

**Further Investigation of water quality and muck at
Saginaw Bay Parks and Beaches**

Report

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Summary

In order to evaluate the microbial quality of Saginaw Bay, multiple indicator bacteria were tested for and 3 source tracking tools were employed to determine the source of the pollution. Indicators such as *Escherichia coli*, *Enterococci*, coliphage, *Clostridium perfringens* were used. Source tracking methods included the *esp* human sewage marker, *Bacteroides* (human and cow markers) and human Adenovirus. The recent sampling event showed high levels of fecal contamination in waters containing suspended algae (muck) and in the solids material on the shore. Evidence was found that both human and cattle are sources of pollution in Saginaw Bay. Specifically, the water sample at site 4 tested positive for human derived *bacteroides* and adenovirus. Attention to the public health messages and good hygienic procedures is needed while recreating in these areas and when coming in contact with muck, hands should be washed.

Introduction

The purpose of this study was to further evaluate the microbiological quality of the algal debris and muck as well as water at the shoreline of Saginaw Bay, using a variety of fecal bacterial indicators and viruses as well as to evaluate and develop protocols for extraction and testing of DNA from the samples for the purpose of microbial source tracking. Analyses included the conventional water quality indicator *Escherichia coli*, as well as the alternative indicators *Enterococci*, the *esp* sewage marker in the *Ent faciem*, coliphage, *Clostridium perfringens*, human and cow markers in the *Bacteroides* and human Adenovirus.

Table 1 below describes the indicators and their applications. All of these indicators are found in the feces of humans and animals and are referred to as fecal indicators and are used to indicate fecal or wastewater contamination of water and potential for waterborne disease and presence of pathogens. They are also all found in septic tank effluents, combined sewer overflows, animal fecal wastes including birds and storm water (picking these indicators up from sediments, soil, wildlife).

- *E. coli* and *Enterococci* most commonly used bacteria, likely re-grow in the environment, during summer months (eg on algae and in soil and sand).
- *Clostridium* bacterium anaerobic spore former, does not re-grow, but is environmentally stable and generally survives routine chlorination of wastewater. It is not found in as high of concentrations in wastewater as the bacteria mentioned above, but survives longer.
- The coliphage (virus indicator, infects *E.coli*) does not re-grow in the environment and it's survival in the environment is finite compared to the bacteria and usually indicates more recent pollution.
- Finding all of these particular indicators shows greater fecal impact than finding just one and all are found in human, animal and bird fecal wastes.

Table 2 describes the microbial source tracking (MST) targets and indicators. In this case, gene targets are amplified and detected using molecular techniques, the polymerase chain reaction method, PCR, to determine the presence or absence of these indicators in environmental samples. *Bacteroides* are anaerobic bacteria found in high concentrations, mainly in the colon of

mammals and birds. Human and animal feces and sewage gene targets have been evaluated and been shown to have host specificity and usefulness for microbial source tracking for the cow, human and swine (SEE Rose review on MST). Adenoviruses are DNA viruses (90-100nm in size) which can cause infections, including respiratory illnesses and gastroenteritis in humans. They are able to survive for prolonged periods outside the body due to their ability to withstand adverse pH conditions, UV and other physical agents in the environment (21). Human Adenoviruses have been found to be more abundant in coastal waters impacted by sewage and septic tanks and are being used as an MST target.

These tools were used to evaluate water and muck collected from the shores of Saginaw Bay in August 2007.

