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Antibiotic Resistance, Gene Transfer, and Water Quality Patterns Observed in Waterways near CAFO Farms and Wastewater Treatment Facilities

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Abstract We examined water quality indicators (pH, temperature, turbidity, total phosphorus, and fecal coliform density) and bacterial antibiotic resistance (prevalence, conjugative transfer, and genetic linkage of resistance elements) at locations impacted by confined animal feeding operations (CAFOs) and compared them to nearby reference sites. Sites located upstream and downstream of two wastewater treatment facilities were also compared. Sites near CAFO farms had poor water quality (elevated total phosphorus and turbidity), while water quality remained relatively good downstream of wastewater treatment plants. High proportions of antibiotic-resistant bacteria were observed at all study sites, and frequent conjugative transfer of resistance was observed in laboratory assays. Out of a total of 830 environmental bacterial isolates, 77.1% were resistant to only ampicillin, while 21.2% were resistant to combinations of antibiotics including ampicillin (A), kanamycin (K), chlorotetracycline (C), oxytetracycline (O), and streptomycin (S). Multi-drug-resistant bacteria were significantly more common at sites impacted by CAFO farms. In conjugation assays, 83.3% of the environmental isolates transferred one or more antibiotic resistance genes to a laboratory strain of *Salmonella typhimurium*. A subset of multi-drug-resistant (A, C, and O) isolates was screened for specific tetracycline resistance genes and class I and II integrons. None of the screened isolates (n=22) were positive for integrons, while 13 isolates contained resistance genes for *tet* (B) and *tet* (C). Our results indicate that CAFO farms not only impair traditional measures of water quality but may also increase the prevalence of multi-drug-resistant bacteria in natural waters.

Keywords Antibiotic resistance · Conjugation · Confined animal feeding operations (CAFOs) · Fecal coliforms · Water pollution · Water quality

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1 Introduction

Pollution by human and animal wastes is a common threat to water resources. Untreated human sewage frequently enters waterways as point source discharges (intentional or accidental) or via combined sewer overflows (CSO) from wastewater treatment plants (WWTPs; Stoner 2005; Whitlock et al. 2002; Wiggins et al. 1999). For example, from January 1, 2002, to December 31, 2005, WWTPs across Michigan reported having 2,542 CSO events, totaling 270 billion liters of discharged sewage (Michigan Department of

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Environmental Quality 2004a, b, 2005). Animal waste seeps into surface water as non-point source pollution as a result of housing large numbers of animals in confined areas, or as runoff from liquid manure applications onto croplands (a primary method of disposing of the waste generated by confined animal feeding operations (CAFOs); Carpenter et al. 1998; Esiobu et al. 2002).

Both human and animal wastes add nutrients (e.g., nitrogen, phosphorus; Carpenter et al. 1998; Tabbara 2003) as well as fecal coliform bacteria (Esiobu et al. 2002) to receiving waters. Excessive nutrients promote eutrophication, which decreases water transparency, creates foul odor and taste, depletes oxygen, causes fish kills, reduces biodiversity, and decreases esthetic, recreational, and property values along waterways (Boesch et al. 2001; Carpenter et al. 1998; Wetzel 2001). To reduce eutrophication, nutrient levels are often controlled via regulation and mitigation treatments (e.g., Carpenter et al. 1998; Cooke et al. 2005; Wetzel 2001). Human and animal wastes may also contaminate waterways with pathogenic bacteria (Burton and Engelkirk 2004; Guan and Holley 2003; Pell 1997). To avoid the difficulty and expense of direct testing for all possible types of pathogenic bacteria transmittable by feces, standard microbiological examination of water centers on determining the abundance of indicator bacteria, such as the fecal coliform bacteria (American Public Health Association 1998; Griffin et al. 2001). These bacteria are commonly found in the gut and feces of warm-blooded animals, and their presence in water environments can indicate contamination with human or animal feces (and by extension, contamination of water by pathogenic bacteria possibly present in feces; American Public Health Association 1998; Ash et al. 2002; Chao et al. 2003; Griffin et al. 2001). The greater the fecal coliform count, the greater the probability there is of contracting diseases from waterborne pathogenic bacteria (Mitchell and Stapp 1997). In Michigan, the Michigan Department of Natural Resources and Environment (MDNRE, formerly Michigan Department of Environmental Quality) monitors surface waters for a specific fecal coliform bacterium (*Escherichia coli*), rather than the entire fecal coliform group (Michigan Department of Environmental Quality 1999). In contrast, some other states in this region monitor fecal coliforms in surface waters. Both Ohio and Illinois require that surface waters used for extensive contact (e.g., swimming) have no more than 10% of samples exceeding a maximum fecal coliform count of 400 colony-forming units per 100 ml (Illinois Pollution Control Board 2002).

Antimicrobial agent resistance is an emerging global concern to both public and veterinary health. Antibiotics are heavily used to treat disease in both humans and animals, and there is a pattern of antibiotic resistance and transfer emerging among bacterial populations in proportion to the use of antibiotics, especially in agriculture (Levy 1997; Oppegaard et al. 2001). Antibiotics have been added to animal feed as growth promoters for some time, and the animal production industry has been identified as a potential reservoir for resistant *Enterobacteriaceae* (Witte 1997). The use of antibacterial drugs for prophylactic or therapeutic purposes in humans and for veterinary and agricultural purposes has provided selective pressure favoring the survival and spread of resistant organisms (Hooper 2002; Mascaretti 2003; Taber 2002). These resistant bacteria may transfer their resistance to previously non-resistant pathogenic bacteria or directly infect humans with bacterial diseases that cannot be treated by conventional antimicrobial therapies (Khachatourians 1998). The potential for antibiotic exposure and resistance development in human and animal gastrointestinal tracts, coupled with relatively great abundance in waters contaminated with human and animal waste, makes the fecal coliform bacteria a logical focal group for studies of antibiotic resistance and transfer in aquatic environments.

Despite active research investigating how antibiotic resistance enters and is maintained in the environment (D'Costa et al. 2006; Esiobu et al. 2002; Graves et al. 2002; Iwane et al. 2001; Rice et al. 1995; Sayah et al. 2005; Schwartz et al. 2003; Whitlock et al. 2002; Witte 1997), determining the mechanisms by which bacteria can transfer resistant genes within and across species (Arana et al. 1997, 2001; Bell et al. 1980; Oppegaard et al. 2001; Salyers et al. 2002), and identifying and characterizing new antibiotic resistance genes (Aminov et al. 2001, 2002; Chopra and Roberts 2001; Davies 1997; Gevers et al. 2003; Roberts 2005) and integrons (France et al. 2005; Hall 1997; Hall et al. 2003), the global ecological impact of antibiotic resistance and risks to human and veterinary health are yet to be determined. In the USA, there are

no national or state water quality regulations requiring testing or reporting on antibiotic-resistant organisms (American Public Health Association 2005; Michigan Department of Environmental Quality 1999; US Environmental Protection Agency 2002).

