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Antibiotic-Resistant Fecal Bacteria, Antibiotics, and Mercury in Surface Waters of Oakland County, Michigan, 2005–2006

Scientific Investigations Report 2007–5242

U.S. Department of the Interior
U.S. Geological Survey

Cover image: View of River Rouge near Birmingham, Mich.

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By Lisa R. Fogarty, Joseph W. Duris, Suzanne L. Crowley, and Nicole Hardigan

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Conversion Factors and Abbreviated Water-Quality Units

Multiply	By	To obtain
square mile (mi^2)	2.590	square kilometer (km^2)
ounce, fluid (fl. oz)	29.57	milliliter (mL)
microliter (µL)	0.001	milliliter (mL)
cubic foot per second (ft^3/s)	0.02832	cubic meter per second (m^3/s)
ounce, avoirdupois (oz)	28.35	gram (g)
milligram per liter (mg/L)	1,000	micrograms per microliter (µg/µL)

Temperature in degrees Celsius (°C) may be converted to degrees Fahrenheit (°F) as follows:

$$°F = (1.8 \times °C) + 32$$

Concentrations of bacteria in water are given in colony-forming units per 100 milliliters (CFU/100 mL).

Concentrations of antibiotics are given in micrograms per liter (µg/L) and micrograms per milliliter (µg/mL).

Concentrations of mercury are given in nanograms per liter (ng/L).

Concentrations of analytical reagents are given in millimolar (mM) or micromolar (µM).

Concentrations of other chemical constituents of water are given in milligrams per liter (mg/L).

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Antibiotic-Resistant Fecal Bacteria, Antibiotics, and Mercury in Surface Waters of Oakland County, Michigan, 2005–2006

By Lisa R. Fogarty, Joseph W. Duris, Suzanne L. Crowley, and Nicole Hardigan

Abstract

Water samples collected from 20 stream sites in Oakland and Macomb Counties, Mich., were analyzed to learn more about the occurrence of cephalosporin-resistant *Escherichia coli* (*E. coli*) and vancomycin-resistant enterococci (VRE) and the co-occurrence of antibiotics and mercury in area streams. Fecal indicator bacteria concentrations exceeded the Michigan recreational water-quality standard of 300 *E. coli* colony forming units (CFU) per 100 milliliters of water in 19 of 35 stream-water samples collected in Oakland County. A gene commonly associated with enterococci from humans was detected in samples from Paint Creek at Rochester and Evans Ditch at Southfield, indicating that human fecal waste is a possible source of fecal contamination at these sites. *E. coli* resistant to the cephalosporin antibiotics (cefoxitin and/or ceftriaxone) were found at all sites on at least one occasion. The highest percentages of *E. coli* isolates resistant to cefoxitin and ceftriaxone were 71 percent (Clinton River at Auburn Hills) and 19 percent (Sashabaw Creek near Drayton Plains), respectively. Cephalosporin-resistant *E. coli* was detected more frequently in samples from intensively urbanized or industrialized areas than in samples from less urbanized areas. VRE were not detected in any sample collected in this study. Multiple antibiotics (azithromycin, erythromycin, ofloxacin, sulfamethoxazole, and trimethoprim) were detected in water samples from the Clinton River at Auburn Hills, and tylosin (an antibiotic used in veterinary medicine and livestock production that belongs to the macrolide group, along with erythromycin) was detected in one water sample from Paint Creek at Rochester. Concentrations of total mercury were as high as 19.8 nanograms per liter (Evans Ditch at Southfield). There was no relation among percentage of antibiotic-resistant bacteria and measured concentrations of antibiotics or mercury in the water. Genetic elements capable of exchanging multiple antibiotic-resistance genes (class I integrons) were detected in several samples, indicating that the resistance carried by these organisms may be transferable to other bacteria, including disease-causing bacteria.

Introduction

The effects of urbanization on water quality in streams in Oakland County were evaluated during 2001–2003 by the U.S. Geological Survey (USGS), in cooperation with the county (Aichele, 2005). Results from that study showed that concentrations of fecal indicator bacteria in many streams throughout the county exceeded Michigan's recreational water-quality standards. An initial followup investigation of the fecal indicator bacteria showed the presence of multiple-antibiotic-resistant fecal coliform bacteria, vancomycin-resistant enterococci (VRE), and indicators of pathogenic *Escherichia coli* (*E. coli*) (Fogarty and others, 2005). This initial bacteria study was done in August and September 2003, at a time of very low streamflows.

Antibiotic-resistant bacteria have become of increasing public health concern because of the emergence of multiple antibiotic-resistant pathogens, such as multiple-drug-resistant *E. coli* and *Salmonella* spp. (Walsh, 2003) and VRE (Cetinkaya and others, 2000). Recent studies have identified antibiotic-resistant bacteria and genes responsible for antibiotic resistances in surface waters in the United States (McArthur and Tuckfield, 2000; Ash and others, 2002; Pruden and others, 2006), but very little is known about the occurrence and patterns of these resistant bacteria during recreational months in Oakland County. Because the enteric bacteria *E. coli* and enterococci have previously been detected in high concentrations and have been isolated with resistance to several human antibiotics, the USGS, in cooperation with the Oakland County Health Department, further evaluated the spatial and temporal distribution of cephalosporin-resistant *E. coli*, VRE, and an enterococci gene that is commonly associated with disease-causing enterococci and also is used as a marker for human fecal pollution (Shankar and others, 1999; Scott and others, 2005). The co-occurrence of antibiotics and mercury concentrations in these waters also was evaluated in the current study to determine whether bacterial antibiotic resistances were related to these chemicals and whether these chemicals might aid in the transfer and maintenance of antibiotic resistances in the environment.

The purpose of this study was to learn more about the occurrence of resistant bacteria and their relation to antibiotics or metals in streams during typical recreational months in Michigan (April through September) in order to provide important information to the public and communities that use these waters.

Purpose and Scope

This report describes the occurrence and distribution of antibiotic-resistant fecal bacteria, antibiotics, and mercury concentrations in surface-water samples collected in Oakland and Macomb Counties, Mich. This report is a followup to the previous USGS-Oakland County study (Fogarty and others, 2005) and includes information on (1) concentrations of fecal indicator bacteria (fecal coliform bacteria, *E. coli*, and enterococci), antibiotics, and mercury in river-water samples collected from September 2005 through August 2006, (2) percentage of *E. coli* isolates resistant to cephalosporin antibiotics and percentage of enterococci resistant to vancomycin, (3) detection of transferable genetic elements (class I integrons and vancomycin-resistance genes), and (4) presence of an enterococci gene used to indicate human fecal waste. In addition, this report includes a comparison of data obtained from Oakland County to results obtained from analyses of samples collected throughout the Clinton River Watershed as part of the USGS National Water-Quality Assessment Program mercury topical study for Lake Erie and Lake St. Clair (Brigham and others, 2003).

Description of Study Area

Oakland County, Mich., comprises 910 mi^2 in southeastern Michigan. The county is largely suburban, with a population of approximately 1.2 million in 2005. Approximately 42 percent of the land in Oakland County is residential, 6.3 percent commercial and industrial, and 3.2 percent agricultural. The heaviest residential and commercial land use is in the southeastern part of the county (fig. 1). Seven of the fourteen sites studied in 2003 (Fogarty and others, 2005) were sampled again in 2005 and 2006 (fig. 1, table 1) for followup microbial and chemical analysis. These sites are in the Clinton River, Flint River, and River Rouge Watersheds (table 1 and fig. 1). Land-use characteristics for waters draining to these sites were reported by Aichele (2005) and are summarized in table 2. Four of the seven sampling sites are in the Clinton River Watershed. The Clinton River at Auburn Hills (USGS station number 04161000) is the only Oakland County study site downstream from a known wastewater discharge.

The Clinton River Watershed extends from Oakland County into Macomb County and includes small parts of Genesee and Lapeer Counties. A total of 16 sites in the Clinton River Watershed (table 3 and fig. 2) were studied as part of the USGS National Water-Quality Assessment (NAWQA) for Lake Erie and Lake St. Clair. Similar to Oakland County, the southern part of Macomb County is a predominantly urbanized area with residential areas extending north. Land in the northeast part of the watershed is mainly agricultural (fig. 2).

Antibiotic-Resistant Bacteria in the Environment

Antibiotics have been detected at low concentrations (in the nanograms per liter range) in waters across the United States (Kolpin and others, 2002). Little is known about the effects of nontherapeutic levels of antibiotics in the environment on human health, aquatic ecology, or microbial communities. Antibiotic-resistant bacteria have been well documented in the environment (Ash and others, 2002; Park and others, 2003; Roe and others, 2003), but antibiotic resistance to newer antibiotics that are currently being used to treat humans has not been. Little is known about the presence of antibiotics in stream waters in Oakland County. The initial 2003 study did not investigate the presence of antibiotics, nor were the authors able to find any information regarding other studies that measured antibiotics in the environment in this area of Michigan.

Table 1. Sample-collection sites for the Oakland County, Mich., study, 2006.

[USGS, U.S. Geological Survey]

Map number	USGS station number	Station name	County	Watershed
1	04148035	Kearsley Creek at Mill Street at Ortonville	Oakland	Flint
2	04160800	Sashabaw Creek near Drayton Plains	Oakland	Clinton
3	04161000	Clinton River at Auburn Hills	Oakland	Clinton
4	04161540	Paint Creek at Rochester	Oakland	Clinton
5	04161580	Stony Creek near Romeo	Macomb	Clinton
6	04166000	River Rouge at Birmingham	Oakland	Rouge
7	04166200	Evans Ditch at Southfield	Oakland	Rouge

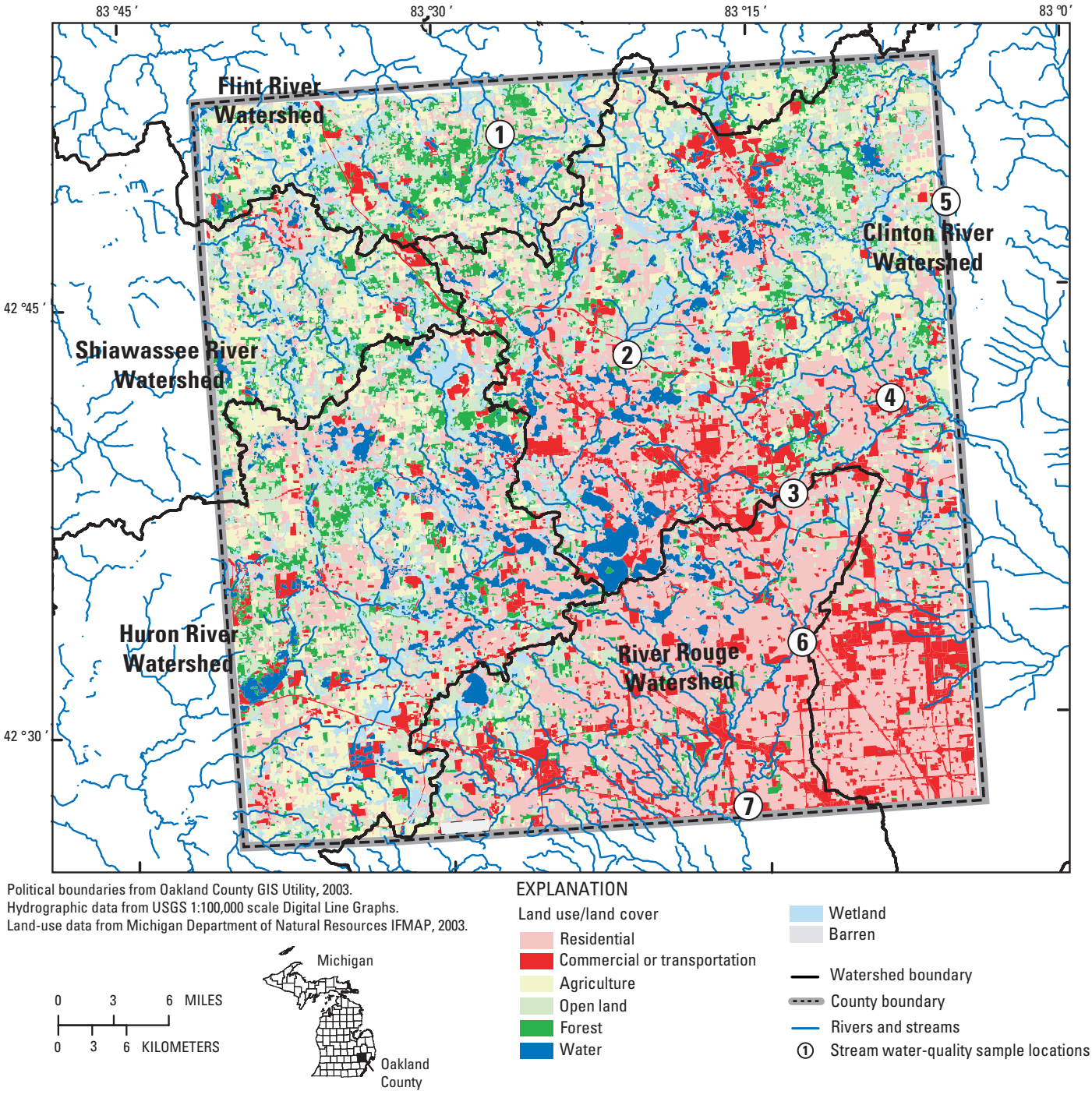

Figure 1. Sampling locations for study sites and land use/land cover for Oakland County, Mich.

Table 2. Land-cover percentages for Oakland County study sites, 2000[1].

[USGS, U.S. Geological Survey]

USGS station number	Built[2]	Residential	Commercial	Agricultural	Open space	Forest	Water	Wetland	Total	
									Built	Unbuilt
4148035	1	32.8	1.6	12.1	17.8	15.5	3.9	15.4	35.3	64.7
4160800	6.8	36.1	7.3	3.9	15.7	5.1	6	19.1	50.2	49.8
4161000	6.4	44.3	12.5	2.5	9.3	4.8	10.3	10	63.1	36.9
4161540	5.4	43.9	3.1	9.2	15.3	7.1	4	11.9	52.4	47.6
4161580[a]	3	24.7	2.4	13.2	22.1	16	4.3	14.2	30.2	69.8
4166000	5.7	70	10.7	0.4	2.8	2.3	4.8	3.4	86.3	13.7
4166200	8.1	65.7	21	0	1.8	2.8	0	0.7	94.8	5.2

[1]Data summarized from Aichele (2005).