Table 1. Indicators and their Applications

Indicator	Definition	Drinking Water Standards (limits/100mL)	Recreational Use Standards* (limits/100mL)	Advantages of Use as an Indicator	Disadvantages of Use as an Indicator
Total Coliforms	<p>Members of the family Enterobacteriaceae (includes E. coli)</p> <p>Make up 10% of intestinal organisms in humans and animals</p> <p>Facultatively anaerobic, gram-negative, nonsporing, rod-shaped bacteria (14)</p> <p>Ferments lactose</p>	<p>Not more than 1 positive sample found per month (applies to Public Water Systems serving 25-33,000 people)</p> <p>Not more than 5.0% of samples can be total coliform-positive in a month (applies to Public Water Systems serving >33,000 people)</p>		<p>Used as an indicator of bacteriological quality in drinking water assessment according to the Total Coliform Rule. (USEPA 816-F-01-035) (15)</p> <p>Lose viability in fresh water at a slower rate than most other intestinal bacterial pathogens</p> <p>Will usually be detected in waters impacted by animal feces or sewage</p>	<p>“Coliforms” represent a large class of microbes, including bacteria that don’t come from the intestinal tract. Therefore, it is critical to use more specific indicators, such as fecal coliforms, as indicators of potential risk</p> <p>Waters containing total coliforms may not have been impacted by fecal contamination due to the wide variety of potential sources of total coliform contamination.</p> <p>Coliform bacteria can re-grow</p> <p>Not directly related to human health risk</p>

Table 1. Indicators and their Applications (cont)

Indicator	Definition	Drinking Water Standards (limits/100mL)	Recreational Use Standards* (limits/100mL)	Advantages of Use as an Indicator	Disadvantages of Use as an Indicator
<i>E. Coli</i>	<p>A type of coliform bacteria that naturally occurs in the human intestinal tract.</p> <p>Many strains exist – only a few of these are pathogenic (14)</p>	<p>ZERO</p> <p>(water must be boiled before consumption if E. coli is present)</p>	<p>FRESHWATER ONLY</p> <p>PRIMARY CONTACT RECREATION – GENERAL (3):</p> <p>< 126cfu/100mL(based on geometric mean)</p> <p>PRIMARY CONTACT RECREATION - MICHIGAN (8):</p> <p>< 130cfu/100mL (based on a geometric mean)</p> <p>< 300cfu/100mL (for a single sample from a designated beach area)</p>	<p>Used as an indicator of bacteriological quality in both drinking and recreational waters.</p> <p>Found to have a high correlation with gastroenteritis associated with bathing in fresh water (6)</p> <p>Source tracking methods have been developed (7)</p>	<p>May grow in the soil in tropical locations.</p> <p>Found to be poorly correlated with gastroenteritis in marine waters (5).</p> <p>E. coli presence does not correlate with the presence of enteric viruses and parasites.</p>

Table 1. Indicators and their Applications (cont)

Indicator	Definition	Drinking Water Standards (limits/100mL)	Recreational Use Standards* (limits/100mL)	Advantages of Use as an Indicator	Disadvantages of Use as an Indicator
<p>Enterococci</p>	<p>A gram-positive non-spore forming member of the Streptococci bacteria</p> <p>Commonly found in the feces of humans and other warm-blooded animals</p> <p>Many strains are not harmful, however the presence of enterococci is an indication of the possible presence of enteric pathogens.</p>	<p>NONE</p>	<p style="text-align: center;"><u>FRESHWATER</u></p> <p>PRIMARY CONTACT RECREATION – GENERAL (1): < 33cfu/100mL (based on a geometric mean)</p> <p>< 61cfu/100mL (based on a single sample)</p> <p style="text-align: center;"><u>MARINE WATER</u></p> <p>PRIMARY CONTACT RECREATION - GENERAL (1): < 35cfu/100mL (based on a geometric mean)</p> <p>< 104cfu/100mL (for a single sample at a designated beach area)</p>	<p>Used as an indicator of bacteriological quality of recreational waters.</p> <p>Enterococci may die at a slower rate than fecal coliforms in water and sediments, providing more reliable indications of possible recent pollution (7).</p> <p>Multi-site epidemiological studies have shown that enterococci have a higher correlation with gastroenteric disease related to swimming in both fresh and marine waters than fecal coliforms (5).</p>	<p>Can regrow in the environment</p> <p>Less data is available</p>

Table 1. Indicators and their Applications (cont)