In order to understand how CAFOs and WWTPs affect bacterial antibiotic resistance and transfer, we simultaneously conducted traditional water quality measurements (pH, temperature, turbidity, total phosphorus, and fecal coliform density) and microbiological and molecular techniques for determining patterns of antibiotic resistance and potential transfer of resistance genes at CAFO-impacted and unimpacted sites and at sites upstream and downstream of WWTPs. We also examined the relationships between traditional water quality parameters and conducted a preliminary analysis of the genetic location and mode of transfer of antibiotic resistance elements.

2 Materials and Methods

2.1 Study Design and Site Descriptions

Ten sites were selected for this study, based on the presence of previously documented point source and non-point source contamination (see following section) and proximity to public access (Table 1). Six sites were located in an agricultural region near Hudson, MI. Three of the agricultural sites were waterways approximately 1–2.5 km from different CAFO farms and were classified as agriculturally impaired (AI) based on the following criteria: (1) the waterways received direct runoff from fields sprayed with liquid manure from CAFO farms; (2) previous observations of fecal coliform densities greater than 1,000 colony-forming unit (CFU)/100 ml (Khachatourians 1998; J. Pernicano personal communication); (3) ratings of "poor" on biological (macroinvertebrate) assessments of these waterways (Michigan Department of Environmental Quality 2003a); and (4) the MDNRE listing these waterways on their non-attainment and total maximum daily load lists (Michigan Department of Environmental Quality 2004b). Three additional agricultural sites located in the same farming region were classified as agriculturally unimpaired (AUI) since they did not receive direct runoff from manure-sprayed fields and were not located near a CAFO farm. Two of these three sites (sites SJT8 and SJC7) have been classified by MDNRE as "least impacted" streams in this location and have been previously identified as reference streams (Michigan Department of Environmental Quality 2003a). Four additional sites were selected approximately 1–1.5 km both upstream and downstream of the Ann Arbor, MI, and Chelsea, MI, WWTPs in the Huron River watershed. Like all WWTPs, these facilities occasionally release partially treated and untreated sewage. The most recent such episode prior to this study was the release of ~50 million liters of sewage by the Ann Arbor WWTP in August 2003 (Michigan Department of Environmental Quality 2003b).

2.2 Sampling and Physical and Chemical Water Quality Procedures

The initial and final sampling dates (June 3, 2004, and August 9, 2004, respectively) were chosen during distinct dry periods (zero precipitation measured within the proceeding 72 h), and the middle three sampling periods (June 16, 2004, July 7, 2004, and July 18, 2004) occurred after rainstorms where >1 cm of precipitation was recorded within 24 h (Michigan State Climatology, http://climate.geo.msu.edu/Semcog/SEMdaily/2004/, SEMCOG Daily Precipitation Summary). Site VH25 was completely dry by the end of the study, so this site was sampled on only four out of five possible dates.

At each site, a handheld probe was used to measure pH and temperature (YSI 63, Yellow Springs Instrument Co., Yellow Springs, OH). Turbidity was measured in the field using a Hach 2100P portable turbidimeter. For total phosphorus measurements, 100 ml of water was collected into an acid-washed Nalgene bottle and stored at −20°C for later analysis using the protocol of Wetzel and Likens (2000). For fecal coliform isolation, 100 ml of water was collected into two sterile 50-ml Falcon conical tubes and kept on ice (<8 h) until it was processed in the laboratory (Harwood et al. 2000). All water samples were obtained by submerging the collection container approximately 1 cm below the water surface before opening the lid to collect the sample.

Fecal coliform density was determined as follows: water samples of various volumes (100, 1.0, 0.1, and 0.01 ml) were brought to a final volume of 100 ml with 0.01 M phosphate-buffered saline (0.138 M

Table 1 Site descriptions: designations, locations, and average discharge

Site name	Study site designation	Watershed	Latitude/ longitude	Road crossing	Average discharge[a] (cubic meters per second)
Vanderhoff-Haley (VH19) Rice Lake Drain	AI	River Raisin	41° 49′ 69″ N 084° 13′ 24″ W	Haley Rd.	ND
Vreba-Hoff I (VH21) Medina Drain	AI	Bean-Tiffin	41° 48′ 17″ N 084° 17′ 58″ W	Ingall Hwy	ND
Vreba-Hoff II (VH25) Lime Creek	AI	Bean-Tiffin	41° 47′ 37″ N 084° 22′ 39″ W	Lime Lake Rd.	ND
Hazen Creek (HC1)	AUI	River Raisin	41° 55′ 69″ N 084° 15′ 71″ W	Burton Rd.	ND
Unnamed Tributary to St. Joseph Creek (SJT8)	AUI	Bean-Tiffin	41° 52′ 29″ N 084° 25′ 20″ W	Waldron Rd.	ND
St. Joseph Creek (SJC7)	AUI	Bean-Tiffin	41° 52′ 70″ N 084° 25′ 20″ W	Waldron Rd.	ND
Mill Creek upstream from WWTP (MC1)	UPWWTP	Huron River	42° 19′ 54″ N 084° 1′ 06″ W	McKinley Rd.	2.2
Huron River upstream from WWTP (HR1)	UPWWTP	Huron River	42° 20′ 19″ N 083° 52′ 30″ W	Zeeb Rd.	19.2
Mill Creek downstream from WWTP (MC2)	DNWWTP	Huron River	42° 19′ 33″ N 083° 58′ 77″ W	Chelsea-Dexter Rd.	2.2
Huron River downstream from WWTP (HR2)	DNWWTP	Huron River	42° 15′ 23″ N 083° 37′ 30″ W	Huron Parkway	19.2

AI agriculturally impaired, *AUI* agriculturally unimpaired, *UPWWTP* upstream from wastewater treatment plant, *DNWWTP* downstream from wastewater treatment plant, *ND* no data for that body of water

[a] Source: USGS National Water Information System: Web Interface, http://nwis.waterdata.usgs.gov/mi/nwis/annual/ (Ann Arbor gauging station, site number 04174500) and Mill Creek near Dexter, MI (site number 04173500)

NaCl and 0.0027 M KCl, pH 7.4 at 25°C) and filtered onto a 0.45-μm filter (Pall Life Sciences, East Hills, NY) using a vacuum system as described by Mitchell and Stapp (1997) and the Standard Methods for the Examination of Water and Wastewater (American Public Health Association 1998). The filter membranes were transferred to Petri plates (BD Falcon, BD Biosciences, Bedford, MA) containing fecal coliform selective agar (modified fecal coliform, mFC; BBL, BD Biosciences, Franklin Lakes, NJ) without antibiotics and were incubated at 44.5°C in a humidified incubator for 24 h. For the first sampling date (June 3, 2004), 100-, 0.1-, and 0.01-ml volume water samples were used; filtration of 100 ml yielded colonies with confluent growth, while the low-volume samples (0.1 and 0.01 ml) had no growth. As a result, fecal coliform densities from June 3, 2004, were not considered in any further analyses, and volumes of 100 and 1 ml were analyzed for the remaining four sampling dates (June 17, 2004, July 7, 2004, July 18, 2004, and August 9, 2004). After incubation, bacterial density (CFU/per 100 ml) was determined for each site on each sampling using the colony counts from either the 100- or 1-ml filtered plate, depending on which plate produced countable isolated colonies.

