

1-1-2007

# Impact of the 25th Street Combined Sewer Overflow on the Ohio River

Travis M. Bailey  
bailey53@marshall.edu

Follow this and additional works at: <http://mds.marshall.edu/etd>

 Part of the [Water Resource Management Commons](#)

---

## Recommended Citation

Bailey, Travis M., "Impact of the 25th Street Combined Sewer Overflow on the Ohio River" (2007). *Theses, Dissertations and Capstones*. Paper 450.

This Thesis is brought to you for free and open access by Marshall Digital Scholar. It has been accepted for inclusion in Theses, Dissertations and Capstones by an authorized administrator of Marshall Digital Scholar. For more information, please contact [zhangj@marshall.edu](mailto:zhangj@marshall.edu).

**Impact of the 25<sup>th</sup> Street Combined Sewer Overflow on the Ohio River**

**Thesis Submitted to  
The Graduate College of  
Marshall University**

**In partial fulfillment of the  
Requirements of the degree of  
Master of Science  
Biological Science**

**By**

**Travis M. Bailey**

**Charles C. Somerville, Committee Chair  
Frank L. Binder, Committee Member  
Ronald E. Gain, Committee Member**

**Marshall University**

**July 31, 2007**

## ABSTRACT

The 25<sup>th</sup> Street Combined Sewer Overflow was analyzed for total coliforms, *Escherichia coli*, antibiotic-resistant coliforms and *E. coli*, and routine water chemistry. The objectives of this study were to enumerate antibiotic resistant bacteria near a CSO, determine the impact of a small quantity CSO on Ohio River water quality during a storm event, and to correlate antibiotic resistance with conventional water quality measurements. The data indicate that resistant bacteria exist in both river water and wastewater, and that this CSO can not be identified as a source of resistant strains. Rain events do cause a detectable and transient change in water quality due to CSO release. Smaller rain events had a prolonged negative impact on river water quality relative to large rain events due to cleansing of the CSO by large volumes of stormwater. Biological indicators were found to be better markers of CSO impact than standard chemical analytes.

## ACKNOWLEDGMENTS

Several individuals have assisted in completion of this research project who I wish to acknowledge. Special thanks to Charlene DeBord and Scott Kelley who assisted in the research and analysis on all the samples. Warren Loudermilk is acknowledged for his help for all the sampling especially the early morning wet weather event.

Dr. Charles Somerville and Dr. Frank Binder also deserve acknowledgement. During the past five years they have offered a tremendous amount of help and support.

A special thank you to my wife, Bonnie, for all the hard work and support she has given to me in reaching this point in my education. I truly appreciate everything everyone has done to assist in the research and the writing of this paper.

## Table of Contents

Abstract	ii
Acknowledgements	iii
Table of Contents	iv
Chapter 1	1
Literature Review	1
History/Engineering	1
Bacterial Indicators	4
Antibiotic Resistant Bacteria Sources	7
Antibiotic Resistance as a New Water Quality Parameter	10
Antibiotic Resistant Genes	11
Lack of CSO Studies	12
Study Objectives	12
Chapter 2	14
Materials and Methods	14
Site Description	14
Sampling Protocol	15
Bacteriological Sampling	18
Chapter 3	21
Results	21
Dry Weather 1	21
Dry Weather 2	21
Dry Weather 3	21
Wet Weather 1	26
Wet Weather 2	29
Wet Weather 3	32
Correlations	35
Chapter 4	43
Discussion and Conclusion	43
Dry Weather 1	43
Dry Weather 2	43
Dry Weather 3	44
Summary of Dry Weather Data	45
Wet Weather 1	46
Wet Weather 2	48
Wet Weather 3	50
Overall Conclusion	53
Chapter 5	55
Summary	55
Literature Cited	57
Tables and Figures	61
Appendices	63

# **Chapter 1**

## **Introduction**

### **Combined Sewer Overflows**

The City of Huntington's combined sewer systems (CSSs) are sewers that are designed to collect rainwater runoff, domestic sewage, and industrial wastewater in the same collection system. CSSs transport all of the sanitary sewage to the treatment plant located at the southwest region of city limits, where it is treated and then discharged to the Ohio River at river mile 313.2 (Latitude 38°24'04", Longitude 82°31'44"). Heavy rain events in the area increase wastewater volume in the combined sewer system to the point where they exceed the capacity of the collection system. The Huntington CSSs are designed to temporarily overflow the additional water directly into the Ohio River relieving the system of the excessive volume. The overflow pipes are called combined sewer overflows (CSOs). They do not only contain storm water but also untreated human and industrial waste. The fact that untreated human and industrial wastes are released into the Ohio River is a major environmental and public health issue.

The original sewer system at Huntington, West Virginia was constructed in the early part of the twentieth century, primarily in the 1940's. Its purpose was to direct all sewage away from the community directly to the Ohio River to ensure the health of the public (33). In 1964 Huntington built the only waste water treatment plant in the city to decrease the amount of raw sewage going to the Ohio River. The original waste water treatment plant provided only primary treatment and chlorine disinfection. Primary

treatment entails allowing those substances in wastewater that readily settle or float to be separated from the water being treated. The Huntington plant accomplished it using bar screens, aerated grit tanks and primary settling tanks with clarifiers. In 1984 the plant was upgraded from a primary treatment facility to its current secondary treatment and chlorine disinfection process. Secondary treatment is used to convert dissolved or suspended materials into a form more readily separated from the water being treated. The secondary treatment system at the Huntington plant is a biological treatment process followed by secondary clarifiers that allow the solids to settle out from the water being treated then sent for chlorination and dechlorination.

The conversion from discharging sewage directly to the Ohio River to the original primary treatment facility was accomplished using the existing wastewater collection system. The installation of regulator stations (mechanical way of controlling flow) or diversion chambers (non mechanical way of controlling flow) was made prior to the outfall to direct the sewage to the new treatment plant. If the sewage/storm water exceeds the capacity of that specific regulator station or diversion chamber (2.5 times higher than dry weather flow) the excess water is diverted to the receiving body of water, usually the Ohio River. There are 25 total CSOs in the Huntington area: 14 are on the Ohio River; six on the Guyandotte River, a large tributary of the Ohio River; and five on smaller tributaries of the Ohio River.

The 25<sup>th</sup> Street Diversion chamber (Figure 1) was the outfall sampled for this study because the West Virginia Department of Environmental Protection (WVDEP) found its location to be of importance. The 25<sup>th</sup> Street outfall is located less than one mile from the city's Drinking Water Intake (DWI). It is actually less than 1000 feet from

the DWI. It was sampled to determine its impact on the Ohio River and primarily the DWI.

The WV American Water Company's plant was inspected and staff members were interviewed before the sampling events began to obtain their opinion of water quality when the local area had rain events that led to releases at CSOs. The company had recently moved their river intake 300 feet out into the river channel due to barges hitting the previous placement. Since the move they could not tell through their analysis when our system would outfall. They did not have to alter their process due to the weather conditions.

Published research on CSOs and their impacts on water quality, particularly relating to bacterial indicators, is limited. Most studies revolve around the impacts of agriculture or wastewater treatment plants on receiving streams. During rain events, CSOs are a point source contributor of bacteria in our waterways and little is being done to determine if they have a discernable impact on the quality of the receiving body. Due to the lack of research on CSOs, employees of the Huntington Sanitary Board were required to write a sampling protocol (Appendix A) and seek WVDEP approval of the standard operating procedure (SOP).

The US Environmental Protection Agency (USEPA) and state environmental agencies have mandated that Publicly Owned Treatment Works (POTWs) decrease the number of CSOs in their communities. The goal is for communities to either limit the number of outfall events to six a year, capture 85% of the discharged water, or all outfalls must meet water quality limits. All POTWs are submitting a Long Term Control Plan (LTCP) on what they are planning to do to meet the proposed requirements.



Many cities on the Ohio River such as Louisville, KY and Cincinnati, OH are spending billions of dollars to prevent outfalls but still not certain if they will be able to meet water quality limits. Other cities are spending millions to hundred of millions of dollars on their CSO problem passing the cost onto the rate payers. Studies such as this one are required to understand the actual impact of CSOs on river water quality.

### **Bacterial Indicators of Water Quality**

Coliform bacteria have long been used as a means of monitoring water quality. Even though coliforms are not the predominant gut flora in warm blooded animals and most strains are not pathogenic, they are relatively easy and inexpensive to cultivate. Also, because coliforms are present at 10 million or more cells per gram of fecal matter, gut-adapted strains are useful as indicators of recent fecal contamination.