[2]Undifferentiated urban land, not clearly identified as commercial or residential.

[a] Site location is in Macomb County, Mich.

Table 3. Clinton River Watershed study sites, 2006.

[USGS, U.S. Geological Survey; map number shown in fig. 2]

Map number	USGS station number	Site name	County
2	04160800	Sashabaw Creek near Drayton Plains	Oakland
8	04160900	Clinton River near Drayton Plains	Oakland
3	04161000	Clinton River at Auburn Hills	Oakland
4	04161540	Paint Creek at Rochester	Oakland
5	04161580	Stony Creek near Romeo	Macomb
9	04161800	Stony Creek near Washington	Macomb
10	04161820	Clinton River at Sterling Heights	Macomb
11	04163030	Red Run at Warren	Macomb
12	04163400	Plum Brook at Utica	Macomb
13	04164000	Clinton River near Fraser	Macomb
14	04164100	East Pond Creek near Romeo	Macomb
15	04164110	East Pond Creek at Powell	Macomb
16	04164300	East Branch Coon Creek at Armada	Macomb
17	04164350	Highbank Creek at 32 Mile Road near Armada	Macomb
18	04164500	North Branch Clinton River near Mt. Clemens	Macomb
19	04164800	Middle Branch Clinton River at Macomb	Macomb

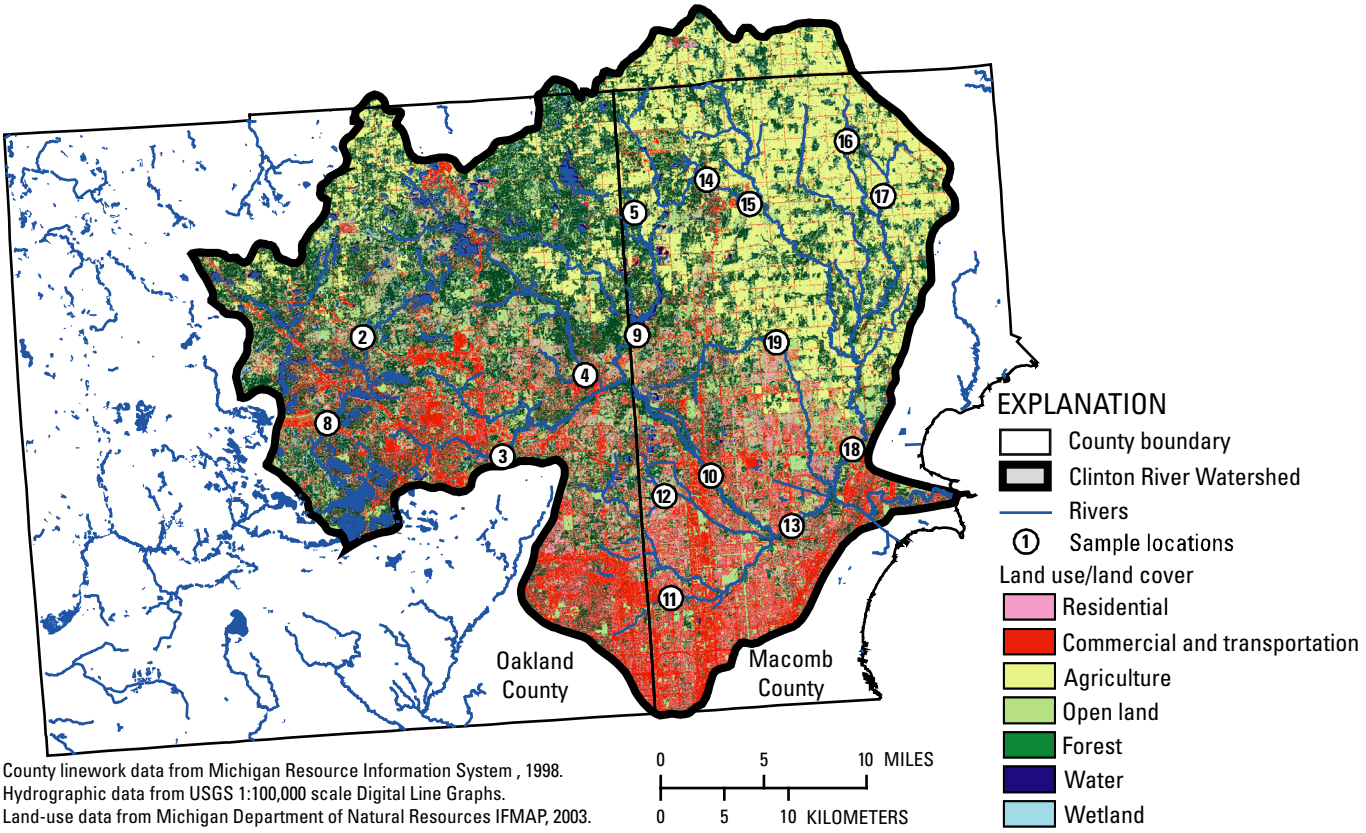

Figure 2. Sampling locations for study sites and land use/land cover for Clinton River Watershed study, Mich.

One means by which an organism may develop antibiotic resistance is through gene transfer. Bacteria originally sensitive to an antibiotic may acquire resistance genes from resistant organisms that have been exposed to antibiotics. Genes responsible for antibiotic resistance are commonly carried on transferable genetic elements (such as plasmids and class I integrons, described in a review by Roy, 1999) that can carry multiple genes—including not only genes for antibiotic resistance but also genes responsible for resistance to heavy metals, detergents, solvents, or other quaternary ammonia compounds. Thus, it is possible that antibiotic-resistance genes may be selectively maintained and transferred in the environment as a result of exposure of microorganisms to either antibiotics, heavy metals, or other chemicals constituents (Bass and others, 1999; Ug and Ceylan, 2003). Previous studies identified several wastewater organic compounds, including detergents and solvents, in stream waters in Oakland County (Aichele, 2005). Study limitations prevented further investigation of any potential link of these chemicals to antibiotic resistances in this current 2005–2006 study, but it should be noted that such compounds also may influence antibiotic resistance and gene transfer in stream-water samples.

In this study, cephalosporin-resistant *E. coli* and VRE were targeted for analysis in selected stream waters of Oakland County. *E. coli* is typically sensitive to all cephalosporin antibiotics, and resistance is often a result of an acquired gene that is responsible for resistance to a large group of similar antibiotics (beta-lactam (β-lactam) antibiotics). β-lactam resistance in *E. coli* has been identified as a resistance problem for patients suffering with infectious diseases (Levy, 2005). This study evaluated the environmental occurrence of *E. coli* resistant to levels of cefoxitin and ceftriaxone, which have been determined to be clinically significant. In other words, organisms causing infections that are resistant to those levels tested would be difficult to treat with conventional antibiotics. Cefoxitin and ceftriaxone are antibiotics that are typically prescribed only for humans, but they may have some veterinary uses. They belong to the cephalosporin antibiotic group, which has widespread applications (Hornish and Kotarski, 2002; Aarestrup, 2006). Cephalosporin antibiotics break down relatively quickly and therefore are not typically found in environmental waters. However, microorganisms may develop resistance at their source, where antibiotic concentrations might be higher, or by acquisition of an antibiotic-resistance gene that

was carried on a genetic element and transferred to that organism via exposure to a different chemical. For example, if a microorganism is in an environment contaminated with heavy metals, the organism may obtain a genetic element from other organisms that survive because they carry a gene responsible for resistance to those metals. If a gene responsible for antibiotic resistance also was present on the same element, it too would be transferred to the organism.

Mercury has been identified as a problem in several streams and lakes in southeast Michigan. Several lakes in Oakland County and parts of the Clinton River and River Rouge have been listed as Areas of Concern or have a total maximum daily loads (TMDLs) for mercury either because of accumulation of mercury in fish tissues or because waters fail to meet the Michigan water-quality standard (Michigan Department of Environmental Quality, 2006). Mercury-resistance genes are commonly associated with elements that bacteria can readily transfer among each other. In addition to carrying genes for mercury resistance, the elements that can transfer between organisms also may contain genes for antibiotic resistance (Ug and Ceylan, 2003; Baker-Austin and others, 2006). The presence of mercury may encourage the transfer of the element (which contains the mercury-resistance gene) to other organisms for survival in a mercury-contaminated environment. The organism also may acquire the antibiotic-resistance gene also being carried on this element, resulting in a resistance to antibiotic without being exposed to it. This implies that the presence of mercury may be involved in the maintenance and transfer of antibiotic resistance in these environments as well. In particular, Baker-Austin and others (2006) discuss shared structural and functional characteristics of bacteria and antibiotic- and metal-resistance systems, including a resistance mechanism for mercury and β-lactam antibiotics (including the cephalosporins).

Vancomycin-resistant enterococci (VRE) were evaluated in this study. Vancomycin is a potent antibiotic used strictly in human medicine and is considered an antibiotic of last resort when other treatments fail. Vancomycin was never approved for use in livestock production, but avoparcin, another antibiotic similar to vancomycin, was used in European countries for livestock production for many years. VRE has become a significant problem in some European countries, where it is believed the use of avoparcin may be partially responsible (Kruse and others, 1999; Kühn and others, 2005). Avoparcin was never approved for use in the United States; however, VRE has recently been identified as a major health issue in U.S. hospitals. VRE has also been detected in enterococci isolates obtained from canines, poultry, and wastewaters in the United States (Harwood and others, 2001; Simjee and others, 2002; Poole and others, 2005). Resistance to vancomycin is often attributed to genes that can be transferred among similar organisms, and molecular assays can often be used to identify the presence of these genes in a given sample (Dutka-Malen and others, 1995). Other studies have identified a gene in enterococci isolates that has been demonstrated to be associated solely with human fecal waste (Shankar and others, 1999; Scott and others 2005). This gene is commonly found in isolates that have caused infections in humans; therefore, it may be not only an indicator of human fecal pollution but also a marker for a human pathogen.

Microbial antibiotic resistance is a recognized clinical problem because of limited treatment options (McGowan and Tenover, 2004; Levy, 2005; Centers for Disease Control and Prevention, 2007), but little is known about the role the environment also may have on antibiotic resistances of clinical concern. Improving the understanding of the distribution of antibiotic-resistant bacteria, as well as the genes responsible for antibiotic resistance in bacteria isolated from the environment, will improve the ability to assess the effects these waters may have on human health. In addition, relating these resistance patterns to other environmental contaminants will improve our understanding of the sources and (or) maintenance of resistance in the environment.

Analytical and Sampling Methods

Physical, chemical, and bacterial water quality were determined for five different sampling dates (September 19, 2005, and April 26, June 28, August 9, and August 23, 2006) at 7 sites in Oakland County and an additional 12 sites in the Clinton River Watershed for two of those sampling dates (April 26 and August 23, 2006). In addition, during a rainstorm (on June 28, 2006), a sample was collected for bacteria analyses from a drain that discharges into Evans Ditch at Southfield. Not including replicates or blanks, a total of 59 samples were collected for total mercury determinations, 60 for bacterial analyses, and 17 for antibiotic analyses. A summary of samples collected and analyzed is presented in table 4.

E. coli and enterococci were isolated from stream water and were tested for the ability to grow in the presence of antibiotics. *E. coli* and enterococci isolates that were capable of growing on media with antibiotic concentrations considered significant for human health (Clinical Laboratory and Standards Institute, 2002) were reported as a percentage of isolates resistant to the antibiotic. Isolates were further tested for growth on media containing mercury to determine resistance (organisms capable of growth at 50 μM of mercury were considered resistant). Molecular analyses were used to identify antibiotic-resistance genes, or elements that may carry resistance genes, in isolates from stream-water samples. These results were then compared to antibiotic and mercury concentrations that were measured in similar stream-water samples. More detailed explanations of these methods are given in the following sections.

Table 4. Summary of field measurements (dissolved oxygen, pH, specific conductance, and streamflow) and total mercury, antibiotic, and bacteria samples collected September 19, 2005, through August 23, 2006, in Oakland and Macomb Counties, Mich.

[Shading indicates that measurements were made or samples were collected on the corresponding date]

USGS station number	Field measurements					Total mercury					Antibiotics					Bacteria/antibiotic resistance				
	9/19	4/26	6/28	8/9	8/23	9/19	4/26	6/28	8/9	8/23	9/19	4/26	6/28	8/9	8/23	9/19	4/26	6/28	8/9	8/23
04148035	■	■	■	■	■	■	■	■	■	■		■			■	■	■	■	■	■
04160800	■	■	■	■	■	■	■	■	■	■		■			■	■	■	■	■	■
04161000	■	■	■	■	■	■	■	■	■	■		■		■	■	■	■	■	■	■
04161540	■	■	■	■	■	■	■	■	■	■		■		■	■	■	■	■	■	■
04161580	■	■	■	■	■	■	■	■	■	■		■			■	■	■	■	■	■
04166000	■	■	■	■	■	■	■	■	■	■		■		■	■	■	■	■	■	■
04166200	■	■	■	■	■	■	■	■	■	■		■			■	■	■	■	■	■
Drain			■															■		
04160900		■			■		■			■							■			■
04161800		■			■		■			■							■			■
04161820		■			■		■			■							■			■
04163030		■			■		■			■							■			■
04163400		■			■		■			■							■			■
04164000		■			■		■			■							■			■
04164100		■			■		■			■							■			■
04164110		■			■		■			■							■			■
04164300		■			■		■			■							■			■
04164350		■			■		■			■							■			■
04164500		■			■		■			■							■			■
04164800		■			■		■			■							■			■

Field Measurements

Field measurements of water temperature, dissolved oxygen, specific conductance, and pH were made near the sampling point with a multiparameter water-quality meter. USGS streamgages at the sampling site were used to calculate streamflow as described in Rantz and others (1982) with the exception of Kearsley Creek at Ortonville. Streamflow at the Kearsley Creek site was measured by means of an acoustic Doppler velocimeter (Kraus and others, 1994).

