Project Report to Minnesota Department of Agriculture

Development of a DNA Marker Gene System for E. coli from Cows, Pigs, and Turkeys and Use of Small Watersheds to Monitor Bacteria Loadings and Effects of Mitigation Practices

Clean water Legacy Funds

Project Leader: Adam Birr, Minnesota Department of Agriculture (MDA)

PIs: Michael J. Sadowsky and James Anderson, Department of Soil, Water, and Climate, University of Minnesota

The following studies comprise part of the M.S. Thesis of Richard C. Sawdey for the Graduate Program in Water Resources Science (WRS) at the University of Minnesota.

Abstract

Fecal contamination of waterways represents a consistent threat to public health. In Minnesota's agricultural regions, cattle grazing operations are abundant, often leading to direct and indirect contamination of waterways. In this study, we assessed the spatial and temporal dynamics of bovine fecal loading to a small creek system in Southeastern Minnesota, The Little Jordan Creek (LJC). The purpose of this study was to: (1) determine the scale and source of fecal loading to the creek in 2008; and (2) subsequently employ a best management practice (BMP) at the beginning of and throughout the 2009 sampling season to mitigate fecal loading to the system. The USEPA method 1603 was used to determine the scale of *E. coli* presence at five locations in 2008 and 2009, and conventional polymerase chain reaction (PCR) and quantitative PCR assay were used to determine fecal inputs into the LJC. Our analyses revealed that the total *E. coli* counts across all sampling locations in 2008 exceeded state and federal water quality standards, with the highest levels observed where the creek leaves the property. Three major rain events were sampled in 2008, and *E. coli* numbers spiked considerably on these dates. A bovine-specific, *Bacteroidales*-like, marker gene (Bac3) was detected on all sampling dates in 2008, with an overall mean across sites of 1,433 copies for the season. Best Management practice (BMP) remediation plans were initiated in 2009 to reduce direct cattle exposure to the creek, rehabilitate upland areas, and curb over-grazing. The mean *E. coli* level in 2009 across sampling locations and dates was 859 CFU *E. coli*/100ml. Unlike 2008, the Bac3 marker gene was not detected on each sampling date, and had an overall mean detection of 998 copies across dates and sites in 2009. The human marker was not detected by conventional PCR in 2008 or 2009. While the *E. coli* levels in 2009

were lower than observed in 2008, it is difficult to surmise the BMP's initial role, as fewer rain events were sampled in 2009 and the BMP was not completed before the culmination of the sampling season. It is likely that during significant rain events naturalized *E. coli* present in creek sediments are liberated due to increased flow, and observed CFU counts raise accordingly. As a result, USEPA method 1603 may not accurately characterize the scale of fecal loading to stream systems during significant rain events. Further, rain events may skew long-term *E. coli* count data used to determine impairments. Observed CFU *E. coli* did not correlate well with detection of the bovine marker gene, with the exception of sampling locations that had direct cow exposure. The copy number of the bovine marker gene decreased considerably in 2009 at the sampling location exiting the property, suggesting that a decrease in cattle presence in the stream corridor may have helped to achieve lower amounts of fecal loading to the system at this location, and may be successful if applied on a watershed scale.

Introduction

Fecal loading of aquatic environments by animals and humans is of concern for public and environmental health. The deposition of fecal bacteria into waterways is thought to be one of the main routes by which humans are exposed to pathogens. The microbiological contamination of waterways is amongst the most commonly listed water quality impairment in the U. S. (USEPA, 2005). The federal Clean Water Act (CWA) requires that individual states provide the U. S. Environmental Protection Agency (USEPA) with an aggregate quality assessment of its waterways every biennium (section 305(b) list) in effort to identify waters that do not meet state and federal quality standards (the section 303(d) list). Once deemed impaired, states must conduct a Total Maximum Daily Load (TMDL) assessment in order to mitigate the impairment and restore the water body to acceptable quality. Successful fecal coliform TMDL implementation strategies require microbial source tracking (MST) technologies that accurately and efficiently characterize the host-specific source(s) of bacterial loading.

Traditional MST methods require the use of large known source libraries. While it has been shown that smaller libraries have higher average rates of correct classification (ARCC), they are not as representative at a watershed scale as larger libraries (Moore et al., 2005; Wiggins et al., 2003). The construction of large libraries is very labor intensive, and despite their size, they often fail to identify the host origins of environmental isolates (Hassan et al., 2005; Stoeckel et al., 2004). Furthermore, known-source libraries are limited by the temporal and geographical variation of target bacterial genotypes, variation in the diets of host animal groups, and the naturalization of target organisms to aquatic environments (Byappanahalli et al., 2003; Byappanahalli et al.,

2006; Gordon, 2001; Hartel et al., 2002, 2003; Ishii et al., 2006; Olapade et al., 2006; Power et al., 2005; Scott et al., 2003; Whitman et al., 2003, 2005).

To rectify the shortcomings of library- based MST methodologies, investigation of the use of non-culture based, library independent methods represents the current focus of MST technology development. Such methods aim to circumvent the use of large known source libraries by identifying host-specific bacterial gene targets (markers) that are found in the fecal material of animals. To date, genetic markers have been developed and reported to identify several host-specific fecal inputs into aquatic environments. Bernhard and Field have investigated the use of 16S rRNA-based genes from *Bifidobacterium* and *Bacteroides-Prevotella* that can discriminate between human and ruminant fecal input sources to waterways (Bernhard and Field, 2000(a), 2000(b)). Further, Dick and coworkers have successfully used subtraction hybridization to identify host specific *Bacteroides* 16S rRNA markers, and in a subsequent study, investigated *Bacteroidales* 16S rRNA gene sequences to identify swine and fecal loading due to horses (Dick et al., 2005(a), 2005(b)). These marker genes have been subsequently used by others to examine fecal loading to waterways due to human activities (Bower et al., 2005). Further, Hamilton and coworkers used subtraction hybridization to identify duck and goose specific *E. coli* marker genes (Hamilton et al., 2006). Similarly, Shanks and coworkers used a gene fragment enrichment (GFE) technique to identify cow specific *Bacteroidales*-like marker genes (Shanks et al., 2006, 2008).

In this study, we examined the spatial and temporal dynamics of bovine fecal loading to a small creek system (the Little Jordan) in Southeastern Minnesota. The watershed is home to a host of agricultural practices, and cattle grazing is prevalent in

direct upland areas of the creek. Fecal coliform and *E. coli* plate count data was obtained using USEPA method 1603 to determine potential regulatory impairments (USEPA, 2002). A quantitative PCR protocol was developed for the bovine *Bacteroidales*-like 16S rDNA marker Bac3 (Shanks et al., 2006) to aide in determining the scale of bovine fecal contributions to the Little Jordan Creek system. Results from the Bac3 qPCR assay and plate count analysis were compared, and minimal correlation was observed during rain events. Our results suggest that USEPA method 1603 may not be an appropriate tool for classifying the scale of fecal pollution during wet periods.

Materials and Methods

Sampling location description.

Field investigations were performed on the Little Jordan Creek, near Chatfield, Minnesota (Figure 1). Cattle grazing is prevalent within the Little Jordan watershed, and animals are frequently allowed unrestricted access to the creek for the majority of the grazing season. In 2008, five sampling sites were selected in cooperation with a local landowner to assess bovine fecal loading to the waterway. The strategy of location selection was to include areas of the creek that were thought to be significantly impacted by grazing practices, and to those thought to have less cattle impact. In 2008, sites 1 and 3 were selected as areas that would not be significantly influenced by the landowners grazing practices. Sites 2, 4, and 5 all were expected to have high exposure to cattle. In 2009, three new sites were selected upstream to determine potential sources of fecal inputs based on observations from the 2008 sampling season. In 2009, the main spring

source of the creek, site 8, was sampled, along with two additional sites (7 and 9). These were selected based on geographical proximity to the creeks source.

While the creek is relatively small, it is actively managed by the Minnesota Department of Natural Resources (MNDNR) as a designated trout stream. Although there is an abundance of agricultural practices within the watershed area, there is also considerable arboreal growth and very few impervious surfaces. As a result, only large scale rain events had noticeable affects on stream level and turbidity.

Environmental sample collection.

In 2008, samples were collected from April to September. For initial samples, through July 14, 2008, three 700 ml samples were collected per sampling location along the Little Jordan Creek, near Chatfield Minnesota. However, beginning on July 31, 2008, six 1000 ml samples were collected to ensure adequate availability of water for analyses. Samples were collected in either sterile 710 mL capacity Whirl-Pak bags (eNasco, Fort Atkinson, WI) or sterile polypropylene copolymer Nalgene bottles (Thermo Fisher Scientific Inc., Waltham, MA). Time was given to allow stream sediments to settle after entry, and high flow areas at each location were chosen to retrieve sample. Samples were placed on ice during return to laboratory, and held at 4ºC until processed the next morning. Temperature readings were obtained at each location with a digital thermometer during each sampling occasion. Transparency tube (T-tube) measurements were also taken to determine the scale of sediment suspension in the water column (MPCA, 2005).

Filtering strategy and generation of plate count data.

Samples were processed according to EPA Method 1603, using modified membrane-thermotolerant *Escherichia coli* agar medium (modified mTEC) using Millipore S-Pak 0.45 µm sterile gridded filters (Millipore, Billerica, MA) (USEPA, 2002). In addition to using mTEC medium to obtain *E. coli* plate count data, mFC medium (Difco, Detroit, MI) was also used to determine total fecal coliform counts. Three replicate 100, 10, and 1 ml volumes of each sample from each site were filtered and each dilution was performed in duplicate per sample replicate. However, due to occasional sample loss, the replicate numbers obtained from each sampling site sometimes varied. The 10 and 1 ml volumes were placed in 10 and 9 ml of phosphate buffered saline (Sambrook, Fritsch, and Maniatis, 1989), respectively to achieve uniform cell distribution on filters. Filters were placed onto the surface of mTEC and mFC media and incubated at 37 ºC for 2 hours to recover cells, and then at 44.5ºC for 24 hours (Ishii et al., 2006). After incubation, blue colonies on plates were counted. Filtration volumes that yielded colony numbers between 30 and 300 were selected and used to report plate count data. The duplicate counts per volume filtered were averaged for each replicate. Subsequently the average was taken of the three duplicate outputs, and this number was reported as the total count per respective site.

Molecular methods.

Water samples (700 to 1000 ml) for DNA analysis were filtered through 0.45µm sterile filters (Millipore, Billerica, MA). Filters were stored at -80ºC until further processing. Filters were cut into small pieces using sterile razor blades and DNA was extracted from entrapped microorganism using the MO-BIO Power Soil DNA Extraction

Kit (MO-BIO Laboratories, Carlsbad, CA). The concentration of extracted DNA in each sample was determined using an Eppendorf (nanodrop)Bio-Photometer (Eppendorf AG, Hamburg, Germany). DNA concentrations of samples were normalized to 3 ng/µl, such that 5 µl of sample could be added to each PCR tube to obtain 15 ng of total DNA per reaction.

Marker gene presence in environmental samples was determined by using conventional PCR assays targeting total Bacteroides (AllBac) and the human-specific 16S rRNA HF8 gene cluster (HF183) (Bernhard and Field, 2000(b); Layton et al., 2006). A qPCR method was also developed to target a bovine-specific, *Bacteroidales*-like, 16SrRNA gene marker, Bac3 (Table 1) (Shanks et al., 2006). The thermal and chemical conditions used for PCR of environmental DNA extracts for the universal (AllBac) and human-specific (HF183) PCR assays were conducted as previously described (Bernhard and Field, 2000(b); Layton et al., 2006) with the exception of the HF183 assay, which was done using an annealing temperature of 63˚C.