There are three main tests that are used for water quality purposes to define if a stream has been contaminated with sewage. Total coliform, fecal coliform, and *E. coli* are the three tests for water quality. Total coliform bacteria is the most general group which includes fecal coliform and *E. coli*.

Total coliform bacteria are generally harmless and commonly found in the environment. The presence of total coliform in surface water does not indicate contamination.

Fecal coliform bacteria are a sub-group of the total coliform group. They are normal flora of the intestines of humans and are found in great numbers in the feces of people and animals. The presence of these organisms can indicate possible

contamination of the surface water and there is a greater potential that pathogenic bacteria are present.

*E. coli* is a sub-group of the fecal coliform group. Most *E. coli* are harmless and found in great quantities in the intestines of people and warm-blooded animals. Fecal contamination of surface water is indicative with the presence of *E. coli*.

Standard Methods: For the Examination of Water and Wastewater, 18<sup>th</sup> Ed. explains membrane filtration (MF) technique as the direct plating for the detection and estimation of coliform densities, while the Most Probable Index (MPN) index is the number of coliform bacteria that, more probably than any other number, would give the results shown by the laboratory examination; it is not an actual enumeration (15).

The actual organism used and the concentration in the water are used to determine whether the water is contaminated depends on individual states' laws and regulations. Below are the allowable limits for fecal indicator organisms in the surface water of West Virginia and its neighboring states.

### **WV DEP Limits**

#### 8.13 Fecal Coliform:

Maximum allowable level of fecal coliform content for Primary Water Contact Recreation (either MPN or MF) shall not exceed 200/100 ml as a monthly geometric mean based on not less than 5 samples per month; nor to exceed 400/100 ml in more than ten percent of all samples taken during the month.

8.13.1 Ohio River main stem (zone 1) - During the non-recreational season (November through April only) the maximum allowable level of fecal coliform for the Ohio River

(either MPN or MF) shall not exceed 2000/100 ml as a monthly geometric mean based on not less than 5 samples per month (37).

### **OH EPA Limits**

For the months of May to October, the maximum allowable level of fecal coliform bacteria shall not exceed two hundred per one hundred ml as a monthly geometric mean based on not less than five samples per month; nor exceed four hundred per one hundred ml in more than ten per cent of all samples taken during the month. For the months of May to October, measurements of *Escherichia coli* bacteria may be substituted for fecal coliform. Content shall not exceed one hundred thirty per one hundred ml as a monthly geometric mean, based on not less than five samples per month, nor exceed two hundred forty per one hundred ml in any sample. For the months of November to April, the maximum allowable level of fecal coliform bacteria shall not exceed two thousand per one hundred ml as a geometric mean based on not less than five samples per month (27).

### **KY DEP Limits**

Section 7. Recreational Waters.

(1) Primary contact recreation water. The following criteria shall apply to waters designated as primary contact recreation use:

- (a) Fecal coliform content or *Escherichia coli* content shall not exceed 200 colonies per 100 ml or 130 colonies per 100 ml respectively as a geometric mean based on not less than five (5) samples taken during a thirty (30) day period. Content also shall not exceed 400 colonies per 100 ml in twenty (20) percent or more of all samples taken during a thirty (30) day period for fecal coliform or 240 colonies per 100 ml for *Escherichia coli*. These limits shall be applicable during the recreation season of May

1 through October 31. Fecal coliform criteria listed in subsection (2)(a) of this section shall apply during the remainder of the year.

(b) pH shall be between six and zero-tenths (6.0) to nine and zero-tenths (9.0) and shall not change more than one and zero-tenths (1.0) pH unit within this range over a period of twenty-four (24) hours.

(2) Secondary contact recreation water. The following criteria shall apply to waters designated for secondary contact recreation use during the entire year:

(a) Fecal coliform content shall not exceed 1,000 colonies per 100 ml as a thirty (30) day geometric mean based on not less than five (5) samples; nor exceed 2,000 colonies per 100 ml in twenty (20) percent or more of all samples taken during a thirty (30) day period.

(b) pH shall be between six and zero-tenths (6.0) to nine and zero-tenths (9.0) and shall not change more than one and zero-tenths (1.0) pH unit within this range over a period of twenty-four (24) hours (22).

## **Antibiotic Resistant Bacteria**

### **Sources**

The first antibiotic resistance mechanism was identified a little more than 65 years ago in 1940 (29,34). It involved an enzyme that inactivates penicillin in *Escherichia coli* (34). The first reports of multiple antibiotic resistance were made in the 1970s, and the past decade organisms have been described which are resistant to all known antimicrobial agents (34). The health concern that antibiotic resistant and multiple antibiotic resistant bacteria present is amplified by the phenomenon of horizontal gene transfer. DNA

coding for antibiotic resistance may be transferred by conjugation between microorganisms under rich nutritional conditions such as those found in sewage and in the human gastrointestinal tract (25)

Antibiotic resistant bacteria and antibiotics are discharged in various amounts into the environment as a result of the increasing and often indiscriminate use of antibiotics in medical, veterinary, and agricultural practices (12, 17). River waters are the main receptacle for these pollutants since they receive the sewage of urban runoff (12). Since the ability of many different species of bacteria, including those that cause disease in humans, to resist the inhibitory actions of antimicrobial agents has become a global problem, interest in the distribution of antibiotic resistant bacteria in the environment has grown. The first report of antibiotic resistant bacteria in the Ohio River was presented in 2002 (35). These findings indicate that surface waters are a significant reservoir of antibiotic resistance determinants that is not adequately mapped by standard water quality analyses.

The emergence of antibiotic resistant bacteria is alarming because of the lack of new antibiotics being discovered and marketed. It was once thought that the primary reservoir for antibiotic resistance was hospitals but now it has been found that it extends from agricultural farm lands to fish hatcheries to sewage treatment plants (3, 9, 12, 29).

Animal agriculture operations and fish farms use antibiotics to prevent infectious diseases caused by bacteria or protozoa, to decrease the amount of feed used, and to increase the rate of weight gain in livestock (9). Antibiotic production has increased from 2 million pounds in 1954 to over 50 million pounds per year presently in the US alone (11). It is estimated that more than 70% of the annual antibiotic output is fed to

chickens, pigs, and cows for non-therapeutic purposes (11, 19). It is estimated that 25%-75% of the antibiotics administered to feedlot animals may be excreted unaltered in feces (5). The amending of farm fields with animal manure and processed biosolids from water and wastewater treatment plants is a common agricultural practice, and contaminants absorbed to these amendments may concentrate in the soil over time (29). When a rain event occurs the antibiotics fate may ultimately be transported into a receiving body of water. Surface waters such as rivers often become a downstream community's drinking water source.

Entry of antimicrobials into streams and rivers from human wastewater treatment plant effluent is the most direct route of contamination, and municipalities with hospitals may discharge both antibiotics and resistant bacteria to surface receiving waters. *E. coli* in effluent from modern German activated sludge sewage treatment plants were found to be resistant to several antimicrobials (29). Ozonation of wastewater was recommended to increase the degradation of veterinary and human antimicrobials (29). It has been found that certain antimicrobials in wastewater have a greater than 90% removal rate due to strong absorption to sludge and inactivation by chlorine (29).

The use of antibiotics in medicine, veterinary practice and agriculture has aroused concern about the incidence and spread of antibiotic resistance among bacterial populations. However, it is now clear that medicine and agriculture are not the only contributors to environmental reservoirs of antibiotic resistance since the incidence of resistance appears to increase during sewage and water treatment (1, 20). Antibiotics used by humans are discharged to the sewer systems together with urine and feces and enter the sewage treatment plant. The fate of antibiotics in a sewage treatment plant, as

for any xenobiotics, can be described as follows: the drug may be mineralized by microorganisms to carbon dioxide and water, or the drug or its metabolites may be persistent in the sewage treatment system. In the case of persistent compounds, the antibiotic or its metabolites may be lipophilic which implies that a part of the substance will be retained in the sludge and could later be distributed to agricultural fields and eventually leach into a receiving body of water. Persistent compounds may, instead, be polar, in which case they can easily reach the aquatic environment through the effluent of the plant (16). Nevertheless, little information is available on the frequency and distribution of antibiotic resistant bacteria and how that distribution relates to other water quality indexes along a highly urbanized river receiving a large amount of effluents from wastewater treatment plants (18). Wastewater treatment plants may be releasing antibiotic resistant bacteria directly, and/or they may be releasing sufficient bioactive compounds to allow for selection of resistant strains *in situ*. In either case the aquatic environment acts as a reservoir of resistance genes that may be transferred. The water environment may play a part in providing resistance genes that may be transferred to non-resistant strains, and provides a route of distribution for those strains that are already resistant (18).