Collection of Samples for Chemical Analyses

For total mercury and antibiotic analyses, grab samples were collected according to USGS protocols (Wilde and others, 1999). Water samples collected for total mercury analysis were collected in an acid-rinsed Teflon bottle and kept on ice until all samples had been collected for the day, at which time samples were preserved with 10 mL of a 6 *N* hydrochloric acid solution (Olson and DeWild, 1999) and shipped to the USGS Wisconsin Mercury Research Laboratory for analysis. Samples for antibiotic analysis were collected in a Teflon bottle cleaned in the laboratory prior to sample collection, as specified in Wilde (2004). The sample was placed on ice and kept cool until the next day, when it was filtered through a 0.7-µm glass fiber filter into three 150-mL glass amber bottles and packed on ice for shipment to the USGS Organic Geochemistry Research Laboratory for analysis (laboratory schedule LCAN (Lindsey and others, 2001; Kolpin and others, 2002)). The antibiotics that were analyzed for are listed in table 5.

Collection of Samples for Bacteria Analyses

Samples were collected and processed for fecal coliform bacteria, *E. coli,* and enterococci, following protocols of the U.S. Environmental Protection Agency (2000) and American Public Health Association and others (1998). A single grab sample was collected from the center of river flow in a 1-L sterile bottle and kept on ice or cooled at 4°C until samples could be processed (18–24 hours following collection). Water samples were filtered through a 0.45-µm membrane filter in 100-, 10-, and 1-mL volumes. Fecal coliform bacteria were enumerated on mFC medium; *E. coli* were differentiated by transferring the fecal coliform colonies to NA-MUG medium. Enterococci were enumerated on mEI medium. Growth on filters from the 100-mL sample for both mFC and mEI was rinsed from the filters with a sterile phosphate buffered saline solution (except for samples collected on April 26, 2006, in which all colonies from 100 mL were isolated and preserved). The samples were spun in a centrifuge to separate the cells from the liquid. The cells were then resuspended in 1 mL of phosphate-buffered saline containing 20 percent glycerol and stored at -80ºC. Those preserved cells are hereafter referred to as the "FC or MEI culture stocks." DNA was extracted from these cultures by a process of freezing and thawing a pellet of cells from 100 µL of the FC or MEI culture stock that had been resuspended in 100 µL of TE buffer (10 mM tris and 1 mM EDTA, pH 8.0).

E. coli and enterococci isolates were transferred to Mueller-Hinton and brain-heart infusion broth, respectively, in 96-well plates, grown overnight, preserved with glycerol, and stored at -80ºC. Isolates were transferred to fresh media and

Table 5. List of antibiotics for which samples were analyzed in the Oakland County study.

[Analyzed by U.S. Geological Survey Geochemistry Organics Research Laboratory]

Beta-lactams	**Sulfonamides**	**Others**
Amoxicillin	Sulfachloropyridazine	Carbadox
Ampicillin	Sulfadiazine	Lincomycin
Cefotaxime	Sulfadimethoxine	Trimethoprim
Cloxacillin	Sulfamerazine	Ormetoprim
Oxacillin	Sulfamethazine	
Penicillin G	Sulfamethoxazole	
Penicillin V	Sulfathiazole	
Macrolides	**Tetracyclines and degradation products**	
Azithromycin	Chlortetracycline	
Erythromycin	Anhydro-chlortetracycline	
Erythromycin-H_2O	Demeclocycline	
Roxithromycin	Doxycycline	
Tylosin	Minocycline	
Virginiamycin	Oxytetracycline	
	Tetracycline	
Quinolines	Anhydro-tetracycline	
Ciprofloxacin	Alpha apo-oxytetracycline	
Clinafloxacin	Beta apo-oxytetracycline	
Flumequine	Epi-anhydro-chlortetracycline	
Lomefloxacin	Epi-chlortetracycline	
Norfloxacin	Iso-chlortetracycline	
Ofloxacin	Epi-Iso-chlortetracycline	
Oxolinic acid	Epi-oxytetracycline	
Sarafloxacin	Epi-anhydro-tetracycline	
	Epi-tetracycline	

grown overnight prior to antibiotic resistance testing. Antibiotic solutions were made up according to the Clinical Laboratory Standards Institute protocols (2002) in the following concentrations: cefoxitin, 16 and 32 µg/mL; ceftriaxone, 32 and 64 µg/mL; erythromycin, 8 µg/mL; ofloxacin, 8 µg/mL; and vancomycin, 16 µg/mL. Growth medium containing antibiotic solutions was dispensed into 96-well plates in 100-µL volumes. All *E. coli* isolates were transferred to 96-well plates containing 100 µL of Mueller-Hinton media with cefoxitin or ceftriaxone antibiotic solutions. Enterococci isolates were transferred to 96-well plates containing brain-heart infusion broth with vancomycin. *E. coli* isolates from samples collected April 26 and August 23 were also tested against erythromycin and ofloxacin. Isolates were incubated at 37ºC for 18–24 hours (48 hours for vancomycin), after which any growth was recorded as a resistant isolate. American Type Culture Collection (ATCC) strains *E. coli* ATCC 25922 and *Enterococcus faecalis (E. faecalis)* ATTC 29212 were used as controls for antibiotic analyses, as described by the Clinical Laboratory and Standards Institute (2002).

E. coli isolates were tested for resistance to mercury by plating isolates to Luria-Bertani (LB) media containing 50 µM mercuric chloride. Isolates were incubated overnight at 37ºC. Any growth was recorded as a resistant isolate.

E. coli isolates resistant to mercury, erythromycin, or ofloxacin (from April and August 23, 2006, samples) were chosen for molecular analyses to identify genes responsible for antibiotic resistance. Polymerase chain reaction (PCR) was performed on all mFC culture stocks and selected isolates from April 26 and August 23, 2006, to detect three different components of class I integrons, the 5 prime to 3 prime conserved region containing gene cassettes (Lévesque and others, 1995), the integrase gene (Koeleman and others, 2001), and the sulfonamide-resistance gene (Lévesque and others, 1995). A positive control (ECOR-03, *E. coli* reference collection, Michigan State University, East Lansing, Mich.) and negative control *E. coli* strain (ATCC 25922) were run with each PCR reaction. All enterococci isolates resistant to 16 µg/mL vancomycin were tested by PCR for the presence of *van*A, a transferable gene responsible for high-level vancomycin resistance, following protocols described in Fogarty and others (2005). DNA extracted from MEI culture stocks was analyzed for the presence of the *van*A following protocols similar to those previously described (Fogarty and others, 2005) by

using100 µL of the stock culture instead of an isolate. A positive-control enterococci strain (ATCC 700221) containing the *van*A gene and a negative control (no DNA) was included with each PCR reaction. MEI cultures were also analyzed for the *esp* gene from *Enterococcus faecium* and *E. faecalis*. The *esp* gene encodes a surface protein that is found on human pathogenic strains of *Enterococcus*, and it is commonly used to identify sources of human fecal pollution. All reagents were from Applied Biosystems (Foster City, Calif.). Primers and the positive control strain (E53) used for the *esp* analyses are described in Shankar and others (1999). Each 15-µL reaction contained (final concentration) 1× Gold buffer, 2.5 mM MgCl2, 0.2 mM dNTP's, 0.5 µg/µL BSA, 0.5 µM of the forward and reverse primers, 0.05 unit/µL of Amplitaq Gold Polymerase and between 1 and 100 ng of template DNA.

Quality Assurance and Quality Control

Additional samples were collected for quality assurance and quality control at all steps for all analyses in this study. A sequential replicate sample was collected for antibiotics, mercury, and bacterial analyses on one occasion during this study. Field blanks were collected at one site for antibiotic analyses, two sites for mercury analyses, and one site for bacterial analyses in accordance with Wilde and others (1999). In addition, laboratory blanks (sterilized phosphate buffer saline filter in 100-mL volumes) were also analyzed for each sampling event for all bacterial analyses. A laboratory split replicate was also processed by filtering equal volumes of sample water onto two different filters and processing as described above. Concentrations of all constituents of interest in the field blanks were less than the method detection limits for all constituents. Concentrations in all bacterial-analysis lab blanks also were less than the method detection limit (less than 1 cell per 100 mL). Analytical results for mercury were identical for replicate samples collected at the Clinton River Auburn Hills on August 9, 2007 (1.17 ng/L). Analytical results for replicate samples collected at the Clinton River at Auburn Hills on August 9, 2006, for antibiotics were fairly consistent (table 6). Differences detected in both the bacterial field and laboratory replicates are discussed in the following section.

Results of Data Analyses

Fecal indicator bacteria were enumerated and the percentages of cephalosporin-resistant *E. coli* and VRE were determined and related to antibiotic and mercury concentrations in the water. Field measurements made for each of the seven sites on all five sampling dates are listed in table 7.

Fecal Indicator Bacteria Enumeration

Fecal coliform bacteria, *E. coli,* and enterococci were quantified for samples collected on five separate occasions from September 2005 to August 2006. These results are shown in table 8. State of Michigan recreational-water quality standards for all surface waters in the State are in effect from May 1 to October 31 (Michigan's water-quality standard R323.1062, Rule 62). The Michigan water-quality standard for a single sampling event specifies three or more samples from which a geometric mean is calculated.

Bacteria concentrations reported in this study resulted from enumeration of a single sample rather than a calculation of the geometric mean of three samples. However, the Michigan recreational-water quality standard of 300 colony-forming units (CFU) per 100 mL and partial contact-water quality standard of 1,000 CFU/100 mL for a single sampling event can be used as a frame of reference for samples collected in this study. Figure 3 shows the concentrations for all the indicator bacteria measured and the recreational standard of 300 CFU/100 mL for *E. coli*. Of the 35 samples collected for Oakland County, 19 exceeded this standard. Every site exceeded this standard on at least one sampling date. No sample collected on April 26, 2006, exceeded 300 CFU/100 mL. Two samples (Evans Ditch and Kearsley Creek) collected June 28, 2006, exceeded 1,000 CFU/100 mL. The high levels of *E. coli* and enterococci frequently measured in these samples are indicative of fecal pollution. High levels of these indicator organisms have been shown to be correlated with increased risk of illness (U.S. Environmental Protection Agency, 1986).

Table 6. Concentrations of antibiotics detected in replicate samples collected at Clinton River at Auburn Hills, Mich., August 9, 2006.

[Concentrations are in micrograms per liter]

Sample	Azithromycin	Erythromycin	Erythromycin-H_2O	Ofloxacin	Trimethoprim
Clinton River at Auburn Hills	<0.005	0.064	0.063	0.138	0.013
Replicate	0.108	0.067	0.064	0.136	0.014

Table 7. Field measurements for samples collected at Oakland County, Mich., study sites, September 2005 through August 2006.

[USGS, U.S. Geological Survey; ft^3/s, cubic feet per second; °C, degrees Celsius; mg/L, milligram per liter; µS/cm, microsiemens per centimeter; NA, not available; **, instantaneous discharge measurement, daily mean value not available]

USGS station number	Site name	Sample-collection date	Daily mean discharge (ft^3/s)	pH	Water temperature (°C)	Dissolved oxygen (mg/L)	Specific conductance (µS/cm)
04148035	Kearsley Creek at Mill Street at Ortonville	09/19/05	4.1**	7.9	14.2	8.5	842
		04/26/06	25**	8.4	8.2	9.6	446
		06/28/06	11**	8.2	17.6	7.4	739
		08/09/06	8.3**	8.3	17.0	8.2	751
		08/23/06	4.2**	8.3	18.0	NA	862
04160800	Sashabaw Creek near Drayton Plains	09/19/05	2.0	7.9	16.5	7.8	708
		04/26/06	21	8.7	9.7	9.2	579
		06/28/06	10	8.0	23.1	8.5	713
		08/09/06	3.2	8.0	22.0	8.0	697
		08/23/06	2.6	8.3	17.6	6.4	682
04161000	Clinton River at Auburn Hills	09/19/05	25	7.6	19.0	7.7	1,108
		04/26/06	124	8.4	15.3	10.0	657
		06/28/06	131	8.1	23.4	6.9	838
		08/09/06	39	8.3	22.1	7.5	904
		08/23/06	26	7.8	21.0	9.0	1,110
04161540	Paint Creek at Rochester	09/19/05	14	8.0	15.5	9.5	794
		04/26/06	69	8.4	13.0	10.0	499
		06/28/06	38	8.4	19.1	8.3	707
		08/09/06	20	8.7	17.2	8.4	746
		08/23/06	12	8.4	19.0	NA	885
04161580	Stony Creek near Romeo	09/19/05	2.2	8.0	15.1	9.3	713
		04/26/06	21	7.5	7.7	10.0	626
		06/28/06	5.3	8.5	20.0	7.8	640
		08/09/06	3.3	8.4	17.6	8.0	637
		08/23/06	3.3	8.0	17.5	8.0	724
04166000	River Rouge at Birmingham	09/19/05	7.8	8.0	18.9	8.4	1,086
		04/26/06	30	8.4	13.8	9.7	788
		06/28/06	14	8.1	22.8	8.1	1,030
		08/09/06	6.4	8.2	23.4	7.0	1,020
		08/23/06	6.4	8.1	21.9	7.9	1,070
04166200	Evans Ditch at Southfield	09/19/05	1.6	7.7	17.5	5.2	1,690
		04/26/06	5.0	8.4	9.9	11.3	1,570
		06/28/06	5.6	8.0	20.0	7.4	1,790
		08/09/06	1.2	8.1	20.5	5.1	2,780
		08/23/06	1.3	8.2	19.4	5.7	2,340

Because samples were collected as a single sample, it is important to understand the bacteria variation that may occur in these samples. Sequential replicate samples collected from the Clinton River on August 9, 2006, show very little difference in fecal coliform bacteria or *E. coli* concentrations (table 8). There was a greater difference in the enterococci concentration from this sample. Single samples were also processed as laboratory split replicates (table 8). Again, differences were detected, but they do not change the interpretation in regards to the recreational water-quality standards. It is important, however, to understand these are single samples that may not be a full representation of the conditions at the site. Yet the analyses give an indication of how high fecal bacteria concentrations may be in these waters.

Table 8. Fecal indicator bacteria concentrations for samples collected at Oakland County, Mich., study sites, September 2005 through August 2006.