2.3 Bacterial Isolation and Antibiotic Resistance Susceptibility

Individual colonies were picked from distinctly isolated typical colonies as recommended by the Standard Methods for the Examination of Water and Wastewater (American Public Health Association 1998). Approximately 30 isolates per site per sampling date were analyzed. To test for multiple antibiotic resistance, individual bacterial isolates were replica-plated onto tryptic soy agar (TSA; BD Biosciences, Franklin Lakes, NJ) supplemented with one of five antibiotics: ampicillin (A), kanamycin (K), streptomycin (S), chlorotetracycline (C), and oxytet-

racycline (O) (Sigma-Aldrich, St. Louis, MO). These antibiotics are commonly used in both human and veterinary medicine and demonstrate different mechanisms and pathways (inhibiting cell wall synthesis [ampicillin] vs. protein synthesis [streptomycin, kanamycin, and the tetracyclines]; Esiobu et al. 2002). The antibiotic concentration for all drugs was 20 μg/ml for samples processed from the initial measurement date (June 3, 2004). The antibiotic concentrations were increased to 30 μg/ml for the remainder of the sampling dates (June 17, 2004, July 7, 2004, July 18, 2004, and August 9, 2004) to ensure complete inhibition of the control bacterial strain in the conjugation assay. Some authors consider these to be high antibiotic concentrations for screening environmental bacteria (D'Costa et al. 2006), but they are similar to concentrations used in other screening studies (i.e., 5–80 μg/ml; Harwood et al. 2000; Wiggins et al. 1999). The plates were incubated at 37°C for 24 h. Bacterial isolates were scored as positive for resistance if there was visible, tangible growth. No growth or trace colony development was recorded as antibiotic susceptible. All resistant isolates were preserved in tryptic soy broth (TSB, BD Biosciences, Franklin Lakes, NJ) supplemented with the appropriate antibiotic and 10% glycerol and frozen at −80°C.

2.4 Conjugation Testing

An S-resistant *Salmonella typhimurium* strain, EM1000, was used as the recipient in all conjugation testing. EM1000 was originally obtained as SGSC452 from the *Salmonella* Genetic Stock Center (Calgary, Alberta, Canada; Bullas and Ryu 1983). All environmental isolates not resistant to S were tested as donor strains in individual conjugation assays. Isolates demonstrating multiple-drug resistance were processed and tested separately for the ability to transfer each type of drug resistance gene. For example, after the replica-plating assay, if a particular isolate grew on an A plate and also on a separate K plate, the isolate was subjected to two conjugation assays. In the first assay, the transconjugants would be selected on a dual antibiotic plate containing A and S, and in the second assay, the selective plate would include K and S.

An A- and K-resistant *E. coli* strain, SM10-Tn*pho*A, was used as a positive control donor for testing the efficiency of conjugation. SM10-Tn*pho*A was obtained originally from John Mekalanos (Taylor et al. 1989). Prior to conjugation, individual bacterial cultures were grown overnight at 37°C in TSB containing 30 μg/ml of the appropriate antibiotic (S for EM1000; A and K for SM10-Tn*pho*A; and either A, K, C, or O for the environmental isolates). The bacterial cells were pelleted by centrifugation and resuspended in fresh TSB lacking antibiotic. Conjugation experiments were conducted as described by Bell et al. (1983). Briefly, equal volumes (0.1 ml) of stationary-phase cells from the donor strain (an individual environmental isolate or the SM10-Tn*pho*A positive control donor) and recipient strain (EM1000) were mixed in a 1.5-ml tube with 0.8 ml of antibiotic-free TSB and incubated at 37°C for 2–4 h. After the incubation, mating mixtures were plated onto TSA containing dual antibiotics (S and either A, K, C, or O all at 30 μg/ml) and incubated for 24 h at 37°C to select for transconjugants. Transconjugants of interest were preserved in TSB supplemented with the appropriate antibiotic and 10% glycerol and frozen at −80°C.

Negative conjugation control experiments lacking either the donor or recipient bacteria were included in all conjugation experiments to check for mutation of either strain to antibiotic resistance. No mutation to specific antibiotic resistance was seen with any donor strains or the universal *S. typhimurium* EM1000 recipient strain.

2.5 PCR Screening for Tetracycline Resistance Genes

Environmental isolates positive for tetracycline resistance and their respective transconjugants were screened for specific tetracycline resistance genes using the polymerase chain reaction (PCR). The tetracycline resistance genes tested in this study included *tet* (B), *tet* (C), *tet* (E), *tet* (H), *tet* (Y), and *tet* (Z) based on primer sets from Aminov et al. (2002); *tet* (M) and *tet* (W) from Aminov et al. (2001); and *tet* (K) and *tet* (L) from Gevers et al. (2003). The presence of class I and II integrons was screened using primer sets from France et al. (2005). A typical PCR reaction was performed with a 50-μl mixture of the following reagents: 1-μl DNA template (50 ng) or bacterial cell suspension (one colony suspended in 10 μl H_2O), 0.2-μM primers, and 45-μl Platinum PCR Supermix (Invitrogen Corporation, Carlsbad, CA) supplemented

with $MgCl_2$ to a final concentration of 2.0 mM. PCR amplification was performed as follows: 95°C for 5 min (one cycle), 94°C for 30 s, 50°C for 30 s, 72°C for 30 s (25 cycles), and 72°C for 7 min (one cycle) using an MJ Research PTC-200 thermocycler (Bio-Rad Laboratories, Hercules, CA). If amplification products were not detected in the first assay, the samples were subjected to another round of amplification using the same cycling protocol with the exception of reducing the annealing temperature to 45–48°C. PCR amplification products were analyzed by gel electrophoresis on a 2.5% (wt/vol) agarose gel (NuSieve; FMC Bioproducts, Rockland, MD) and stained with ethidium bromide. The Promega 1 KB Plus Ladder (Promega Co., Madison, WI) was used for verifying DNA fragment sizes. Amplification products were purified by the use of a Qiagen QIAquick PCR purification kit (Qiagen Inc., Valencia, CA) and sequenced at the University of Michigan DNA Sequencing Core (Ann Arbor, MI). The DNA sequences generated from environmental isolates were compared to known sequences using the Basic Local Alignment Search Tool family of programs from NCBI (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=pubmed&list_uids=15215342&dopt=Citation; McGinnis and Madden 2004).