Indicator	Definition	Drinking Water Standards (limits/100mL)	Recreational Use Standards* (limits/100mL)	Advantages of Use as an Indicator	Disadvantages of Use as an Indicator
<i>Clostridium perfringens</i>	<p>Obligate anaerobic gram-positive bacteria that forms endospores and does not carry out dissimilatory sulfate reduction (14)</p> <p>Found in sewage and highly impacted waters (7).</p> <p>An opportunistic pathogen that produces enterotoxin.</p>		<p>FRESHWATER (standards for the state of Hawaii)</p> <p>< 50cfu/100mL</p> <p>MARINE WATER (standards for the state of Hawaii)</p> <p>< 5cfu/100mL</p> <p>(guidelines used by the state of Hawaii based on research by Dr. Roger Fujioka et al. at the University of Hawaii)</p>	<p>C. perfringens spores could be an index parameter for the occurrence of persistent intestinal pathogens like viruses and (oo)cysts of protozoa (7).</p> <p>Useful in such specific situations as the examination of chlorinated waters or industrial waters that may contain compounds lethal to non-spore forming bacterial indicators, samples that cannot be processed within 12 hours and the detection of recent as well as long term inputs of fecal pollution (4)</p>	<p>May be too conservative an indicator (7)</p> <p>Found in low concentrations</p>
Coliphage	<p>Viruses (also known as Bacteriophages) whose hosts are strains of the bacteria E. coli (13).</p> <p>Found wherever fecal contamination occurs.</p>	ZERO (suggested by the ground water rule)	<p>< 100 pfu/100mL</p> <p>(based on previous studies by Dr. Joan Rose, USF)</p>	<p>A good indicator of Enteroviruses due to similar seasonal variation, propensity for removal and resistance to environmental stress (7).</p>	<p>Coliphage is not specific to human sewage</p>

* The indicators used and standards enforced differ from state to state for recreational waters. For information about a particular state refer to reference #2.

Table 2. Molecular Assays using PCR

<u>Indicator</u>	<u>Definition</u>	<u>Issues as an MST Indicator</u>
Enterococcus facieum esp	A protein in the enterococci bacteria specific to humans.	Based on a cultivation test followed by PCR. Found in untreated sewage, 100% of the time, detects only live bacteria.
Adenovirus	A DNA virus found in both animals and humans. The virus has host specificity and animal and humans types can be distinguished.	PCR can be used to target the human specific Adenovirus. This indicates possible presence of human sewage and live virus represents a health risk to recreators. Found in high numbers in raw human sewage(19). Method used detects live and dead viruses. Can detect only live with cell culture.
Bacteroides (human and cow)	An anaerobic bacteria present in the gastrointestinal tract of humans. When found in water, indicates fecal pollution (23).	The human and cow gene sequences have been identified and are currently some of the most widely used source tracking targets. Methods used detect live and dead bacteria.

Table 3 shows the fresh water quality guidelines for waters used for recreation. Due to concerns of *E. coli* and Enterococci regrowth, Hawaii uses *C. perfringens* as one of their standards as traditionally does not display evidence of regrowth in the environment, especially under tropical conditions such as in Hawaii.

Table 3. Fresh water quality guidelines and standards (CFU/100 mL) for areas of recreational use.

	Geometric Mean ^a	Single Sample Maximum
US. EPA		
Enterococci	33	61
<i>E. coli</i>	126	235
MDEQ		
<i>E. coli</i>	130	300
Hawaii		
<i>C. perfringens</i>		50

^a based on five or more samples equally spaced over a 30-day time period
MDEQ: Michigan Department of Environmental Quality

Methods

Six water samples were collected directly at the shoreline where the waves were breaking onto the sand, containing large amounts of algae and debris. No samples were collected from waters at depths out from the shore used for swimming. Six samples were also collected from Muck debris along the shoreline.