[USGS, U.S. Geological Survey; CFU/100 mL, colony forming units per 100 milliliters]

USGS station number	Site name	Sample-collection date	Fecal coliform bacteria (CFU/100 mL)	*E. coli* (CFU/100 mL)	Enterococci (CFU/100 mL)
04148035	Kearsley Creek at Mill Street at Ortonville	09/19/05	330	310	750
		04/26/06	128	98	50
		06/28/06	3,200	1,300	720
		08/09/06	208	208	390
		08/23/06	809	357	280
04160800	Sashabaw Creek near Drayton Plains	09/19/05	850	830	360
		04/26/06	45	36	20
		06/28/06	410	410	176
		08/09/06	250	204	243
		08/23/06	982/720[a]	483/520[a]	370/260[a]
04161000	Clinton River at Auburn Hills	09/19/05	300	140	280
		04/26/06	68	42	29
		06/28/06	1,173	847	590
		08/09/06	78/79[b]	72/68[b]	230/146[b]
		08/23/06	1,282	278	99
04161540	Paint Creek at Rochester	09/19/05	510	410	540
		04/26/06	39	24	19
		06/28/06	760	330	410
		08/09/06	280	204	330
		08/23/06	490	300	300
04161580	Stony Creek near Romeo	09/19/05	340	310	560
		04/26/06	48	42	21
		06/28/06	290	150	250
		08/09/06	430	430	590
		08/23/06	29	2	6
04166000	River Rouge at Birmingham	09/19/05	570	200	460
		04/26/06	27	24	22
		06/28/06	1,673	540	680
		08/09/06	690	640	360
		08/23/06	1,535	935	370
04166200	Evans Ditch near Southfield	09/19/05	840	830	360
		04/26/06	71	51	37
		06/28/06	5,600	1,400	8,600
		08/09/06	720	60	542
		08/23/06	2,600/1,000[a]	700/639[b]	2,300/1,600[b]
	Drain at Evans Ditch	06/28/06	4,100		1,900

[a] Replicate lab sample—single sample filtered in replicate.

[b] Replicate field sample—replicate samples collected in field.

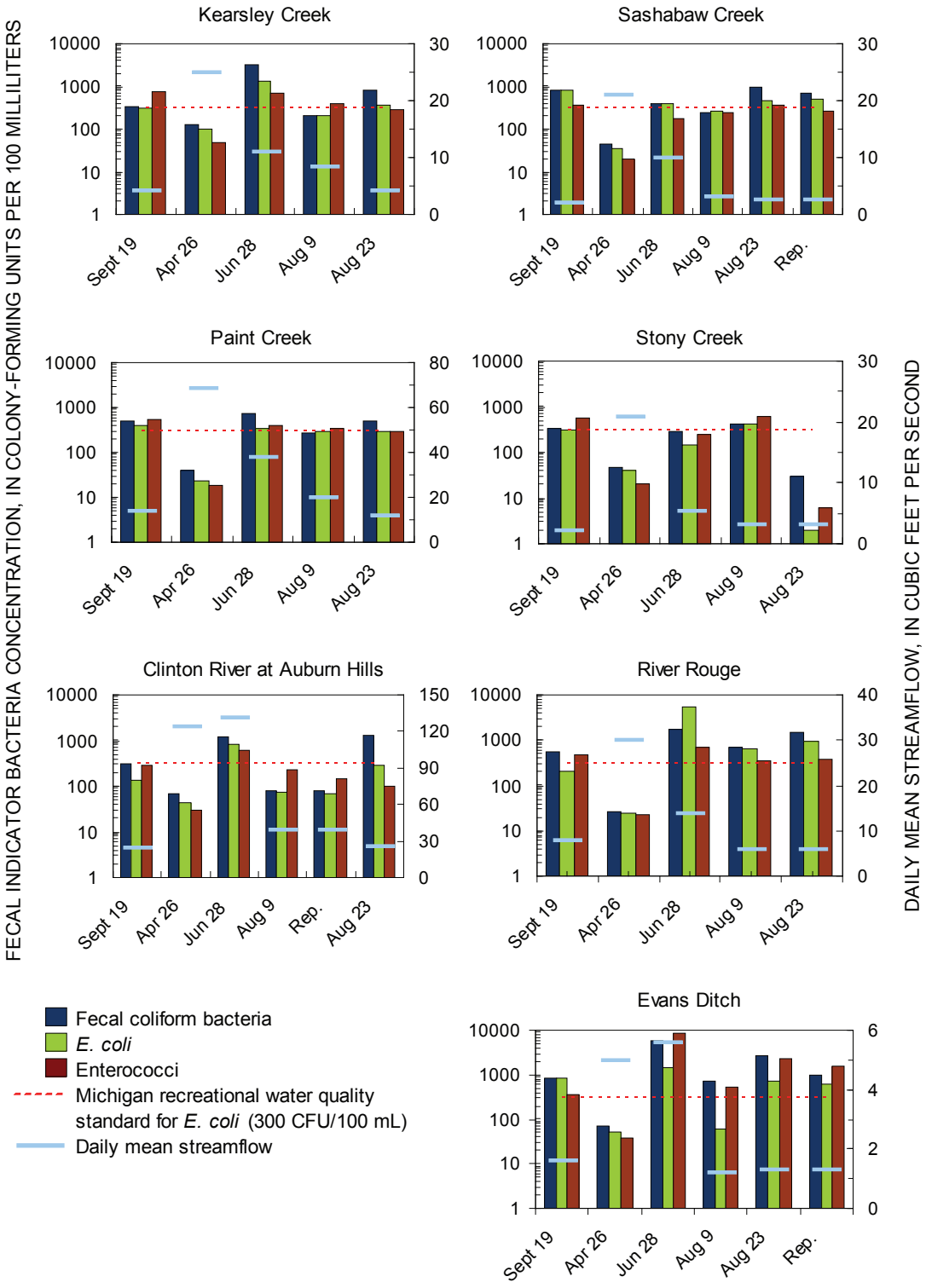

Figure 3. Fecal indicator bacteria concentrations for samples collected September 19, 2005, and April 28, June 28, August 9, and August 23, 2006, at Oakland County study sites.

Vancomycin-Resistant Enterococci and Human-Fecal-Enterococci Gene

Vancomycin-resistant enterococci were previously detected in surface-water samples collected in Oakland County in 2003 (Fogarty and others, 2005). In the current 2005–2006 study, no enterococci isolated out of the stream water were resistant to vancomycin at the same concentration tested in 2003 (16 µg/mL). The mEI cultures were tested for the presence of two genes responsible for vancomycin resistance, *van*A and *van*B. These cultures presumably represent a larger enterococci population than the number of single isolates tested (ranging from 3 to 96 isolates per sample, depending on the number of well-isolated colonies that grew on a single plate). The *van*A gene was not detected in any of the MEI cultures, whereas in 2003 the *van*A gene was identified is several enterococci isolates from Kearsley Creek and Evans Ditch. The source or sources of the vancomycin-resistant isolates in 2003 are unknown. Owing to the absence of vancomycin-resistant organisms in 2005–2006, it does not appear that the source is a persistent problem at those sites where VRE was once detected.

All mEI cultures were analyzed for the presence of a human fecal enterococci gene (*esp*). The *esp* gene is commonly associated with enterococci known to cause human illness and has also been used to identify human fecal pollution in the environment (Betancourt and Fujioka, 2006). The *esp* gene was detected in two stream samples collected June 28, 2006—Paint Creek and Evans Ditch—and in the Evans Ditch drain sample collected during a rain event on that day. Samples in which the *esp* gene was found had enterococci concentrations greater than 400 CFU/100 mL. However, this gene was not detected in all samples with concentration greater than 400 CFU/100 mL. The presence of this gene indicates a source of human fecal pollution at these two sites.

Occurrence of Cephalosporin-Resistant *E. coli*

In the study conducted in 2003, cephalosporin-resistant fecal coliform bacteria and *E. coli* were frequently isolated from streams in Oakland County (Fogarty and others, 2005). As a result, two cephalosporin antibiotics tested in that study were chosen for analysis in the 2005–2006 study: cefoxitin, a second-generation cephalosporin antibiotic, and ceftriaxone, a third-generation cephalosporin antibiotic. Both antibiotics are approved for human use only; however, cefoxitin is legally used to treat infections in cats and dogs, and similar antibiotics are given to livestock. *E. coli* isolates were grown on antibiotic concentrations representing intermediate resistance (16 µg/mL for cefoxitin, 32 µg/mL for ceftriaxone) and high-level resistance (32 µg/mL for cefoxitin, 64 µg/mL for ceftriaxone). Levels of resistance are often used to determine severity of resistance, which is sometimes controlled by different types of genes. The greater the level of resistance, the more difficult treatment could be. “Intermediate-” and “high-level” resistance is defined by the Clinical Laboratory Standards Institute (Clinical Laboratory and Standards Institute, 2002).

E. coli resistant to cefoxitin and/or ceftriaxone were isolated at all sites (table 9). Out of the 35 samples collected, 24 samples contained *E. coli* resistant to high-level cefoxitin, and 13 samples contained *E. coli* resistant to high-level ceftriaxone. Isolates resistant to high-level cefoxitin were detected at all seven Oakland County study sites. High-level ceftriaxone resistance was detected at all sites except Paint Creek and Stony Creek. High level resistance to cefoxitin was detected in 50 percent of *E. coli* isolates, whereas intermediate-level resistance was detected in 75 percent of the isolates. High-level and intermediate ceftriaxone resistance was detected in 6 percent and 41 percent of *E. coli* isolates, respectively. Isolates resistant to both ceftriaxone and cefoxitin were detected in 10 of the 35 samples analyzed. Samples from all sites except Paint Creek and Stony Creek contained *E. coli* resistant to both cephalosporin antibiotics. *E. coli* resistant to one or more cephalosporin antibiotics commonly carry a gene that results in resistance to not only the cephalosporin antibiotics but also a large class of antibiotics (β-lactam antibiotics) commonly used to treat infections. Infections caused by organisms with this type of resistance may be very difficult to treat because of a lack of treatment options.

Because *E. coli* are not typically resistant to these antibiotics, it is interesting to see such widespread dissemination of these resistances in this area. Further studies would need to be done to determine whether the *E. coli* that was isolated out of the environment resembles *E. coli* that are common to human or animal intestinal systems or that cause human illnesses. This would provide a better understanding of the potential effects these organisms have on human health.

Significant differences in the percentage of resistant isolates between replicates samples were observed in the sample set (table 9). Difference in the percentage of antibiotic-resistant isolates was as high as 45 percent. This may be a reflection of heterogeneity of the microbial community in water samples. It is important to understand that, like the fecal-indicator bacteria concentrations, the sample used to determine the percentage of resistant isolates is just a snapshot of the stream site and may not be a true representation of conditions at the stream site over time.

Studies by the National Antimicrobial Resistance Monitoring System (NARMS) have detected very few human pathogenic *E. coli* isolates with resistance to either cefoxitin or ceftriaxone (Centers for Disease Control and Prevention, 2004). Despite being approved only for human medicine, a greater percentage of *E. coli* isolated from veterinary samples were found to be resistant to either cefoxitin or ceftriaxone (U.S. Department of Agriculture, 2007) than in the human pathogenic *E. coli* study. The results shown in the NARMS studies do not rule out human fecal waste as a source of cephalosporin-resistant *E .coli,* because only pathogenic *E. coli* were tested in the NARMS studies. However, the NARMS studies do indicate *E. coli* from fecal sources other than humans may be a source of cephalosporin-resistant *E. coli.*

Table 9. Percentage of *E. coli* isolates resistant to tested antibiotics for samples collected at Oakland County, Mich., study sites, September 2005 through August 2006.

[USGS, U.S. Geological Survey; CFX, cefoxitin; AXO, ceftriaxone; HLR, high-level resistance to both cefoxitin and ceftriaxone resistant; ERY, erythromycin; OFL, oflaxicin; int., intermediate resistance; NT, not tested]

USGS station number	Site name	Sample collection date	Number of isolates tested	Percentage of *E. coli* isolates resistant						
				CFX int.	CFX high	AXO int.	AXO high	HLR	ERY high	OFL high
04148035	Kearsley Creek at Mill Street at Ortonville	09/19/05	31	0	0	3	0	0	NT	NT
		04/26/06	74	11	0	26	0	0	0	0
		06/28/06	29	17	17	3	3	3	NT	NT
		08/09/06	16	25	6	0	0	0	NT	NT
		08/23/06	31	23	10	6	3	0	13	0
04160800	Sashabaw Creek near Drayton Plains	09/19/05	83	8	0	13	0	0	NT	NT
		04/26/06	30	7	0	53	0	0	0	0
		06/28/06	16	38	31	19	19	19	NT	NT
		08/09/06	27	7	7	0	0	0	NT	NT
		08/23/06	37/47[a]	54/13[a]	49/13[a]	0/0[a]	0/0[a]	0/0[a]	5	0
04161000	Clinton River at Auburn Hills	09/19/05	14	14	0	43	7	0	NT	NT
		04/26/06	36	11	3	31	3	0	0	0
		06/28/06	7	71	71	0	0	0	NT	NT
		08/09/06	72/68[b]	39/3[b]	29/3[b]	11/0[b]	3/0[b]	3/0[b]	NT	NT
		08/23/06	16	44	38	13	6	6	13	0
04161540	Paint Creek at Rochester	09/19/05	41	5	0	17	0	0	NT	NT
		04/26/06	22	5	0	32	0	0	0	0
		06/28/06	33	21	18	0	0	0	NT	NT
		08/09/06	30	13	10	10	0	0	NT	NT
		08/23/06	30	3	3	0	0	0	0	0
04161580	Stony Creek near Romeo	09/19/05	31	19	0	32	0	0	NT	NT
		04/26/06	39	3	0	38	0	0	0	3
		06/28/06	36	19	14	3	0	0	NT	NT
		08/09/06	43	19	2	0	0	0	NT	NT
		08/23/06	2	50	0	0	0	0	0	0
04166000	River Rouge at Birmingham	09/19/05	19	42	5	21	16	5	NT	NT
		04/26/06	23	26	4	17	4	4	0	0
		06/28/06	54	31	30	11	7	7	NT	NT
		08/09/06	56	4	4	0	0	0	NT	NT
		08/23/06	45	31	31	0	0	0	4	0
04166200	Evans Ditch at Southfield	09/19/05	61	15	0	34	2	0	NT	NT
		04/26/06	45	13	2	42	4	2	0	0
		06/28/06	14	71	64	7	0	0	NT	NT
		08/09/06	62	5	5	5	2	2	NT	NT
		08/23/06	46/7[a]	74/29[a]	67/29[a]	24/0[a]	0/0[a]	0/0[a]	13	0
	Drain at Evans Ditch	06/28/06	4	100	100	0	0	0	NT	NT

[a] Replicate lab sample—single sample filtered in replicate.