### **Antibiotic Resistance as a New Water Quality Parameter**

There is a growing understanding that antibiotic resistant bacteria represent significant environmental contaminants and calls have been made for antibiotic resistance to be considered when establishing bacteriological water quality criteria (4).

The public health implications of drug resistant coliforms in water supplies suggest that the prevalence of these drug resistant bacteria require reevaluation of water quality standards as well as more advanced purification of sewage prior to discharge into the environment (21). Little is yet known about the antibiotic resistance patterns of Gram-negative bacteria that occur in the environment (21).

### **Medical/Genetic**

The genetic traits that determine antibiotic resistance are being investigated as much or more than the actual source of these organisms. Research focuses on many factors that could result in resistance to antibiotics. Some studies focus on specific genes, others investigate exposure times to antibiotics, while other studies seek to determine the prevalence of horizontal gene transfer in the environment.

Grabow identified resistance (R) factors in many Gram-negative bacteria isolated from sewage, river, drinking and sea water, and suggests that such resistance does have an impact on the survival of these organisms in water (2, 14). Resistance (R) factors are nucleic acid elements which confer resistance to antibacterial agents such as antimicrobial drugs, ultraviolet light, bacteriocins, bacteriophage, heavy metals and arsenic compounds (14). Plasmids containing R factors are commonly found in bacteria isolated from the intestinal tract and from human and livestock feces. Resistant bacteria in turn, have been found to be contaminants of river water, especially at sites near sewage outfalls (13). Within these environments the transmission of R determinants may occur in less than one minute and resistance can be spread rapidly among bacteria (2, 14).



Understanding the selection for resistance to antibiotics by bacteria in natural and modified stream environments, e.g., below major sources, may be important to managing streams for human health (24). It is unclear what the selective advantage of resistance to antibiotics is in unpolluted streams, and it may even be a selective disadvantage in unpolluted streams (24). It has been found that an R factor for metals in a stream can be correlated to multiple antibiotic resistances for the same organisms (6).

### **Lack of CSO Studies**

Research on the sources of antibiotic resistant bacteria in the environment is limited to agricultural operations and wastewater treatment plants. No studies have been published to date that involve domestic raw sewage or storm water runoff from urbanized areas. Many communities on the Ohio River have multiple CSOs discharging to the river during wet weather events. This water is untreated and little is known about the identities or concentrations of any of the pollutants present in stormwater. Some of these CSOs can discharge millions of gallons of polluted water within hours. The loading potential of CSOs on a receiving body can be severe. Little has been done to determine the impact CSOs have on a receiving body and nothing is yet known about how they impact the level of antibiotic resistance in the receiving waters.

### **Study Objective**

The emergence of new contaminants such as pharmaceuticals and hormones has caused great concern regarding the quality of surface waters and their impact on public health. Research to date has focused on the presence or absence of these contaminants

but little has been done to identify specific sources. Antibiotic resistance is becoming an area of significant concern, but most studies have focused on agricultural operations.

Recent data from our laboratory suggests that other sources should be considered.

The objectives of this study were: i) to determine the concentration of antibiotic resistant coliforms and *E. coli* in the environment near a CSO in both dry and wet weather conditions; ii) to determine if a small quantity CSO has an impact on Ohio River near shore water during a storm event; and iii) if the level of antibiotic resistance could be correlated with any conventional water quality metrics in a CSO.

## Chapter 2

### Materials and Methods

#### Site Description

The 25<sup>th</sup> Street CSO (Permitted Outfall #014) is a mechanical type of diversion chamber, also called a regulator. The drainage area for the regulator chamber is quite small, 51.4 acres of urban realty which is 75% impermeable and predominately domestic. The 25<sup>th</sup> Street Regulator (N 38°26'00" W 82°24'53") is located at the Ohio River mile point 306.6. The area is occupied by the Ohio River Terminal which loads and unloads coal being transported by barge. The Guyandotte River is the nearest tributary located approximately one mile upstream of the sample site.

The 25<sup>th</sup> Street East Regulator has a 30" inlet pipe in the West Manhole (Figure 1) that carries domestic wastewater. The water is directed in a 90 degree angle to the right through the outlet pipe into the South Manhole. The wastewater enters the 48" Interceptor and is directed towards the wastewater treatment plant.

The CSO and weirs are located in the West Manhole, the steel sluice gate is in the South Manhole, and the counter weight is found in the North Manhole. All of these parts are essential for the overflow to function properly.

During a significant rainfall, stormwater enters the collection system and mixes with sanitary sewage. The combined influent enters the regulator via the 30" inlet pipe. As the flow increases it exceeds the capacity of the 12" outlet pipe. The combined water then flows over the first weir and enters the weep hole (Overflow Inlet in Fig. 1) leading to the counter weight. As the counter weight fills with water it will close the steel sluice

gate in the South Manhole. Once the sluice gate closes all flow is diverted towards the Ohio River via the 32" overflow pipe.

Eventually the rain will subside and flow will decrease. As the flow decreases the counter weight drains, slowly opening the sluice gate allowing the sewer to enter the main system.

### **Sampling Protocol**

A dry weather sampling event was attempted when there had been a 48 hour period of dry weather and a forecast of an additional 24 hours of no precipitation. Once the decision had been made, a team of three (3) individuals traveled to the 25<sup>th</sup> Street Regulator (N 38°26'00" W 82°24'53"), Ohio River mile point 306.6. It was determined that Outfall # 014 would have three sample points; one approximately 100 feet upstream of the outfall (N 38°26'051" W 82°24'841") and 100 feet downstream of the outfall (N 38°26'002" W 82°24'924") and one inside the West Manhole for Outfall #014. The upstream and downstream samples were collected approximately 100 feet off the river bank using a boat. Upstream and downstream samples were collected as grab samples once during every dry weather sampling event to obtain historical data on the Ohio River near the outfall location. The manhole at Outfall #014 was sampled four times within the 24 hour dry weather sampling event. Composite samples (40 CFR 403.12 b. (5) (iii)) such as; BOD, COD, TSS, hardness, ammonia-nitrogen, fluoride, copper, zinc, lead, and nickel were collected by using a Sigma 950 automated sampler. Grab samples were collected with clean (using a nitric acid solution and an acetone rinse) stainless steel cups with new string for each sample site. Dry weather sampling was required to quantify dry

weather flow quality characteristics. Three 24 hour composite sampling events were collected at each site and analyzed during the six month study period.

A wet weather event was characterized by 48 hours of dry weather before a storm of a tenth of an inch or more of rain within an hour. A rain gauge was set on site to confirm the proper amount of rain had fallen. Once the proper amount of rain was reached and the outfall began to spill the sampling event started. The sanitary sewage and stormwater would start flowing over the weir and discharging into the Ohio River. The stormwater and sewage samples from the manhole were collected simultaneously with the upstream and downstream samples. The upstream and downstream sites were sampled from the river bank using a stainless steel bucket and sterile rope. The river samples were not taken by boat due to safety precautions. The buckets were thrown out into the river and brought back in to fill all the bottles. The buckets were rinsed out before each grab. All bottles were labeled with the proper time, date, intended analysis, preserve, lab number, and sampler. The bottles were then brought back to the central base and cooled to 4° C. The manhole was sampled using a Sigma 900, automated sampler. The list of pollutants assayed for during each event can be found in Table 1.

The potential pollutants that were assayed for in composite samples during dry weather sampling were assayed for in grab samples during wet weather events. The justification for the change in sampling techniques was to demonstrate whether a CSO's discharge eventually becomes more or less contaminated or if its level of contamination was constant.

Dry weather sampling was done three times according to the WV DEP approved protocol (Appendix A). The first sampling event started 22 June 2005. The first sample

for the manhole was at 3:45 pm. Three additional grab samples at the manhole were taken at approximately eight hour intervals after the first grab. The remaining grab times for the manhole were 11:45 pm, 8:25 am, and 3:45 pm. The latter two samples were collected on 23 June 2005. The upstream and downstream samples were taken on June 23, 2005 at 3:45pm.

The second dry weather sampling event started on 27 June 2005. The first grab time for the manhole was 4:23 pm. The upstream and downstream samples were also taken at that time. The grab sampling times were 11:35pm, 8:45am, and 1:40pm. The latter two samples were collected on 28 June 2005.