Optimization of the Bac3 qPCR assay was done by initially using the thermal protocol as described by Shanks et al. (2006), using 2x iTaq SYBR Green Supermix With ROX (Bio-Rad Laboratories, Hercules, CA), and 200 nM of each primer. Thermal and chemical optimization was necessary to improve qPCR reactions using the Applied Biosystems ABI PRISM 7000 Sequence Detection System (Foster City, CA). The cycling protocol was adjusted to include a 2 min step at 50˚C before initial denaturation and enzyme activation, and excluded a final 72˚C extension step. Primer concentrations were tested between 200nM and 300nM and annealing temperatures were tested between 60˚C and 61˚C. The optimized Bac3 qPCR assay consisted of the following protocol: 2

min at 50˚C, followed by a 10 min cycle at 95˚C for enzyme activation and denaturation, followed by 40 cycles of denaturing at 95˚C for 15 sec, and annealing and primer extension steps of 1 min at 61˚C. Following amplification, the dissociation of samples was measured between 65ºC and 95ºC. The optimized reaction mixture (25 μl) contained 2x iTaq SYBR Green Supermix With ROX (Bio-Rad Laboratories, Hercules, CA), and 300 nM of each primer. For standard curve generation, 3 x 10^5, 1 x 10^5, 3 x 10^4, 1 x 10^4, 3 x 10^3, 1 x100^3, 3 x10^2, 1 x10^2, 30, 10, or 3 copies of the 166 bp Bac3 gene fragment (which was previously cloned into the StrataClone PCR cloning vector pSC-A-amp/kan (Stratagene, La Jolla, CA), were obtained from stock tubes frozen at -80˚C) was added to individual reaction mixtures. Each environmental sample run included a standard curve run to ensure calibration accuracy. For environmental sample runs, 15 ng of extracted DNA was added to the reaction mixture. The qPCR assays were performed in an Applied Biosystems ABI PRISM 7000 Sequence Detection System (Foster City, CA), using optically clear 96 well microplates. Samples were run in triplicate to determine qPCR variability. The mean of the qPCR output of triplicate samples were averaged.

Determination of qPCR detection.

Simple detection was defined as the circumstance when at least one of the triplicate samples gave a copy number average above the limit of detection. Numerical detection was defined as the circumstance when ≥ 2 of 3 copy number averages from each DNA sample were above limit of detection, and the subsequent average of those replicates was above limit of detection.

Statistical analysis.

Mean CFU counts and mean total copy number were calculated from sample replicates for plate count analysis and the qPCR output, respectively. The standard errors of the means were also calculated and the ANOVA program (www.physics.csbsju.edu/stats/anova.html), at $\alpha = 0.05$, was used to assess the statistical significance of spatiotemporal variation in CFU counts and total Bac3 copy number in the Little Jordan Creek. Pair-wise comparisons were subsequently done by determining Fisher's Least Significant Difference (LSD) value between sample means.

Results

Plate count data.

In the Little Jordan watershed, there are three landowners actively grazing cattle, providing the animals with unrestricted access to the creek. At least one landowner employs no rotational grazing strategy, allowing significant degradation of riparian vegetation. Recent rain events, proximity of cows to the creek, and water temperature were major factors influencing the *E. coli* and fecal coliform plate count data observed. In 2008, three significant rain events occurred within a 48 h period prior to sampling (June 9, July 13, and July 31). Cattle or fecal material were often observed in and adjacent to the creek. Early in the 2008 sampling season, cows were distributed amongst the entire property where sampling occurred. As the season progressed, grass based foodstuffs became limited, and the landowner began gathering cattle near site number 4 to feed. In 2009, cattle appeared to be grazing in a similar manner on the property sampled in 2008. A rotational grazing strategy was designed in the summer of 2009 in cooperation with

one of the three landowners. While cows were still observed in and adjacent to the creek in 2009, it was with less frequency than in 2008. Water temperature averages during both sampling seasons gradually increased until August, and fell off slightly as September progressed.

While grazing patterns were difficult to assess in 2008, the predictability of either an increase or decrease in observed plate counts at sites 4 and 5 was high, as all fecal inputs to the creek have the potential to influence counts at these locations. In 2009, grazing patterns at designated locations were easier to predict, and the same phenomena were observed. Site number 1 in 2008 was located on the landowner's upstream property line, and received the least exposure to his cattle throughout the season. In 2009, site number 7 was located on the property of a third landowner actively grazing cattle, but not participating in the study.

Results in Figure 2 shows count data of *E. coli* and fecal coliforms (as CFU per ml) at each date and site during the 2008 sampling season. The data shown in Figure 2 indicate that recent rain events, stockpiled manure, and the proximity of cows to the creek were likely major factors influencing the relatively high counts observed. The Minnesota State standard of 126 CFU *E. coli* per 100 ml water was exceeded at each sampling location throughout the sampling season. Site 1 consistently had the lowest counts on both media, with the exception of rain-event sampling dates (June 9, July 13, and July 31), where counts averaged 1,007.5 and 880.7 CFU/100ml for fecal coliforms and *E. coli*, respectively. The site 1 exceeded the state standard 55.6% of the dates sampled. Surprisingly, site 2 averaged the second highest counts at 2,401.4 and 2,549.7 CFU/100ml for fecal coliforms and *E. coli*, respectively, and exceeded the 126 CFU *E.*

coli/100ml standard 77.7% of sampling visits. Site 3 exceeded the standard 87.5% of sampling visits, averaging 1,764.9 and 1,337.8 CFU/100ml for fecal coliform and *E. coli*, respectively; and sites 4 and 5 exceeded the standard each sampling visit. Average fecal coliform and *E. coli* counts at site 5 were, 3,902.7 and 4038.4 CFU/100ml for fecal coliform and *E. coli*, respectively, were the greatest measured at any site during the 2008 sampling season. Site 4 averages were the third highest with 2,178.6 and 2,393 CFU/100ml fecal coliform and *E. coli*, respectively. Interestingly, average *E. coli* counts exceeded fecal coliform averages at sites 2, 4 and 5. Same site sample replicates had minimal variation, and significant differences determined by Fisher's LSD are indicated by alphabetical association (Tables 2-3).

While precipitation during the 2009 sampling season was comparable to what was observed in 2008 (Table 4), it was largely distributed throughout the summer, and fewer significant rain events occurred. As a result, one rain event sampling occurred in 2009 (August 17). Results in shown in Figure 3 represent total *E. coli* and fecal coliform counts per date and site during the 2009 sampling season. In 2009, proximity of cows to the stream and the rain event proved to be the largest factors contributing to fluctuations of numbers of fecal coliforms and *E. coli.* The Minnesota State standard was exceeded at each sampling location at least once in 2009. Site 8, the creeks main spring source, averaged the lowest numbers of both fecal coliform and *E. coli* by a large margin at 116 and 67 CFU/100ml, respectively. Moreover, site 8 exceeded the state standard 22.2% of sampling dates. Interestingly, site 7 averaged the highest counts for both fecal coliform and *E. coli*, at 2627.6 and 1607.1 CFU/100ml, respectively, and exceeded the standard 88.9% of sampling date. Site 5 followed averaging 2021.1 and 1235 CFU/100ml for fecal

coliform and *E. coli* respectively, and each exceeded the standard on nine sampling dates in 2009. Observed counts at site 4-2 averaged 1846.9 and 920.2 CFU/100ml for fecal coliforms and *E. coli*, respectively, and this site exceeded the state standard on 77.8% of the dates sampled. Site had the second lowest counts at 649.7 and 467.4 CFU/100ml for fecal coliforms and *E. coli*, respectively. Fecal coliform counts averaged higher at each sampling location than *E. coli* counts in 2009. Statistically significant differences in *E. coli* and coliform count averages at the sites and across dates were tested by using Fisher's LSD, and are shown in Tables 5 and 6.

onventional PCR to determine presence of all Bacteroides and human derived Bacteroides.

Two conventional PCR primer sets were used in 2008 and 2009 to examine DNA's extracted from water at the Little Jordan Creek study location. The general probe AllBac was used to determine the presence of all *Bacteroides sp.* strains (Layton et al., 2006), while the human-specific probe HF183 was used to identify potential inputs of human derived *Bacteroides* sp. (Bernhard and Field, 2000(b)). Each replicate DNA extraction tube obtained in 2008 and 2009 was subjected to both of these assays. The AllBac PCR assay identified *Bacteroides* targets in all samples processed, indicated that the DNA extracted was capable of being used for PCR. In contrast, the HF183 marker genes was not observed in any of the environmental samples obtained during the study, indicating that human inputs to the LJC were likely not contributing to elevated fecal loading of this waterway.

Generation of qPCR calibration curves for the Bac3 primer set.

Before environmental samples could be analyzed for concentration of the Bac3, Bovine-specific target gene, optimization of the chemical and thermal protocols for qPCR was necessary. Initially, the thermal protocol as described by Shanks et al. (2006) was used. The primer concentration initially tested was 200 nM as per manufacturers recommendations (Bio-Rad Laboratories, Hercules, CA). The standard run at these conditions yielded an indeterminable detection limit and low amplification efficiency of 71.8% (E=-1+10$^{(-1/slope)}$) (Figure 4, Panel A). The Shanks (2006) protocol included a final 72˚C extension step, which was used during the quantification stage in this reaction. Because the majority of target product had likely been extended prior to this step, amplicon quantification was likely inaccurate using this step. Accordingly, a second qPCR run was done excluding the 72˚C extension step. This resulted in a detection limit of 1,000 copies (R^2=0.9983), and a slight increase in amplification efficiency to74.2%. Analysis of the dissociation curve for this experiment revealed that a secondary product was amplified. This was likely due to primer- dimerization, and amplification of this false target. To alleviate this problem, the annealing temperature was increased to 61˚C. Increasing the annealing temperature to 61˚C improved the detection limit, to 300 copies (R^2= 0.9945), and decreased the amount of secondary product observed in the dissociation curve. However, the amplification efficiency decreased to 69.4%. To improve the amplification efficiency, the primer concentration in subsequent reactions was increased to 300nM. These conditions proved to be optimal, as amplification efficiency improved to 89.9% and the detection limit dropped to 100 copies (R^2=0.9947) (Figure 4, Panel B).

Presence of the Bac3 bovine-specific marker gene in environmental samples.

The optimized Bac3 qPCR assay was used to determine the amount of bovine-specific marker genes in environmental DNA extracts obtained from the aforementioned sampling locations in 2008 and 2009. The Bac3 qPCR assay identified the presence of bovine-specific *Bacteroidales*-like target genes in every sample obtained during the 2008 season (Figure 5, Panel A). The variability in copy numbers among replicates was high using this assay (data not shown). As a result, site copy number averages spanning the 2008 sampling season were difficult to interpret. The highest observed copy number was at site 2, where the assay quantified 25,058 copies of the *Bacteroidales*-like target. Due to this, site 2 averaged the highest copy number during the 2008 sampling season. Surprisingly, site 1 had the second highest copy number average in 2008 (1,397 copies), despite the fact that the site was initially thought to have limited impact due to grazing cattle. This was followed by copy number averages of 1,226, 1,131, and 922 at site 5, 3, and 4, respectively. Interestingly, site 5 had the greatest copy number of the bovine marker gene on 66.7% (6/9) of the sampling dates, and site 4 had higher copy numbers than sites 1, 2, and 3 on 55.6% (5/9) of sampling dates. Thus, sites 4 and 5 generally had greater numbers of the Bac3 marker gene than sites 1, 2, and 3 on the majority of sampling dates. This correlated well with the presence of a cattle feeding area located adjacent to site 4, and upstream of site 5.