[b] Replicate field sample—replicate samples collected in field.

The resistance seen in the veterinary isolates was not directly attributed to cephalosporin antibiotic use in that animal. Therefore, it is possible that other environmental or natural factors may result in the presence of cephalosporin-resistant bacteria in the environment.

Spatial and Temporal Patterns in the Percentage of Cephalosporin-Resistant *E. coli*

Despite the difficulties in interpreting the value of the percentage resistance due to the results in replicate samples, there were still interesting patterns in the percentage of cephalosporin-resistant *E. coli* isolates temporally and spatially. Sites in less urbanized areas (sites with less than or 51 percent developed area (table 2): Kearsley Creek, Sashabaw Creek, Paint Creek, and Stony Creek) had no detectable cefoxitin- or ceftriaxone-resistant *E. coli* in September 2005 and April 2006, but cefoxitin- and/or ceftriaxone-resistant *E. coli* were detected in summer months (June through August 2006) (fig. 4). This pattern did not hold true for the more urbanized sites (sites with greater than 51 percent developed area (table 2): Clinton River, River Rouge, and Evans Ditch), at which there appeared to be more consistent detection of resistant *E. coli* throughout the study period (fig. 4).

Rainfall varied slightly throughout the county prior to sample collections (fig. 5), and only one sample was collected during a precipitation event, on June 28, at Evans Ditch. There was no correlation with 24- or 72-hour preceding rainfall and the percentage of cephalosporin-resistant *E. coli* detected, nor was there was any linear relation between discharge and percent resistant *E. coli* at any site. In other words, it does not appear that rainfall controls the levels at which cephalosporin-resistant *E. coli* are found in streams in the study area.

It is important to remember that the percentage of resistance is inherently variable in the sample (as seen in the replicate samples); therefore, these results only indicate possible patterns in such resistance and would require more intensive sampling to confirm.

Antibiotic Concentrations and Cephalosporin-Resistant *E. coli*

Antibiotic concentrations were measured in water samples collected on multiple occasions during this study (table 10). There was no apparent relation between the antibiotics detected in the water to the percentage of cephalosporin-resistant *E. coli* in the sample. Cephalosporin antibiotics were not detected in any sample. Cephalosporin antibiotics degrade quite quickly in the environment and are not frequently detected in environmental samples. However, multiple antibiotics were detected in samples from the Clinton River at Auburn Hills: erythromycin, ofloxacin, sulfamethoxazole, and trimethroprim were detected in all three of the samples, and azithromycin was also detected in two of the five samples from this site. The only other water sample collected that had detectable antibiotics was the August 9, 2006, Paint Creek sample (tylosin).

All *E. coli* isolates from April 26 and August 23, 2006, samples were tested for resistance to high levels of erythromycin (8 μg/mL) and oflaxicin (8 μg/mL), because both antibiotics were detected in the Clinton River at Auburn Hills samples. Erythromycin-resistant *E. coli* were not detected in any of the April samples but were detected at five of the seven sites in August. Erythromycin-resistant *E. coli* was detected at the Clinton River at Auburn Hills site, but no difference in frequency of occurrence was observed compared to other sites at which erythromycin was not detected in the water (table 10). Although no antibiotic tested was detected at the Stony Creek site, ofloxacin-resistant *E. coli* isolates were detected only at Stony Creek near Romeo in April.

Antibiotics that are detected in the water are not related to the antibiotic-resistant *E. coli* isolated from these waters. Therefore, antibiotics in the water are not a reliable indictor for the co-occurrence of antibiotic-resistant *E. coli.*

Mercury- and Cephalosporin-Resistant *E. coli*

Several studies have linked mercury pollution to antibiotic resistance (McArthur and Tuckfield, 2000; Baker-Austin and others, 2006). Mercury concentrations were measured in this study to determine whether there was any apparent relation between mercury concentrations in the area and antibiotic-resistant bacteria. Mercury concentrations in the water are listed in table 11. Samples collected from Clinton River at Auburn Hills and Evans Ditch frequently exceeded the Michigan water-quality criterion for mercury of 1.3 ng/L. The Clinton River site is just downstream from a wastewater-treatment-plant discharge, and the Evans Ditch site is in a heavily industrialized area.

Samples from seven study sites (Kearsley Creek, Sashabaw Creek, Paint Creek, Clinton River, River Rouge, and Evans Ditch) contained *E. coli* isolates resistant to mercury in either September or April. Mercury-resistant *E. coli* were not detected in samples collected in June or August. In September, mercury-resistant *E. coli* isolates were detected at Paint Creek (2 percent), Sashabaw Creek (1 percent), and River Rouge (26 percent). In April, resistant isolates were detected at Kearsley Creek (4 percent), Stony Creek (3 percent), Clinton River (3 percent), and Evans Ditch (7 percent). In September, 1 and 26 percent of *E. coli* isolates from Sashabaw Creek and River Rouge, respectively, were resistant to both mercury and cephalosporin antibiotics. In April, isolates from the Clinton River (3 percent) and Evans Ditch sites (2 percent) were resistant to both mercury and cephalosporin antibiotics. Overall, the percentage of mercury-resistant isolates is not related to the mercury concentrations detected in that sample (fig. 6), nor does it seem as if this percentage is related to the median or mean concentrations of mercury over the study period. There are, however, some patterns at individual sites.

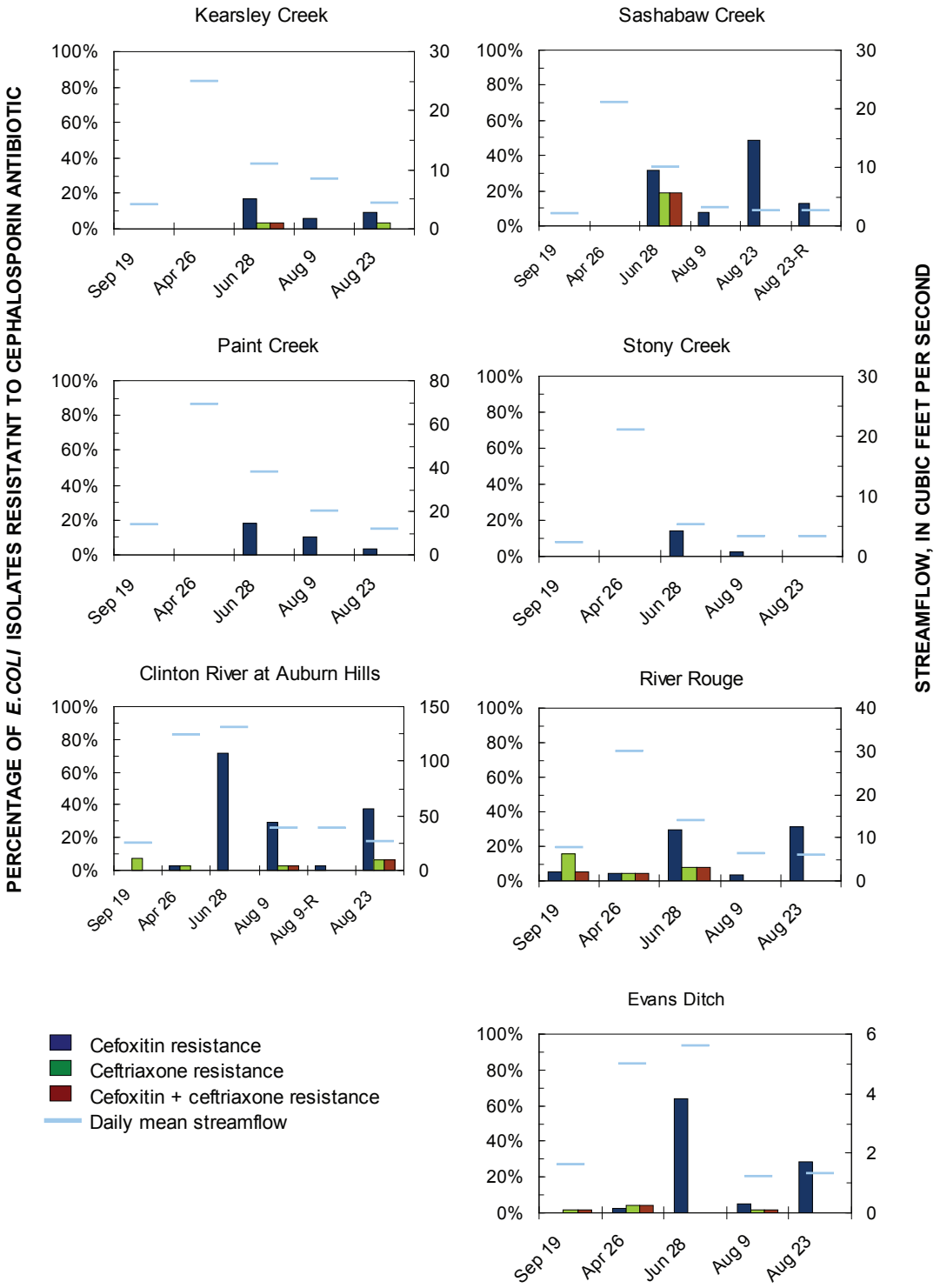

Figure 4. Percentage of cephalosporin-resistant *E. coli* isolates and daily mean streamflow on five sampling dates at study sites, Oakland County, Mich.

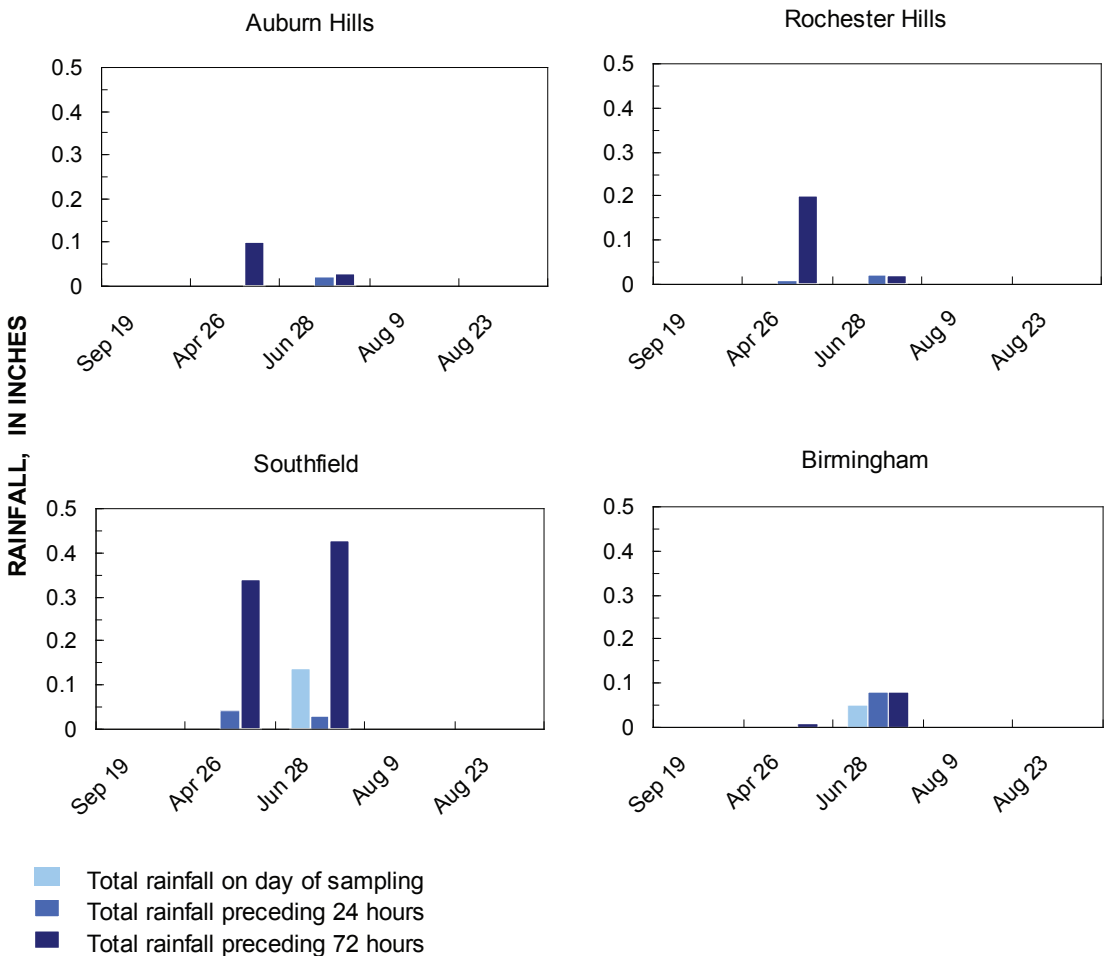

Figure 5. Precipitation totals on the day of sample collection and in the preceding 24 and 72 hours at locations in Oakland County, Mich.

For example, at River Rouge at Birmingham, ceftriaxone-resistant *E. coli* was detected only on September 19, April 26, and June 28, in which mercury concentrations were greatest; and at Evans Ditch near Southfield, the highest percentage of cefoxitin-resistant *E. coli* occurred along with the highest concentrations of mercury.

Although the results of this study cannot confirm a relation between mercury concentrations in the water to the percentage of mercury- or cephalosporin-resistant *E. coli* in the water, there are some indications that mercury may be related to resistances under specific conditions in areas with greater urban or industrialization. This result agrees with results seen in a study by McArthur and Tuckfield (2000), who found antibiotic-resistant bacteria at an industrially perturbed and an undisturbed stream; however, in that study, the industrially perturbed stream had much higher proportion of antibiotic-resistant bacteria. There was no significant difference between the Oakland County industrialized and nonindustrialized samples.