The final dry weather sampling event started on was 7 September 2005. The first grab at the manhole was taken at 9:45 am. The upstream and downstream grabs were collected at approximately 10:50 am. The following three grabs for the manhole were at 4:45 pm, 11:20 pm, and finally 8: 45am. The final sample was collected on 8 September 2005.

The first wet weather event occurred on 16 August 2005. The outfall started purging and the initial grab for all three sites was taken immediately after at 2:30pm. The following three grab samples were taken at 2:45 pm, 3:00 pm and then 3:20 pm. This was considered a short outfall event due to the time of the actual purging.

The second wet weather event lasted longer due to the intensity of the storm. The second sampling occurred 7 October 2005. The sampling event included six total grabs samples to be taken, but only four grab samples were taken for *E. coli* and antibiotic resistance. The times of the grab samplings for all three sites were 3:40am, 3:55am, 4:15am, and 4:30am.

The final wet weather event occurred 29 November 2005. The system began to purge at 2:30 am. The three following grabs were at 2:50 am, 3:10 am, and finally at 3:25 am. The upstream and downstream sites were sampled simultaneously as the manhole times.

During the six months of sampling a flow meter (Isco 4150 Flowlogger) was placed in the influent pipe and overflow pipe to record flows for dry and wet weather conditions. The meters were monitored twice a week for battery life, desiccant status, and downloading. A rain gauge (Sigma 900) was used to measure lengths of dry periods and the intensity of storm events.

### **Bacteriological Sampling**

During June 2005 thru November 2005 samples were enumerated for total coliforms, *E. coli*, ciprofloxacin-resistant coliforms, ciprofloxacin-resistant *E. coli*, erythromycin-resistant coliforms, erythromycin-resistant *E. coli*, tetracycline-resistant coliforms, and tetracycline-resistant *E. coli*. The antibiotics used in this study were noted as emerging contaminants by Kolpin *et al.* (2002, 19) during a nationwide reconnaissance of the occurrence of pharmaceuticals, hormones, and other organic wastewater contaminants in water resources. The river samples for total coliform and *E. coli* were assayed as collected. Wastewater samples were diluted to a 1:100 solution with sterile deionized water prior to analyses. The samples were then inoculated with 0.1 ml of an individual antibiotic stock and thoroughly mixed (Appendix C).

Total coliforms and *E. coli* were enumerated using IDEXX (Westbrook, ME) Colilert® reagent and Quanti-Tray®/2000 incubations trays according to the manufacturer's directions. Antibiotic resistant coliforms and *E. coli* were enumerated by the same methods for total coliforms and *E. coli* except that individual antibiotics were added to samples prior to transfer to the incubation trays. Ciprofloxacin (Cellgro, Herndon, VA) was added to IDEXX Colilert® medium at a final concentration of 4 µg/ml. Erythromycin (Fisher Scientific, Fair Lawn, NJ) was added to the medium at a final concentration of 8 µg/ml. Tetracycline (Fisher Scientific, Fair Lawn, NJ) was added to the medium at a final concentration of 12.5 µg/ml. All Colilert® cultures were incubated at 35°C for 24 hours. MPN estimations were made by counting the number of positive wells after incubation.

Samples were taken using sterile stainless steel dip cups on the Ohio River near mile point 306.6. Site specific sampling cups were marked and used each time for the upstream and downstream grabs. The outfall samples were collected using a Sigma 900 (MCS) automated sampler. Sodium thiosulfate (Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>) was added to each 100 ml sample to neutralize any residual chlorine.

Each location (inside CSO, upstream, and downstream) was sampled four times regardless of the intensity of the rain event. Fecal coliform, total coliform, *E. coli*, and antibiotic resistant coliforms and *E. coli* were enumerated from each grab sample. All samples were placed on ice and cooled to 4°C prior to cultivation. The samples were taken to Marshall University and analyzed within six hours of collection.

Fecal coliforms were enumerated by filtering suitable aliquots of water through disposable 0.45 µm cellulose filters (Nalgene, Rochester, NY). Aliquots of water were



determined based on the turbidity at each sampling site. Less water was filtered with increased turbidity. Membranes were incubated on m-FC medium (Gelman Laboratory, Ann Arbor, MI) at 44.5°C in an incubator at the wastewater treatment plant for 24 hours. Blue colonies were counted and recorded as fecal coliform colony forming units (CFU) per 100 ml.

The antibiotics used in this study were noted as emerging contaminants by Kolpin *et al.* (2002, 19) during a nationwide reconnaissance of the occurrence of pharmaceuticals, hormones, and other organic wastewater contaminants in water resources.

Total coliforms, *E. coli*, and antibiotic resistant coliforms and *E. coli* were analyzed using the EPA approved Idexx Quanti-Tray/ 2000® method. Reagent powder packs were added to aliquots (100 ml) of whole river water and diluted wastewater, transferred to the 97 well Quanti-Tray®, sealed, and incubated for 24 hours at 37° C. After incubation, clear wells were negative for coliform bacteria and positive wells turned yellow due to the break down of 2-Nitrophenyl-β-Dgalactopyranoside (ONPG). Wells that fluoresced under UV light were positive for *E.coli* since these bacteria express an enzyme capable of hydrolyzing 4-Methylumbelliferyl-β-D-glucuronide (4-MUG).

## Chapter 3

### Results

#### Dry Weather # 1

The first dry weather sampling event occurred on 22 and 23 June 2005. The upstream and downstream samples were done as single grabs. The results from the enumeration of total coliforms, *E. coli*, antibiotic resistant coliforms, and antibiotic resistant *E. coli* for both sites and the composite samples for the manhole site are shown in Table 3.1.

Erythromycin resistant counts were markedly higher than tetracycline or ciprofloxacin resistant counts, both in river water samples and raw sewage.

#### Dry Weather # 2

The second dry weather sampling event was completed on 27 and 28 June 2005. The upstream and downstream samples were taken at the time of the first grab for the manhole. The coliform and *E. coli* data from the upstream, downstream, and manhole composite samples are listed in Table 3.2.

#### Dry Weather # 3

The third and final dry weather sampling event started 7 September 2005 and was completed on 8 September 2005. This time the upstream and downstream grabs were not taken simultaneously with the manhole. The manhole's first grab was approximately an hour before a boat could be accessed and the upstream and downstream samples were collected. The upstream, downstream, and manhole data for the final dry weather is listed in Table 3.3.

The upstream and downstream results concluded that erythromycin resistant *E. coli* has a higher percentage of existence in the environment than other antibiotic resistant bacteria.

**Table 3.1: Total coliforms and *Escherichia coli* counts in dry weather # 1 samples (22 and 23 June 2005).**

	TOTAL COLIFORM				TOTAL E. COLI			
	CONTROL	TET +	ERY+	CIPRO +	CONTROL	TET +	ERY+	CIPRO +
	CFU/100ml	(%)	CFU/100ml	(%)	CFU/100ml	(%)	CFU/100ml	(%)
UPSTREAM # 1 2:15 PM	1986.3	13.4 (0.67%)	1203.3 (60.58%)	1 (0.05%)	40.8	1 (2.45%)	26.2 (64.22%)	1 (2.45%)
DOWNSTREAM # 1 3:00 PM	1203.3	5.2 (0.43%)	1299.7 (108.01%)	1 (0.08%)	25.6	< 1 (3.91%)	15.8 (61.72%)	< 1 (3.91%)
MANHOLE # 1								
3:45PM	816.4	2 (0.24%)	727 (89.05%)	1 (0.12%)	517.2	2 (0.39%)	344.8 (66.67%)	< 1 (0.19%)
11:45PM	172.2	< 1 (0.58%)	135.4 ((78.63%)	< 1 (0.58%)	137.6	< 1 (0.73%)	114.5 (83.21%)	< 1 (0.73%)
8:25AM	259.5	7.5 (2.89%)	209.8 (80.85%)	< 1 (0.39%)	158.5	3.1 (1.96%)	117.8 (74.32%)	< 1 (0.63%)
3:45PM	980.4	1 (0.10%)	770.1 (78.55%)	7.3 (0.74%)	648.8	1 (0.15%)	648.8 (100.00%)	2 (0.31%)

