In Cooperation With Bowling Green State University and the Wood County Health Department

Results from a Microbial Source-Tracking Study in the Portage River Watershed, 2008

By Christopher M. Kephart and Rebecca N. Bushon

Contents

Tables

Results from a Microbial Source-Tracking Study in the Portage River Watershed, Ohio, 2008

By Christopher M. Kephart and Rebecca N. Bushon

Abstract

In Northwest Ohio, an influx of concentrated animal feeding operations has prompted local agencies to examine the effects of these industrial farms on water quality in the upper Portage River Watershed. The utility of microbial source tracking tools as a means of characterizing sources of fecal contamination in the watershed was evaluated. In 2007 and 2008, scientists with the U.S. Geological Survey, Bowling Green State University, and the Wood County Health Department collected and analyzed 17 environmental water samples and 13 source samples for *Bacteroides*-based host-specific DNA markers. *Bacteroides* are a group of enteric bacteria that are being used in microbial source tracking (MST), in hope that host-associated DNA markers could be used to indicate potential sources of fecal contamination in the environment. At the majority of the environmental sites tested, MST marker results corroborated the presumptive fecal contamination sources. Results from this demonstration study support the utility of using MST with host-specific molecular markers to characterize the sources of fecal contamination in the Portage River Watershed.

Introduction

The upper Portage River Watershed in Northwest Ohio has been an agricultural area influenced by runoff from row crops and small-scale livestock operations and discharges from septic systems and small wastewater treatment plants. Recently, however, the area has been targeted for construction and operation of large dairy farms. As of July, 2007, two large-scale dairies are already operating and three more are proposed. Local officials and the public are concerned that these concentrated animal feeding operations (CAFOs) will result in the degradation of water quality. There is little information, however, on the influence of existing fecal sources on water quality (including the two large-scale dairies) within the watershed.

The relative contributions of contaminants from CAFOs, septic systems, and treated wastewater within the Portage River Watershed need to be understood before any watershed protection steps can be initiated. Contaminants include fecal-origin pathogens, wastewater chemicals (including hormones and antibiotics), nutrients, and suspended sediment. Research has supported that the best approach to characterize the sources of fecal contamination in a watershed is to establish multiple lines of evidence (Boehm and others, 2003; Francy and others, 2006). This is done by identifying the spatial distribution of bacterial and chemical indicators, understanding the hydrologic factors that affect their distribution, and applying molecular source tracking (MST) techniques. Among researchers, the use of culture-independent host-specific molecular markers is gaining acceptance as the preferred MST tool (Santo Domingo and others, 2007). Molecular

markers for MST are a research tool only, and their utility for forensic assessment of fecal sources in a specific location must be demonstrated before being applied.

The U.S. Geological Survey (USGS), in cooperation with Bowling Green State University (BGSU) and the Wood County Health Department (WCHD), have taken the first step towards understanding the relative contributions of these contaminants by identifying the source-tracking tools that can best be used in the watershed. Source and environmental water samples were analyzed for *Bacteroides* DNA markers of general fecal contamination, as well as markers that are associated with human and bovine feces. The *Bacteroidales* are a dominant group of enteric bacteria that may have co-evolved with their hosts. For this reason, their host specificity has been investigated for use in microbial source tracking (Bernhard and Field, 2000). In this study, samples were analyzed by means of quantitative polymerase chain reaction (qPCR), which results in a relative quantity of each DNA marker. The results from this study may be used as preliminary information for a larger, long-term source-tracking study to identify and assess relative source contributions of fecal contamination in the changing Portage River Watershed.

Purpose and Scope

The overall purpose of this demonstration study was to identify MST tools that can best be used to understand the relative contributions of fecal contaminant sources in the Portage River Watershed. A total of 17 environmental water samples and 13 known source samples (treated wastewater, septage, and bovine slurry) were tested for the presence and relative quantity of MST markers by qPCR. Four *Bacteroides*-based markers were evaluated: two human-specific and one bovine-specific MST markers, and one marker of general fecal contamination.

