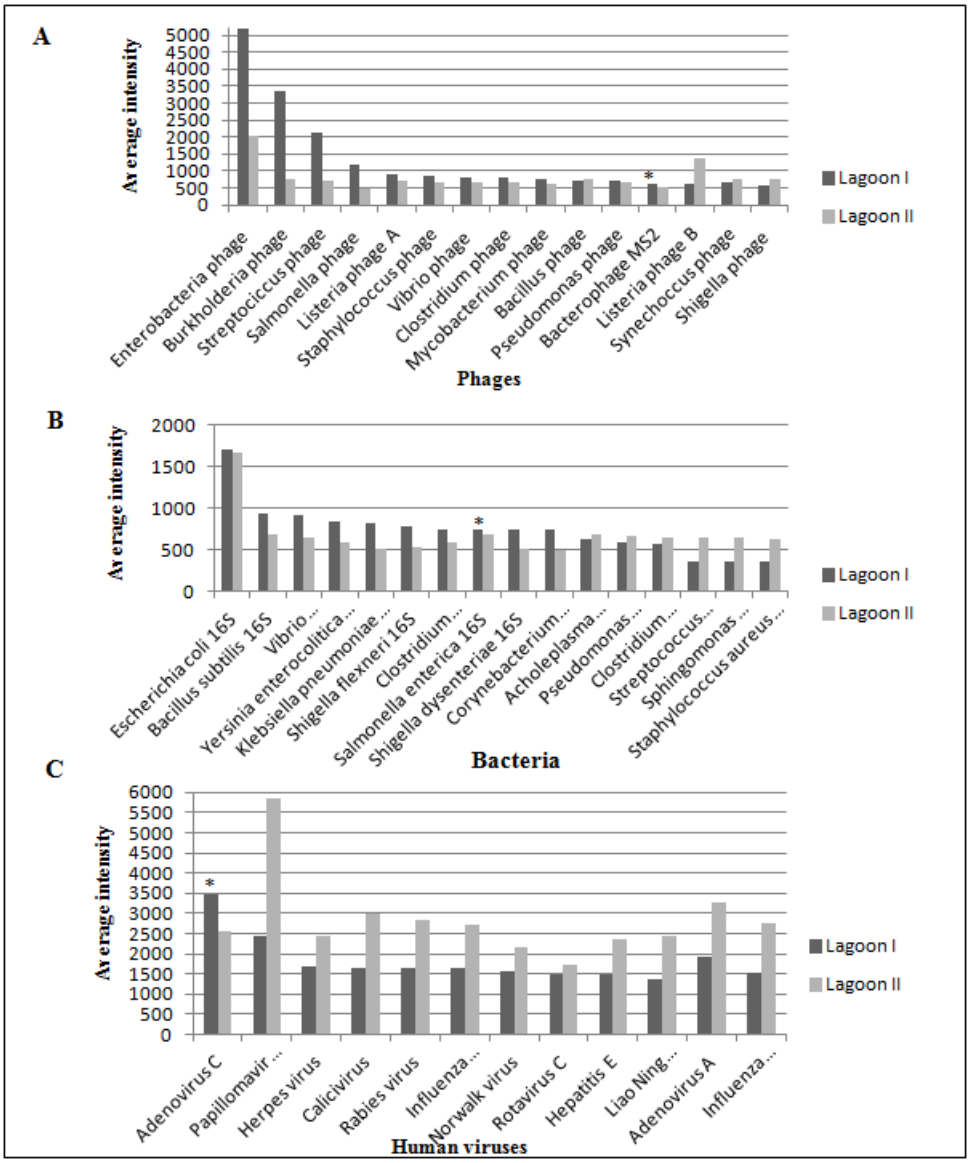


Figure 6 The top-ten signature signals detected in Lagoons I and II.
A) Average signal intensity values of bacteriophage signatures in Lagoons I and II.
B) Average signal intensity values of bacterial signatures in Lagoons I and II.
C) Average signal intensity values of human virus signatures in Lagoons I and II. Bars represent the average signal intensity of all probes designed for a particular organism. An asterisk indicates significant differences between Lagoons I and II ($p \leq 0.05$). Lack of appropriate controls prevented such analyses for all signatures (see text). ElectraSense™ Application Software and CombiMatrix Blist Software were used for background subtraction and data analysis.