**Table 3.2: Total coliforms and *Escherichia coli* counts in dry weather # 2 samples (26 and 27 June 2005).**

	TOTAL COLIFORM								TOTAL E. COLI							
	CONTROL		TET +		ERY+		CIPRO +		CONTROL		TET +		ERY+		CIPRO +	
	CFU/100ml	(%)	CFU/100ml	(%)	CFU/100ml	(%)	CFU/100ml	(%)	CFU/100ml	(%)	CFU/100ml	(%)	CFU/100ml	(%)	CFU/100ml	(%)
UPSTREAM # 2 4:30 PM	461.1	<1	(0.22%)	209.8	(45.50%)	< 1	(0.22%)	5.2	< 1	(19.23%)	3	(57.69%)	< 1	(19.23%)		
DOWNSTREAM # 2 4:40 PM	365.4	4.1	(1.12%)	95.7	(26.19%)	< 1	(0.27%)	4.1	< 1	(24.39%)	5.2	(126.83%)	< 1	(24.39%)		
MANHOLE # 2																
4:23PM	> 2419.6	5.2	(0.21%)	> 2419.6	(100.00%)	< 1	(0.04%)	686.7	4.1	(0.60%)	686.7	(100.00%)	< 1	(0.15%)		
11:35PM	2419.6	1	(0.04%)	1732.9	(71.62%)	< 1	(0.04%)	1986.3	1	(0.05%)	1553.1	(78.19%)	< 1	(0.05%)		
8:45AM	2419.6	1	(0.04%)	1119.9	(46.28%)	< 1	(0.04%)	1299.7	1	(0.08%)	547.5	(42.13%)	< 1	(0.08%)		
1:40PM	1986.3	8.5	(0.43%)	1732.9	(87.24%)	5.2	(0.26%)	816.4	5.2	(0.64%)	727	(89.05%)	< 1	(0.12%)		

**Table 3.3: Total coliforms and *Escherichia coli* counts in dry weather # 3 samples (7 and 8 September 2005).**

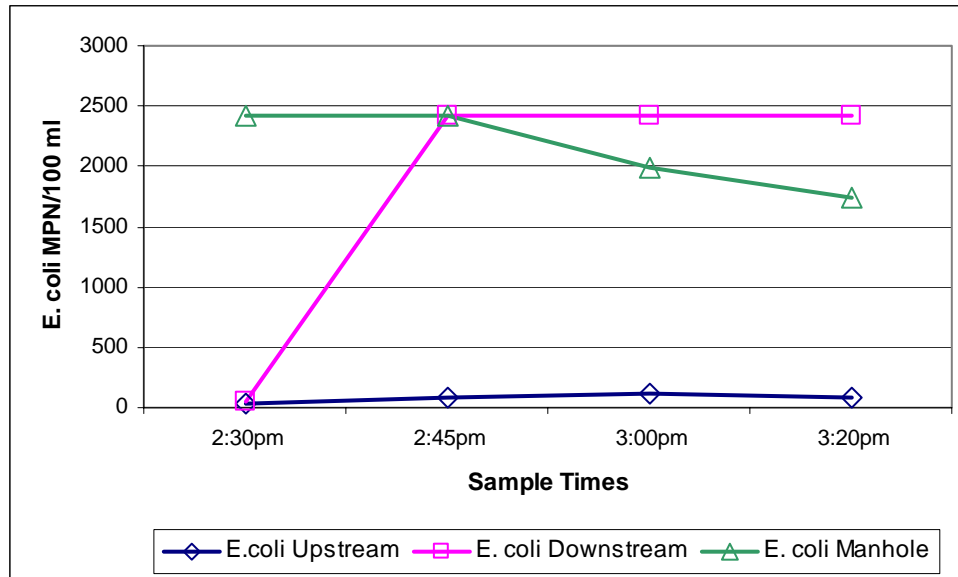
	TOTAL COLIFORM								TOTAL E. COLI							
	CONTROL		TET +		ERY+		CIPRO +		CONTROL		TET +		ERY+		CIPRO +	
	CFU/100ml	(%)	CFU/100ml	(%)	CFU/100ml	(%)	CFU/100ml	(%)	CFU/100ml	(%)	CFU/100ml	(%)	CFU/100ml	(%)	CFU/100ml	(%)
UPSTREAM # 3 10:54 AM	2419.6	13.4	(0.55%)	1413.6	(58.42%)	1	(0.04%)	69.7	4.1	(5.88%)	18.5	(26.54%)	< 1	(1.43%)		
DOWNSTREAM # 3 10:46 AM	2419.6	28.5	(1.18%)	1413.6	(58.42%)	1	(0.04%)	109	8.4	(7.71%)	69.5	(63.76%)	< 1	(0.92%)		
MANHOLE # 3																
9:45AM	> 2419.6	44.3	(1.83%)	> 2419.6	(100.00%)	61.3	(2.53%)	> 2419.6	1	(0.04%)	2419.6	(100.00%)	< 1	(0.04%)		
4:45PM	> 2419.6	98.8	(4.08%)	> 2419.6	(100.00%)	172.3	(7.12%)	1732.9	4.1	(0.24%)	172.5	(9.95%)	< 1	(0.06%)		
11:20PM	1986.3	6.3	(0.32%)	1299.7	(65.43%)	16	(0.81%)	1203.3	< 1	(0.08%)	517.2	(42.98%)	< 1	(0.08%)		
8:45AM	2419.6	7.5	(0.31%)	1553.1	(64.19%)	4.1	(0.17%)	1986.3	2	(0.10%)	920.8	(46.36%)	< 1	(0.05%)		

## **Wet Weather # 1**

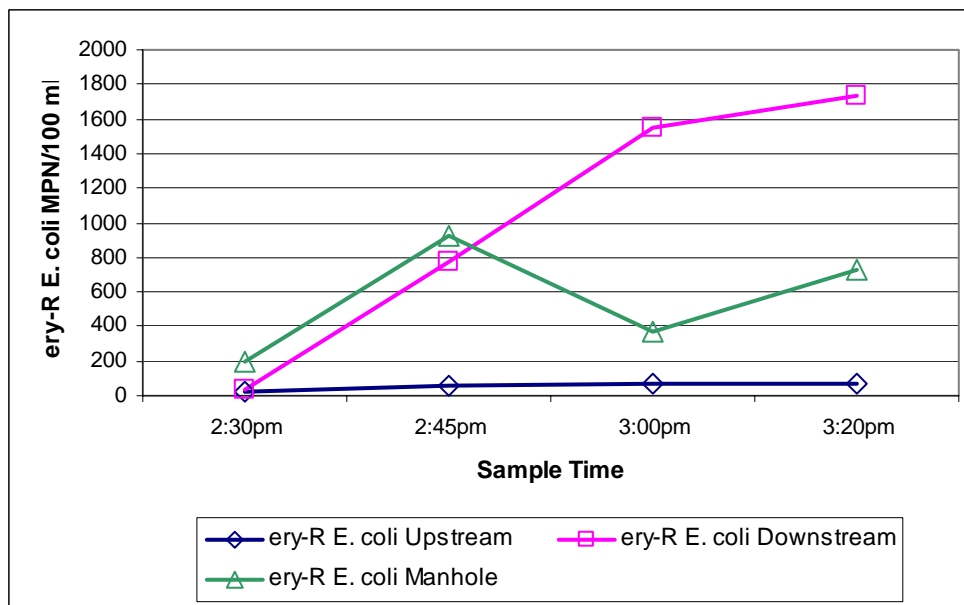
The first wet weather sampling event occurred on 16 September 2005. Samples were collected at 2:30 pm, 2:45 pm, 3:00 pm, and 3:20 pm. The changes in *E. coli* counts over time at the upstream and downstream sites are shown in Fig. 3.1. The upstream and downstream sites have similar *E. coli* counts at the beginning of the event, but the downstream counts increase rapidly and remain high relative to the upstream site throughout the sampling cycle.

The changes in erythromycin resistant *E. coli* counts over time at the upstream and downstream sites are shown in Fig. 3.2. The upstream and downstream sites have similar counts of erythromycin-resistant *E. coli* counts at the beginning of the event, but the downstream counts increase rapidly and remain high relative to the upstream site throughout the sampling cycle.

Tetracycline resistant *E. coli* increased steadily the entire event shown in Fig. 3.3. Ciprofloxacin resistant *E. coli* was done but no colonies were found.