Integron Detection

Class I integrons are genetic structures that may contain several different resistance genes that could be transferred among similar bacteria. A schematic of this structure is shown in figure 7. Class I integrons have been implicated in the transmission and spread of multidrug resistance in disease-causing organisms (Lévesque and others, 1995; Rosser and Young 1999; Gruteke and others 2003; Singh and others, 2005). The presence of this element in organisms isolated from the environment suggests a possible mechanism for the transfer of antibiotic resistances in stream waters to disease-causing organisms.

Table 10 Antibiotics detected in samples collected September 2005 through August 2006 at Oakland County study sites.

[USGS, U.S. Geological Survey; µg/L; micrograms per liter; <, less than; **bold,** detectable concentration]

USGS station number	Site name	Sample-collection date	Antibiotic concentration (µg/L)						
			Azithro-mycin	Erythro-mycin	Erythro-mycin-H_2O	Ofloxacin	Sul-fameth-oxazole	Trimeth-oprim	Tylosin
04148035	Kearsley Creek at Mill Street at Ortonville	04/26/06	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
		08/23/06	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
04160800	Sashabaw Creek near Drayton Plains	04/26/06	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
		08/23/06	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
04161000	Clinton River at Auburn Hills	04/26/06	**0.151**	**0.052**	**0.019**	**0.008**	**0.046**	**0.013**	<0.005
		08/09/06	**0.108**	**0.067**	**0.064**	**0.026**	**0.136**	**0.014**	<0.005
		08/23/06	<0.005	**0.055**	**0.053**	**0.043**	**0.277**	**0.006**	<0.005
04161540	Paint Creek at Rochester	04/26/06	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
		08/09/06	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	**0.006**
		08/23/06	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
04161580	Stony Creek near Romeo	04/26/06	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
		08/23/06	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
04166000	River Rouge at Birmingham	04/26/06	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
		08/09/06	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
		08/23/06	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
04166200	Evans Ditch at Southfield	04/26/06	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
		08/23/06	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005

Table 11. Mercury concentration in samples collected September 2005 through August 2006 at Oakland County study sites.

[USGS, U.S. Geological Survey; ng/L, nanograms per liter; **bold**, concentration exceeds Michigan's Rule 57 water-quality criterion of 1.3 ng/L]

USGS station number	Site name	Mercury concentration (ng/L)						
		Median	Mean	9/21/2005	4/26/2006	6/28/2006	8/9/2006	8/23/2006
04148035	Kearsley Creek at Mill Street at Ortonville	0.53	0.68	0.49	0.68	**1.36**	0.53	0.34
04160800	Sashabaw Creek near Drayton Plains	0.99	0.90	0.99	**1.31**	1.12	0.6	0.46
04161000	Clinton River at Auburn Hills	1.82	**1.76**	**1.82**	**1.95**	**2.66**	1.17	1.18
04161540	Paint Creek at Rochester	0.57	0.69	0.57	0.95	1.07	0.45	0.40
04161580	Stony Creek near Romeo	0.6	0.46	0.6	1.22	0.68	0.47	0.42
04166000	River Rouge at Birmingham	1.04	1.10	1.04	**1.41**	1.28	1	0.79
04166200	Evans Ditch at Southfield	**3.83**	**7.78**	**3.68**	**3.42**	**19.8**	**8.18**	**3.83**

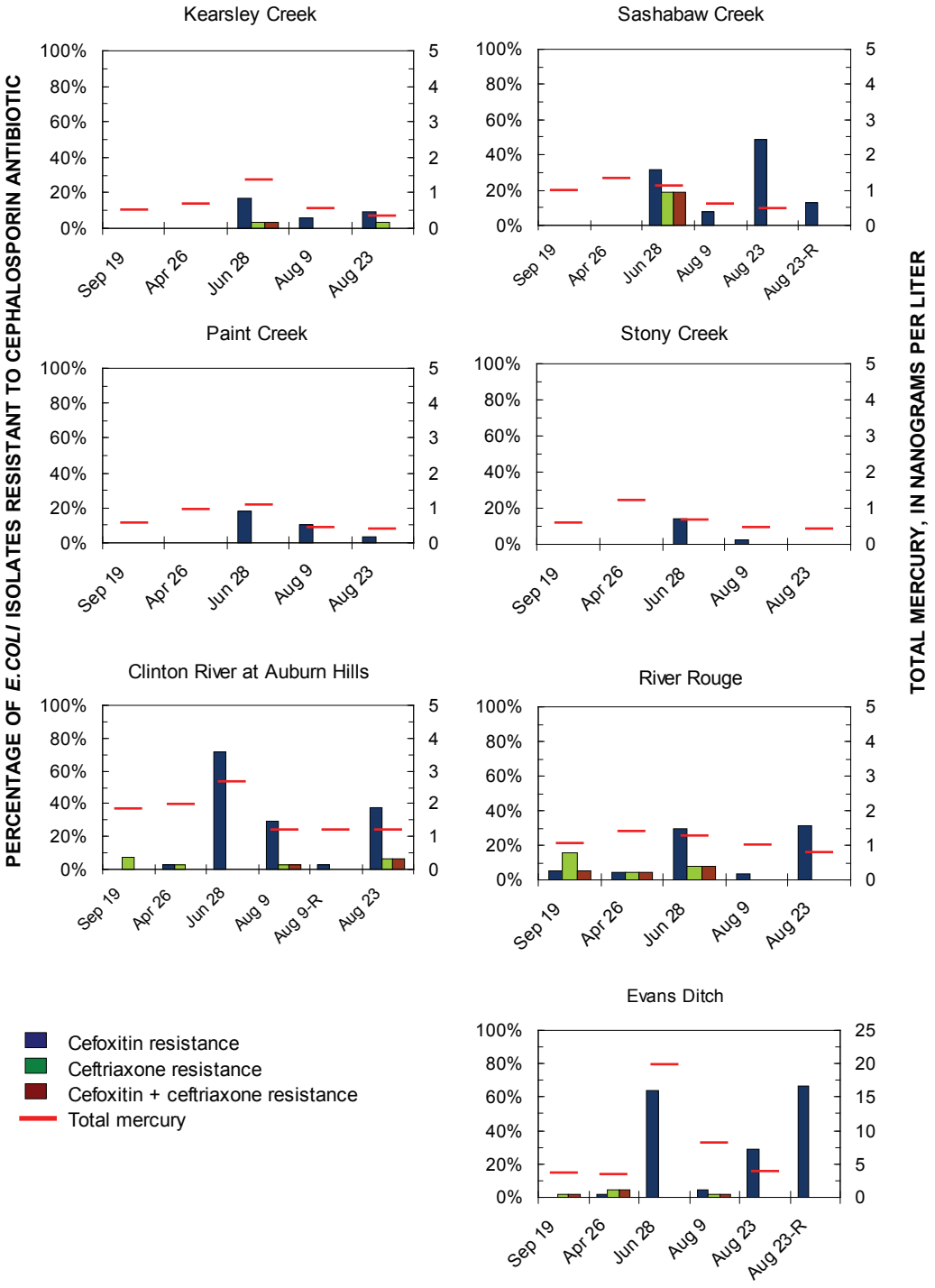

Figure 6. Percentage of cephalosporin-resistant *E. coli* isolates and total mercury concentration for samples collected on five sampling dates at Oakland County study sites.

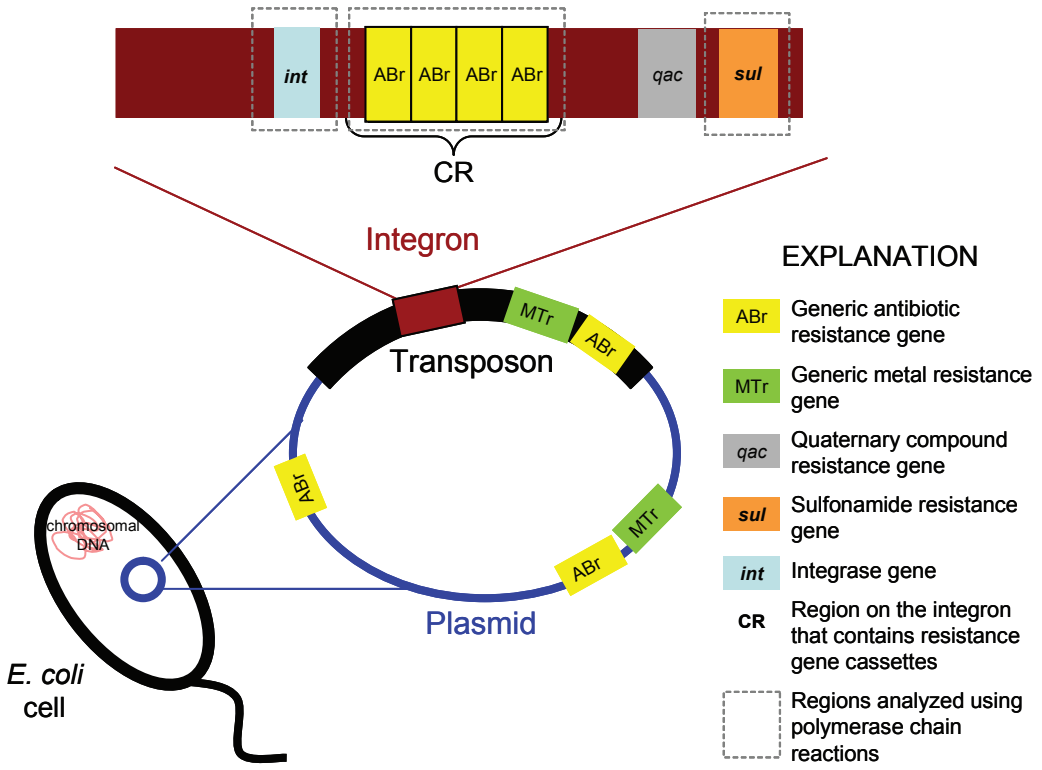

Figure 7. Schematic of the relation of transferable genetic elements: plasmid, transposon, integron, and resistance genes. Plasmids are circular pieces of DNA that can move from cell to cell. Plasmids commonly contain genes for antibiotic resistance or other resistance such as to heavy metals, as well as transposons, which also may contain resistance genes. In particular, integrons—located on the transposon—may carry multiple resistance genes and are commonly associated with a sulfonamide resistance gene (sul) and a gene for resistance to quaternary ammonia compounds (qac). The presence of the integrons can be detected by use of polymerase chain reaction (PCR) to amplify particular regions on the integrons.

The presence of class I integrons at study sites was determined by use of PCR to detect key components of the integron: the integrase gene (*int*1) required for incorporation of gene cassettes, a sulfonamide resistance gene (*sul*1) commonly found at the end of integrons, and the cassette region (CR) in which resistance genes are incorporated. All FC cultures from September, June, and August were analyzed for the presence of these three components of the integron (CR, *int*1, and *sul*1). *E. coli* isolates tested were those from April and August 23 that were resistant to either erythromycin or ofloxacin, and all mercury-resistant *E. coli* isolated from April were analyzed for the present of CR and *int*1. The *sul*1 gene was not analyzed for in these isolates.

The presence of CR, *int*1, and *sul*1 is a clear indication of the presence of integrons in these samples. Of the 29 FC samples analyzed, 8 contained all three components of the integron (table 12). All three components were detected at least once from all sites but Sashabaw Creek. The presence of a *sul*1 gene has been shown to be commonly associated with integrons but is not critical to integron function; however, the *int*1 gene is required for the incorporation of gene cassettes onto the integron. Therefore, samples such as the Clinton River at Auburn Hills (Aug. 9), in which no *sul*1 gene was detected but the *int*1 and CR were detected, still indicate the potential for antibiotic resistance and transferability of resistance within fecal coliform populations.

Because the FC cultures represent a large community of organisms, individual *E. coli* isolates were selected on the basis of resistance to erythromycin, ciprofloxacin, or mercury for integron analyses. Fourteen mercury-resistant *E. coli* isolated from April were tested for the presence of integrons; one resulted in a positive CR and *int*1 detection (Evans Ditch). Of the 16 erythromycin- or ofloxacin-resistant *E. coli* isolates tested, 3 isolates—all from Evans Ditch—were positive for the CR. No antibiotics were detected at the Evans Ditch site.

Further analysis would be needed to determine which genes are present to determine if resistance to cephalosporin or other antibiotics is the result of a gene carried on the integrons or if the presence of the integrons is only a coincidence. These results indicate that integrons are present in these waters and could be a mechanism for transfer of resistance to other organisms, including pathogens. Further analyses would need to be done to determine precisely the resistance genes contained on the class I integron structure and the ability to transfer to pathogens.

Table 12. Results of polymerase chain reactions (PCR) for integron structures in samples collected September 2005 through August 2006 at Oakland County study sites.

[USGS, U.S. Geological Survey; PCR, polymerase chain reaction; *int*1, integrase gene; *sul*1, sulfonamide resistance gene; 5´-3´, 5 prime to 3 prime conserved region; +, positive reaction; -, negative reaction; NT, not tested]

USGS station number	Site name	Sample-collection date	PCR products		
			*int*1	*sul*1	integron (5′-3′)
04148035	Kearsley Creek at Mill Street at Ortonville	09/19/05	-	NT	-
		06/28/06	+	+	+
		08/09/06	+	+	+
		08/23/06	-	-	-
04160800	Sashabaw Creek near Drayton Plains	09/19/05	-	NT	-
		06/28/06	+	-	-
		08/09/06	-	-	-
		08/23/06	-	+	-
04161000	Clinton River at Auburn Hills	09/19/05	+	NT	-
		06/28/06	+	+	+
		08/09/06	+	-	+
		08/23/06	+	-	-
04161540	Paint Creek near Rochester	09/19/05	-	NT	-
		06/28/06	+	+	+
		08/09/06	+	+	-
		08/23/06	+	+	+
04161580	Stony Creek near Romeo	09/19/05	+	NT	-
		06/28/06	-	-	-
		08/09/06	+	-	-
		08/23/06	+	+	+
04166000	River Rouge at Birmingham	09/19/05	+	NT	-
		06/28/06	+	-	+
		08/09/06	-	-	-
		08/23/06	+	+	+
04166200	Evans Ditch at Southfield	09/19/05	+	NT	+
		06/28/06	+	+	-
		08/09/06	+	+	+
		08/23/06	+	-	-
	Drain at Evans Ditch	06/28/01	+	+	+

Results From Clinton River Watershed Study

To determine whether results from streams of Oakland County were representative of conditions across a broader region, samples were collected throughout the Clinton River Watershed on April 26 and August 23, 2006. Fecal indicator bacteria concentrations, mercury concentrations, and the percentage of cephalosporin-, vancomycin-, and mercury-resistant isolates were analyzed for an additional 12 sites in the watershed (fig. 2). *E. coli* and enterococci concentrations for sites in Oakland County were similar to concentrations at sites outside of Oakland County (figs. 8 and 9).