Methods

Source sampling. Sample collection of potential sources (treated wastewater, septage, and cattle slurry) was done on November 6, 2007 by USGS, BGSU, and WCHD to ensure that the selected DNA markers were found in potential sources. Two wastewater samples (primary-treated influent and final effluent) were collected from two local treatment plants (designated as WWTP-1 and WWTP-2). Samples were collected from the septic tanks of six households in the watershed. Two replicate cattle slurry samples were collected from the primary settling lagoon at a local dairy farm. A composite goose fecal sample was also collected from a golf course in the watershed to confirm that human or bovine-specific markers would not be detected.

Wastewater, septage, and bovine slurry samples were collected in sterile polypropylene bottles using a grab-sampling technique described in Myers and others, 2007. A total of 20 goose fecal samples were composited into a sterile 50-mL centrifuge tube using sterile toothpicks. All source samples were preserved on ice and were transported to the USGS for further analysis.

Stream sampling. Stream samples were collected on June 26, 2008 and September 15, 2008 at the sites described in Table 1. The June samples were collected following a recent rain event. The June samples were collected by USGS and BGSU and the September samples were collected by BGSU. Samples were collected in sterile polypropylene bottles using a grab-sampling technique

described in Myers and others, 2007. All stream samples were preserved on ice and were transported to BGSU and USGS for further analysis.

Sample analyses. Source samples were analyzed by the USGS within 24 hours of collection by membrane filtration on modified mTEC agar (U.S. Environmental Protection Agency, 2006). Stream samples were analyzed by BGSU within 24 hours of collection for *E. coli* by a most-probable number (MPN) technique using a defined-substrate medium in Quanti-Tray/2000 wells (Idexx, Westbrook, Maine). At USGS, all samples were filtered and stored in the freezer for subsequent DNA marker analysis. All samples were analyzed for three *Bacteroides* DNA markers.

- General fecal marker, AllBac (Layton and others, 2006)
- Human fecal marker, qHF183 (Bernhard and Field, 2000)
- Bovine fecal marker, BoBac (Layton and others, 2006)

The source samples were analyzed for an additional human marker, *Bacteroides thetaiotaomicron* (Btheta) (Carson and others, 2005).

Quantitative PCR. Quantification was done by use of standard curves calculated from threshold cycles observed for decimal dilutions of plasmid-borne DNA target. Standard concentrations were calculated based on the total concentration of DNA in the plasmid solution and the known size of the target-containing plasmid. Characteristics of the standard curves are presented in Table 2.

Each qPCR run included a standard curve, extraction blank (negative control at the extraction step), and a no-template control (negative control at the qPCR step). The upper and lower limits of quantification (ULQ and LLQ, represented as the dynamic range in Table 2) were based on the range of standards that contributed to the linear portion of the standard curve. In all cases, the standard curve remained linear at both the highest standard and the lowest standard.

No-template control and extraction blank data were used to measure a limit of detection (LOD). The no-template controls and extraction blanks sometimes showed non-specific fluorescent signal during late cycles. In these cases, the mean and 99% confidence interval among cycle thresholds were calculated. To guard against false-positive results, the target concentration that corresponded with detection at the lower 99% confidence interval of multiple detections was used as the LOD. A cycle threshold higher than the LOD was not considered credible evidence that the sample contained detectable quantities of the marker.

The LLQ and the LOD were used to qualify low-concentration data. In cases where the LLQ was greater than the LOD, results higher than the LLQ were not qualified. Results between the LLQ and the LOD were qualified detectable, not quantified (DNQ). Results below the LOD were purged and considered nondetects. Conversely, when the LOD was greater than the LLQ, results higher than the LOD were not qualified. Results below the LOD were purged and considered nondetects.

Exogenous internal standard. DNA encoding red-fluorescent protein dsRed2 (Matz and others, 1999) was used as an internal standard that is not expected to be detected naturally in the fresh water environment. Approximately 2.5×10^6 cells of *E. coli* containing a dsRed2 plasmid

were added to each filtered sample immediately before extraction. Recovery of dsRed2 marker was measured by qPCR and used as a measure of matrix inhibition. Cycle threshold values of all samples were within 2 cycles of the positive control and were not considered to be inhibited.

Results

A total of 13 source samples and 17 stream samples were analyzed for DNA markers. Tables 3 and 4 list the results for the source samples and stream samples, respectively. These tables show the quantity of the marker in copies per 100 milliliters, which were based on multiple runs of composite standard curves of known concentrations of each marker.