In contrast to what was found in 2008, the Bac3 qPCR assay did not identify the bovine-specific *Bacteroidales*-like target gene at each sampling date in 2009 (Figure 5, Panel B). However, like 2008, replicate copy number variability was high. The greatest copy number, 7,911, was observed at site 9 on September 2, 2009. This resulted in site 9

having the highest overall copy number average in 2009. Site 4-2 averaged the second highest copy number, 1,315, in 2009. This was followed by averages of 1,241, and 734 for sites 7 and 5, respectively. As expected, site 8 averaged the lowest number of bovine-specific marker genes, 189, observed in 2009. Relatively speaking, site 7 had the highest copy number on 37.5% (3/8) of sampling dates, while site 5 did not yield the highest observed copy number at any sampling date during the 2009 season.

Discussion

Plate count data.

Total fecal coliform counts on the April 2 and April 28, 2008 sampling dates (the first two of the season) were comparatively low, but variation between sites was seen as expected (Figure 2). Low numbers were likely due to several factors, including low water temperatures and low initial influence of cow manure in immediate upland areas throughout the property. In addition, during this early part of the season animals were not given direct access to the stream corridor. Cows were largely confined to the area near site 5, likely explaining the highest counts observed at this site on both dates. Counts from the April 28 sampling date were on average higher than the previous samples, likely due to an increase in water temperature, and the fact that cows were allowed to graze at a closer proximity to the stream near site 5.

On May 12, 2008 the cows were distributed amongst the entire property with no apparent grazing strategy. This was the first observed occasion that all cows were given

unrestricted access to the stream. The *E. coli* plate count data for May 12, 2008 likely reflects this observation, as counts were significantly higher at sites 2 to 5, with sites 4 and 5 exceeding the state standard of 126 CFU/ 100mL for the first time. By May 12, 2008 the water temperature had risen since, averaging 11º C with the lower range observed in upstream locations, and the higher range observed downstream. This is true for all temperature data gathered during the 2008 sampling season.

On May 28, 2008, the second sampling date, the cows were again largely distributed amongst the whole property, with the exception of site 5. The land parcel proximate to the road had been sold, and cows that were grazing in the immediate upland areas adjacent to site 5 had been moved upstream of the property border. This did not appear to affect the numbers of fecal coliform and *E. coli* present at this location, however, as the plate count data were higher than on the May 12 sampling date. This was true for all sites, and likely due to an increase in the average water temperature to 13º C. It should be noted, that for both the May 12 and May 28 sampling dates, high counts were observed at site It was thought that this was likely due to significant amounts of sediment obtained in water samples at this location due to low flow rates. Based on this, it was decided that future samples would be taken from just over the fence-line, where the flow rate was significantly higher.

The June 9 sample marked the first date that sampling occurred within 72 h of a significant rain event (8.9 centimeters of rain in the 48 h prior to sampling (http://www.climate.umn.edu/). The water level had risen 25.4 centimeters since May 28, and high turbidity measurements were observed. The water level was determined by drop-down style measurement from two culverts exiting the property downstream of site

5. The effect of the rain event was manifested in elevated plate count data. Interestingly, counts were similarly high for all sites, including site 1, which had previously been consistently low. This is perhaps due to runoff carrying additional fecal material to sections of the stream that would regularly not be influenced, combined with increased flow disturbing sediment and liberating bacteria that would normally not be identified by the water sampling technique used. By this date that average water temperature had risen to approximately 14.5º C.

By the June 25 sampling date the average water temperature had risen significantly to 17º C. Despite this, *E. coli* counts were less, on average, from those seen on the June 9th rain event sampling, and from the May sampling events at sites 4 and 5. This trend may be explained by the absence of cows from the immediate upland areas of these sites sometime after the May 12 sampling date, coupled with a reduction in stream turbidity, which had decreased considerably from the June 9, 2008 sampling date.

With the exception of site 2, *E. coli* counts for each of the other four sites exceeded the state water quality standard on the July 14 sampling date. Count data at sites 1 and 3 were high, potentially due to the 2.6 cm rainfall that fell during the previous 72 h. Coliform and *E. coli* counts continued to be greater than expected in the higher flow area near site During this time period the cows were largely confined to an area near Site 4, where the landowner had began to supply feed. Water temperature averages were the same as on June 25, and water level and turbidity were average (average turbidity is ~55 cm of clarity when using a Transparency Tube. The average water level was ~165 cm below the culvert).

On July 31, 2008 a significant rain event (~2.5 cm) began upon arrival to the sampling location. Plate counts for this sampling event were the greatest recorded to this date. Turbidity and water levels exceeded observed averages, and water temperatures were the greatest recorded, averaging over 18ºC. As before, cows were largely confined to a pen area near site 4. Interestingly, on this date plate counts of fecal coliform bacteria and *E. coli* were the second lowest recorded at site 4. This may be in part due to the riparian vegetation that had grown in the upland areas adjacent to site 5, as the new landowner had rotated cows out of this area since mid-June. However, plate count results were similar at all sites, likely due to sediment re-suspension that distributed attached bacteria into sampling areas.

Fewer cows were observed on the property on the August 15 sampling than was previously documented, and all cattle were confined to pen area near site 4. Discussion with the landowner revealed that they were being moved to another property to be sold. Because the average water temperature was approximately 18ºC on this date, the decrease in fecal coliform and *E. coli* plate counts were likely due to the reduction in herd size, and a lack of recent precipitation. There was no *E. coli* plate count data for sites 3 and 4 on this sampling date, due to significant sample loss upon transport to laboratory.

On September 11, 2008, cattle confined to pen area near site 4 were fewer in number than observed on the August 15 sampling date. Water temperatures had dropped to an average of 16 ºC, and the decrease in water temperature, coupled with a lack of recent precipitation likely explains the decrease in fecal coliform counts as compared to the August 15 sampling date. Interestingly, site 3 continued to exceed the state *E. coli* standard, ruling out fecal coliform and *E. coli* contributions from sediment, as the

sampling location had been moved to a high flow area. Hence, there are likely unknown fecal inputs to this small tributary that contributed to the higher than expected plate count values observed here. The variation in plate count values observed at each sampling site on September 11 was in line with what was expected, with the exception of site

The last sampling event for the 2008 season occurred on September 28, and at this time less than ten cows were being reared in the pen area near site 4. Average water temperatures were 15.5 ºC and the plate count data for this date showed very similar values at each sampling location. This is likely due to a small rain event (~0.95 cm) that began upon arrival to the site. Peak rain event plate count values averaged lower on September 28 than on the rain event sampling dates in July and on June 9.

Plate count data was also obtained for the 2009 sampling season. The first sampling of the 2009 season occurred on April 30, and three new sampling locations were chosen in 2009 upstream of the 2008 locations (Figure 1). This was in part due to a new property owner and loss of access to some of the old sites. The average water temperature on April 30 was 10.8 ºC, and cows were observed in the creek and in immediate upland area adjacent to site 5. Fecal material that had been recently excreted was found near site 7. Total fecal coliform and *E. coli* counts reflected these observations, as these two sites yielded the highest counts for both respective analyses. *E. coli* counts at both locations exceeded 126 CFU/100ml.

While fecal material was not observed near site 7 on May 12, approximately 60 head of cattle were counted upstream of site 9. Cattle were also present in upland areas adjacent to site 5, yet fewer were observed than on the April 30 sampling date. Water temperature averaged 11.0ºC, and fecal coliform and *E. coli* counts were highest at sites 9

and 5, both exceeded 126 CFU/100ml where cows were observed in the creek. Counts were lower at site 7, as it appeared cows had been vacant from the area for some time. Expectedly, counts at site 8 were low.

Water temperature had risen, averaging 16ºC, by the June 1 sampling date. Very little rain had fallen in the watershed, as water levels were identical for each of the first three sampling dates. Cattle were observed in, and adjacent to, the creek upstream of site 9, and were widely distributed upstream of site 4-2. Plate counts for both fecal coliforms and *E. coli* were the highest observed at sites 7 and 4-2 to that date in the 2009 sampling season, and were likely elevated due to an increase in water temperature. While no cattle or fecal material was observed near site 7, grazing occurred in that general area, which may explain the higher counts observed on June 1. Cattle were absent from site 5, which may explain the slight drop in average CFU's observed here for *E. coli*.

On June 15 the water temperature averaged 11ºC, and the watershed had received 0.53 cm of rain 48 h prior to sampling. Also, 4.1 cm of rain fell on the watershed on June 8. This precipitation had residual affects on stream level, as it had risen ~ 4 cm since the May 12 sampling date. While cattle were not observed in proximity to any of the five sampling locations, cow fecal material was still abundant in areas upstream of site 9, so it is likely cows had been in that area recently. As a consequence of this, fecal coliform and *E. coli* counts were the highest at site 9 on June 15. While the increase in precipitation may have had a moderate affect on overall counts, as they averaged higher than May 12, counts were not considerably higher, likely due to minimal sediment re-suspension as determined by turbidity measurements.

Interestingly, by June 29 the water temperature average had fallen to 12.6ºC and site 8 continued to have low numbers of both fecal coliform and *E. coli*. Fecal coliform and *E. coli* counts at site 9 also decreased on this date, and this was likely due to the absence of cattle from this area and lack of fecal material observed in upland areas.

By the July 13 sampling date the watershed had received only trace amounts of precipitation in the weeks previous to sampling. Water temperature had risen on average to 15ºC and fecal material from cattle was observed adjacent to site 7, which likely contributed to the high fecal coliform and *E. coli* counts observed. At site 9, however, counts continued to fall, as cattle remain absent from this area. There were few cows observed in the upland area near site 5, and counts increased there in comparison to the June 29 sampling date. In contrast, counts at site 4-2 decreased slightly, potentially due to that the lack of cattle at site 2.

The watershed received 8 cm of precipitation on July 25, and this was expected to increase counts of coliforms and *E. coli*. However, due to a lack of precipitation to this date, it was likely that the infiltration capacity of soil in the watershed was high. Thus, this precipitation had little affect on stream level and flow on July 29, as the water level was 8.9 cm lower than that measured on June 15. Plate counts for both fecal coliform and *E. coli* were, on average, lower than that observed on July 1 While the water temperature average remained relatively high, 16ºC, no cows were observed in areas adjacent to any of the sampling locations. Interestingly, *E. coli* counts at site 8, which is the spring source, exceeded 126 CFU/100ml. Fecal coliform and *E. coli* counts at the four remaining sites decreased slightly relative to other dates, likely due to the absence of cattle in adjacent upland areas.

Similarly, the area received 1.7 cm of precipitation on August 16. But unlike the other sampling dates, the August 17 sampling date was preceded by rain within a 24 h period. For the first time during the 2009 sampling season, stream turbidity measurements were observed below 60 cm, indicating significant sediment suspension in the water column. Water temperature averaged 16.3ºC, the highest in 2009. Cattle were observed near site 7 and upstream of site 5. Consistent with this, plate counts at sites 7, 4-2, and 5 averaged the highest recorded during the 2009 season. Fecal coliform and *E. coli* counts at site 9 increased for the first time since June 15, likely due to increased sediment re-suspension, and counts were still relatively high at site 8.