No VRE was detected in any water samples collected on these two dates. The percentage of cefoxitin- and ceftriaxone-resistant *E. coli* and total mercury concentration are shown in figure 10. At sites outside Oakland County, the percentage of cephalosporin-resistant *E. coli* and mercury concentrations were as high as or higher than those at sites in Oakland County, with the percentage of antibiotic resistance and mercury concentrations being higher in the more industrial or urbanized areas.

The percentage of cefoxitin-resistant *E. coli* appears to be less in April then in August (fig. 11). April samples were collected from 24 to 72 hours after small rainfall events in the area (fig. 5), whereas August samples were collected during a very dry period. As a result, April streamflows were much higher than those in August. Wet weather (and as a result, more runoff into the streams) may tend to decrease the resistant *E. coli* populations. However, these results are not supported by the more intensive sampling done in Oakland County, in which the percentage of resistance was not correlated with rainfall or streamflow.

Very few ceftriaxone-resistant *E. coli* were isolated in the larger watershed study (fig. 12). All resistant isolates were from sites in the more urban or industrialized areas in the watershed (figs. 2 and 12). The highest percentage of ceftriaxone-resistant *E. coli* was found at Red Run and Clinton River at Fraser (fig. 12). At Red Run, the ceftriaxone-resistant *E. coli* were detected only in April, and at Clinton River at Fraser, the percentage of ceftriaxone-resistant *E. coli* was greater in April than in August. These results are opposite to the percentage of cefoxitin-resistant *E. coli* results presented earlier, an indication of different sources of *E. coli* for these two time periods.

Results of this watershed-focused study demonstrate that the cephalosporin-resistant *E. coli* results for Oakland County are not highly local phenomena. In fact, similar results were found throughout a watershed spanning multiple counties. The results of the watershed-based study also confirm earlier findings that areas with greater urban or industrial development pose an increased probability of detecting cephalosporin-resistant *E. coli* when compared to less developed areas.

View of Sashabaw Creek near Drayton Plains, Mich.

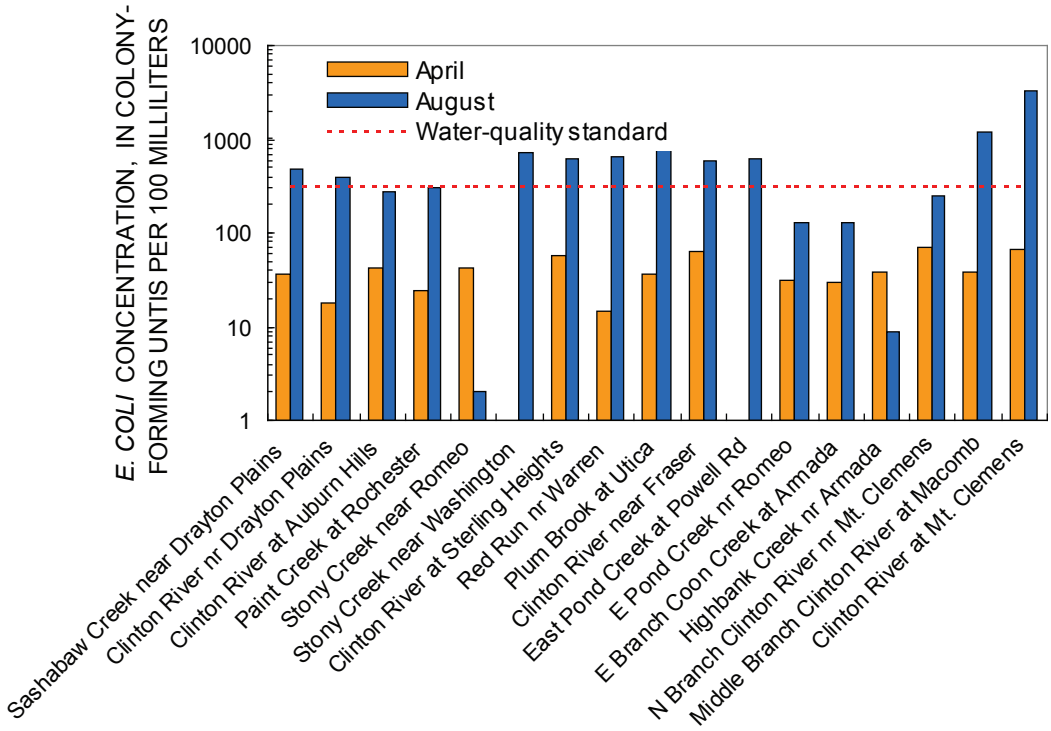

Figure 8. *E. coli* concentrations for samples collected April 26 and August 23, 2006, at Clinton River Watershed study sites.

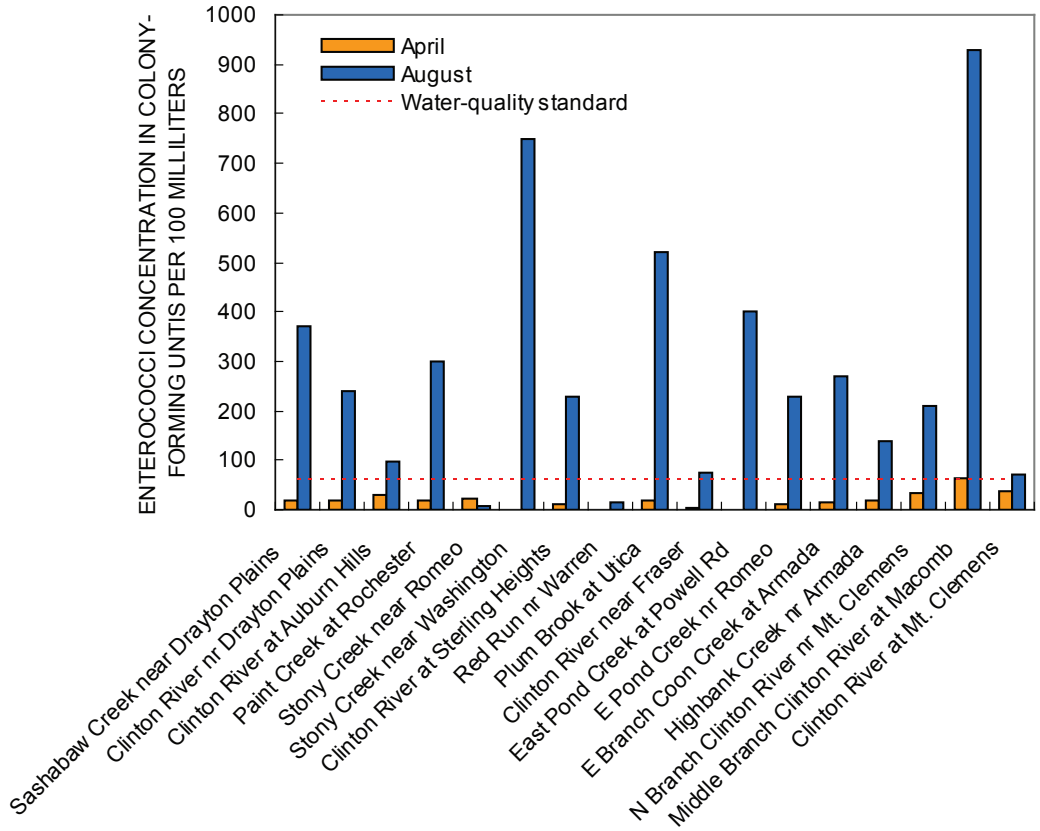

Figure 9. Enterococci concentrations for samples collected April 26 and August 23, 2006, at Clinton River Watershed study sites.

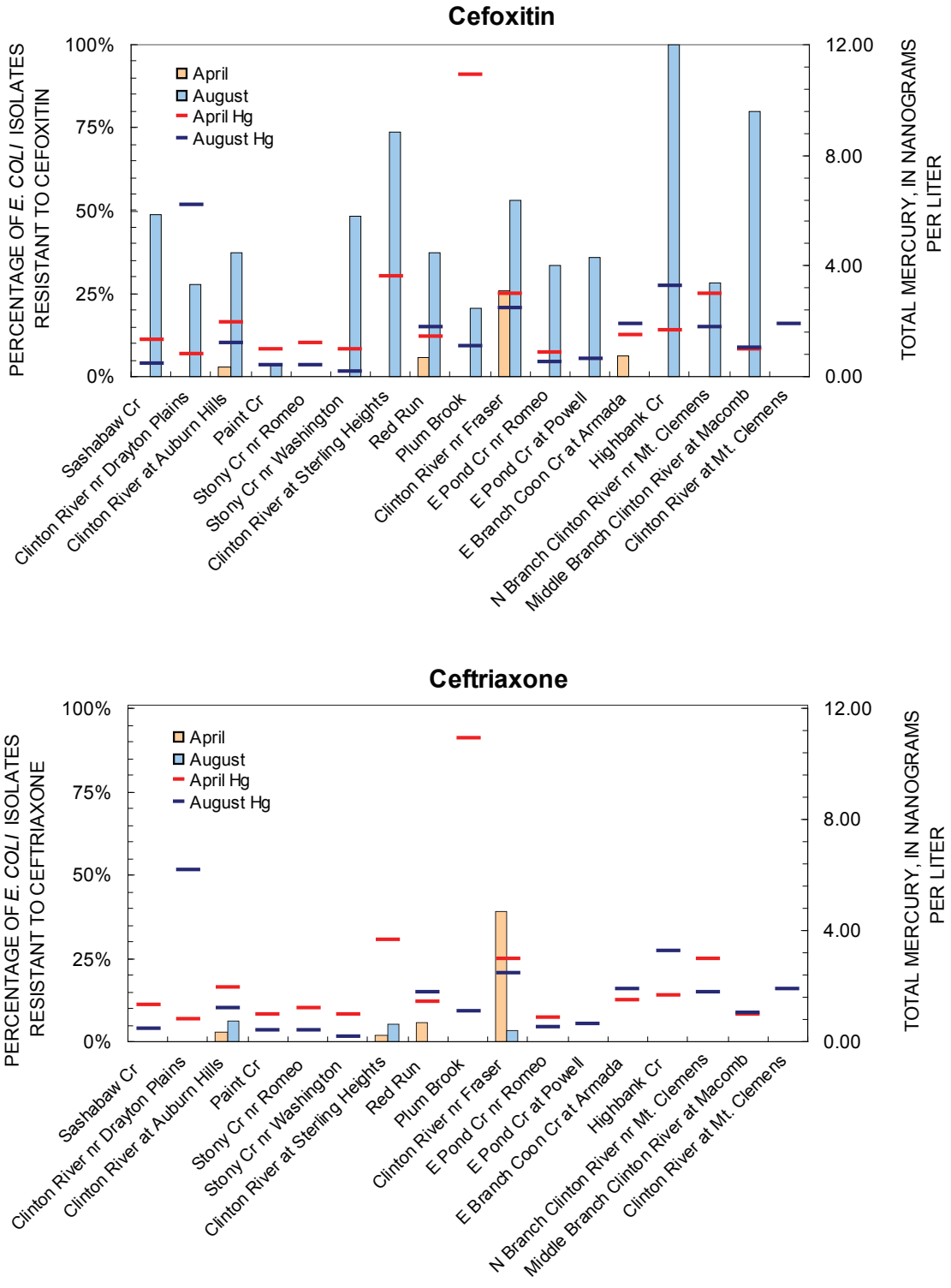

Figure 10. Percentage of *E. coli* isolates resistant to cefoxitin (top) and ceftriaxone (bottom) with respect to total mercury concentration for Clinton River Watershed study sites.

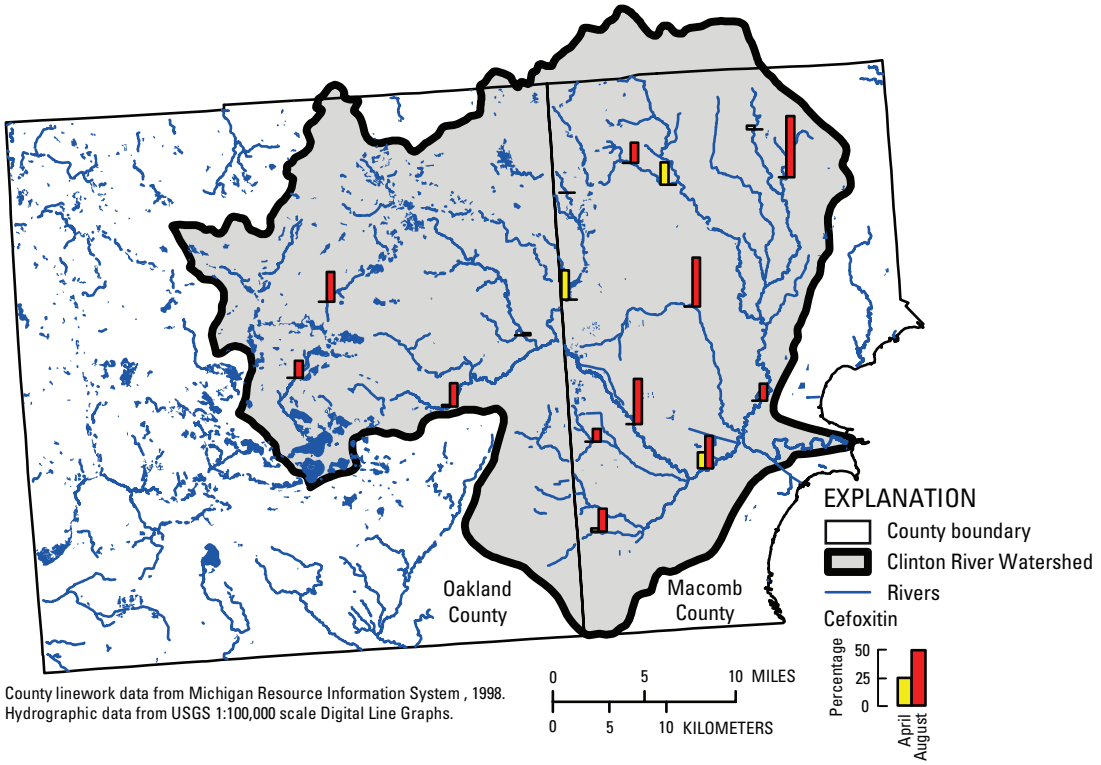

Figure 11. Percentage of *E. coli* isolates resistant to cefoxitin for samples collected April 26 and August 23, 2006, at the Clinton River Watershed study sites.

