Hope College – MST Methods Summary

Sample collection: Stream samples were obtained in the field by hand using sterile (autoclaved) 1-liter polypropylene storage bottles. Collected samples were placed on ice immediately and delivered to the laboratory for analysis and long-term storage.

***E. coli* analysis:** Detection of E. coli in water samples was performed by growth in IDEXX Colilert® medium. Colilert® contains the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide. Hydrolysis of the substrate by *E. coli*-specific enzymes is detected by illumination of the culture with long-wave ultraviolet light. For quantitation, a standard volume of water sample was diluted to 100 mL in sterile water, to which was added the contents of one packet of Colilert®. Due to the levels of *E. coli* in the samples, in general 10 mL of environmental sample was diluted to 100 mL for the assay. Each sample with medium/reagent was poured into an IDEXX Quanti-Tray®/2000 tray, sealed, and incubated at 35 C. [i] After 24 hours, most-probably number (MPN) of E. coli colony-forming units (cfu) was determined by counting tray wells showing fluorescence upon UV illumination based on the IDEXX MPN table. For samples that were diluted 1:10 as described, the measured MPN values were multiplied by ten to compensate for dilution. Thus, reported *E. coli* measurements are equivalent to cfu/100 mL.

Long-term storage: In order to preserve material from water samples for later molecular analysis, 600 mL of each sample collected were filtered through .45-micron filters (147 mm diameter). The filters were transferred into 50-mL plastic centrifuge tubes containing 40 mL of sterile water. Tube containing the filters were vortexed at high speed for 5 minutes, then agitated on a rocking platform in the cold room for 30 minutes to release material adhered to the filter. The filters were removed and the tubes were centrifuged for 20 minutes at 4,500 x g. Liquid was decanted from the tubes, leaving 0.5 – 1 mL of supernatant remaining. Pelleted material was resuspended in the remaining supernatant and transferred into a sterile 1.5 mL cryostorage tube and stored at -80 C.

DNA isolation: Following storage, samples chosen for source-tracking analysis were thawed. DNA was isolated from the contents by isolation and purification using Qiagen's QIAamp® DNA mini kit reagents and spin columns. Isolation of DNA was performed as described in the manufacturer's instructions; in brief, material collected by filtration was subjected to increasing temperatures in the presence of lysozyme to induce lysis of bacteria. Following lysis, material was applied to the binding column by centrifugation. Columns were washed and purified DNA was eluted using buffers supplied in the kit. In most cases, concentration of DNA was measured using a Qubit® (Invitrogen) fluorometer using Broad Range DNA measurement reagents. DNA yields were 1 – 5 micrograms DNA per mL in a volume of 200 µL of water.

Detection of general and host-specific *Bacteroides*: Quantitative polymerase chain reaction (qPCR) based on amplification of ribosomal DNA (rDNA) sequences was used to assess the presence and amount of genetic material originating from *Bacteroides* in purified DNA. Unlike E coli and other coliforms, organisms of the *Bacteroides* genus and related bacteria are obligate anaerobes. As such they cannot replicate in the environment and may be a more direct marker of the presence of fecal material. For qPCR, a single non-host specific reverse primer (262R) was used matched to one of a presumed host-

specific forward (32F, HF183, CF128, PF163). The numbering system for the primers is based on their analogous position in the E. coli rDNA gene; F designates a primer oriented in the forward direction relative to the transcription of the rDNA gene and R designates a reverse primer. H denotes a human specific host, C denotes cow specific, and P denotes pig specific.