Table 4. Sampling sites and ID

Collection ID	Beach Name	Collection Date	Water Sample ID	Muck Sample ID
452	Bird County Park	8/13/07	W1	M1
455	Caseville County Park	8/13/07	W2	M2
460	Oak Beach County Park	8/13/07	W3	M3
462	Port Crescent State Park (camping)	8/13/07	W4	M4
463	Port Crescent State Park (Day use)	8/13/07	W5	M5
464	Sleeper State Park	8/13/07	W6	M6

Samples were analyzed using the Colilert® kits by IDEXX. *Enterococci* were analyzed using membrane filtration according to USEPA Method 1600 (*Enterococci*) (9). Coliphage were analyzed via a modification of USEPA Methods 1601 and 1602 (10,11). (Table 5 includes the media and conditions for monitoring these bacteria)

Table 5. Media and Methods used for Indicator Testing

Test	Media	Incubation	Reference
<i>E.coli</i>	Colilert®	24-28 hours at 37°C	APHA Standard Method 9223B (2)
Enterococci	mEI agar	24 hours at 41°C	USEPA Method 1600 (9)
<i>Clostridium perfringens</i>	mCP agar	24 hours at 45°C in anaerobic chamber	Bisson <i>et al</i> (1979)
Coliphage	Tryptic Soy Agar	16 – 24 hours at 37°C	USEPA Method 1601/1602 (10, 11)

Sample Collection:

Grab samples were collected by county environmental health specialists and placed into sterile containers, on ice for transport to the laboratory. Samples were collected on August 13th, 2007 and samples were received by Michigan State University personnel on August 14th, 2007. Samples were immediately processed once received.

Bacterial indicators: Membrane Filtration

Volumes for bacteriological analysis via membrane filtration (*Clostridium perfringens*, and *Enterococci*) ranged from one milliliters of a 10⁻¹ dilutions to 100ml. Serial dilutions were made from log phase cultures of *Enterococcus faecalis* and *E. coli* C3000 for use as positive controls. One milliliter from each of the 10⁻⁶, 10⁻⁷, and 10⁻⁸ dilutions was filtered in duplicate.

Plates were incubated for 24 hours at 41°C for *Enterococci* analysis,. Plates for *Clostridium perfringens* analysis were incubated for 24 hours at 45°C in anaerobic chamber. Solid samples were diluted 10 g into 100ml and assayed similarly.

Bacterial indicators: IDEXX

In order to measure *E.coli* 100 mL samples were assayed in a Quanti-Tray/2000 (WQT-2K) using the IDEXX (Westbrook, Maine) Colilert (98-21375-00) If Bacterial counts exceeded the detection limits of the systems, samples were diluted 1/100 with a sterile phosphate buffered water solution, and the dilution reprocessed. Assays were conducted by adding sample to a powdered reagent (Colilert for *E.coli*) in a sterile container, mixing until reagent was dissolved, and then sealing in a Quanti-Tray/2000 plate. Colilert samples were placed in a 36.5°C incubator for 24 hours.

Coliphage Analysis

Agar overlays were utilized to detect coliphage present in the samples. Filtered volumes of the water sample were used to enumerate coliphage. Two types of overlays were conducted one using *E.coli* F⁺ amp as a host, the other using *E.coli* C3000. The F amp is known as male specific coliphage and infect the host at the F-pili. Somatic coliphage (C3000) infect the host bacteria at the outer cell wall. The F amp phage is an RNA virus and may survive more like some of the human enteric RNA viruses.

Overlays: For each sample, 20 mls were syringe-filtered through a 0.45 micron filter. 0.5mls of host and 2 ml of sample were added to melted top agar before mixing and pouring onto a tryptic soy agar plate (TSA). Two negative control plates were made, one with each host, by adding 1.5mls host to the top agar, mixing and pouring onto a TSA plate. A positive control was run for each host type by adding 1.5mls host to the top agar, mixing and pouring onto a TSA plate. Stock MS-2 phage was spotted onto the hardening agar layer. For each sample, 5 overlays of each host type were performed. Overlays were incubated at 37C for 24 hours, and then assessed for plaque formation.