In the source samples, the AllBac general fecal marker was detected in all 13 samples at concentrations ranging from 3,500 to 3,700,000,000 copies per 100 milliliters. The BoBac bovine-specific marker was detected at high concentrations, as expected, in the two cattle samples. BoBac was detected at considerably lower levels in both wastewater influent samples. The Btheta human-specific marker was only detected in 6 of the 10 human-source samples. It was detected at levels below the LLQ in 4 human-source samples and all of the non-human samples (2 cattle and 1 goose). The Btheta marker was not used for analysis of the stream samples and does not appear to be a useful human-associated marker for this area. The qHF183 human marker was detected at levels above the LLQ in 9 of the 10 human-source samples, with one septic sample detected at a level below the LLQ. This marker was detected in one of the cattle samples, but at levels below the LLQ.

In the Poe Ditch 1 samples, collected upsteam of a WWTP, concentrations of AllBac, BoBac, and qHF183 were higher on both collection dates than in Poe Ditch 2 samples, collected downstream of the WWTP. At Poe Ditch 2, the concentrations of BoBac and qHF183 markers were among the highest observed in the stream samples collected during this study. This may be attributed to the high flow of the WWTP effluent possibly disturbing contaminants in the bed-sediments of the ditch.

At the Huffman Ditch site, it was thought that septic sources were of possible influence. This expectation was corroborated by the highest concentration of qHF183 observed in this study in the June sample. This sample was collected following a significant rain event and also had the highest concentrations of *E. coli* and AllBac. An unexpected result was that the highest concentration of BoBac was also observed in this sample. This finding demonstrates that other sources of fecal contamination may be influencing this site, especially after rainfall.

The Unnamed Tile 1 and 3 sites were known to drain fields that do not receive manure application; results show concentrations of BoBac and qHF183 that were either below the LLQ or not detected. Unnamed Tile 2 was thought to drain a manure-applied field; results show concentrations of BoBac in the June sample.

In the Bays Ditch and Tile sites, there was less confidence in the presumptions about the inputs of contamination to the sites. Concentrations of the BoBac and qHF183 markers were generally low, if detected, with the exception of high levels of qHF183 in the Bays Tile 2 site, which was thought to have septic inputs.

The Ostego Pike Ditch sample was thought to have mixed sources of contamination. The BoBac marker was detected below the LLQ and the qHF183 marker was detected at a low concentration. The Rangeline Ditch site was included to serve as a control site, not expected to have high levels of contamination. The concentration of the AllBac marker was the lowest measured in the stream samples for this study. The BoBac marker was not detected in this sample and the qHF183 marker was detected at levels below the LLQ.

References

Bernhard, A.E., and Field, K.G., 2000, 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.

Boehm, A.B., Fuhrman, J.A., Mrše, R.D., and Grant, S.B., 2003, Tiered approach for identification of a human fecal pollution source at a recreational beach—Case study at Avalon Bay, Catalina Island, California: Environmental Science & Technology, v. 37, p. 673–680.

Carson, C.A.; Christiansen, J.M.; Yampara-Iquise, Helen; Benson, V.W.; Baffaut, Claire; Davis, J.V.; Broz, R.R.; Kurtz, W.B.; Rogers, W.M.; and Fales, W.H., 2005, Specificity of a *Bacteroides thetaiotaomicron* marker for human feces: Applied and Environmental Microbiology, v. 71, no. 8, p. 4945–4949.

Francy, D.S., Bertke, E.E., Finnegan, D.P., Kephart, C.M., Sheets, R.A., Rhoades, J., and Stumpe, L., 2006, Use of spatial sampling and microbial source-tracking tools for understanding fecal contamination at two Lake Erie beaches: U.S. Geological Survey Scientific Investigations Report 2006-5298, 29 p., available at *http://pubs.water.usgs.gov/sir2006-5298/*

Layton, Alice; McKay, Larry; Williams, Dan; Garrett, Victoria; Gentry, Randall; and Sayler, Gary, 2006, Development of *Bacteroides* 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water: Applied and Environmental Microbiology, v. 72, no. 6, p. 4214–4224.