Plasmid DNA was purified from environmental isolates and their transconjugants using the Qiagen QIAprep plasmid purification system. The following modifications in the procedure were performed to optimize purification of low-copy plasmids and cosmids: (1) volumes of buffers P1, P2, and N3 were doubled, (2) the optional PB wash step was included, and (3) H_2O heated to 70°C was used to elute DNA from the QIAprep membrane.

2.6 Identification of Environmental Bacteria

Each bacterial isolate that screened positive for a particular tetracycline resistance gene was identified using the RapiD 20 E system (bioMerieux, Inc., Durham, NC). Individual isolates were inoculated on media containing trypticase soy agar with 5% sheep blood BBL (Becton Dickinson, Franklin Lakes, NJ) and incubated overnight at 37°C. A cell suspension was prepared using one to four well-isolated colonies diluted in 2 ml 0.85% NaCl to a turbidity equivalent to 0.5 McFarland. The cell suspension was immediately used to inoculate a RapiD 20 E test strip and incubated for 4 h at 37°C. The test results were entered into the RapiD 20 E Analytical Profile Index, and the species identification was provided at the bioMerieux *apiweb* site (https://apiweb.biomerieux.com/servlet/Authenticate?action=prepareLogin).

2.7 Statistical Analysis

Differences between the AI and AUI sites in (1) the proportion of samples which exceeded established water quality thresholds for total phosphorus (0.1 mg/l, Lind 1985), turbidity (40.1 NTU, Mitchell and Stapp 1997), and fecal coliform density (400 CFU/100 ml, Michigan Department of Environmental Quality 1999), (2) the proportions of single- vs multi-drug resistant bacterial isolates, and (3) the proportions of bacterial isolates able to transfer their resistance during conjugation assays were tested using Fisher's exact test (Zar 1999). Identical comparisons were examined for sites upstream and downstream of WWTPs. Spearman's rank correlation was used to examine the relationships among turbidity, temperature, total phosphorus, pH, and fecal coliform density across all samples and sites. For correlation analyses, fecal coliform densities plated as too numerous to count (TNTC) were coded as an arbitrarily large number greater than 7,900 CFU/100 ml (the largest recorded value). The SYSTAT computer package (v 10.2, SYSTAT Inc., Chicago, Illinois) was used to perform all statistical analyses for this study.

3 Results

3.1 Physical, Chemical, and Biological Water Quality

Temperature, pH, and turbidity were similar among the agricultural streams and within the WWTP sites (Table 2). Turbidity tended to be increased by rain events, especially in the agricultural streams. AI sites were one to eight times more phosphorus rich than all other sites. Fecal coliform densities ranged from 70 to >7,900 CFU/100 ml. For some measurements, particularly those taken during rain events and at AI sites, quantitative fecal coliform densities could not be determined as a result of confluent colony growth regardless of the water dilution (Table 3). The fecal coliform densities at agricultural sites ranged from 700 to TNTC, while fecal coliform densities in

Table 2 Water chemistry ranges across sampling dates for agriculturally impaired and agriculturally unimpaired sites

Sample site group	Sampling date	pH	Temperature (°C)	Turbidity (NTU)[a]	Total phosphorus (mg/l)[b]
AI	Initial (June 3, 2004)	7.19–7.53	18.6–23.4	9.09–15.80	0.12–0.25
	Rain event 1 (June 17, 2004)	6.89–7.66	19.5–20.5	114.00–846.00	0.50–0.65
	Rain event 2 (July 7, 2004)	6.52–7.14	21.5–22.6	9.30–56.60	0.10–0.34
	Rain event 3 (July 18, 2004)	7.06–7.56	18.4–18.9	5.36–72.90	0.13–0.32
	Final (August 9, 2004)	6.82–7.54	16.6–19.7	14.60–33.70	0.36–0.76
AUI	Initial (June 3, 2004)	7.44–7.98	20.7–21.5	2.22–3.36	0.02–0.06
	Rain event 1 (June 17, 2004)	7.23–7.57	19.3–19.7	21.40–96.70	0.10–0.15
	Rain event 2 (July 7, 2004)	7.64–8.18	19.0–21.5	13.20–28.90	0.07–0.12
	Rain event 3 (July 18, 2004)	7.71–8.28	16.9–17.7	4.92–28.80	0.02–0.07[d]
	Final (August 9, 2004)	7.37–8.09	14.0–16.8	2.65–11.60	0.00–0.05
UPWWTP	Initial (June 3, 2004)	8.18–8.19	15.3–17.8	3.04–4.26	0.02–0.03
	Rain event 1 (June 17, 2004)	7.99–8.11	20.5–22.5	6.89–7.47	0.02–0.03
	Rain event 2 (July 7, 2004)	7.95–8.20	22.5–24.4	5.72–17.50	0.02–0.05
	Rain event 3 (July 18, 2004)	7.84–8.13	19.5–23.8	5.26–10.50	0.02–0.03
	Final (August 9, 2004)	8.00–8.04	18.9–23.6	4.20–5.22	0.00–0.01
DNWWTP	Initial (June 3, 2004)	7.98–8.28	16.6–18.7	3.38–3.47	0.03–0.04
	Rain event 1 (June 17, 2004)	7.91–8.12	21.8–22.9	3.89–6.43	0.02–0.04
	Rain event 2 (July 7, 2004)	7.95–7.95	21.7–25.4	3.63–17.20	0.03–0.07
	Rain event 3 (July 18, 2004)	7.80–8.18	21.4–24.6	3.88–10.50	0.02–0.03
	Final (August 9, 2004)	8.09–8.28	21.6–23.9	3.16–5.37	0.01–0.03

AI agriculturally impaired, *AUI* agriculturally unimpaired, *UPWWTP* upstream from wastewater treatment plant, *DNWWTP* downstream from wastewater treatment plant, *NTU* nephelometric turbidity units

[b] Rating according to Mitchell and Stapp (1997): excellent=0–10, good=10.1–40, fair=40.1–150, poor=>150

[c] Phosphorus levels. Rating >0.1 mg/l is considered poor water quality according to Lind (1985)

waterways near WWTPs ranged from 70 to 2,300 CFU/100 ml. The highest fecal coliform densities were measured after rain events and occurred most frequently at the AI sites (Table 3). Turbidity, total phosphorus concentration, and fecal coliform density were highly correlated (turbidity–phosphorus $n=49$, $r_s=0.790$, $p<0.001$; turbidity–fecal coliform $n=39$, $r_s=0.688$, $p<0.001$; phosphorus–fecal coliform $n=39$, $r_s=0.684$, $p<0.001$), with AI sites tending to have the greatest values for these three parameters. Turbidity, total phosphorus concentration, and fecal coliform density were all negatively correlated with pH (turbidity–pH $n=49$, $r_s=-0.371$, $p<0.01$; phosphorus–pH $n=49$, $r_s=-0.606$, $p<0.001$; fecal coliform–pH $n=39$, $r_s=-0.471$, $p<0.01$). AI streams were significantly more likely than AUI streams to exceed a total phosphorus threshold of 0.1 mg/l ($p<0.001$) and marginally more likely to exceed a turbidity threshold of 40.1 NTU ($p=0.08$, Table 4). There was no difference in the total phosphorus or turbidity exceedance frequencies between sites upstream and downstream of WWTPs (p always >0.05). Although the AI sites tended to have greater fecal coliform densities than the AUI sites, there was no difference in the frequency of fecal coliform densities >400 CFU/100 ml ($p>0.05$), nor was there any difference observed in fecal coliform exceedance frequencies between sites upstream and downstream of WWTPs ($p>0.05$, Table 4).