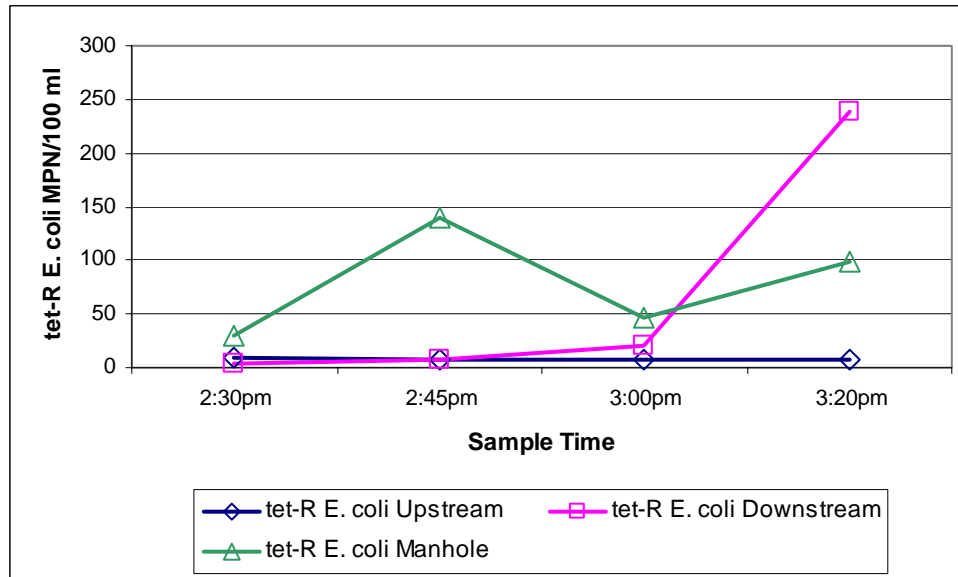


**Figure 3.1.** Counts (MPN per 100 ml) of *E. coli* upstream, downstream, and in the manhole of the 25<sup>th</sup> Street CSO during the first wet weather sampling event, 16 September 2005.



**Figure 3.2.** Counts (MPN per 100 ml) of erythromycin-resistant *E. coli* upstream, downstream, and in the manhole of the 25<sup>th</sup> Street CSO during the first wet weather sampling event, 16 September 2005.



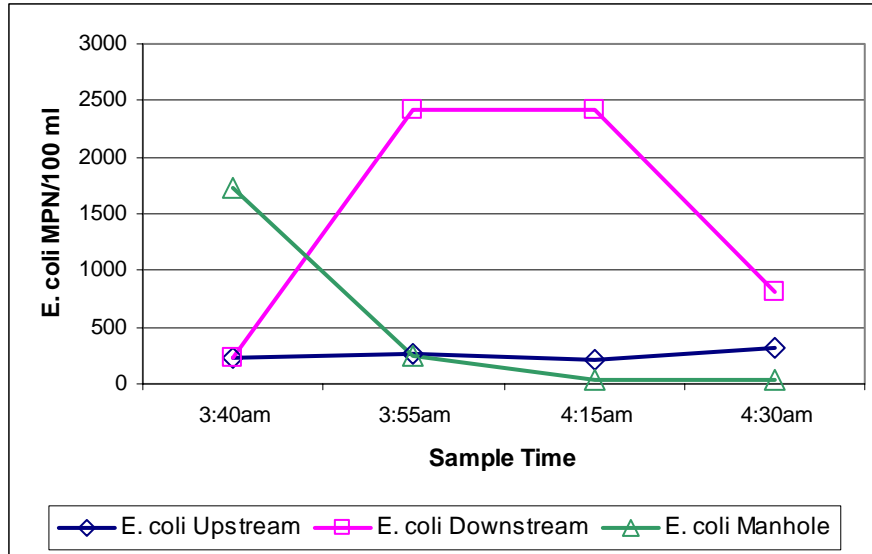


**Figure 3.3.** Counts (MPN per 100 ml) of tetracycline-resistant *E. coli* upstream, downstream, and in the manhole of the 25<sup>th</sup> Street CSO during the first wet weather sampling event, 16 September 2005.

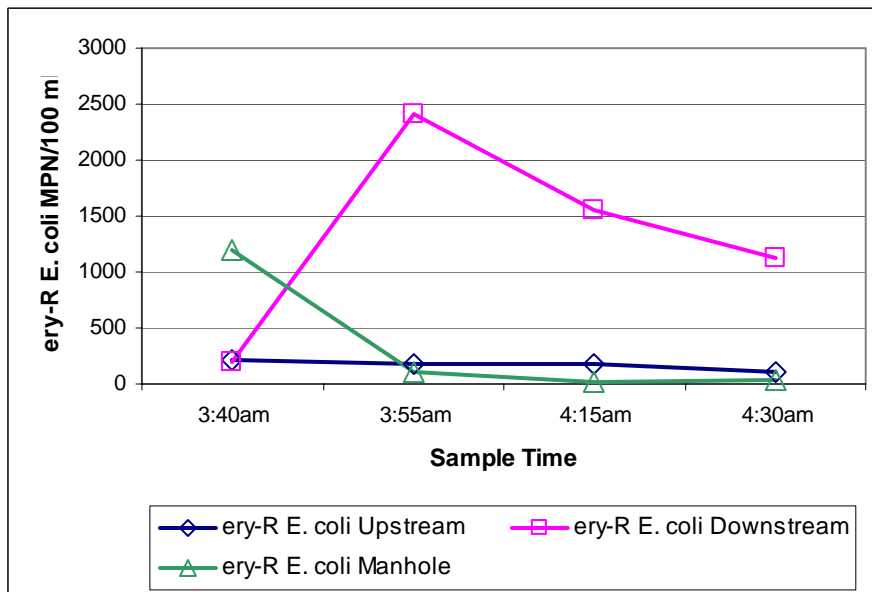
## **Wet Weather # 2**

The second wet weather event occurred during the morning of 7 October 2005. The grab times for the event were 3:40 am, 3:55 am, 4:15 am, and the final grab was at 4:30 am. The numbers (MPN/100 ml) of *E. coli* at the upstream and downstream sample sites at each time during this event are shown in Fig. 3.4. The numbers of *E. coli* detected per 100 ml of river water were higher than during the first wet weather event, but the overall patterns were similar. The changes in *E. coli* numbers during the rain event were much smaller at the upstream site than at the downstream site. Also, the transient nature of the CSO impact is shown more clearly in this set of samples than in the first wet weather event.

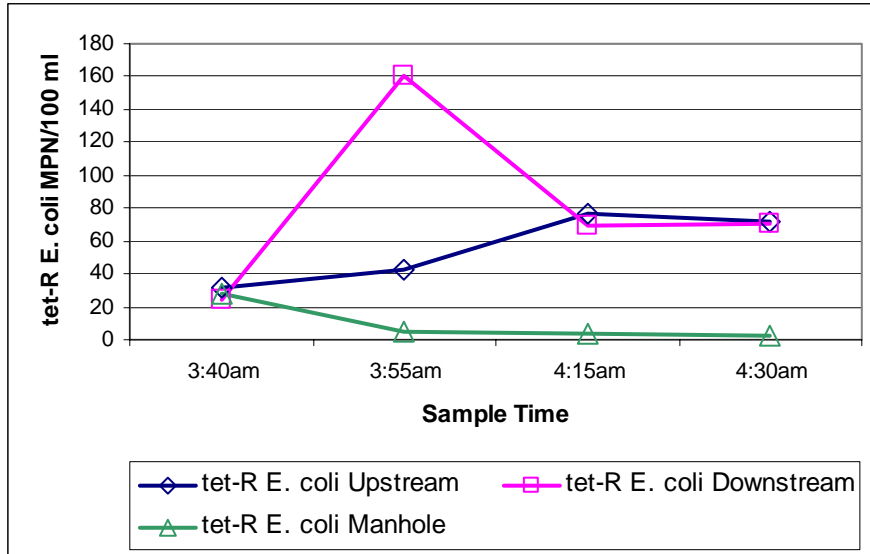
The numbers (MPN/100 ml) of erythromycin-resistant and tetracycline-resistant *E. coli* at the upstream and downstream sample sites at each time during the second wet weather event are shown in Fig. 3.5 and 3.6 respectively . The upstream and downstream counts are very similar at the beginning of the wet weather event, but the downstream numbers rise rapidly in a short time. As was noted for the *E. coli* counts during this event, the spike in erythromycin-resistant *E. coli*, relative to the upstream site, is transient.



**Figure 3.4.** Counts (MPN per 100 ml) of *E. coli* upstream, downstream, and in the manhole of the 25<sup>th</sup> Street CSO during the second wet weather sampling event, 7 October 2005.



**Figure 3.5.** Counts (MPN per 100 ml) of erythromycin-resistant *E. coli* upstream, downstream, and in the manhole of the 25<sup>th</sup> Street CSO during the second wet weather sampling event, 7 October 2005.