Matz, M.V., Fradkov, A.F., Labas, Y.A., Savitsky, A.P., Zaraisky, A.G., Markelov, M.L., and Lukyanov, S.A., 1999, Fluorescent proteins from nonbioluminescent Anthozoa species: Nature Biotechnology, v. 17, p. 969–973.

Myers, D.N., Stoeckel, D.M., Bushon, R.N., Francy, D.S., and Brady, A.M.G., 2007, Fecal indicator bacteria: U.S. Geological Survey Techniques of Water-Resources Investigations, book 9, chap. A7, section 7.1 (version 2.0), available from http://pubs.water.usgs.gov/twri9A/.

Santo Domingo, J.W., Bambic, D.G., Edge, T.A., and Wuertz, Stefan, 2007, Quo vadis source tracking? Towards a strategic framework for environmental monitoring of fecal pollution: Water Research, v. 41, no. 16, p. 3539–3552.

U.S. Environmental Protection Agency, 2006, Method 1603—*Escherichia coli* (*E. coli*) in water by membrane filtration using modified membrane-thermotolerant *Escherichia coli* agar (modified mTEC): Washington, D.C., Office of Water, EPA–821–R–06–011 [variously paginated].

Table 1. Site locations and descriptions.

Site name	Latitude	Longitude	Site description[a]
Poe Ditch 1	41.38513	-83.61185	Upstream of WWTP
Poe Ditch 2	41.38511	-83.61086	Downstream of WWTP
Huffman Ditch	41.33953	-83.59162	Thought to have septic inputs
Unnamed Tile 1	41.24818	-83.77061	Field with no manure application
Unnamed Tile 2	41.24817	-83.77083	Field with possible manure application
Unnamed Tile 3	41.25075	-83.76598	Field with no manure application
Bays Ditch 1	41.26963	-83.73242	Thought to drain manure-applied field
Bays Ditch 2	41.26461	-83.72824	Thought to drain manure-applied field and have septic inputs
Bays Tile 1	41.26963	-83.73100	Thought to drain manure-applied field
Bays Tile 2	41.26962	-83.72825	Thought to have septic inputs
Ostego Pike Ditch	41.24782	-83.78533	Mixed sources
Rangeline Ditch	41.28486	-83.76642	Not expected to have high levels of fecal contamination

[a]Descriptions are based on presumptions, input from local agencies, and historical data collected by BGSU and WCHD. Discussions of possible sources are observations and are not based on scientific evidence.

Table 2. Standard curve characteristics for AllBac, BoBac, Btheta, and qHF183 microbial-source tracking (MST) markers.

MST marker	Number of compiled curves	Dynamic range	Range of amplification efficiency (percent)	Range of R^2 values	Limit of detection
AllBac	11	$12 – 1.2 \times 10^7$	82 - 102	0.985 – 0.999	290
BoBac	11	$61 – 6.1 \times 10^7$	82 - 101	0.992 – 0.999	ND*
Btheta	4	$57 – 5.7 \times 10^7$	84 – 91	0.992 – 0.999	ND*
qHF183	23	$23 – 2.3 \times 10^7$	84 - 108	0.991 – 1.000	ND**

*The marker was never detected in a blank

**The marker was detected in one of 10 blanks; however, it was considered an outlier and was removed from the dataset because it was atypical and the replicate result was not detected.

Table 3. DNA marker results for source samples, Portage River Watershed, Ohio.

Sample name[a]	*E. coli* (colony-forming units per 100 milliliters)	AllBac general marker	BoBac bovine marker	Btheta human marker	qHF183 human marker
		Copies per 100 milliliters			
WWTP-1 influent	760,000	130,000,000	14,000	27,000	3,700,000
WWTP-1 effluent	300	4,000	ND	DNQ	45
WWTP-2 influent	980,000	230,000,000	9,900	72,000	2,900,000
WWTP-2 effluent	1,000	3,500	ND	DNQ	480
Septic 1	980,000	790,000,000	ND	1,700	840,000
Septic 2	<1,000	340,000,000	ND	DNQ	600,000
Septic 3	210,000	260,000,000	ND	1,100	240
Septic 4	1,000	380,000,000	ND	DNQ	DNQ
Septic 5	29,000	460,000,000	ND	1,900	110,000
Septic 6	46,000	150,000,000	ND	6,500	580,000
Cattle 1	9,100,000	3,700,000,000	110,000,000	DNQ	DNQ
Cattle 2	--	2,500,000,000	55,000,000	DNQ	ND
Goose	--	11,000,000	ND	DNQ	ND

[a] Sample names defined in methods section.