3.2 Antibiotic Resistance Patterns

A total of 830 fecal coliform isolates were collected from mFC agar plates and replica-plated on TSA supplemented with one of five antibiotics. Overall, 98.3% of the fecal coliform isolates selected were resistant to at least one antibiotic (Table 5). Fourteen of the isolates (1.7%) did not grow on any of the

Table 3 Fecal coliform densities for each grouping of sites across sampling dates from filtered plates (CFU/100 ml)

Sites	Measurement dates[a]			
	Rain event #1 June 17, 2004	Rain event #2 July 7, 2004	Rain event #3 July 18, 2004	Final August 9, 2004
AI				
VH19	TNTC	TNTC [b]	900	2,800
VH21	TNTC	3,200	1,600	600
VH25	4,400	TNTC	4,900	No data[c]
AUI				
HC1	5,900	3,100	7,900	2,400
SJT8	2,900	2,300	2,400	700
SJC7	5,800	800	2,900	700
UPWWTP				
MC1	800	2,300	2,000	600
HR1	79	700	88	198
DNWWTP				
MC2	198	2,100	1,500	900
HR2	174	1,800	70	300

For specific site abbreviations, see Table 1.

AI agriculturally impaired, *AUI* agriculturally unimpaired, *UPWWTP* upstream from wastewater treatment plant, *DNWWTP* downstream from wastewater treatment plant, *TNTC* too numerous to count

[a] The Initial sample date (June 3, 2004) yielded zero colony growth for the 0.01- and 0.1-ml samples and TNTC=confluent growth for the filtered 100-ml water samples. The protocol was adjusted after June 3, 2004, to include a 0.1-, 1-, and 100-ml filtered sample

[b] Mann–Whitney *U* test shows AI sites significantly greater than AUI sites for rain event 2 ($p=0.046$); TNTC: confluent growth on filters growing on media plates (0.1, 1.0, and 100 ml)

[c] Dry creek, unable to sample water on this date

antibiotic plates, 640 isolates (77.1%) were resistant to only A, and the remaining 176 resistant isolates (21.2%) were resistant to two or more antibiotics. The only single-drug resistance pattern observed was to A, and all the multi-drug isolates included A resistance with the exception of one isolate that was KCO resistant (Table 5).

The AI sites had a significantly greater proportion of isolates that were resistant to multiple antibiotics (41.6% vs. 16.5%) and a lower proportion of isolates resistant to only ampicillin (58.4% vs. 83.5%) than the AUI sites ($p<0.001$, Tables 4 and 5). The sites upstream and downstream of WWTPs had similar proportions of isolates resistant to only ampicillin (81.0% vs. 89.1%) and multi-drug resistant (19.0% vs. 10.9%) ($p>0.05$, Tables 4 and 5).

3.3 Conjugal Transfer of Antibiotic Resistance Genes

Conjugation assays were conducted on 735 non-S-resistant isolates. Of the 640 single-drug resistant isolates (all were A-only resistant), 456 (71.3%) produced viable resistant transconjugants. For the multi-drug-resistant isolates ($n=95$), 27.4% were able to transfer all resistance determinants (AK, AO, and ACO), and 81.1% were able to transfer at least one multi-drug resistance pattern (AK, AO, AC, ACO, and AKCO) with A being the most prevalent resistance determinant transferred (Table 6). The proportion of isolates from each site category able to transfer resistance in laboratory conjugation assays ranged from 60.0% to 85.6% (Table 4) and did not differ significantly between either the AI and AUI

Table 4 Patterns of water quality and multi-drug resistance at study sites

Site groups[a]	Water quality			Multi-drug resistance and gene transfer	
	Turbidity rating[b] (%) 40.1 >150 NTU	Phosphate rating[c] (%) >0.1 (mg/l)	Fecal coliform densities[d] (%) >400 CFU/100 ml	Multi-drug resistance[e] (%)	Gene transfer of one or more resistance genes[f] (%)
AI	35.7[g]	100	100	41.6	80.0
AUI	6.7[g]	26.7[g]	100	16.5	86.5
UPWWTP	0	0	62.5[g]	19.0	76.0
DNWWTP	0	0	50[g]	10.9	60.0

NTU nephelometric turbidity units

[a] AUI, UPWWTP, and DNWWTP (15 water samples collected); AI (14 samples; VH25 site was dry at final collection)

[b] Rating according to Mitchell and Stapp (1997): excellent=0–10, good=10.1–40, fair=40.1–150, poor=>150

[c] Phosphorus levels. Rating >0.1 mg/l is considered poor water quality, according to Lind (1985)

[d] Fecal coliform density for second to fifth sampling dates, initial coliform density samples are not included in this comparison because of a dilution change in the protocol, see Section 2.

[e] AI (n=185), AUI (n=328), UPWWTP (n=147), DNWWTP (n=156)

[f] Ninety-five multi-drug resistant isolates (all non-streptomycin) were tested for gene transfer: the percent is the number of isolates showing positive for gene transfer of one or more resistance genes per number of isolates tested in each grouped site = AI (24/30), AUI (32/37), UPWWTP (14/18), DNWWTP (6/10)

[g] These water quality indicators reached their highest ratings during rain events

sites (p>0.05) or the sites upstream and downstream of WWTPs (p>0.05).

A subset of AO, AC, and ACO multi-drug-resistant isolates (n=22) and their transconjugants were further screened for the presence of specific tetracycline resistance genes and class I and II integrons. In all, 13 isolates were *tet* positive: six isolates were identified carrying the *tet* (B) gene, five with *tet* (C), and two with both *tet* (B) and *tet* (C). No class I or II integrons or *tet* (E, H, K, L, M, Y, W, or Z) genes were detected. The 13 *tet*-positive isolates were individually identified as *E. coli* by the RapiD 20 E system (Table 7). The identity of all *tet* (B) and *tet* (C) PCR products was confirmed by DNA sequence analysis.