1) Bacteriophages:

Most of the bacteriophage signals detected in our samples were associated with Enterobacteria species. This was not surprising given that the Enterobacteriaceae family dominates the bacterial populations in both lagoons. Inoviridae, Siphoviridae and Myoviridae were the most abundant phage families in the lagoons, showing some differences in signal intensities between the two lagoons. Furthermore, dsDNA viruses from the order Caudovirales dominated both lagoons where there were fewer signatures of ssDNA or ss/dsRNA viruses. This finding was expected since most of the phages that have been reported in the literature so far are dsDNA viruses (Abedon 2007). Our results also showed that phages scored higher signal intensities in both lagoons as compared to bacterial populations (Figure 6). This finding is supported by many studies recording the abundances of phages in environmental samples (Alhamlan et al. 2012; Rosario et al. 2009; Cantalupo et al. 2011).

Correlations between the presence of phages and their hosts in such systems should be explored further because it is important to understand the roles of phages in the microbial ecology of waste lagoons, particularly how phages alter the makeup of bacterial populations. It has been suggested that the presence of phages in wastewater can help eradicate or reduce

the presence of pathogens during wastewater treatment and thus can be used as therapeutic agents (Wiggins and Alexander 1985; O'Flynn et al. 2004). Another possibility for productively utilizing the abundance of bacteriophages in wastewaters is in identifying viral indicators for evaluation of water quality. It has been proposed that bacteriophages be employed as bioindicators (Payment and Franco 1993; Harwood et al. 2005). Our results support this idea of pursuing a new direction toward development of viral indicators of fecal contamination.

2) Bacteria:

Microarray data revealed a broad spectrum of potential bacterial pathogens to be present in the lagoon samples. Indeed, using the Phage/Host array we detected signatures of mostly the Enterobacteriaceae family (Figure 6). These signatures included those for E coli, Yersinia, Salmonella, Shigella, Klebsiella, and Enterobacteria. Most of the signal intensities for these bacteria were higher in Lagoon I than in Lagoon II. This can be explained by the nature of Lagoon I (anaerobic environment) that provides a good environment for proliferation of facultative anaerobes such as members of the Enterobacteriaceae. Also, considering that Lagoon I was more heavily contaminated with dairy wastes including manure, it should harbor many more bacteria than Lagoon II. We further observed that bacterial populations (hosts) were slightly different in Lagoon I as compared to Lagoon II. As expected, Lagoon I exhibited more signatures of facultative anaerobic bacteria such as E coli, V. parahaemolyticus, Y. enterocolitica, K. pneumonia, S. flexneri and others while Lagoon II exhibited positive signatures of more aerobic bacteria such as P. aeruginosa, S. pyogenes and S. paucimobilis.

Livestock operations and their associated waste management practices are a prominent source of microbiological contaminants to the environment. Bacteria are among the most common microbial contaminants that are detected in such operations (Barwick RS et al. 2000). The bacteria that were detected in the present study (e.g. E. coli, Salmonella, Clostridium, Staphylococcus, Vibrio, etc.) are causative agents for serious diseases such as gastrointestinal infections, salmonellosis, typhoid fever, dysentery and others (Liang et al. 2006). In addition, more than 150 microbial pathogens are known to be transmitted to humans via water; thus, the development of a rapid and sensitive methods such as that described here to screen and monitor for the presence of problematic bacteria is clearly necessary (USEPA 1998).

3) Viruses:

Viruses scored the highest signal intensities in the lagoons, suggesting that viruses dominated the microbial populations in the lagoons. This finding was not surprising since abundances of viruses in aquatic environments have been reported to exceed those of bacteria by 5-25 times (Bergh et al. 1989; Borsheim, Bratbak, and Heldal 1990). Signatures of various human viruses were observed in both lagoons using the PanVira™ microarray (Figure 6). These signatures belonged to the families Adenoviridae, Papillomaviridae, Herpesviridae, Caliciviridae, Rhabdoviridae, Orthomyxoviriade, Rioviridae, Hepeviridae and Reoviridae and were detected in both lagoons; however, they differed in their intensities. The signal intensities for these probes were higher in Lagoon II for most of the families than in Lagoon I. Our data support previous studies that indicate the presence of such viruses in wastewater treatment systems (Okoh, Sibanda, and Gusha 2010; Carter 2005; McLellan et al. 2010; Cantalupo et al. 2011; Symonds, Griffin, and Breitbart 2009; Bosch 1998; Schaub and Oshiro 2000; Lodder and de Roda Husman 2005). These viruses have been detected in wastewater throughout the world; however, their frequencies of occurrence and their actual potential for pathogenesis have not been well studied (Puig et al. 1994). Indeed, to date there have been few studies conducted specifically to examine viral populations in wastewater lagoons, leaving this field largely unexplored. The association of outbreaks of waterborne viruses with wastewater has raised important concerns for public health and emphasizes the need for further research (Gerba and Smith 2005).

The presence of human viruses in our samples was not surprising based upon what we know of the structure of the lagoon system. The lagoons are not closed systems and are open to access by animals such as birds (e.g., starlings and water fowl) and other aquatic animals and plants which might act as vectors to move outside contaminants into the lagoons. Also, humans work very closely with dairy cows (e.g., through milking and feeding operations). As a result, human viruses might be transferred by human-cow contact. All these factors would have to be evaluated further to trace the actual sources of contamination and to better define the risks of human diseases that might be mediated by dairy lagoon waters. More than 150 known enteric pathogens have been detected in untreated wastewater, and every year there are new enteric pathogens discovered (Gerba and Smith 2005).

In this study we detected Influenza A and B viral signatures in both lagoons. This observation needs specific follow-up since there has been significant recent concern regarding the possibility of influenza outbreaks mediated by highly dangerous strains such as H1N1 (Swine flu) (Dawood et al. 2009). Moreover, our results showed the presence of caliciviruses and Norwalk virus signatures in both lagoons, indicating yet another potential health risk. Indeed, most of the outbreaks of acute water-borne infections in the United States are due to norovirus infections (Atmar and Estes 2006). These viruses are disseminated by the fecal-oral route and have the ability to infect humans with low infectious doses (ID_{50} = 10 virions) if ingested through contaminated water (Keswick et al. 1985; Shin and Sobsey 2003; Donaldson et al. 2008). Thus, these viruses are on the EPA's "contaminants candidate list" and need to be closely monitored (Schaub and Oshiro 2000). Microarray technology shows great promise for this purpose.

Another observation from our viral signature analyses was the presence of Human Papillomavirus (HPV), which scored its

highest signal intensity value in Lagoon II. This was further examined using PCR and sequence analysis which showed that the actual amplicon sequence did not match any HPV sequence in the present database. This suggests that there are unidentified viruses present in the lagoons that share sequence similarities with HPV and that these also need further study.

4) Microarray Result Confirmation:

In order to corroborate some of the positive microarray results, conventional PCR and sequencing were utilized. Our strategy for choosing specific array-positive pathogen signatures to perform confirming PCR was mostly based on the availability of positive controls. The positive controls were needed to assure that the presence or absence of pathogen was real and that absence of "hits" was not due to the presence of unknown inhibitors in the samples. The presence of the following viral and bacterial signatures was confirmed by PCR amplification and sequencing: Bovine enterovirus (AY831697.1), Phage cdtI (AB285204.1), Bacteriophage HK109 (AJ298550.1), Enterobacteria phage MS2 (EF204940.1), Staphylococcus Phage MR11 (AB370268.1), Streptococcus thermophilus phage OBJ (in Lagoon 1 only), Salmonella phage mig-3 (AF020804), Bacillus licheniformis (EU221362.1), Virgibacillus (AM237397.1), Bacillus 16S (HM061660.1), Clostridium 16S (AB539900.1), Bacillus subtilis (AB542912.1), and Escherichia coli (CP001969.1). Nucleotide sequences of each amplicon were BLAST searched and identified based on similarity to sequences in GenBank.