General (non-host specific) Bacteroides: 32F = AACGCTAGCTACAGGCTT [ii]

Human specific forward: HF 183 = ATCATGAGTTCACATGTCCG [iii]

Cow specific forward: CF128 = CCAACYTTCCCGWTACTC (Y = C or T; W = A or T) [iv]

Pig specific forward: PF163 = GCGGATTAATACCGTATGA [v]

All-purpose reverse primer: 262R = TACCCCGCCTACTA TCTAATG [vi]

Primers were obtained from Eurofins/MWG-Operon and dissolved in water at a working stock concentration of 10 µM.

qPCR was performed using Bio-Rad SsoAdvanced™ SYBR Green Supermix. For each reaction, 1 µL of Qiagen-purified DNA was combined with 1 µL of forward and reverse primer in a volume of 10 µL, to which were added 10 µL of SYBR Green mastermix. Reactions were carried out in either 96-well plates or 8-tube strips. Once mixed, reactions were placed in a Bio-Rad CFX96™ real-time PCR instrument. Reaction conditions were: 10 min at 95 C pre-heat; 30 sec at 95 C, 30 sec at 57 C, and 1 min at 72 C for 40 cycles, and melt curve detection from 65 C to 95 C.

Threshold cycle values (C_t) were recorded for those reactions that generated an optically detectable amplification product in 36 cycles or fewer. qPCR results were taken as valid if the melt curve for the product was at the correct temperature, indicative of amplification of the desired target.

If an amplification reaction gave an ambiguous result (that is, a melt curve displaying an off-target denaturation temperature), the product was analyzed by gel electrophoresis. Electrophoresis was performed on 2% agarose or 12% cross-linked polyacrylamide,[vii] stained using ethidium bromide, and imaged on a UV transilluminator.

In samples for which valid qPCR results were obtained, the C_t for the environmental sample was compared to a standard C_t value using the ΔC_t method[viii]. Positive control standard for the general (32F) and human specific (HF183) was purified from sewage obtained from the local sewage treatment plant. Control for cow (CF128) amplification was purified from cow manure from a local dairy. Amounts of each (general, human, and cow) was expressed as parts per million relative to the appropriate standard, calculated as ppm = $2^{\{-(C_{t,sample}-C_{t,standard})\}} \times 10^6$. For example, if an environmental sample upon amplification with human-specific primers yielded a C_t of 35.0 compared to a C_t from the sewage standard of 25.0, the amount of human-specific fecal material was reported as $2^{\{-(35.0-25.0)\}} \times 10^6$ = 976 ppm. Because the sewage and cow manure standards were diluted (1:1000) prior to qPCR analysis, the corresponding ppm concentrations in the environmental samples were further multiplied by 10^{-3}.

[i] "Quanti-tray/2000 Procedure."
[ii] Bernhard and Field, "Identification of Nonpoint Sources of Fecal Pollution in Coastal Waters by Using Host-Specific 16S Ribosomal DNA Genetic Markers from Fecal Anaerobes."
[iii] Bernhard and Field, "A PCR Assay To Discriminate Human and Ruminant Feces on the Basis of Host Differences in Bacteroides-Prevotella Genes Encoding 16S rRNA."
[iv] Ibid.
[v] Dick et al., "Host Distributions of Uncultivated Fecal Bacteroidales Bacteria Reveal Genetic Markers for Fecal Source Identification."
[vi] Seurinck et al., "Detection and Quantification of the Human-specific HF183 Bacteroides 16S rRNA Genetic Marker with Real-time PCR for Assessment of Human Faecal Pollution in Freshwater."
[vii] Ausubel, *Short Protocols in Molecular Biology*.
[viii] Livak and Schmittgen, "Analysis of Relative Gene Expression Data Using Real-time Quantitative PCR and the 2(-Delta Delta C(T)) Method."

Results – Hope college MST study in Black River and Pine Creek.

Microbiology:

Measurement of viable *E. coli* by IDEXX Colilert® reagent revealed high bacterial counts (exceeding 300 cfu/100 mL standard) throughout the watershed and over most of the sampling events. At most sites sampled, *E. coli* amounts peaked in early summer and dropped into late summer/fall. Slightly higher *E. coli* levels were found following rain events, though with a less dramatic difference between rain and dry samplings than have been seen in a different west Michigan watershed (Lake Macatawa and tributaries in Ottawa County, MI) as tested in this laboratory and others. No particular "hot spots" were observed, suggesting that the *E. coli* is of non-point source origin.