To characterize the potential location and mode of horizontal transfer of *tet* (B) and *tet* (C) genes between the environmental isolates and the conjugative recipient, plasmid DNA was purified and PCR-amplified using primer sets for *tet* (B) and *tet* (C) (see Table 7). All eight isolates positive for the *tet* (B) gene showed the presence of *tet* (B) on plasmid DNA, as did their respective transconjugants, with the exception of two isolates (VH19-7R1 and VH25-16R1). For these two isolates, their transconjugants could not be subcultured and maintained on tetracycline-selective media (at 30 μg/ml C or O). Repeated conjugation experiments for isolates VH19-7R1 and VH25-16R1 failed to produce any viable colonies selected on tetracycline plates despite the evidence that the donor isolates contain plasmid DNA with the *tet* (B) gene. In this study, chromosomal DNA was not definitively screened in the absence of plasmid DNA.

For the *tet* (C) determinant, three different patterns of gene transfer were observed (Table 7): (1) In two out of eight isolates (SJ7-5-I and HC-1-R2), the *tet* (C) gene was identified with plasmid DNA in both the donor isolates and their transconjugants; (2) *tet* (C) was identified in total DNA from isolates VH19-8R3, VH19-11F, and MC2-9R1 and their transconjugants, but not associated with any plasmid DNA; and (3) for isolates MC1-22R4 and MC1-1F, the *tet* (C) gene was present in total DNA from the isolate but was unidentifiable in any plasmid DNA or total DNA from the transconjugant even though

Table 5 Percentage of antibiotic-resistant fecal coliform isolates from individual study sites

Antibiotic[a]	Individual study sites									
	VH19 (AI) n=58	VH21 (AI) n=57	VH25 (AI) n=70	HC1 (AUI) n=142[b]	SJT8 (AUI) n=93	SCJ7 (AUI) n=93	MC1 (UPWWTP) n=72	HR1 (UPWWTP) n=75	MC2 (DNWWTP) n=84	HR2 (DNWWTP) n=72
A	43.1%	54.4%	74.3%	88.7%	82.8%	76.3%	84.7%	77.3%	88.1%	90.3.4%
Total % by site for amp	AI=58.4%			AUI=83.5%			UPWWTP=81.0%		DNWWTP=89.1%	
AK	5.2%	5.3%	20.0%	4.2%	10.8%	10.8%	2.8%	9.3%	3.6%	4.2%
AO	0	0	0	2.8%	0	0	0	0	0	0
AC	0	0	0	0	0	1.1%	1.4%	0	0	0
AS	24.1%	7.0%	2.9%	3.5%	2.2%	5.4%	1.4%	4.0%	3.6%	2.8%
AKS	0	0	0	0	0	0	2.8%	2.7%	0	0
ACO	8.62%	3.5%	2.9%	0	3.2%	3.2%	6.9%	1.3%	2.4%	2.8%
KCO	0	0	0	0	0	0	0	1.3%	0	0
AOS	0	1.8%	0	0	0	0	0	0	0	0
COS	3.5%	0	0	0	0	0	0	0	1.2%	0
AKCO	0	1.8%	0	0	0	0	0	1.3%	0	0
AKOS	1.7%	0	0	0	0	0	0	0	0	0
ACOS	5.1%	0	0	0.7%	1.1%	3.2%	0	2.7%	1.2%	0
AKCOS	8.6%	26.3%	0	0	0	0	0	0	0	0
Total% multiple resistance	56.9%	45.6%	25.7%	11.2%	17.2%	23.7%	15.3%	22.7%	11.9%	9.7%
Total% by site for multi-drug	AI=41.6%			AUI=16.5%			UPWWTP=19.0%		DNWWTP=10.9%	

Summary: total N=830; 816/830=98.3% isolates resistant to at least one antibiotic; 640/830=77.1% resistant to amp only; 176/830=21.2% multi-drug resistant

A ampicillin, *K* kanamycin, *O* oxytetracycline, *C* chlorotetracycline, *S* streptomycin

[a] Values tabulated for a single antibiotic (e.g., A) indicate isolates resistant only to that individual antibiotic; values tabulated for a particular combination of antibiotics indicate isolates resistant to all the listed antibiotics and only the listed antibiotics (i.e., isolates tabulated as AKS resistant would not also be included in the AS category)

viable tetracycline-resistant colonies appeared after conjugation.

For the two isolates carrying both *tet* (B) and *tet* (C) genes (VH19-11F and MC2-9R1), both *tet* genes were identified in total DNA (in the isolates and transconjugants). The *tet* (B) gene was also amplified from plasmid DNA isolated from both isolates and their transconjugants, but this did not occur with the *tet* (C) gene (Table 7). Figure 1 shows gel PCR amplification products produced using *tet* (B) and *tet* (C) primer pairs with DNA from bacterial cells and isolated plasmid from isolate MC2-9R1 and its transconjugant, MC2-9R1T2-1.

4 Discussion

Chemical water quality at the WWTP sites was good; all WWTP samples had total phosphorus concentrations <0.1 mg/l, which is considered acceptable or unpolluted (Lind 1985; Mitchell and Stapp 1997), and turbidities of <40.1 NTU, which is considered excellent to good (Mitchell and Stapp 1997). Fecal coliform densities at the WWTP sites were marginal, as 56% of samples surpassed the 10% exceedance threshold (<400 CFU/100 ml). No statistically significant differences were detected in water chemistry measurements and fecal coliform densities between

Table 6 Resistance pattern observed after gene transfer for non-streptomycin isolates

Antibiotic resistance pattern	Number of isolates[a]	Resistance pattern genetically transferred via conjugation	Number of isolates transferring resistance[b]
Single-drug resistance			
A	640	A	456 (71.3%)
		No transfer	184 (28.8%)
Multi-drug resistance (n = 95)			
AK	61	A	25 (41.0%)
		K	2 (3.3%)
		AK	18 (30.0%)
		No transfer	16 (26.2%)
AO	4	A	1 (25.0%)
		O	2 (50.0%)
		AO	1 (25%)
AC	2	A	2 (100%)
ACO	25	A	5 (20.0%)
		AC	3 (12%)
		ACO	7 (28%)
		AO	2 (8%)
		CO	3 (12%)
		O	4 (16%)
		No transfer	1 (4%)
KCO	1	No transfer	1 (100%)
AKCO	2	A	1 (50%)
		No transfer	1 (50%)

A ampicillin, *K* kanamycin, *O* oxytetracycline, *C* chlorotetracycline, *S* streptomycin

[a] Number of isolates, lacking streptomycin resistance, that were tested for gene transfer, total 735

[b] Number of multi-drug isolates: (1) transferring one or more resistance genes 77/95=81.1%; (2) transferring all resistance genes 26/95=27.4%

the upstream and downstream sites. These results suggest that little, if any, WWTP point source pollution occurred during the study period. MDNRE (Michigan Department of Environmental Quality 2004a) confirmed that no untreated sewage was discharged from either the Chelsea or the Ann Arbor WWTPs during or immediately preceding the study period.