In conclusion, our microarray studies provided a broad picture of potential pathogens (i.e., sequence signatures) that are likely to be present in dairy lagoons. Follow up experiments to confirm more of the positive candidates are needed. We believe that the use of ECD-based microarray techniques in combination with PCR and qPCR (see below) is sensitive, accurate, and can complement or in some cases replace traditional methods. We confirmed that a protocol employing ECD microarray technology can be used for microbiological monitoring of environmental water samples. In addition, each chip can be stripped and reused several times (at least 5 times), providing significant cost savings for analyses of multiple samples or for multiple time points.

There are limitations of microarray technology, as any other molecular technology, that must be considered. One of these disadvantages is that absolute quantification and confirmation of positive hybridizations are not possible with this technology and each positive hybridization needs to be further confirmed with other techniques to rule out any false positive hybridizations (Everett K.R. et al. 2010). Nevertheless, microarrays can be used as an initial broad approach to detect the primary members of a population in a sample, and then positive hybridizations can be further examined and confirmed with other techniques (i.e., PCR and qPCR).

F. Microbial Pathogens in Dairy Lagoons: qPCR/ qRT-PCR

To determine an organism's load in a sample, a standard curve was created using known amounts of DNA/cDNA of pure strains, ranging from 3×10^5 to 3×10^1 gene copies/µl. Also, positive controls were utilized for qPCR standard curve constructions and to assure the presence or absence of pathogen was real. Table 2 summarizes the qPCR absolute quantification assay results. Quantification of genomic copy number is based on C_t value (also known as quantification cycle, C_q) where a low threshold cycle (C_t) value means a greater amount of nucleic acid present in the sample (Bustin et al. 2009). We observed that Lagoon I had higher copy numbers of detected organisms than did Lagoon II. The threshold C_t values were calculated by detecting the point at which the green fluorescence exceeded the threshold signal. Amplification efficiency was determined from the slope of the log-linear portion of the standard curve ($10^{-/slope}$ -1) following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al. 2009). Efficiencies of our samples ranged from 90.4% to 93.2%. C_t values, slope, and intercept for each run were calculated using linear regression analysis of log (N_0) versus C_t. Optimal slope calculations (-3.5) and coefficient of correlation (R^2) (0.99) were obtained by StepOnePlus qPCR software (v2.0). Melting curves were utilized to confirm assay specificities and agarose gel electrophoresis to confirm amplicon size.

TABLE I A SUMMARY OF QPCR STANDARD CURVE RESULTS

Water sample	Target gene	Number of data points	Efficiency %	Slope	R^2	Gene Copy Number	CT (mean)
Lagoon I	E coli 16s rRNA	5	92.3	-3.50	0.99	94896	14.8
Lagoon II	E coli 16s rRNA	5	92.3	-3.50	0.99	93907	14.8
Lagoon I	Salmonella invA gene	5	93.2	-3.49	0.99	33446	15.7
Lagoon II	Salmonella invA gene	5	93.2	-3.49	0.99	29933	19.3
Lagoon I	Adenovirus C hexon gene	5	90.4	-3.57	0.99	376	26.4
Lagoon II	Adenovirus C hexon gene	5	90.4	-3.57	0.99	22	30.8
Lagoon I	Enterobacteria phage MS2	5	92	-3.55	0.99	3469	23.7
Lagoon II	Enterobacteria phage MS2	5	92	-3.55	0.99	466	26.9

Our data indicated that Lagoon I and Lagoon II were positive for the same tested organisms (E coli 16S rRNA, Salmonella invA gene, Adenovirus C hexon gene, and Enterobacteria phage (MS2)). E coli 16S rRNA exhibited the highest gene copy number ranging from 94.9 x 10^3/µl in Lagoon I to 93.9 x 10^3/µl in Lagoon II, however the C_t values were similar in both lagoons (14.8). Salmonella invA gene was detected with higher gene copy number in Lagoon I (33.4 x 10^3/µl) compared to Lagoon II (29.9 x 10^3/µl), as well as higher C_t value of 15.7 and 19.3, respectively. MS2 phage was detected in Lagoon I with 34.9 x 10^2/µl gene copy number and 23.7 C_t value compared to Lagoon II where only 4.6 x 10^2/µl gene copies/µl and 26.9 C_t value were detected. Finally, Adenovirus C hexon gene exhibited the lowest gene copy number and C_t value. It was detected in Lagoon I with gene copy number of 3.7 x 10^2/µl and 2.2 x 10/µl in Lagoon II and C_t value of 26.4 and 30.8, respectively. Overall, the detection of Adenovirus in the lagoon samples was very low and needs to be further tested. Furthermore, due to expected variation in the viral sequences, more regions of the genome must be targeted (i.e., designing primers for different regions of the genome) to assure accurate detection and quantifications.