The cattle were largely vacated from the field site by the September 2 sampling date. The watershed received no significant precipitation in the week prior to sampling, and water temperature averaged 14ºC. Accordingly, fecal coliform and *E. coli* plate counts were down significantly from the August 17 sampling date, however, counts were still significantly above the 126 CFU/100ml mark at all sites with the exception of site 8.

In the Little Jordan watershed, there are three landowners actively grazing cattle, and providing the animals with unrestricted access to the creek. At least one landowner employs no rotational grazing strategy, allowing significant degradation of riparian vegetation. Water temperature, recent rain events, and the proximity of cattle to the creek were major factors influencing the *E. coli* and fecal coliform plate count data observed. *E. coli* counts often exceeded the regulatory standard of 126 CFU per 100 ml in both 2008 and 2009. While water temperature did appear to influence levels of observed fecal coliform and *E. coli*, increased streamflow resulting in sediment re-suspension and the

proximity of cows to the stream were likely the largest determinants of increased plate count numbers.

While unexpected at first, the relatively uniform plate count data observed during rain events is likely due to the re-suspension of sediment into the stream channel, which causes adherent populations of fecal coliform bacteria and *E. coli* to be liberated from sediments. The consistency at which site 3 showed elevated levels of both indicators was surprising. Upland land use practices are unknown, but are likely contributing to elevated indicator bacteria levels here. On average, sites 4 and 5 yielded had the greatest numbers of fecal coliforms and *E. coli*. This was not surprising, as all inputs to the creek system have the potential to influence levels of indicator bacteria at these locations. Also, cattle were observed near both of these locations, with the greatest frequency throughout the sampling season. In the beginning of the sampling season, cows were largely distributed over the property. However, as grass became limited in various areas of the property, the landowner began to feed the cows in a rearing pen directly adjacent to site 4. As a result, counts began to decrease at sites 1 and 2, and remained consistently high at sites 4 and 5.

In 2009, three new sampling locations were selected to determine counts at locations upstream of the 2008 sites. Site 5 remained the same as sampled in 2008 and site 4-2 was slightly downstream of the 2008 site 4. However, spatial similarities existed in plate count data observations. Sites 7, 4-2 and 5 averaged the highest counts for both fecal coliforms and *E. coli*, as their exposure to cattle was consistently the highest although significantly lower than observed in 2008. Surprisingly, site 7 averaged higher counts than sites 4-2 and 5, likely due to higher levels of cattle exposure in this area. It was known that cattle were grazing in upland areas adjacent to site 7, but the extent of

their presence was previously unknown. Low flow rate was also a factor also potentially contributing to the high counts observed at site 7. This site is upstream of the creeks main source of flow, and just below the spring where the creek begins. Low flow may allow bacteria to accumulate in the stream channel, causing more organisms to be obtained during sampling. Further, fecal material deposited in the creek or on its edge was likely to remain for a longer period of time. Cows were grazing near site 9 early in the season (May through mid June), considerably affecting the levels of indicator bacteria observed there. As expected, once cattle were removed from this area, plate counts decreased at each sampling date at site 9, with the exception of the rain event sampling obtained on August 17. Increased sediment in the stream channel likely caused fecal coliform and *E. coli* counts to spike as a result of adherent organisms being liberated due to increased flow. Interestingly, site 8 exceeded the state water quality standard twice during the 2009 season. The cause for this is largely unknown. Lower than average plate counts per site in 2009 were likely due to a combination of factors. The new sampling locations, with the exception of site 7 had lower overall exposure to cattle than sites 1 and 2 sampled in 2008. Perhaps the largest contributor to lower overall averages in plate count numbers in 2009 was the sporadic nature of storm events and lack of sediment derived turbidity during sampling, coupled with BMP installment prior to and during the sampling season. While precipitation totals were similar between the two seasons (Table 4), rain events in 2009 were more spread out and difficult to predict. Consequently, only one sampling date was within a 24-hour period of a rain event as opposed to three during the 2008 season.

5.2 Bovine-specific (Bac3) qPCR.

In this study, we optimized a qPCR protocol for detecting the bovine-specific *Bacteroidales*-like 16SrRNA gene marker Bac 3 (Figure 4). As previously mentioned, numerical detection was defined as the circumstance when ≥ 2 of 3 copy number averages from each DNA sample were above limit of detection, and the subsequent average copy number of those replicates was above limit of detection. The limit of detection for this assay was 100 copies, that is, this assay had the ability to accurately quantify the Bac3 DNA target down to 100 copies. However, due to the potential of *Bacteroidales* to carry multiple genomes based on growth stage various environmental stressors, copy number is not analogous to cell number. Henceforth, we can not use the qPCR assay developed in this study to determine bovine specific *Bacteroidales* cell number. However, it is reasonable to infer that higher observed copy number translates to greater presence of bovine derived *Bacteroidales*, lending to the conclusion that bovine fecal loading is more substantial. The Bac3 qPCR assay identified the bovine-specific *Bacteroidales* target on every sampling date during the 2008 season (Figure 5). This indicates that cattle manure was likely impacting all sites in this study and were contributing to the total fecal loading of the LJC. Interestingly, the distribution of the bovine-specific marker gene varied by sample at each location. This is likely due to the large variability inherent in sampling stream systems, coupled with the sensitivity of the Bac3 qPCR assay.

It appears there was not a strong correlation between numbers of fecal coliform or *E. coli* and the Bac3 assay output on rain event sampling dates. For example, while the highest plate count numbers observed were during the July 31, 2008 sampling date, during a rain event (~2.5 cm upon arrival), this date had very low qPCR copy numbers

per site, and were below the limit of detection at sites 1, 3, and 4. Two major factors likely contribute to this phenomenon. First, during the rain event, flow in the stream increases, increasing volume, effectively diluting the amount of Bac3 target present in water samples. This is in contrast to what findings of a previous study may suggest, as the detection of the Bac3 marker was reported in stream sediments (Lee et al., 2008). Further, Bower and coworkers (2005) found elevated levels of bovine-specific *Bacteroides* markers in a Lake Michigan harbor following a rain event. Secondly, due to increased flow there is a significant increase in turbidity, or sediment re-suspension. Because populations of fecal coliform bacteria and *E. coli* are bound to sediments and released due to increased turbidity, plate count numbers spike to very high levels. There was a general correlation however, between the highest average CFU and Bac3 copy numbers observed at sites 4 and 5 during the majority of sampling dates (see discussion on site 2 below).

Additionally, because *Bacteroides* does not propagate in the environment, direct or adjacent loading to the stream are likely the major influences on copy number values. For example, the highest copy number observed was at site 2 on the May 12 sampling date. During this time, several cows were standing in the water, and fecal material was abundant on the stream bank, and was observed floating in the river. Total copy numbers generally decreased as cows were being moved from the property late in the sampling season. While the number of Bac3 copies averaged the highest at site 2 over the 2008 sampling season, it was primarily due to the May 12 sampling date alone. Aside from this, however, sites 4 and 5 generally had a higher copy number of the Bac3 marker gene over the 2008 sampling season than all other sites.

During the 2009 season the he Bac3 qPCR assay did not identify the bovine-specific *Bacteroides* marker gene t until May 12 at site 9 (Figure 5). Similarly to 2008, the distribution of the marker across sites and dates was not always consistent. In general, copy number values were lower in 2009 than 2008. This could be due relatively low flow conditions that reduced the distribution of the marker in the stream channel. More importantly, however, fewer cows were present in the watershed in 2009 compared to 2008 (sites 1-3). As anticipated, the copy number of the Bac3 marker gene at site 8, over the sampling season, were the lowest recorded in 2009. This location was 5 m s from the spring seepage. The relatively high numbers observed here on August 17 and September 2 were likely due to the presence of cattle in this area before sampling was conducted. Site 8 is approximately 100 m downstream of site 7, where cattle or fecal material had been observed on several occasions. It is likely that the season average at site 7 was so high as a result of this deposition. Interestingly, the high copy numbers of the Bac3 marker gene observed at site 5 were the second lowest during the 2009 sampling season, perhaps in part due to initiation of a grazing best management practice (BMP) that reduced creek exposure to cattle at sites 9, 4-2 and 5.

Summary of bovine fecal loading to the Little Jordan Creek.

In this study we assessed the spatial and temporal dynamics of bovine fecal loading to a creek system in Southeastern Minnesota. Ample evidence existed that the grazing operations in the Little Jordan Creek watershed were negatively affecting microbiological quality of the creek water, as determined by *E. coli* plate count analysis. *E. coli* numbers often exceeded the Minnesota State Standard of 126 CFU/100ml at

several sampling locations spanning the entire creek. The Bac3 qPCR assay, optimized in this study, revealed abundant presence of the bovine-specific *Bacteroidales* gene marker, confirming fecal loading from grazing cattle. Recent rain events and cow proximity to creek sampling location appeared to be the two most significant factors influencing plate counts and observed Bac3 copy number. These results corroborate well with previous findings that bovine fecal loading to aquatic systems decreases as cattle proximity to the water body increases (Larsen, 1996; Bailey and Welling, 1999; Tate et al., 2003). It is likely that during significant rain events, naturalized *E. coli* present in creek sediments are liberated due to increased flow, and observed CFU counts raise accordingly. As a result, USEPA method 1603 may not accurately characterize the scale of fecal loading to stream systems during significant rain events. Further, rain events may skew long-term *E. coli* count data used to determine impairments.