In contrast, the AI sites located near CAFO farms had indicators of poor water quality compared to reference sites in the same area. Total phosphorus concentrations were much more likely (100% vs. 26.7%) to exceed established thresholds in the AI sites than AUI sites, and turbidity was also somewhat more likely (35.0% vs. 6.7%) to exceed threshold values. Although the frequency of fecal coliform densities exceeding 400 CFU/100 ml did not differ between AI and AUI sites, only the AI sites had fecal coliform densities TNTC, indicating that maximal densities of fecal coliforms occurred at AI sites.

As expected, water quality decreased following heavy precipitation. At all study sites, turbidity levels were elevated after rain events compared to the initial and final measurements taken during dry periods. Increased phosphorus levels were also detected after precipitation in the agriculturally impacted areas, and fecal coliform densities were much higher after precipitation. The strong correlation of turbidity, total phosphorus, and fecal coliform densities suggests a common source for these parameters. Elevated total phosphorus, turbidity, and fecal coliform densities are presumed to be the direct result of runoff from nearby tiled fields sprayed with liquid manure as reported by MDNRE in numerous previous waste discharge infractions by the CAFO farms in close proximity to our AI sites (Michigan Department of Environmental Quality 2003a, 2004b). Our data are consistent with recent biological surveys of the same waterways, which classified the AI sites as poor habitat, supporting only macroinvertebrate and fish communities that

Table 7 Identification of tetracycline (tet) resistance genes in the environmental isolates and their transconjugants

Sample site groups	Environmental isolates (*Escherichia coli*)[a]				Transconjugants (*Salmonella typhimurium*, *strain* EM1000)			
	IDi[b]	Antibiotic resistance pattern on selective plates	Resistance gene identified by PCR (total DNA)	Resistance gene identified by PCR (plasmid DNA)	ID	Resistance pattern on selective plates after conjugation	Resistance gene identified by PCR (total DNA)	Resistance gene identified by PCR (plasmid DNA)
AI	VH19-7R1	ACO	*tet* (B)	*tet* (B)	VH19-7R1T2-1	AC	–	–
	VH25-16R1	ACO	*tet* (B)	*tet* (B)	VH25-16R1T2-1	AC	–	–
	VH19-8R3	ACO	*tet* (C)	–	VH19-8R3T3-4	ACO	*tet* (C)	–
	VH25-6R3	ACO	*tet* (B)	*tet* (B)	VH25-6R3T3-5	ACO	*tet* (B)	*tet* (B)
	VH19-3F	ACO	*tet* (B)	*tet* (B)	VH19-3FT2-1	ACO	*tet* (B)	*tet* (B)
	VH19-11F	ACO	*tet* (B) and *tet* (C)	*tet* (B)	VH19-11FT3-1	ACO	*tet* (B) and *tet* (C)	*tet* (B)
AUI	SJ7-5-I	ACO	*tet* (C)	*tet* (C)	SJ7-5-IT3-1	AO	*tet* (C)	*tet* (C)
	HC-1-R2	ACO	*tet* (C)	*tet* (C)	HC-1-R2T2-2	ACO	*tet* (C)	*tet* (C)
UPWWTP	MC1-17I	ACO	*tet* (B)	*tet* (B)	MC1-17IT2-1	ACO	*tet* (B)	*tet* (B)
	MC1-24I	ACO	*tet* (B)	*tet* (B)	MC1-24IT3-1	ACO	*tet* (B)	*tet* (B)
	MC1-22R4	ACO	*tet* (C)	–	MC1-22R4T1-1	AC	–	–
DNWWTP	MC1-1F	ACO	*tet* (C)	–	MC1-1FT2-1	AC	–	–
	MC2-9R1	ACO	*tet* (B) and *tet* (C)	*tet* (B)	MC2-9R1T2-1	AO	*tet* (B) and *tet* (C)	*tet* (B)

AUI ag. unimpaired, *AI* ag. impaired, *UPWWTP* upstream from wastewater treatment plant, *DNWWTP* downstream from wastewater treatment plant, *A* ampicillin, *O* oxytetracycline, *C* chlorotetracycline

[a] Environmental bacterial isolates were identified to species using the RapiD20 System (bioMerieux)

[b] Isolates are named after the site location, the day when the measurement was taken (*R* rain event, *I* initial, *F* final), and the colony number picked from the plate. Transconjugants are named after the isolate donor, along with T for transconjugant, and the number of colony picked

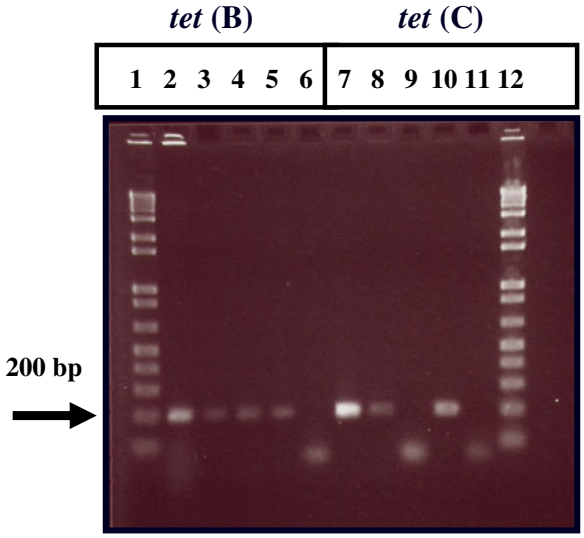

Fig. 1 PCR screening analysis of *tet* (B), *left-side* amplicons (206 bp), and *tet* (C), *right-side* amplicons (207 bp), from a representative tetracycline-resistant bacterial isolate MC2-9R1 and its transconjugant, MC2-9R1T2-1. *Lanes 1* and *12*, 1 KB Plus ladder (Promega); *lanes 2* and *8*, isolate MC2-9R1 cells; *lanes 3* and *9*, isolate MC2-9R1 plasmid DNA; *lanes 4* and *10*, transconjugant MC2-9R1T2-1 cells; *lane 5*, transconjugant MC2-9R1T2-1 plasmid DNA; *lanes 6* and *11*, *Salmonella* strain EM1000 cells (conjugal recipient, negative control); *lane 7*, pBR322 plasmid DNA (positive control for the *tet* (C) gene). The lower bands in *lanes 2*, *6*, *9*, and *11* are DNA primers

could survive in waters with low dissolved oxygen for sustained periods, whereas several of the AUI sites were listed as "reference" or "least impacted" systems in this region (Michigan Department of Environmental Quality 2003a).

Elevated nutrient levels can also improve the efficiency of horizontal gene transfer between species by the bacterial processes of transformation (Davison 1999; Quintiliani et al. 1999; Thomas and Nielsen 2005) and conjugation (Arana et al. 1997, 2001). High fecal coliform densities, as seen in this study, increase the likelihood of humans and animals coming in contact with pathogenic bacteria. Implementing a quantitative risk assessment analysis, as modeled by Hurd (2006), could help identify the "chain of causal events that connect on-farm antibiotic use to additional days of human illness caused by infections with resistant bacteria."