Source tracking:

Organisms within the *Bacteroides* and related genera are obligate anaerobes found in large abundance in the mammalian gut. Unlike *E. coli* and other coliforms, *Bacteroides* cannot grow in the environment. Thus, tests for *Bacteroides* in surface water rely not on culture of live organisms but on molecular detection of the bacteria's genomic DNA. This testing is done by isolation of DNA from an environmental sample and testing that DNA by quantitative PCR (qPCR). Because qPCR relies on detection of particular DNA sequence, selection of appropriate target sequence can distinguish the subtle differences in DNA not only between one bacterial species and another but, in the case of *Bacteroides*, between different sub-strains based on the host mammal species. The DNA sequence targeted in this analysis is within the 16S rDNA gene, the gene for the main RNA component of the ribosome known as the 16S rRNA (ribosomal RNA). 16S rDNA sequences have been compiled for thousands of eubacteria and are the basis for many metagenomic studies. Also unlike *E. coli*, the *Bacteroides* 16S rDNA fingerprint is very short-lived in environmental waters, with a half-life of 1-3 days (decreasing with increasing temperature). Thus the presence of *Bacteroides* 16s rDNA in a water sample is thought to be more indicative of a recent and direct fecal incursion into the water than the presence of *E. coli*.

For this study, qPCR primer sequences were chosen to identify four categories of *Bacteroides*:

1) A "general" or non-host specific primer set was used to indicate fecal contamination by any mammal.

2) A human-specific set which has been shown to have a high degree of selectivity and specificity as an indicator of human host, both in the form of direct fecal isolates and collective municipal sewage.

3) A cow-specific set, which is largely unique to bovine host *Bacteroides* (either from cow feces taken directly or from likely environmental sources such a manure lagoons), with cross-reactivity to other ruminants in many cases.

4) A pig-specific set, which has been confirmed on swine feces and on manure sweepings from a hog farm. It has been less well-validated than the human and cow specific probes.

These primer sets are referred to as general, human, cow, and pig, respectively.

Following work-up and analysis, direct fecal contamination in the Black River and Pine Creek samples, as indicated by *Bacteroides* presence, is overall quite low. Although regulatory standards that correlate directly to *Bacteroides* levels by qPCR do not exist, it seems reasonable to assume a cut-off relative to common suspect fecal contaminants such as municipal sewage or animal facility manure storage lagoons at a dilution of one part to 100,000 (5 $\log_{10}$). This is consistent USEPA guidelines on groundwater [i]. This comparison can be made with qPCR data by comparing C_t values between representative sources and environmental samples.

It must be kept in mind that C_t represents number of "doublings" of target DNA until fluorescence reaches the threshold cycle. Thus, if the C_t for a given environmental sample is four cycles greater than that for the appropriate standard, the relative amount of target DNA sequence is 2 raised to the power of the difference in C_t between sample and standard ($2^{\Delta Ct}$), so 2^4 or 16-fold lower in concentration. For those more accustomed to comparing $\log_{10}$ values, one "log" (a ten-fold dilution) is equal to a ΔC_t of 3.32 cycles (because $2^{3.32}$=10). The 5 x $\log_{10}$ standard mentioned above would correspond to a ΔC_t of 16.6 between standard and sample. Because reliable qPCR results occur in the range of C_t's between 20 and 35, in practice we have pre-diluted our standards by 1:1000 (3 $\log_{10}$). With this dilution, the cutoff becomes 1:100 (2 $\log_{10}$), with the result that a ΔC_t of 6.64 between diluted standard and sample indicates a risk factor of 1:100,000 relative to undiluted standard. Standards used for this work were input from a local municipal wastewater treatment plant for general and human *Bacteroides*, and manure from the storage lagoon of a local dairy farm (located in Jenison, Michigan). For ease of comparison, ΔC_t values were converted to parts per million (ppm) relative to undiluted standard; therefore reported values of 10 ppm or greater correspond to the 1:100,000 cutoff.

Most samples subjected to *Bacteroides* qPCR analysis fell below this cutoff, and where tested but below this limit are recorded as BDL (below detection limit) on the accompanying spreadsheet. Even where detected, *Bacteroides*, whether general or host-specific, showed levels in the tens of ppm relative to standards. Only at three sites did measured *Bacteroides* DNA approach or exceed 100 ppm relative to standard, all three during the July rain event sampling.