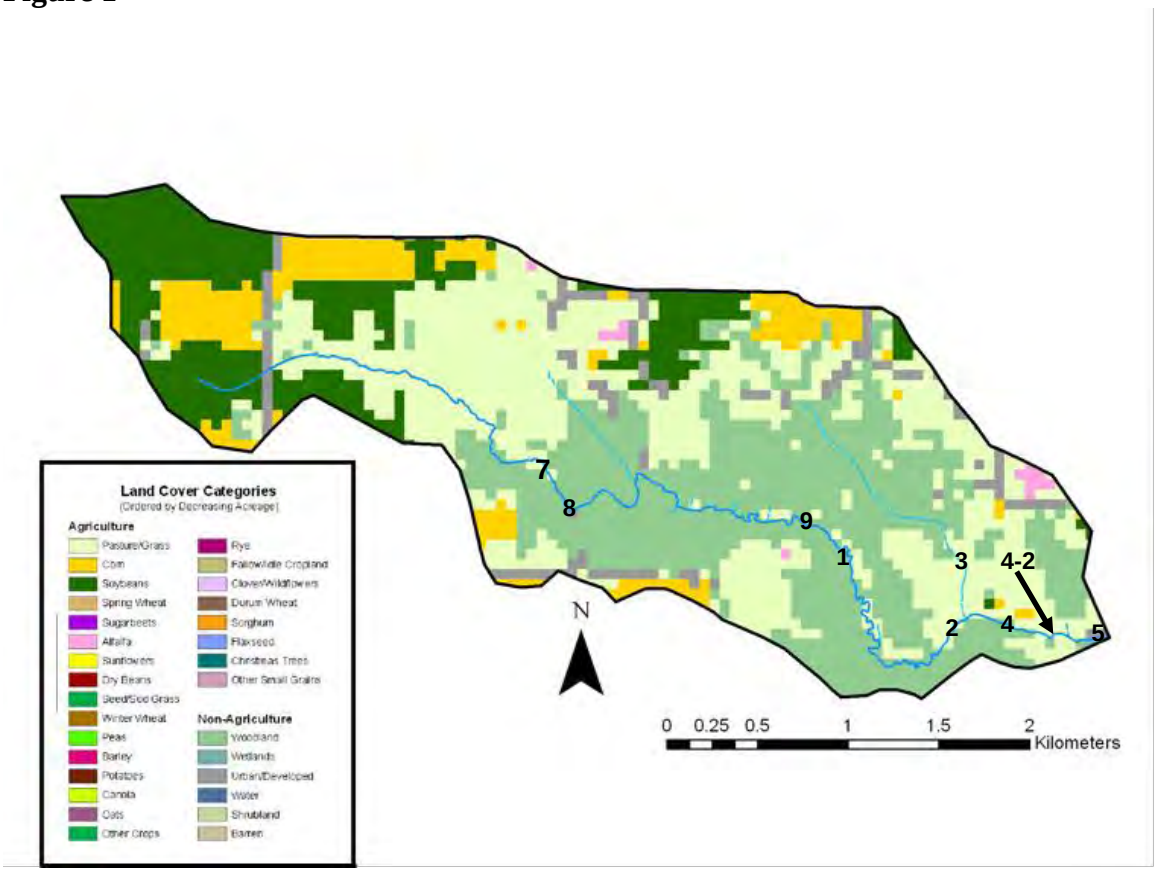
Observed CFU *E. coli* did not correlate well with detection of the bovine marker gene, with the exception of sampling locations that had direct cow exposure. The copy number of the bovine marker gene decreased considerably in 2009 at the sampling location exiting the property, suggesting that decreased cattle access to the creek achieved lower amounts of fecal loading to the system at this location, and would likely be successful on a watershed scale. While the Bac3 qPCR assay effectively identified the scale of the bovine-specific *Bacteroidales* marker gene, work is still needed in accurately characterizing the numerical genome possession of *Bacteroidales* so cell numbers can be determined and scale of bovine fecal loading assessed. Further, fractional characterization of *Bacteroidales* in bovine intestinal flora will be necessary to normalize observed cell numbers in water samples with amount of feces loaded to waterways. Future studies will

also be required to establish epidemiological risk relationships to gene targets derived from fecal anaerobe targets.

While the BMP installed prior to the 2009 sampling season appeared to have desirable affects, cows were on occasion still granted access to the stream corridor. Future mitigation efforts must include all landowners within the watershed, and enforcement mechanisms must be administered to ensure compliance of BMP design.

Tables and Figures

Figure 1



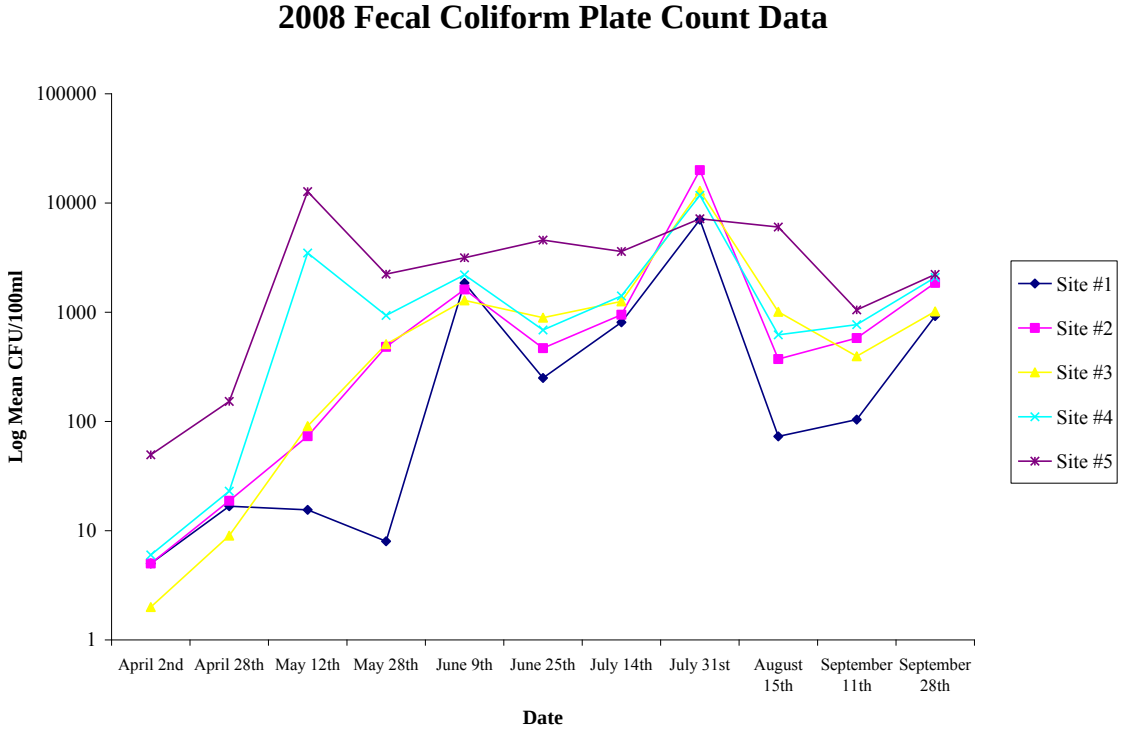
Land-use map of the Little Jordan Creek watershed. Sampling locations are indicated by number. In 2008, sites 1 thorugh 5 were sampled. In 2009, sites 7, 8, 9, 4-2, and 5 were sampled

Table 1 Conventional and real-time PCR primers used in this study.

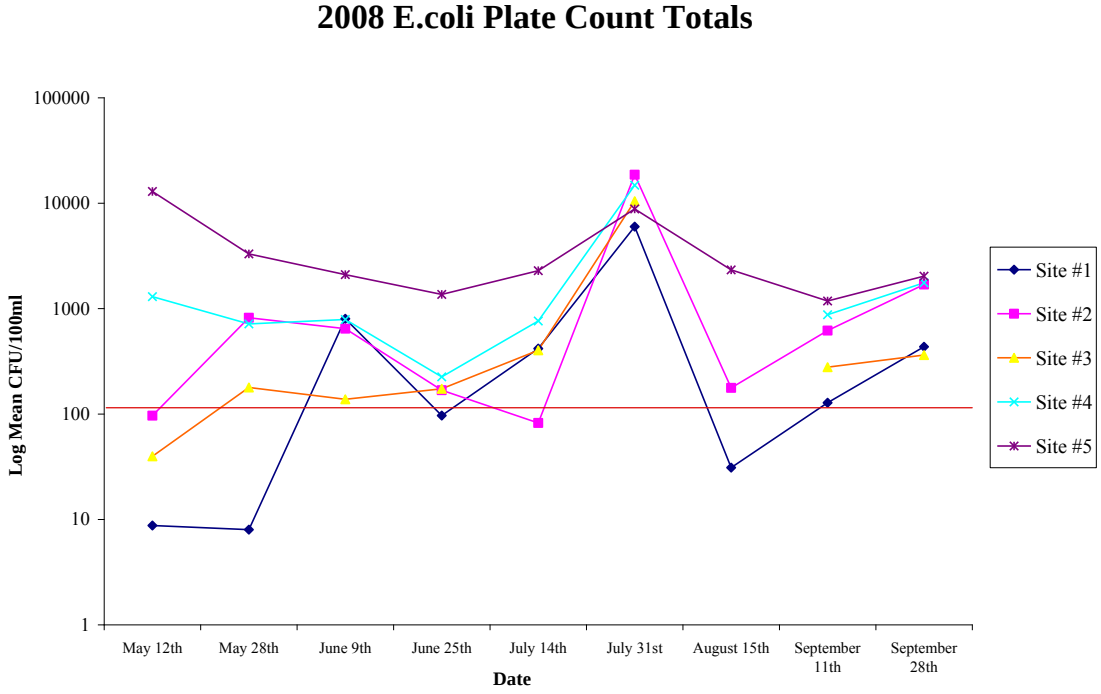
Primer Set	Host	Sequence (5'-3')	Amplicon	Annealing	Cycles

			Lengh (bp)	Temp. (ºC)	
AllBac296F AllBac412R	All Bacteroides	5'-GAGAGGAAGGTCCCCCAC-3' 5'-CGCTACTTGGCTGGTTCAG-3'	106	60	35
HF183F Bac708R	Humans	5'-ATCATGAGTTCACATGTCCG-3' 5'-CAATCGGAGTTCTTCGTG-3'	525	63	35
Bac3F Bac3R	Cattle	5'-CTAATGGAAAATGGATGGTATCT-3' 5'-GCCGCCCAGCTCAAATAG-3'	166	61	40

Figure 2
A.


2008 Fecal Coliform Plate Count Data
Log Mean CFU/100ml
100000
10000
1000
100
10
1
April 2nd
April 28th
May 12th
May 28th
June 9th
June 25th
July 14th
July 31st
August 15th
September 11th
September 28th
Date
Site #1
Site #2
Site #3
Site #4
Site #5

B.



Fecal coliform (A) and *E.* coli (B) plate count data from the 2008 sampling season. Significant rain events had occurred within a 72 hour period previous to sampling on June 9th, July 14th, and July 31st. No *E. coli* counts were performed for site 3 and 4 on August 15th, due to significant sample loss upon transportation to laboratory. Red line on *E. coli* chart indicates state standard level of 126CFU *E.coli*/100ml.

Table 2 Pair-wise comparison of 2008 *E. coli* plate count data by site on each date using ANOVA and Fisher's LSD. Sites across each sampling date followed by the same letter

are not statistically different as determined by ANOVA and Fisher's LSD at $\alpha = 0.05$. No letters are associated with sites 3 and 4 on August 15th due to sample loss (reads across).

	Sites				
Sample Date	1	2	3	4	5
May 12	A	A	A	A	B
May 28	A	A	A	A	B
June 9	A	A	B	A	C
June 25	A	AB	AB	B	C
July 14	A	B	A	C	D
July 31	A	B	C	C	A
August 15	A	A	N/A	N/A	B
September 11	A	B	C	D	E
September 28	A	B	A	B	C

Table 3 Pair-wise comparison by date of 2008 *E. coli* plate count data at each site using ANOVA output to determine Fisher's LSD. Dates across each sampling site that share the same letter were not significantly different at $\alpha = 0.05$ (reads across).