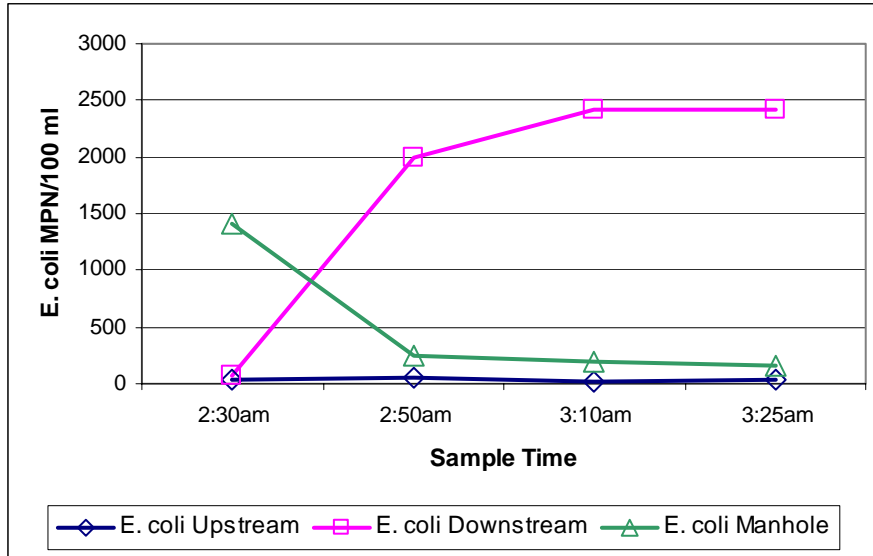
**Figure 3.6.** Counts (MPN per 100 ml) of tetracycline-resistant *E. coli* upstream, downstream, and in the manhole of the 25<sup>th</sup> Street CSO during the second wet weather sampling event, 7 October 2005.

### **Wet Weather # 3**

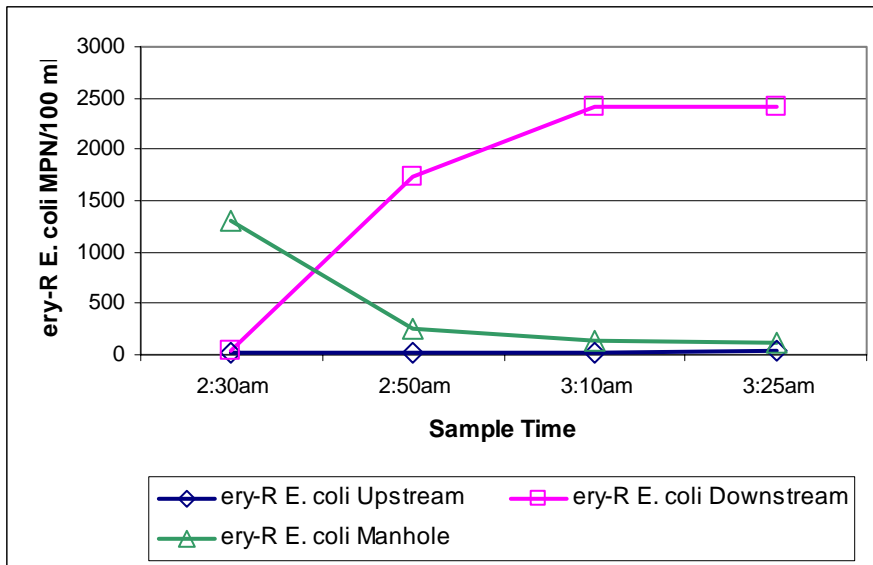
The third and final wet weather event occurred the morning of 29 November 2005. The grab times for all three sites were 2:30 am, 2:50 am, 3:10 am, and 3:25 am. The numbers (MPN/100 ml) of *E. coli* at the upstream, manhole and downstream sample sites at each time during this event are shown in Fig. 3.7. The changes in *E. coli* numbers during the rain event were much smaller at the upstream site than at the downstream site. Also, the counts of *E. coli* in the manhole indicate that the highest impact from the CSO on the river occurs within the first few minutes of the outfall event. The fact that the CSO has a detectable impact on river water quality is demonstrated by the rising *E. coli* counts downstream of the CSO during the rain event.

The numbers (MPN/100 ml) of erythromycin-resistant *E. coli* at the upstream and downstream sample sites at each time during the third wet weather event are shown in Fig. 3.8. The upstream and downstream counts are very similar at the beginning of the wet weather event, but the downstream numbers rise rapidly in a short time. The downstream site went from 41.9 CFU/100ml to greater than 2419.6 CFU/100ml within 55 minutes.

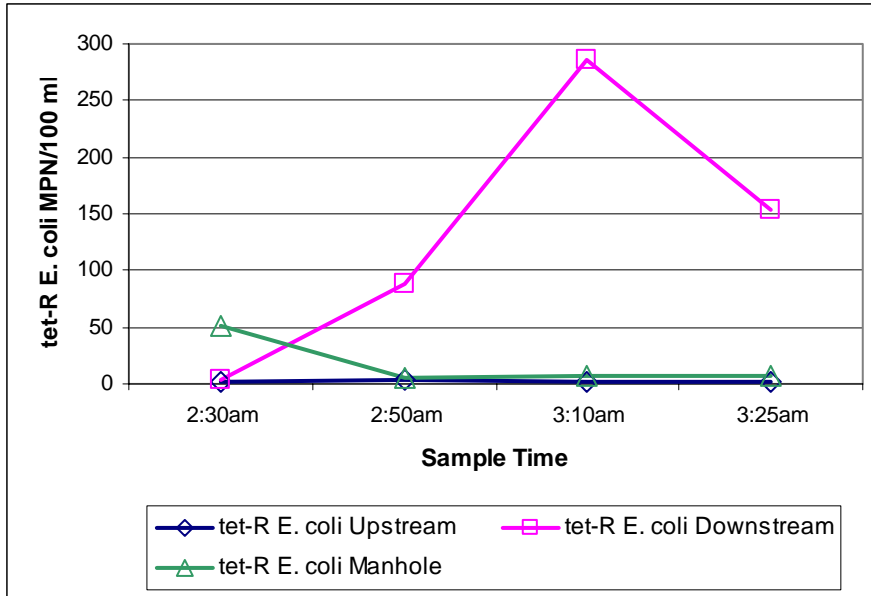
The numbers (MPN/100 ml) of tetracycline-resistant and ciprofloxacin-resistant *E. coli* at the upstream and downstream sample sites at each time during the third wet weather are shown in Fig. 3.9 and 3.10 respectively. The upstream and downstream counts are very similar at the beginning of the event but the downstream numbers rise rapidly in a short time.



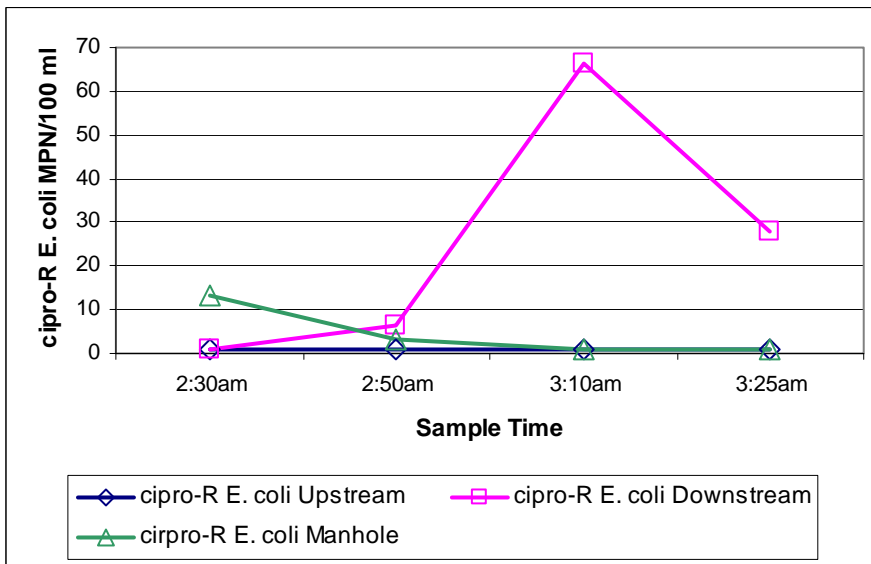
**Figure 3.7.** Counts (MPN per 100 ml) of *E. coli* upstream, in the manhole, and downstream of the 25<sup>th</sup> Street CSO during the third wet weather sampling event, 29 November 2005.