Adenovirus Analysis

Between 1.5 to two L of water samples were filtered through a 90mm, negatively charged HA membrane with pore size of 0.45- μ m (Millipore, Billerica, Mass). After filtration, 100 mL of 0.5 mM H₂SO₄ was filtered to rinse out excess ions, and viral particles were eluted from the filter and stored at - 20°C before further concentration. For further purification and concentration, the elutant was thawed and dispensed into an Amicon Ultra 100K concentrator column (Millipore). Eluate was centrifuged at 4000 x g for 1 min and the resulting filtrate was discarded and column filled with 10 ml PBST (pH 8.0). Column was centrifuged again at 4000 x g for 1 min. After centrifuging, the sample concentrated was removed and stored at -80 °C until further processing. Adenovirus RNA/DNA was extracted using Qiagen RNA viral kit. The sample was concentrated using an Amicon Ultra-15 concentrator column and centrifuged for 20 minutes at 4000x g. Process was repeated until all the eluate is used up. DNA was subsequently extracted using the UltraClean Isolation Kit (Carlsbad, CA). A nested PCR reaction was performed to amplify the hexon gene of all 51 human adenovirus prototype strains. Gel electrophoresis was performed on the PCR product and run on a 1.2% w/v agarose gel at 90 V for approximately one hour.

Bacteroides

Two liters of water sample centrifuged for 30 minutes at 4000 rpm. Mobio mega soil DNA kit was used to extract the DNA from the pellet. PCR amplification was performed on the extracted DNA. Primers for both human and cow bacteroides sequences were used (22). Gel electrophoresis was performed on the PCR product and run on a 1.2% w/v agarose gel at 95 V for approximately one hour.

Muck sample processing

In order to assay the muck samples, the muck was re-suspended and the eluant was collected and assayed like the water samples. Eluant was obtained by weighing out 125 g

of the muck and placing into a sterile blender. Sample was blended for 5 minutes, mixture was strained out using a sterilized cheesecloth.

Muck moisture analysis

Muck samples were dried to assess the moisture content of the material. Table 6 shows the % dry weight of the muck samples. These values are the percent material remaining of the total sample after being placed in an oven for 48 hours at 60°C. Dry weight percentages ranged from 35.7 to 69.5%. The data show Muck sample M5 has highest amount of moisture per gram of solid material.

Table 6. Moisture content of muck samples

Sample	Collection Date	% Dry weight
M1	8/13/07	36
M2	8/13/07	53.8
M3	8/13/07	62.9
M4	8/13/07	49.9
M5	8/13/07	35.7
M6	8/13/07	69.5

Results: Bacterial and Phage indicators

The following tables presented in this section show the results of the various indicator and molecular analysis performed on the water and muck samples.

Table 7 Average bacterial and coliphage indicator results to date from water samples.

Sample	Collection Date	<i>E.coli</i> CFU/100ml (MPN)*	Enterococci CFU/100ml	<i>Clostridium Perfringens</i> CFU/100ml	F specific Coliphage PFU/100ml	Somatic Coliphage PFU/100ml
Water W1	8/13/07	532.6	360	0.883	40	50
Water W2	8/13/07	2419.6	733.3	5.52	2280	2770
Water W3	8/13/07	2076.3	667	4.4	200	230
Water W4	8/13/07	155.7	50	2.0	100	160
Water W5	8/13/07	146.5	85	1.8	130	150
Water W6	8/13/07	74.4	69	2.0	<10	10

*MPN =most probable number calculated from colilert

Table 8 shows the average bacterial and coliphage indicator results to date from muck samples.

Sample	Collection Date	<i>E.coli</i> CFU/100gdw (MPN)*	Enterococci CFU/100gdw	<i>Clostridium Perfringens</i> CFU/100gdw	F specific Coliphage PFU/100gdw	Somatic Coliphage PFU/100gdw**
Muck M1	8/13/07	3.85x10 ⁷	1.29x10 ⁶	2377.77	<222.22	<222.22
Muck M2	8/13/07	3.07x10 ⁴	1.99x10 ⁷	1918.23	<148.7	<148.7
Muck M3	8/13/07	7.59x10 ⁷	2.34x10 ⁵	260.7	<137.22	<137.22
Muck M4	8/13/07	4.00x10 ⁵	6.5 x10 ⁵	118.64	<160.32	<160.32
Muck M5	8/13/07	5.15x10 ⁶	1.41x10 ⁷	46.49	<224.1	672.27
Muck M6	8/13/07	3.63x10 ⁷	1.07x10 ⁷	794.5	<115.11	<115.11