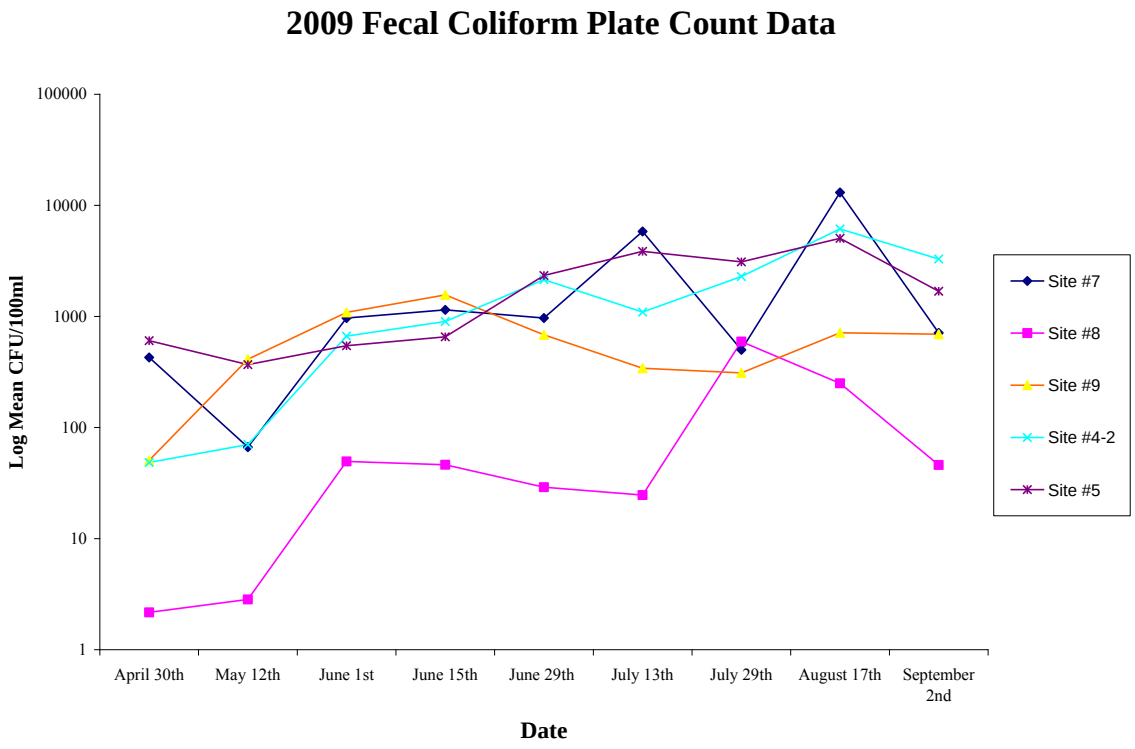
	Sampling Date								
Sites	May 12	May 28	June 9	June 25	July 14	July 31	August 15	September 11	September 28
Site #1	A	A	B	AB	AB	C	AB	AB	AB
Site #2	A	B	B	A	A	C	A	B	D
Site #3	A	A	A	A	A	B	N/A`	A	A
Site #4	A	A	A	A	A	B	N/A`	A	A
Site #5	A	B	B	B	B	C	B	B	B

Table 4 Precipitation comparisons by month for the 2008 and 2009 sampling seasons.

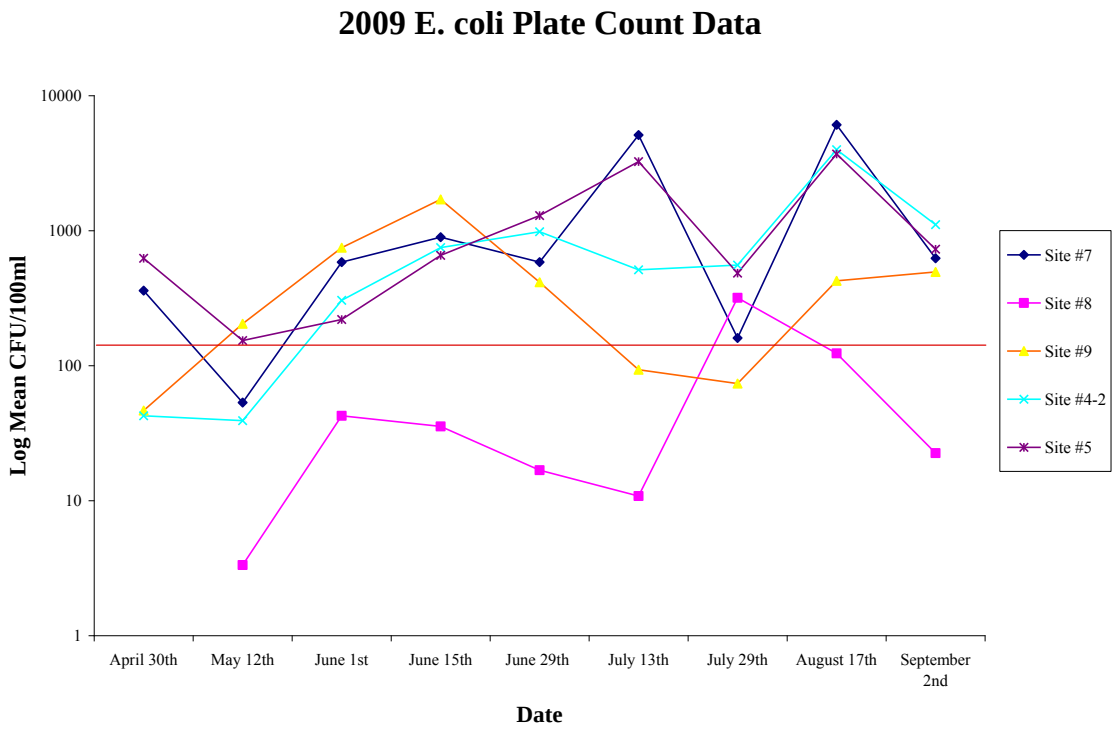
	2008	2009
Month	Precip(cm)	Precip(cm)
April	16.8	7.2
May	10.1	12
June	15.9	9.3
July	7.1	9.4
August	7.3	12.4
September	2	8
Total	60.4	55.3

Figure 3

A.


2009 Fecal Coliform Plate Count Data
Log Mean CFU/100ml
100000
10000
1000
100
10
1
April 30th
May 12th
June 1st
June 15th
June 29th
July 13th
July 29th
August 17th
September 2nd
Date
Site #7
Site #8
Site #9
Site #4-2
Site #5

B.



Fecal coliform (A) and *E.* coli (B) plate count data from the 2009 sampling season. August 17th represents the only sampling date when precipitation had fallen in the watershed within a 24-h period prior to arrival. Red line on *E. coli* chart indicates state standard level of 126CFU *E.coli*/100ml.

Table 5. Pair-wise comparison of 2009 *E. coli* plate count data by site on each date using ANOVA and Fisher's LSD. Sites across each sampling date followed by the same letter

are not statistically different as determined by ANOVA and Fisher's LSD at $\alpha = 0.05$ (reads across)

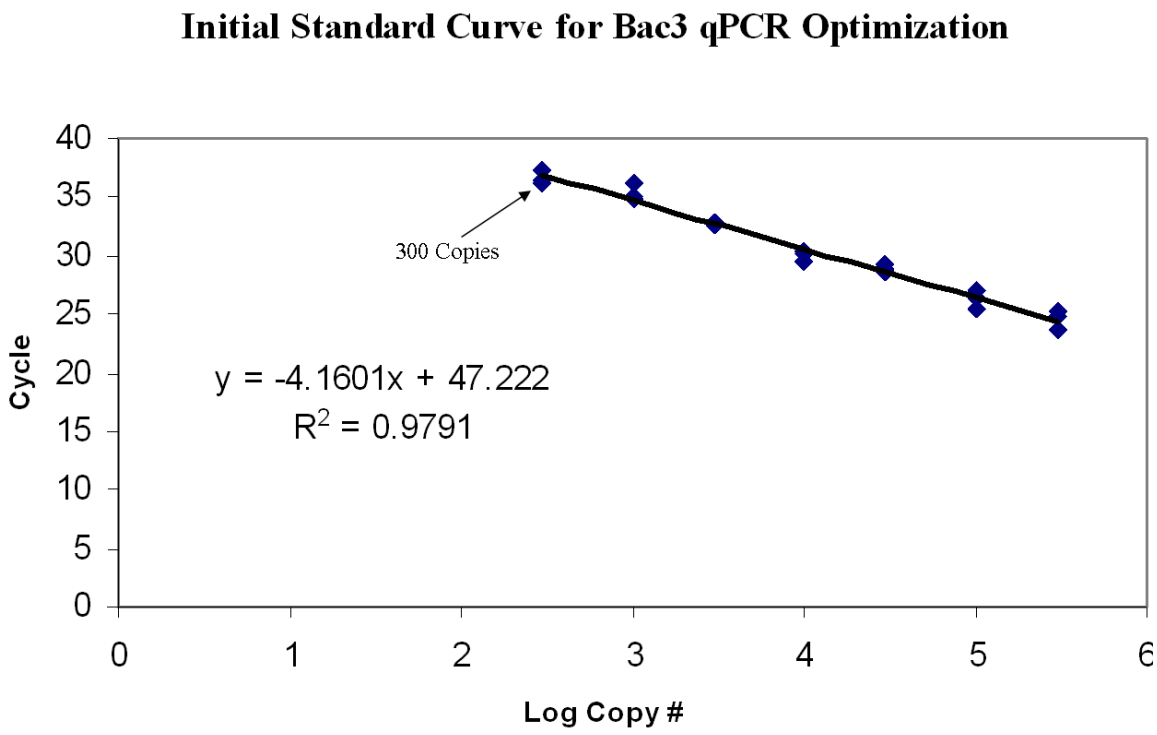
	Sites				
Date	7	8	9	4(2)	5
April 30	A	B	B	B	C
May 12	A	B	C	D	E
June 1	A	B	C	D	E
June 15	A	B	C	AD	D
June 29	A	B	C	D	E
July 13	A	B	B	B	C
July 29	A	B	C	D	E
August 17	A	B	B	C	C
September 2	D	B	AD	C	D

Table 6 Pair-wise comparison by date of 2009 *E. coli* plate count data at each site by use of ANOVA output to determine Fisher's LSD. Dates across each sampling site that share the same letter were not significantly different at $\alpha = 0.05$ (reads across)

	Dates								
Site	April 30	May 12	June 1	June 15	June 29	July 13	July 29	August 17	September 2
7	AB	A	AB	B	AB	C	A	D	AB
8	A	A	B	BC	C	A	D	E	C
9	A	B	C	D	E	A	A	E	E
4(2)	A	A	B	C	D	B	BC	E	F
5	AE	B	B	A	C	D	E	F	A

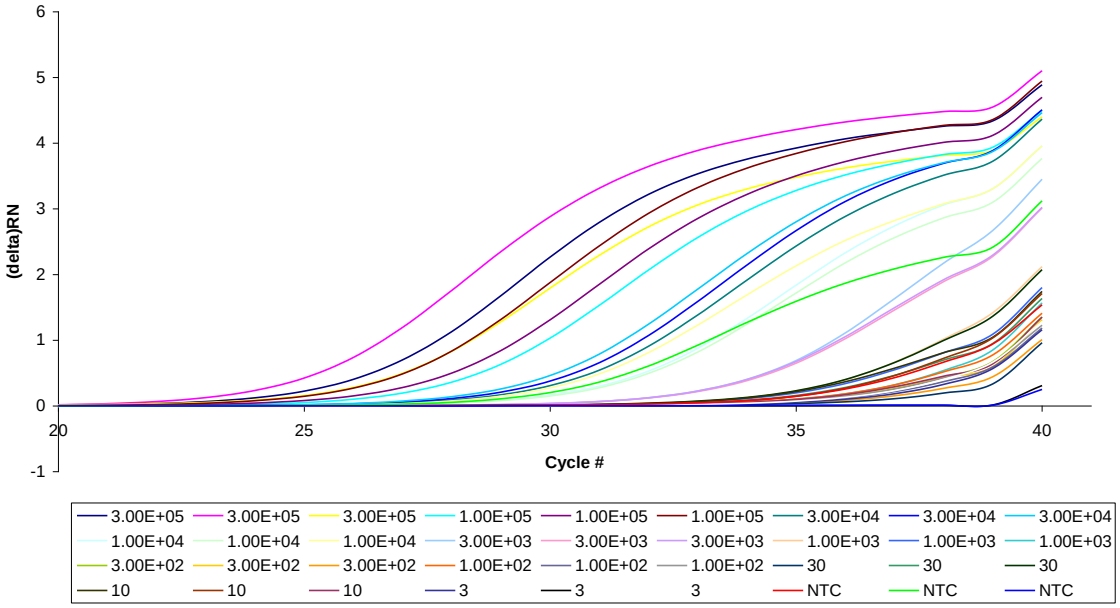
Figure 4 Standard curve, amplification plot, and melting curve for the initial optimization run (A) and final optimization run (B)[1]. The initial optimization run (A) was conducted using conventional thermalcycling conditions as previously described, and 200nM of each primer (Shanks, 2006). The final optimization run (B) was conducted using the following thermalcyling conditions: 2 min at 50˚C followed by a 10 min cycle at 95˚C for enzyme activation and denaturation and then 40 cycles of a denaturing step at 95˚C for 15 seconds, combined with an annealing and primer extension step at 61˚C for 1 min. 300nM of each primer was used in the optimized reaction mixture. Dissociation was measured as described above.