Our results indicated significant differences in the C_t values of Lagoon I and Lagoon II when results for Salmonella invA gene, Adenovirus C hexon gene, and MS2 phage were compared using Student's T-test (P value$\leq$ 0.05). However, no significant differences were observed with E coli 16S rRNA C_t values among the two lagoons (P value > 0.05) suggesting that E coli bacteria are equally abundant in both lagoons. The differences of the load of Salmonella, Adenoviruses, and MS2 phage among the two lagoons could be attributed to the differences of lagoon communities including different physiochemical compositions and lagoon ecologies.

In conclusion, our qPCR assays were in agreement with microarray data, where higher signal intensities translated into higher gene copy numbers by qPCR which supports the microarray's accuracy. Nevertheless, the numbers of organisms that were detected in our lagoon samples were only roughly estimated. More samples are needed before drawing a final conclusion regarding quantitative comparisons.

IV. CONCLUSIONS

Microarray data obtained in this study demonstrated that the detection and identification of bacterial and viral genome signatures in environmental samples (e.g., dairy lagoons) is a viable and rapid approach that complements traditional bioindicator methods. Furthermore, custom-designed ECD microarrays provided us with a specific, sensitive and rapid genomics-based assay method which could be useful for identifying potential pathogens in many types of environmental samples. Since ECD is an emerging technology, we are among the first to confirm the value of this technology for detection of bacterial and viral genetic signatures within agriculture-associated waste treatment systems such as dairy lagoons. Though further studies are required to better understand and characterize the bacterial and viral populations present in such systems, the tools examined here can clearly provide an avenue toward that end. Also, ECD technology certainly should have widespread application to related areas of environmental microbiology. Additional research is needed to identify a specific viral biomarker or group of viral biomarkers that in conjunction with fecal biomarkers will provide the definitive pollution indicator for which we have been searching.

For future research, identifying viral bioindicators is a promising strategy to detect pathogen contaminations in wastewater. Previous studies have indicated that human adenoviruses and enteroviruses are associated with fecal contamination and can be used as bioindicators (Chapron et al. 2000; Puig et al. 1994; Gantzer et al. 1998). Also, animal viruses such as bovine enteroviruses have been used as an indicator of fecal contamination (Ley, Higgins, and Fayer 2002). Finally, bacteriophages, especially coliphages, have been suggested as a fecal bioindicator (Havelaar, Furuse, and Hogeboom 1986; Schaper et al. 2002).

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Supplementary data

Supplemental Table I PCR and qPCR primer sets and PCR conditions used in this study