*MPN =most probable number calculated from colilert

**MPN =most probable number calculated from colilert, converted to gram of wet weight

***Muck sample results given as CFU/ 100 g dry weight

esp marker analysis

DNA was extracted from enterococci colonies that grew on the membrane filters. The polymerase chain reaction was used to amplify the DNA *esp* marker (Scott et al., 2005). Table 9 displays the results of the *esp* marker analysis.

Table 9. *esp* marker results for water and muck samples

Sample ID	<i>Esp</i> presence (human)
Water W1	+
Water W2	+
Water W3	+
Water W4	-
Water W5	-
Water W6	+
Muck M1	+
Muck M2	+
Muck M3	+
Muck M4	-
Muck M5	-
Muck M6	+

* symbol “+” indicates presence, “-” indicates not detected.

Table 10. Results for *Bacteroides* samples in water and muck samples

Sample ID	<i>Bacteroides</i> (human)	<i>Bacteroides</i> (cattle)
Water W1	+	-
Water W2	-	-
Water W3	-	-
Water W4	+	-
Water W5	-	+
Water W6	-	-
Muck M1	+	-
Muck M2	-	-
Muck M3	-	-
Muck M4	-	-
Muck M5	-	+
Muck M6	-	-

* symbol “+” indicates presence, “-” indicates not detected.

Table 11. Results for Adenovirus in water samples.

Sample ID	Mean Concentration (copies/L)
Water W1	<22.39
Water W2	<71.43
Water W3	<32.29
Water W4	99.05
Water W5	<29.18
Water W6	<21.51

All water samples showed presence of bacterial indicators and both types of coliphage. Water samples W1, W2, W3, and W6 contained the human sewage marker for *esp*. All muck samples contained bacterial indicators however only M5 contained coliphage (672.27 PFU/100 g dw somatic coliphage). It should be kept in mind that only small concentrations of Muck could be processed for coliphage thus the detection limit was very high. Muck samples M1, M2, M3, and M6 contained the presence of human sewage marker for *esp*. The *esp* marker has been found in septic tank effluent and in CSO effluents as well as untreated sewage, In previous studies, we have found the marker 100% of the time in untreated sewage and poorly treated secondary sewage which has not received proper disinfection (Kumar, 2007). We have never detected the *esp* in non-human wastes (including cows, sheep, pigs and birds) and in a blind study with samples sent from USGS, only human wastes were positive. However, recently Whitman et al. (2007) has reported that they have detected this marker on occasion in dog feces and bird feces but it is suspected that some of these are *E. fecalis* and not *E. facieum*.

Two sites tested positive for the presence of human derived bacteroides in surface water (W1 and W4) and one muck sample tested positive (M1) as shown in table 10. One water sample tested positive for cattle derived bacteroides at site W5 and one muck sample (M5). Only W4 was found to contain adenovirus. This indicates presence of human sewage. The other sites tested negative for presence of adenovirus and were below the detection limit (table 11). As noted above the bacteriodes and Adenovirus tests look for total DNA and do not distinguish between live and dead. The Enterococci *esp* marker is based on a cultivation test, this improves the sensitivity of the test and also address viable organisms. Statistical assessment of cattle versus the human markers is limited due to the limited number of samples taken.

In conclusion this sampling shows high levels of fecal contamination in waters containing suspended algae (muck) and in the solids material on the shore. There is strong evidence that at least one of the sources is human (whether septic tanks, CSOs or poorly treated sewage is not known) and another is cattle (cow). Specifically, the water sample at site 4 tested positive for human derived bacteroides and adenovirus. Attention to the public health messages and good hygienic procedures is needed while recreating in these areas and when coming in contact with muck, hands should be washed.

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