A.



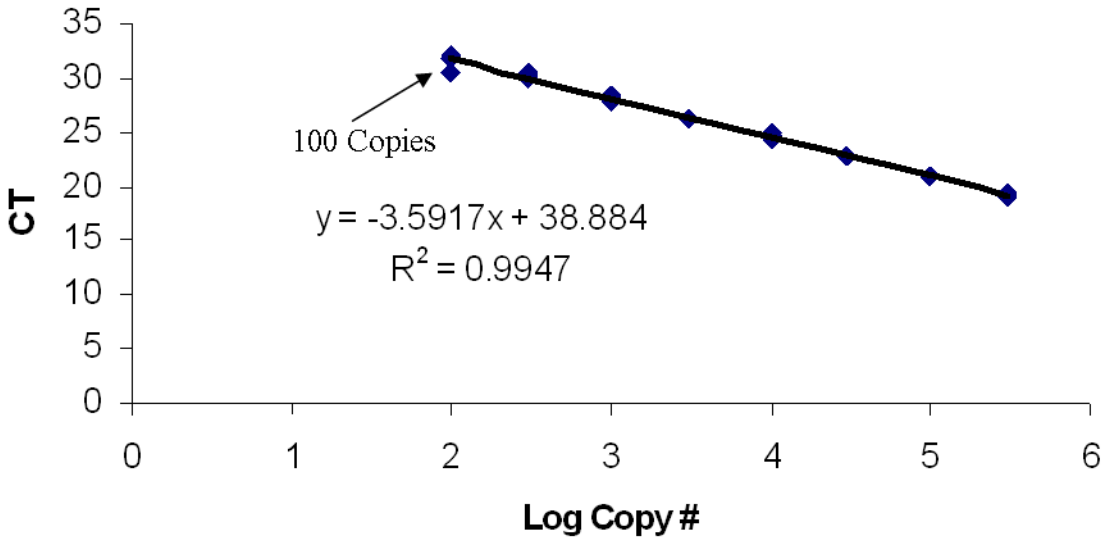
[1] The initial optimization run did not include a dissociation experiment. A melting curve is shown for the final optimization run.

Initial Amplification Plot for Bac3 qPCR Optimization

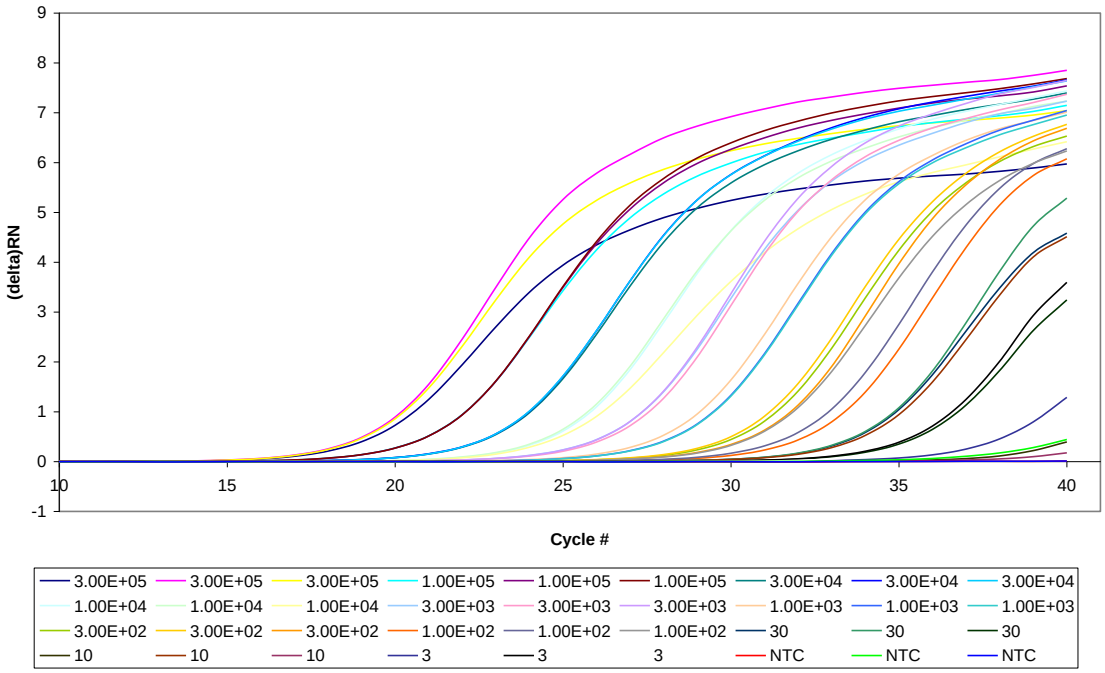


B.

Optimized Bac3 qPCR Standard Curve



Optimized Bac3 qPCR Amplification Plot



Optimized Bac3 qPCR Melting Curve

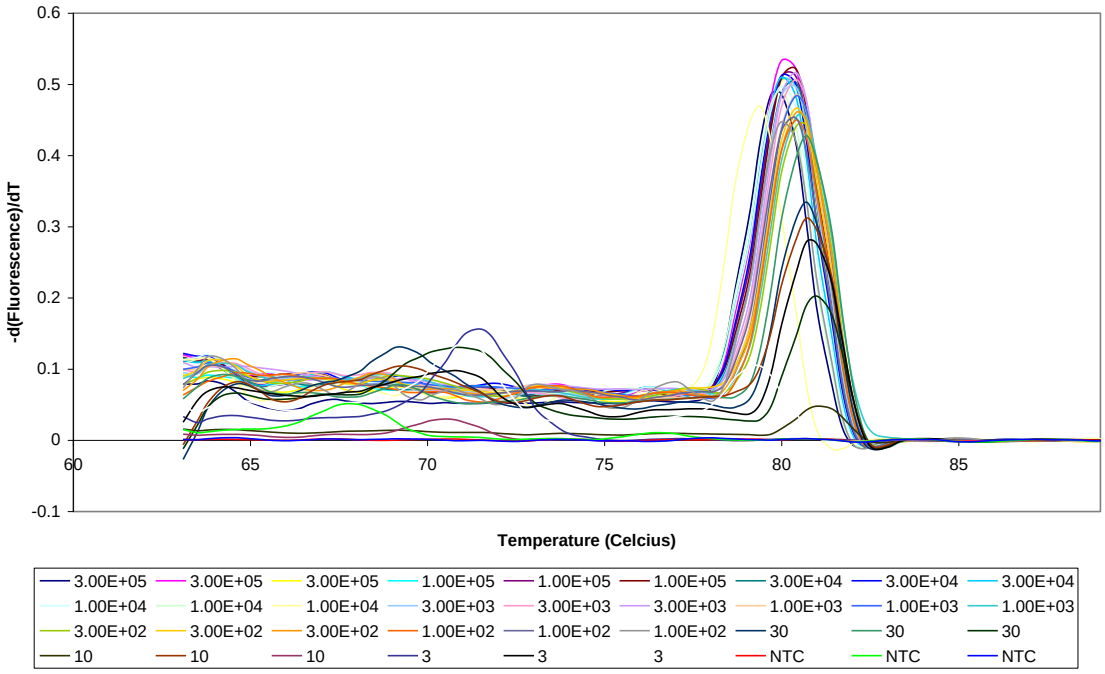
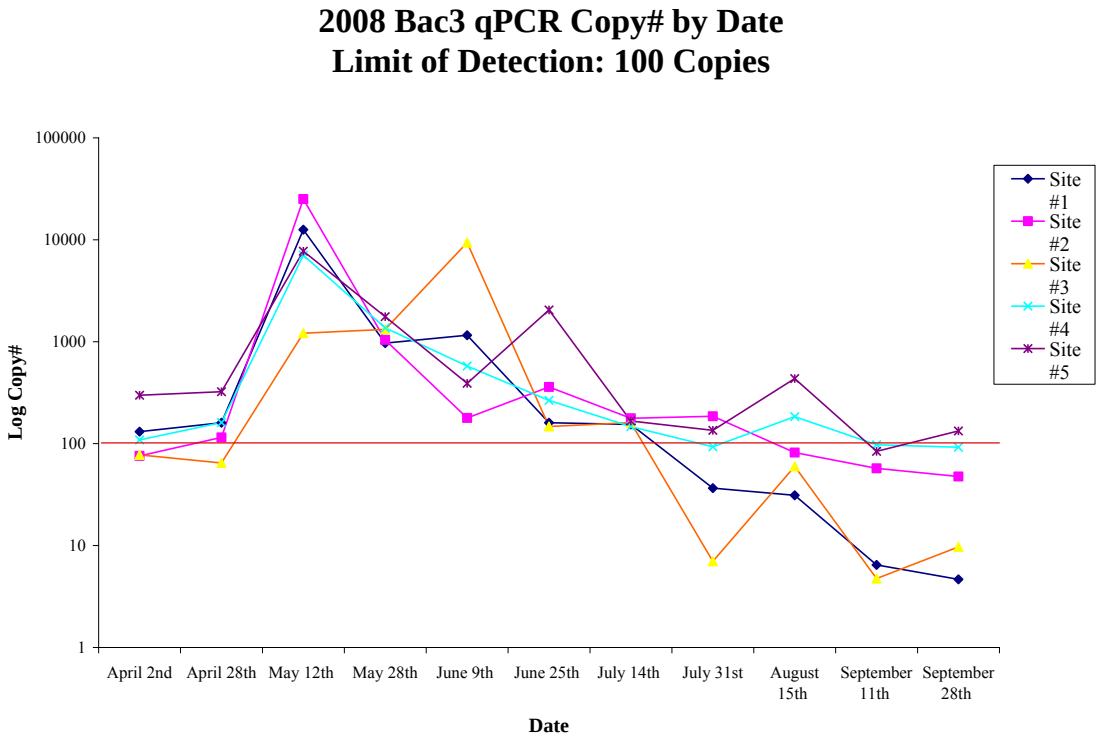
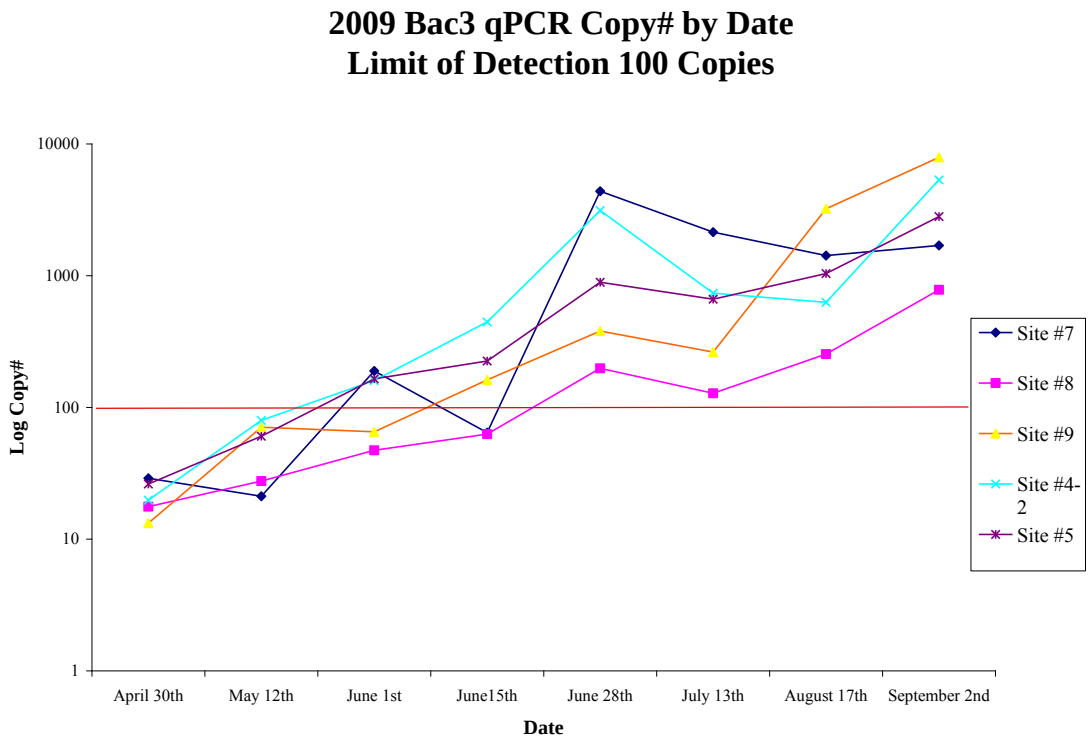