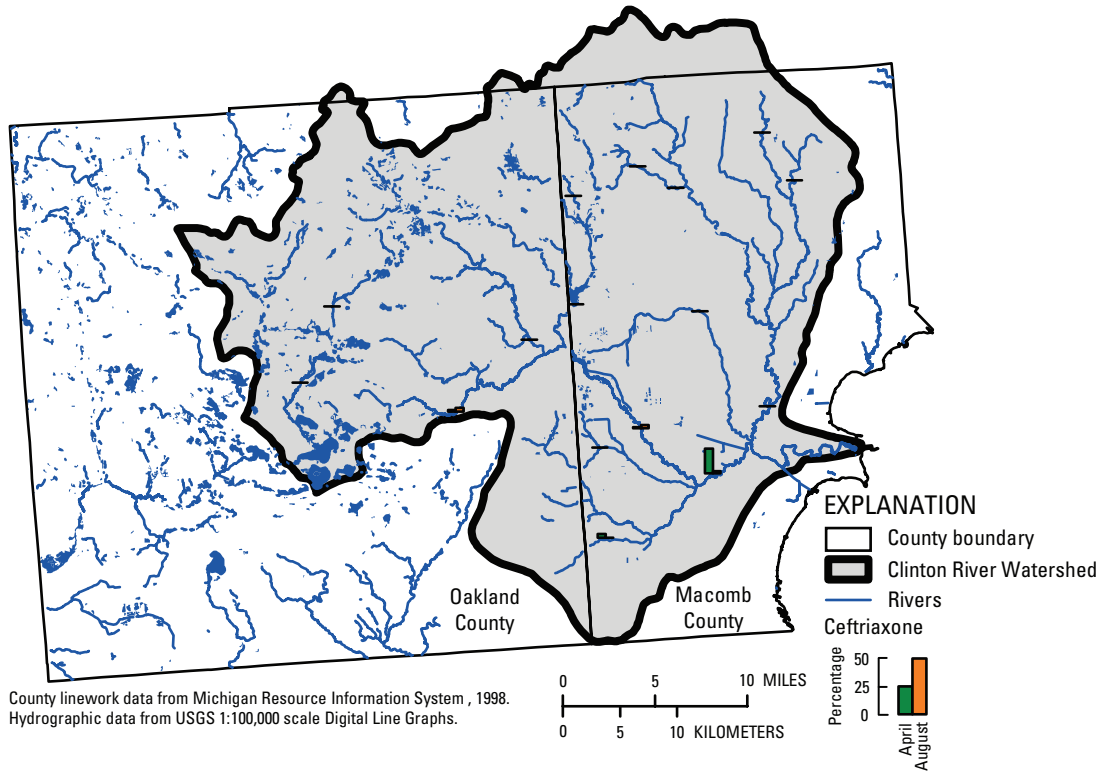

Figure 12. Percentage of *E. coli* isolates resistant to ceftriaxone for samples collected April 26 and August 23, 2006, at the Clinton River Watershed study sites.

Future Studies

This study showed that cephalosporin-resistant *E. coli* could frequently be isolated from streams in Oakland County and the surrounding area. This study was not able to identify the source of resistant isolates. The prevalence of resistant bacteria appears to be highly variable and site dependent. To better understand the significance of the problem, future researchers would need to intensively monitor the sites for not only cephalosporin-resistant *E. coli* but also other potentially significant antibiotic resistance, such as flouroquinolone resistance. In addition, water-quality information, such as concentrations of organic constituents and/or trace metals other than mercury, may help to determine whether water-quality components may contribute to increased resistance. Sediment, which could be a source of mercury exposure to bacteria, was not sampled in this study. Organisms may develop resistance in the sediment and then be resuspended into the water column. A more thorough investigation of potential sources such as wastewater, animal feces, urban runoff, and stormwater is warranted in order to better understand the source and transport mechanisms of antibiotics, antibiotic-resistant bacteria, and other contaminants in Oakland County and surrounding areas. Finally, a better understanding of how cephalosporin-resistant *E. coli* isolated from the environment are related to isolates that cause human illness would help to define the significance these results have to human health.

Summary and Conclusions

Previous water-quality studies in Oakland County, Mich., revealed high fecal indicator bacteria concentrations and the presence of antibiotic-resistant fecal bacteria in stream water. Enterococci resistant to vancomycin and fecal coliform bacteria resistant to multiple antibiotics, including cephalosporin antibiotics, were isolated in stream waters throughout the county.

Vancomycin and cephalosporin antibiotics are used in the clinical setting to treat serious human illnesses. Vancomycin-resistant enterococci and cephalosporin-resistant *E. coli* have been listed as an emerging resistance of human health concern in hospitals. Bacteria normally sensitive to these antibiotics can become resistant by the acquisition of antibiotic-resistance genes by transfer from one organism to another. If bacteria that cause illness become resistant to antibiotics, then treatment of the illness becomes more difficult and treatment options more limited. Because the surface waters in Oakland County serve as an important resource of the county, antibiotic-resistant bacteria in stream waters may pose a risk to human health for those who use these resources.

This current USGS study, in cooperation with the Oakland County Health Department, continued sampling to determine the presence of fecal indicator bacteria, cephalosporin-resistant *E. coli,* and vancomycin-resistant enterococci. One objective of this current (2005–2006) study was to determine whether results obtained in the previous study could be replicated and whether water samples collected under different streamflow conditions and other times of the year would yield similar results. In addition, this current study expanded the sampling to measure antibiotic concentrations and mercury to determine whether these concentrations were related to antibiotic-resistant bacteria also found in these samples. This relation may help explain the link to the mechanism of transfer and maintenance of antibiotic-resistant bacteria in stream waters.

The acquisition of genes responsible for antibiotic resistance may aid in the spread and maintenance of antibiotic-resistant bacteria. Genes responsible for resistance to chemicals, metals, or antibiotics are sometimes linked together on plasmids, transposons, and integrons. Therefore, exposure to any one of the compounds may result in transfer of these elements from organism in the environment that already have it to other organisms who would have otherwise been sensitive. This serves as a survival mechanism for bacteria in the environment. And because these genes are linked together, the receiving organisms could be resistant to all compounds in which there was a resistant gene on the element it received. Both antibiotics and mercury (a heavy metal that has been commonly associated with antibiotic resistance genes) were measured in this study. This is only a short list of compounds that could be linked to antibiotic resistance; therefore, there may be other compounds present at these site that are related to antibiotic-resistance results observed in this study.

In the current study, fecal-indicator bacteria concentrations frequently exceeded the Michigan Recreation Water Quality Standard of 300 colony-forming units per 100 milliliters. High fecal bacteria concentrations are an indicator of possible fecal pollution that may carry harmful pathogens and pose a threat to human health. Fecal bacteria can be exposed to antibiotics at their source and in the environment; therefore, waters with increased fecal bacteria may also contain higher levels of antibiotic-resistant bacteria. Cephalosporin-resistant *E. coli* were detected in all surface waters tested in Oakland County.

Interpretation of the percentage of the population resistant to antibiotics in this study is limited by the inherent variability in laboratory results and the number of samples available for analysis. Therefore, actual concentration at a given site is speculative and should be used only as guidance when interpreting the results of the study.

Despite being detected in previous studies, vancomycin-resistant enterococci (VRE) were not detected at any Oakland County site during this study. Intermediate resistance to vancomycin was detected at two Clinton River Watershed study sites on one sampling date, but the resistant bacteria did not carry genetic elements that could be transferred to other organisms. The source of VRE detected in 2003 is uncertain, and it is possible that specific instances of VRE may occur in the watershed at times and/or places that were not reflected in the current sampling.

E. coli isolated in this study were tested for resistance to two cephalosporin antibiotics used primarily for treatment of human disease (cefoxitin and ceftriaxone). *E. coli* resistant to one or both of these antibiotics were detected at all study sites in Oakland County and sites outside of the county within the Clinton River Watershed. However, there was no indication that an increase in fecal indicator bacteria led to increased percentages of antibiotic-resistant bacteria.

The percentage of cefoxitin- and ceftriaxone-resistant bacteria varied throughout the year at each site in this study. Although analytical variability makes it difficult to interpret the magnitude of variation in the proportion of antibiotic-resistant isolates in environmental samples, resistant *E. coli* were persistently present at the more urbanized sites but were less common at the less urbanized sites, both within in Oakland County and throughout the Clinton River Watershed. This pattern indicates the source of this resistance may be a result of human or urban impacts such as wastewater from wastewater-treatment plants or failed septic systems, urban runoff, industrial discharges, urban animal populations (domestic animals and birds), and so forth.

Rainfall and seasonal patterns were examined to determine whether the percentage of antibiotic-resistance bacteria is a result of runoff or stormwater entering the stream or whether a level of resistance is fairly constant at a site at low flows but is changed by dilution from increased water inputs. The Clinton River Watershed results suggest a possible seasonal or rainfall-related effect on the cefoxitin and ceftriaxone resistance due to the dominant presence on only one sampling date (August for cefoxitin and April for ceftriaxone). However, this general pattern was not observed in the more intensively monitored Oakland County sites, where differences in the percentage of resistant isolates were site specific.

Results of this study indicate that a more intensive, site-specific study would be required to address the source or sources of antibiotic-resistant bacteria in Oakland County streams. It cannot be determined from the current data whether antibiotic-resistant isolates found during the current study entered water bodies directly from wastewater discharge or acquired resistance after being exposed to chemicals or metals in the water or sediment.

The only two sites with detectable antibiotic concentrations were the Clinton River at Auburn Hills, where several antibiotics were consistently detected, and Paint Creek at Rochester, at which only one antibiotic (tylosin) was detected on one sampling date at a very low concentration. Samples that contained detectable concentrations of antibiotics were not necessarily correlated with antibiotic-resistant *E. coli*. Although the percentage of cephalosporin-resistant *E. coli* was more consistent at Clinton River at Auburn Hills than at the Kearsley Creek, Sashabaw Creek, Paint Creek, and Stony Creek sites, the percentage was no greater or more consistent than for samples collected from the other urbanized locations (River Rouge and Evans Ditch). There was no strong evidence that antibiotics detected in the environment are related to cephalosporin, erythromycin, or ofloxacin-resistant *E. coli* in the same environment.

Mercury was detected in samples from every site. Concentrations of total mercury ranged from a low of 0.34 ng/L at Kearsley Creek to a high of 19.8 ng/L at Evans Ditch. Mercury concentrations were greater than the Michigan Rule 57 water-quality standard of 1.3 ng/L at two sites, Clinton River at Auburn Hills and Evans Ditch at Southfield. The highest percentage of cefoxitin-resistant *E. coli* occurred in the same sample as the highest detectable concentration of mercury (Evans Ditch at Southfield, June 28, 2006). Although some of the highest mercury concentrations were detected in samples with the highest percentage of cephalosporin-resistant *E. coli,* there was no linear relation of mercury concentration to percentage of antibiotic-resistant *E. coli*.

The absence of a relation between mercury and antibiotic concentrations in the water indicates that exposure to mercury and antibiotics in the water column may not be responsible for cephalosporin resistance in these samples. However, bacteria, metals, and antibiotics may co-occur in source material (such as a wastewater-treatment effluent) and encourage the transfer of resistance genes among organisms. Because the fate and transport mechanisms of bacteria, metals, and organics may vary, these varied constituents may be differentially dispersed when they enter the stream, which may explain the absence of observable relation between the percentage of antibiotic-resistant organisms and mercury or antibiotic concentration for samples collected in this study.

The detection of genes that are part of the class I integron suggests that antibiotic resistances in bacteria in the environment may be transferred to other bacteria, including pathogens. Class I integron structures may carry and transfer multiple resistance genes from one organism to another. These structures were present in most samples, indicating that they may play a role in the transfer and maintenance of resistance in bacteria isolated from the environment. These structures would need to be further characterized to determine which resistance genes are present and how the environment would aid in the transfer of resistance among bacteria. Specifically, a better understanding on the relation between the presence of integrons and other organic contaminants or metals in both the water column and sediment would help to determine whether antibiotic resistance was being acquired by organisms as a result of contamination in the environment.

Current results do not identify a source of high fecal bacteria or antibiotic-resistant bacteria concentration in Oakland County. There were indications that human activities may, in part, explain the detection of antibiotic resistance because of the more persistent detections in urbanized areas. In addition, a gene that has been used to identify human fecal pollution was detected at sites in the county, suggesting that the sites were being affected by human fecal waste. Nevertheless, effluent from a wastewater-treatment plant did not seem to increase the prevalence of antibiotic-resistant *E. coli* downstream compared to similar sites not directly downstream from a known wastewater input.

Studies by the National Antimicrobial Resistance Monitoring System (NARMS) have detected very few human pathogenic *E. coli* isolates with resistance to either cefoxitin or ceftriaxone. Despite being approved only for human medicine, a greater percentage of *E. coli* isolated from veterinary samples were found to be resistant to either cefoxitin or ceftriaxone than in the human pathogenic *E. coli* study. It is possible that other environmental or natural factors may result in the presence of cephalosporin-resistant bacteria in the environment.

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