Primer	Sequence 5' to 3'	PCR conditions	Reference
Bovine Enterovirus	ACGGAGTAGATGGTATTCCC CGAGCCCCATCTTCCAGAG	95°C for 10 min, followed by 40 cycles of 94°C for 45 s, 56°C for 45 s, 72°C for 1 min, followed by 72°C for 7 min	Ley et al. 2002
E. coli lambda cdtI	GCAGGCTGAAAAACGAAAAG GCCCGGTATTTAGGAACCAT	95°C for 10 min, followed by 35 cycles of 95°C for 1 min, 60°C for 1.5 min, 72°C for 1.5 min, followed by 72°C for 7 min	Asakura et al. 2007
Enterobacteria Phage HK109	TGTCCGTAACGCCATTATCA ATCGAGGCTGGCATAATCAC	95°C for 10 min, followed by 35 cycles of 95°C for 45 s, 58°C for 45 s, 72°C for 1 min, followed by 72°C for 7 min	Nilsson et al, 2001
Enterobacteria Phage MS2	CTGCAAACTTCCAGACAACG AGTCACGTCGCCAGTTCC	95°C for 10 min, followed by 35 cycles of 94°C for 45 s, 60°C for 45 s, 72°C for 1 min, followed by 72°C for 7 min	This study
S. aureus Phage MR1161	GGAGTGGGCAAAGTCGAATA GTCTCCAGGTTCCGGTACAA	95°C for 10 min, followed by 35 cycles of 95°C for 45 s, 60°C for 45 s, 72°C for 1 min, followed by 72°C for 7 min	This study
S. thermophilus phage OBJ	GAATGATACTGCTGGCAGTATTTCGGTTGG CAGTCATGTAGCTATCGATGAAATTCCAACG	95°C for 10 min, followed by 35 cycles of 95°C for 45 s, 60°C for 45 s, 72°C for 1 min, followed by 72°C for 7 min	This study
Salmonella phage mig-3	ACTCCACGTTTGGGTTTCAG CTGGCAATTTTTCAGCACAA	95°C for 10 min, followed by 35 cycles of 94°C for 45 s, 60°C for I min, 72°C for 30 s, followed by 72°C for 5 min	This study
Bacillus licheniformis	GGCTAATACCGGATGCTTGA GAGCCGTTACCTCACCAACT	95°C for 10 min, followed by 35 cycles of 95°C for 45 s, 55°C for 1 min, 72°C for 30 S, followed by 72°C for 5 min	This study
Virgibacillus 16s rRNA	CACGTGGGCAACCTRCCTGTAAGACT GAGAATGGTTTTRTGGGATTT	95°C for 10 min, followed by 35 cycles of 95°C for 1 min, 55°C for 1.5 min, 72°C for 1 min, followed by 72°C for 10 min	Smith et al, 2008
Clostridium 16S rRNA	CCTCAAAGAGGGGAATAGCC ATGTGACCGATCACCCTCTC	95°C for 10 min, followed by 35 cycles of 95°C for 30 s, 55°C for 1 min, 72°C for 30 s, followed by 72°C for 5 min	This study
Bacillus 16S rRNA	GSTTSTTTGAACCGCATGGTTCARACAT GAGAAYAGATTTGTGGGATTGGCTTRAC	95°C for 10 min, followed by 35 cycles of 95°C for 45 s, 55°C for 45 s, 72°C for 1 min, followed by 72°C for 5 min	This study
Escherichia coli 16s rRNA	AGTACTTTCAGCGGGGAGGA CTTGCACCCTCCGTATTACC	95°C for 10 min, followed by 35 cycles of 95°C for 45 s, 56°C for 45 s, 72°C for 1 min, followed by 72°C for 7 min	This study
16S rRNA Universal primers	AAGGAGGTGATCCANCCRCA AGAGTTTGARCMTGGCTCAG	95°C for 15 min, followed by 32 cycles of 95°C for 1 min, 51.4°C for 1.5 min, 72°C for 1.5 min, followed by 72°C for 5 min	Giovannoni, S.J., 1991
E coli 16sRNA	CGGTAATACGGAGGGTGCAA CCTGCGTGCGCTTTACG	95°C for 10 min, followed by 35 cycles of 95°C for 15 s, 55°C for 1 min, 72°C for 1 min, followed by a melt curve ranging from 60-95°C with a ramp temp of .02C/s	This study
Salmonella invA gene	TCGTCATTCCATTACCTACC AAACGTTGAAAAACTGAGGA	95°C for 10 min, followed by 35 cycles of 95°C for 15 s, 55°C for 1 min, 72°C for 30 s, followed by a melt curve ranging from 60-95°C with a ramp temp of .02C/s	Hoorfar et al, 2000
Human Adenovirus C hexon gene	ACTGCCTACAACGCTCTRGC CCRTAGCATGGTTTCATGGG	95°C for 10 min, followed by 45 cycles of 95°C for 15 s, 55.5°C for 1 min, 72°C for 1 min, followed by a melt curve ranging from 60-95°C with a ramp temp of .02C/s	Ebner et al, 2005
Enterobacteria phage MS2	TCGTGCTTTTCGCTGAAGAA GGGTTTCCGTCTTGCTCGTA	95°C for 10 min, followed by 35 cycles of 95°C for 15 s, 60°C for 1 min, 72°C for 30 min, followed by a melt curve ranging from 60-95°C with a ramp temp of .02C/s	This study

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