Figure 5

A.


2008 Bac3 qPCR Copy# by Date
Limit of Detection: 100 Copies
Log Copy#
100000
10000
1000
100
10
1
April 2nd
April 28th
May 12th
May 28th
June 9th
June 25th
July 14th
July 31st
August 15th
September 11th
September 28th
Date
Site #1
Site #2
Site #3
Site #4
Site #5

B.



Total Bac3 qPCR target copy number at each sampling site during the 2008 (A) and 2009 (B) sampling seasons. The limit of detection was determined to be 100 copies. Red line indicates limit of detection (100 copies).

References

Bailey, D.W., and G. R. Welling. 1999. Modification of cattle grazing distribution with dehydrated molasses supplement. J. Range Manage. 52:575-582.

Bernhard, A. E., and K. G. Field. 2000(a). Identification of nonpoint sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes. Appl. Environ. Microbiol. 66:1587–1594.

Bernard, A.E., and Field, K.G. 2000(b). A PCR assay to discriminate human and ruminant feces on the basis of host differences in *Bacteroides-Prevotella* genes encoding 16S rRNA: Applied and Environmental Microbiology, v. 66, no. 10, p. 4571-4574.

Bower, Patricia A., Scopel, Caitlin O.; Jensen, Erika T.; Depas, Morgan M.; McLellan, Sandra L. 2005. Detection of genetic markers of fecal indicator bacteria in Lake Michigan and determination of their relationship to *Escherichia coli* densities using standard microbiological methods. Appl. Environ. Microbiol. 71: 8305-8313

Byappanahalli, M., Fowler, M.; Shively, D.; Whitman, R.L. 200 Ubiquity and persistence of *Escherichia coli* in a Midwestern coastal stream. Appl. Environ. Microbiol. 69 (8), 4549–4555.

Byappanahalli, M.; Whitman, R.L.; Shively, D.A.; Ting, W.T.; Tseng, C.C.; Nevers, M.B. 2006. Seasonal persistence and population characteristics of *Escherichia coli* and enterococci in deep backshore sand of two freshwater beaches. J. Water Health 4 (3), 313–320.

Dick, L.K.; Bernhard, A.E.; Brodeur, T.J.; Santo Domingo, J.W.; Simpson, J.M.; Walters, S.P.; Field, K.G. 2005(a). Host distributions of uncultivated fecal *Bacteroidales* reveal genetic markers for fecal source identification. Appl. Environ. Microbiol. 71, 3184–3191.

Dick, L.K.; Simonich, M.T.; Field, K.G. 2005(b). Microplate subtractive hybridization to enrich for source-specific *Bacteroidales* fecal pollution indicators. Appl. Environ. Microbiol. 71, 3179–318

Gordon, D. M. 2001. Geographical structure and host specificity in bacteria and the implications for tracing the source of coliform contamination. Microbiology 147:1079–1085.

Hartel, P. G.; Summer, J. D.; Hill, J. L.; Collins, J.; Entry, J. A.; Segars, W.I. 2002. Geographic variability of *Escherichia coli* ribotypes from animals in Idaho and Georgia. J. Environ. Qual. 31:1273–1278.

Hartel, P. G.; Summer, J. D.; Segars, W.I. 200 Deer diet affects ribotype diversity of *Escherichia coli* for bacterial source tracking. Water Res. 37:3263–3268.

Hassan, W.M.; Wang, S.Y.; Ellender, R.D. 2005. Methods to increase fidelity of repetitive extragenic palindromic PCR fingerprint-based bacterial source tracking efforts. Appl. Environ. Microbiol. 71 (1), 512–518.

Ishii, S.; Ksoll, W.B.; Hicks, R.E.; Sadowsky, M.J. 2006. Presence and growth of naturalized *Escherichia coli* in temperate soils from Lake Superior watersheds. Appl. Environ. Microbiol. 72 (1), 612–621.

Layton, A.; McKay, L.; Williams. D.; Garrett, V.; Gentry, R.; Sayler, G. 2006. Development of *Bacteroides* 16S rRNA gene TaqMan-Based Real-Time PCR assays for estimation of total, human, and bovine fecal pollution in water. Appl. Environ. Microbiol. 72(6):4214-4224.

Larsen, R.E. 1996. Survival and transport of fecal pathogens in grazed watersheds, p.59-71. In: M.R. George (ed.) Livestock management in grazed watersheds. Univ. Calif. Div. Agric. and Nat. Resourc. Pub. 3381.

Lee, Yong-Jin; Molina, Marirosa; Santo Domingo, Jorge W.; Willis, Jonathan D.; Cyterski, Michael; Endale, Dinku M.; Shanks, Orin C. 2008. Temporal assessment of the impact of exposure to cow feces in two watersheds by multiple host-specific PCR assays. Appl. Environ. Microbiol. 74: 6839-6847

Minnesota Pollution Control Agency. 2005. Using Transparency Tube and total suspended solids data to assess stream turbidity. wq-csm1-01. http://www.pca.state.mn.us/publications/wq-csm1-01.pdf.

Moore, D.F.; Harwood, V.J.; Ferguson, D.M.; Lukasik, J.; Hannah, P.; Getrich, M.; Brownell, M. 2005. Evaluation of antibiotic resistance analysis and ribotyping for identification of fecal pollution sources in an urban watershed. J. Appl. Microbiol. 99, 618–628.

Olapade, O.A.; Depas, M.M.; Jensen, E.T.; McLellan, S.L. 2006. Microbial communities and fecal indicator bacteria associated with *Cladophora* mats on beach sites along Lake Michigan shores. Appl. Environ. Microbiol. 72 (3), 1932–1938.

Power, M.L.; Littlefield-Wyer, J.; Gordon, D.M.; Veal, D.A.; Slade, M.B. 2005. Phenotypic and genotypic characterization of encapsulated *Escherichia coli* isolated from blooms in two Australian lakes. Environ. Microbiol. 7, 631–640.

Sambrook, Fritsch, and Maniatis (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, volume 3, appendix B.12

Scott, T. M.; Parveen, S.; Portier, K. M.; Rose, J. B.; Tamplin, M. L.; Farrah, S. R.; Koo, A.; Lukasik, J. 200 Geographical variation in ribotype profiles of *Escherichia coli* isolates from humans, swine, poultry, beef, and dairy cattle in Florida. Appl. Environ. Microbiol. 69:1089–1092.

Shanks, O.C.; Santo Domingo, J.W.; Lamendella, R.; Kelty, C.A.; Graham, J.E. 2006. Competitive metagenomic DNA hybridization identifies host-specific microbial genetic markers in cow fecal samples. Appl. Environ. Microbiol. 72 (6), 4054–4060.

Shanks, O. C.; Atikovic, Emina; Blackwood, A. Denene; Lu, Jingrang; Noble, Rachel T.; Domingo, Jorge Santo; Seifring, Shawn; Sivaganesan, Mano; Haugland, Richard A. 2008. Quantitative PCR for detection and enumeration of genetic markers of bovine fecal pollution. Appl. Environ. Microbiol. 74: 745-752

Stoeckel, D.M.; Mathes, M.V.; Hyer, K.E.; Hagedorn, C.; Kator, H.; Lukasik, J.; O'Brien, T.L.; Fenger, T.W.; Samadpour, M.; Strickler, K.M.; Wiggins, B.A. 2004. Comparison of seven protocols to identify fecal contamination sources using *Escherichia coli*. Environ. Sci. Technol. 38 (22), 6109–6117.

Tate, K.W.; Atwill, E.R.; McDougald, N.K.; George, M.R. 200 Spatial and Temporal Patterns of Cattle Feces Deposition on Rangeland. J. Range Manage. Vol. 56, No. 5 pp. 432-438

U.S. Environmental Protection Agency. 2002. Method 1603—*Escherichia coli* in water by membrane filtration using modified membrane-thermotolerant *Escherichia coli* agar. EPA821-R-02-23, 9. U. S. Environmental Protection Agency, Washington, DC.

U.S. Environmental Protection Agency. 2005. Microbial Source Tracking Guide Document. EPA-600/R-05/064, 131pp. Office of Research and Development, Washington, DC.

Whitman, R.L.; Shively, D.A.; Pawlik, H.; Nevers, M.B.; Byappanahalli, M.N. 200 Occurrence of *Escherichia coli* and enterococci in *Cladophora* (*Chlorophyta*) in nearshore water and beach sand of Lake Michigan. Appl. Environ. Microbiol. 69, 4714–4719.

Whitman, R.L.; Byers, S.E.; Shively, D.A.; Ferguson, D.M.; Byappanahalli, M. 2005. Occurrence and growth characteristics of *Escherichia coli* and enterococci within the accumulated fluid of the northern pitcher plant (Sarracenia purpurea L.). Can. J. Microbiol. 51 (12), 1027–1037.

Wiggins, B.A.; Cash, P.W.; Creamer, W.S.; Dart, S.E.; Garcia, P.P.; Gerecke, T.M.; Han, J.; Henry, B.L.; Hoover, K.B.; Johnson, E.L.; Jones, K.C.; McCarthy, J.G.; McDonough, J.A.; Mercer, S.A.; Noto, M.J.; Park, H.; Phillips, M.S.; Purner, S.M.; Smith, B.M.; Stevens, E.N.; Varner, A.K. 200 Use of antibiotic resistance analysis for representativeness testing of multiwatershed libraries. Appl. Environ. Microbiol. 69,

3399–3405.