ND, not detected.

DNQ, Detected not quantified.

--. Not analyzed for.

Table 4. DNA marker results for stream samples, Portage River Watershed, Ohio.

Date	Sample name	*E. coli* (most-probable number per 100 milliliters)	AllBac general marker	BoBac bovine marker	HF183 human marker
			Copies per 100 milliliters	Copies per 100 milliliters	Copies per 100 milliliters
6/26/2008	Poe Ditch 1	7,700	5,700,000	1,500	1,000
9/15/2008	Poe Ditch 1	2,100	5,000,000	620	8,400
6/26/2008	Poe Ditch 2	400	17,000,000	9,800	47,000
9/15/2008	Poe Ditch 2	2,800	8,800,000	3,000	56,000
6/26/2008	Huffman Ditch	17,000	150,000,000	30,000	180,000
9/15/2008	Huffman Ditch	330	2,900,000	DNQ	140
6/26/2008	Unnamed Tile 1	380	1,700,000	DNQ	DNQ
6/26/2008	Unnamed Tile 2	3,700	5,200,000	2,800	DNQ
9/15/2008	Unnamed Tile 2	2,300	1,000,000	DNQ	DNQ
9/15/2008	Unnamed Tile 3	280	1,100,000	ND	DNQ
6/26/2008	Bays Ditch 1	2,900	2,200,000	230	960
9/15/2008	Bays Ditch 1	3,800	1,700,000	ND	DNQ
6/26/2008	Bays Ditch 2	6,900	2,600,000	DNQ	2,200
9/15/2008	Bays Tile 1	210	650,000	ND	DNQ
9/15/2008	Bays Tile 2	16,000	13,000,000	970	11,000
6/26/2008	Ostego Pike Ditch	6,100	1,500,000	DNQ	110
6/26/2008	Rangeline Ditch	620	540,000	ND	DNQ

DNQ, Detected not quantified.
ND, Not detected.

LAKE ERIE PROTECTION FUND

SMALL GRANT - FINAL ACCOUNTING

Grant Number: 335-08 v2008

Budget Categories	Orignal Budget	Funds Spent	Current Balance	Matching Funds
A. Salaries & Wages				
Project Director	2818	2818		
Other Professionals	1784	1784		1784
Technical Support	343	343		343
B. Fringe Benefits				
Leave	451	451		451
C. Total Salaries & Benefits (A+B)	$5,396.00	$5,396.00	$0.00	$2,578.00
D. Non-expendable Equipment				
E. Expendable Materials & Supplies				
Laboratory supplies	105	105		105
F. Travel				
QA/QC trip, training	107	107		107
G. Services or Consultants				
USGS Ohio Water Microbiology Lab	6540	6540		2380
H. Computer Costs				
Computer support	344	344		344
I. Publications/Presentations				
USGS Open File Report	750	750		750
J. All other direct costs				
Shipping	200	200		200
Facilities	58	58		58
K. Total Direct Costs (C thru J)	$13,500.00	$13,500.00	$0.00	$6,522.00
L. Indirect Costs				
	1500	1500		8478
Total Costs (K + L)	$15,000.00	$15,000.00	$0.00	$15,000.00

Ohio Lake Erie Commission
One Maritime Plaza, 4th Floor
Toledo, OH 43604
p 419-245-2514
f. 419-245-2519
www.epa.state.oh.us/oleo

I certify that the grant expenitures listed and descriptions of the charges are true and accurate to the best of my knowledge. These expenditures represent approved grant costs that have been previously paid for and for which complete documentation is on file.

		Date
Project Director	[signature]	3/30/2009
Authorizing Agent	Allen E Kunze	3/30/09
Fiscal Agent	Allen E Kunze for Shonnie Tripp	3/30/09