Two of the sites that proved positive were on Pine Creek, PC01 and PC02. The general assay revealed *Bacteroides* DNA at concentrations comparable to sewage at a 500 or 700 ppm dilution. In the host-specific assays, this material matched sequence specific to cow host. In that assay, cow specific DNA is compared to that present in a dairy manure lagoon and revealed an equivalence to lagoon material at a 70 or 300 ppm dilution.

The third positive site was on the Black River, BR06 (also July rain event). The general assay level corresponded to 93 ppm sewage. This sample proved positive for human specific *Bacteroides* and by that assay measured 220 ppm sewage equivalence.

All other sites tested (15 samples tested for general and all three species-specific *Bacteroides*, and a number of further individual tests) were below the 1:100,000 cutoff and are considered of minimal risk by direct fecal contamination. Sites tested with valid below-threshold results are indicated as "BDL" for below detection limit.

In sum, *Bacteroides* genomic DNA was not found in a widespread manner across the sampling sites and sampling events. Where found, at one site it matched human-specific material, and at the other two sites, which are neighboring each other, the *Bacteroides* DNA matched cow (or other ruminant) material. At no sites was DNA matching to *Bacteroides* from pig host. Even where found, the concentration of *Bacteroides* DNA remained at quite low at hundreds of ppm, i.e. less than one part per thousand, relative to likely source contaminants.

[i] "Ground Water Rule Source Assessment Guidance Manual - Guide_gwr_sourcewaterassessments.pdf."

	E. coli cfu/100mL	human		gen		pig	cow	
		Ct	ppm std	Ct	ppm std	Ct	Ct	ppm std
June 28 - dry 1								
PC-01	558							
PC-02	1014							
July 17 - wet 1								
Pine Creek:								
PC-01	>2420	33.94	39	24.50	557	BDL	33.60	69
PC-02	>2420	34.07	36	24.09	740	BDL	31.47	300
PC-03	>2420	35.69	12	28.66	31			
PC-04	>2420						BDL	
Black River								
BR-01	>2420	BDL		BDL		BDL	BDL	
BR-02	1898	35.75	11	33.09	1	BDL	BDL	
BR-03	>2420	BDL		BDL		BDL	BDL	
BR-04	>2420	BDL		BDL		BDL	BDL	
BR-05	>2420	BDL		BDL		BDL	BDL	
BR-06	>2420	31.44	220	27.08	93	BDL	34.87	29
BR-07	>2420	BDL		36.55	0		BDL	
Mill Creek								
MC-01	>2420	36.09	9	30.07	12	BDL	BDL	
August 3 - dry 2								
PC-01	5184	BDL		BDL				
PC-02	1028							
August 18 - wet 2								
Pine Creek:								
PC-01	1007	BDL		BDL		BDL	39.07	2
PC-02	1296	BDL		BDL		BDL	BDL	
PC-03	1587	BDL						
PC-04	1028							
Black River								
BR-01	322	BDL		BDL		BDL	BDL	
BR-02	1654	BDL		BDL		BDL	33.91	55
BR-03	1364	BDL						
BR-04	1690							
BR-05	597							
BR-06	524	BDL						
BR-07	424							
Mill Creek								
MC-01	5577	BDL		BDL		BDL	BDL	
August 30 - dry 3								
PC-01	331	BDL						
PC-02	1208							
September 25 - dry 4								
PC-01	324							
PC-02	499							
October 18 - wet 3								
Pine Creek:								
PC-01	621	BDL		BDL				
PC-02	1235							
PC-03	5796					BDL		
PC-04	719							
Black River								
BR-01	249							
BR-02	709	BDL						
BR-03	985	BDL						
BR-04	497							
BR-05	518							
BR-06	458	BDL						
BR-07	569							
Mill Creek								
MC-01	311							
October 22 - dry 5								
PC-01	72							
PC-02	52							

Fecal standards:

Municipal sewage (pretreatment) at 1:1000 dilution (Ct = 29.26 with human specific primers; 23.66 with general primers)

Cow manure (dairy lagoon) at 1:1000 dilution (Ct 29.74)