Antibiotic-resistant fecal coliform bacteria were found in all water samples collected during the course of this study, with A resistance the most prevalent (77.1% overall, Table 4). This proportion of A resistance in fecal coliform bacteria parallels other river and waterway reports (Ash et al. 2002; McKeon et al. 1995; Ogan and Nwiika 1993). Some studies have reported lower frequencies of A resistance yet still found that resistance to A was the most common form of resistance (Iwane et al. 2001; Niemi et al. 1983). In contrast, other studies of soil, water, and manure samples from farm locations have found tetracycline and other drug resistance patterns in higher proportions than A resistance (Esiobu et al. 2002; Sayah et al. 2005). One plausible explanation for the differences seen in antibiotic resistance patterns is the source of the environmental sample, specifically human waste vs. animal waste. Harwood et al. (2000) determined the antibiotic resistance pattern from fecal coliforms isolated from domestic wastewater and various animal feces using four different concentrations of antibiotics; they showed a significantly greater percentage of A resistance in fecal coliform isolates from human sources (62% at 10 μg/ml A) than animal feces (15%) and a significantly greater percentage of C resistance in cattle feces (58% at 20 μg/ml C) compared to the human sources (35%). This could explain why we observed a significantly lower proportion of A resistance (58.4%) at the AI sites (sites near animal waste contamination) than at the AUI sites (83.5%) and sites upstream (81.0%) and downstream (89.1%) of the WWTPs.

The proportion of multi-drug resistance observed at the AI sites near CAFO farms (41.6%) was almost three times greater than at the AUI sites (16.5%). Certain multiple-resistance combinations were more common at some AI sites than others, perhaps reflecting site-specific antibiotic use patterns. In any case, the high proportion of multi-drug resistance at AI sites suggests that the fecal bacterial populations in these locations were subjected to conditions that fostered the acquisition of multiple-resistance determinants. In addition to likely increases in antibiotic-resistant bacteria from animals fed antibiotic-laden feed (Davies 1997; Lu et al. 2004; Wegener et al. 1999), soil-dwelling bacteria are thought to be a significant reservoir of resistance determinants (D'Costa et al. 2006), and studies on crop soils fertilized with animal manure show that horizontal transfer between fecal and soil bacteria is facilitated by the high nutrient availability of manure (Cooke 1976; Schmitt et al. 2006). We observed that fecal

coliform levels increased after rain events at the AI sites, where periodic spraying of liquid manure was reported in nearby fields (Kauffman and Melmoth 2003; Michigan Department of Environmental Quality 2003a). Natural water environments may allow fecal coliforms a better selective advantage in becoming antibiotic resistant over other habitats such as soil, sand, or sewage effluent (Cooke 1976). Survival rates tend to be higher in bacteria containing plasmids (Arana et al. 1997, 2001; Ash et al. 2002; Schwartz et al. 2003), and the conjugative transfer of plasmids from one bacterium to another tends to occur more readily in aquatic habitats (Lebaron et al. 1993). The high rate of success for gene transfer as seen in our conjugation assays (83.3% overall) suggests that the environmental isolates carried conjugative plasmids or transposons. Of the multi-drug-resistant isolates, most exhibited resistance to combinations of antimicrobial drugs which included A. This is an indication that multiple-resistance genes may coexist on one plasmid (Davison 1999; Quintiliani et al. 1999; Sayah et al. 2005), a single conjugative transposon (Pembroke et al. 2002; Waters 1999), or an integron (Mazel 2004; White et al. 2001). This condition is particularly disconcerting given that exposure to one antibiotic agent may result in resistance to others without previous exposure (Sayah et al. 2005) or cost to bacterial fitness (Aminov et al. 2001).

The conjugation assay in combination with PCR was used to identify genetic patterns of transfer in bacterial populations. Using 10 primer pairs for the tetracycline resistance genes most commonly found in *E. coli* and other Gram-negative bacteria, we detected the presence of the *tet* (B) and *tet* (C) determinants in 13 of 22 ACO-resistant isolates. In strains isolated at all study sites, *tet* (B) was associated with plasmid DNA in isolates and their transconjugants, while *tet* (C) showed three different patterns of gene transfer. The first pattern of gene transfer showed that the *tet* (C) gene was associated with plasmid DNA in isolates and transconjugants from the AUI sites. The second pattern showed that the *tet* (C) gene from the UPWWTP isolates associated exclusively with total DNA in both the original isolates and the transconjugants and not with plasmid DNA; this suggests that the *tet* (C) gene was located on chromosomal DNA or on a large plasmid or cosmid that could not be purified by our methods. The final pattern of gene transfer found the *tet* (C) gene in total DNA in the isolate, but the *tet* (C) gene was undetectable in the resistant transconjugant. These results suggest that the transconjugant in the third pattern could have received a different tetracycline resistance gene from the donor isolate that was not detected by our PCR screening method (e.g., isolates MCI-22R4 and MCI-1F; Table 7). Two of 13 isolates (Table 7; isolates VH19-11F and MC2-9R1) showed the presence of both *tet* (B) and *tet* (C) genes, with *tet* (B), but not *tet* (C), associated with plasmid DNA, suggesting that the *tet* (B) and *tet* (C) genes are not genetically linked. Furthermore, both the differential distribution of the *tet* (B) and *tet* (C) genes in the VH19-11F and MC2-9R1 isolates (Table 7) between total and plasmid DNA suggested that the plasmid DNA preparations were not contaminated with chromosomal DNA. Additional studies with a larger population of tetracycline-resistant isolates would be needed to better characterize these genetic patterns.

Based on current chemical and biological water quality standards (turbidity, total phosphorus concentrations, and fecal coliform densities), study sites near the WWTPs were considered environmentally healthy, yet had high levels of single- and multi-drug-resistant fecal coliform bacteria (>81% for A alone and >10.9% for multi-drug resistance, Tables 4 and 5). Agricultural sites, especially the AI sites near CAFOs, had much lower measures of traditional water quality and also had high levels of single and multi-drug-resistant bacteria, with multi-drug resistance greatest (41.6%) at the AI sites near CAFOs. The risk to human and animal health posed by the high incidence of antibiotic resistance and gene transfer is unknown. Traditional measures of chemical and biological water quality do not appear to be direct surrogates for detection of the prevalence of antibiotic resistance. Those parameters most elevated in the AI sites (e.g., total phosphorus concentrations, turbidity), however, may have some predictive ability for the prevalence of multiple-drug resistance. We echo previous suggestions (Esiobu et al. 2002; Sayah et al. 2005) that testing for antibiotic resistance genes in bacterial strains become part of the standard methods for examining and regulating water quality and wastewater discharge in areas at high risk for pollution from human and animal waste.

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