**Figure 3.8.** Counts (MPN per 100 ml) of erythromycin-resistant *E. coli* upstream, downstream, and in the manhole of the 25<sup>th</sup> Street CSO during the third wet weather sampling event, 29 November 2005.



**Figure 3.9.** Counts (MPN per 100 ml) of tetracycline-resistant *E. coli* upstream, downstream, and in the manhole of the 25<sup>th</sup> Street CSO during the third wet weather sampling event, 29 November 2005.



**Figure 3.10.** Counts (MPN per 100 ml) of ciprofloxacin-resistant *E. coli* upstream, downstream, and in the manhole of the 25<sup>th</sup> Street CSO during the third wet weather sampling event, 29 November 2005.

## Correlations

Correlation coefficients were calculated for all possible pair wise comparisons of the analyte data sets to determine if there was a significant level of correlation between any two sets of measurements. Table 3.4 lists the correlation coefficients. The numbers in blue represent correlations in which  $P = 0.01$  to  $0.05$ . Numbers in red represent a correlation of  $P \leq 0.01$ . Tables 3.5-3.7 show the data accumulated during the dry weather sampling events and Tables 3.8-3.10 illustrate the data accumulated during the wet weather sampling events.



	BOD	TSS	NH3	COD	HARD	Zn	Fecal	Total E. coli	TREC
BOD	1								
TSS	7.18E-08	1							
NH3	0.8013	0.7368	1						
COD	3.57E-09	2.07E-09	0.9006	1					
HARD	0.07512	0.004754	0.1523	0.00066	1				
FL	-4.51E+10	0.6897	1.69E-05	1	1				
Cu	0.8402	0.564	0.6571	1	1				
Zn	9.56E-09	6.41E-09	0.5866	1.78E-06	0.00044	1			
Fecal	0.04938	0.2935	0.4958	1	0.997	0.0779	1		
O&G	0.08312	0.007434	0.9172	0.8655	1	0.8543	0.6821		
Ph	0.001481	0.0002851	0.1859	0.004468	0.00415	0.09874	0.9183		
Temp	0.2947	0.8853	0.1371	0.8099	0.00168	0.8506	0.04624		
DO	0.1923	0.07811	0.01252	0.002954	0.1344	0.03956	0.1885		
Bis 2	0.02535	0.0008434	0.2321	0.8014	0.9163	0.7843	0.000353		
EC Total	0.005141	0.002001	0.7351	0.003197	0.76	0.003385	0.000973	1	
TREC	0.3028	0.4803	0.2651	0.1493	0.06923	0.3625	0.002256	4.48E-06	1
EREC	0.07295	0.2702	0.2147	0.000548	0.9555	0.009224	0.000198	1.93E-09	7.56E-05
CREC	0.5461	0.6379	0.546	1	1	1	1	1	1

**Table 3.4:** Correlations between all analytes measured in the 25<sup>th</sup> Street CSO. Numbers highlighted in red represent a correlation of  $P \leq 0.01$ . Numbers in blue represent  $P = 0.05$  to  $0.01$ .

**Table 3.5:** Chemical analyte concentrations (mg/L) and fecal coliform counts (CFU/100 ml) for dry weather #1 (22 and 23 June 2005).

	BOD	TSS	NH3	COD	HARD	FL	Cu	Zn	Pb	Ni	Fecal	O&G	Phenols	Ph	Temp	DO	Bis 2
UPSTREAM # 1 2:15 PM	2	8.8	7.75	13	150	< 0.2	< 0.006	0.004	< 0.02	< 0.01	EST. 7	< 2.66	< 0.033	8	28	8.4	0.001
DOWNSTREAM # 1 3:00 PM	1	8	< 0.103	13	154	< 0.2	< 0.006	0.004	< 0.02	< 0.01	EST. 20	< 2.66	< 0.033	7.7	28	6.8	0.004
Manhole # 1																	
3:45PM	21	12.4	3.89	80	148		0.015	0.107	< 0.02	< 0.01	30,000	4.52	0.033	7.9	26	4.7	0.0065
11:45PM											8,600	2.66	0.033	7.9	22	5.4	
8:25AM											118,800	2.66	0.033	7.8	22	6.4	
3:45PM											2,800	2.66	0.033	7.7	25	5.7	

**Table 3.6:** Chemical analyte concentrations (mg/L) and fecal coliform counts (CFU/100 ml) for dry weather #2 (26 and 27 June 2005).

		BOD	TSS	NH3	COD	HARD	FL	Cu	Zn	Pb	Ni	Fecal	O&G	Phenols	Ph	Temp	DO	Bis 2
UPSTREAM # 2	4:30																	
PM		< 2	1.4	0.21	12	154	0.15	< 0.006	0.006	< 0.02	< 0.01	EST 13	< 2.66	< 0.033	8.1	30	8.5	0.0009
DOWNSTREAM # 2	4:40																	
PM		< 2	4.6	< 0.107	14	153	0.17	< 0.006	< 0.003	< 0.02	< 0.01	< 1	< 2.66	< 0.033	8.1	29	8.6	0.0009
Manhole # 2																		
4:23PM		48	21	4.1	129	151	0.78	0.027	0.185	< 0.02	0.011	166,320	2.66	0.042	7.5	26	4.27	0.0277
11:35PM												118,800	2.66	0.037	7.4	25	4.3	
8:45AM												231,660	2.66	0.044	7.4	24	4.4	
1:40PM												2,800	2.66	0.035	7.5	27	4.1	

**Table 3.7:** Chemical analyte concentrations (mg/L) and fecal coliform counts (CFU/100 ml) for dry weather #3 (7 to 8 September 2005).

	BOD	TSS	NH3	COD	HARD	FL	Cu	Zn	Pb	Ni	Fecal	O&G	Phenols	Ph	Temp	DO	Bis 2
UPSTREAM # 3 10:54 AM	2	14	< 0.034	21	131	< 0.2	< 0.006	0.012	< 0.02	< 0.01	EST 13	< 2.66	< 0.033	6.9	26	4.95	< 0.0066
DOWNSTREAM # 3 10:46 AM	3	11.75	1.19	66	134	0.21	< 0.006	0.022	< 0.02	< 0.01	140	< 2.66	< 0.033	7.2	26	4.45	< 0.0066
Manhole # 3																	
9:45AM	30	11	5.99	88	138	0.89	0.024	0.16	< 0.02	< 0.01	445,500	8.74	0.033	7.8	24	1.99	0.018
4:45PM											524,700	2.66	0.033	7.5	28	2.63	
11:20PM											148,500	2.66	0.033	7	22	2.8	
8:45AM											163,350	2.66	0.033	7.2	21	2.1	

**Table 3.8:** Chemical analyte concentrations (mg/L) and fecal coliform counts (CFU/100 ml) for wet weather #1 (16 September 2005).

	BOD	TSS	NH3	COD	HARD	FL	Cu	Zn	Pb	Ni	Fecal	O&G	Phenols	Ph	Temp	DO	Bis 2
<b>UPSTREAM # 1</b>																	
2:30 PM	2	19.8	0.11	< 13.7	147	0.22	<0.006	0.011	<0.02	0.028	80	< 2.66	< 0.033	7.6	28	5.8	<0.0076
2:45 PM	2	19.6	0.39	< 13.7	143	0.2	<0.006	0.006	<0.02	<0.01	147	< 2.66	< 0.033	7.5	29	6.01	<0.0071
3:00 PM	<2	15.2	0.74	< 13.7	141	0.2	<0.006	0.011	<0.02	<0.01	120	< 2.66	< 0.033	7.5	29	7.4	<0.0074
3:20 PM	<2	11.2	1.02	< 13.7	139	0.21	0.037	0.034	<0.02	<0.01	390	< 2.66	< 0.033	7.5	29	7.8	
<b>DOWNSTREAM #1</b>																	
2:30 PM	2	14.4	0.14	< 13.7	142	<0.2	<0.006	0.022	<0.02	<0.01	80	< 2.66	< 0.033	7.5	28	5.73	<0.0074
2:45 PM	2	19.6	0.53	< 13.7	141	<0.2	<0.006	0.021	<0.02	<0.01	>1200	< 2.66	< 0.033	7.4	28.2	7.93	<0.0074
3:00 PM	2.25	21.2	0.7	< 13.7	144	<0.2	<0.006	0.016	<0.02	<0.01	>1200	< 2.66	< 0.033	7.5	29	7.4	<0.0074
3:20 PM	2	18.8	0.81	< 13.7	144	<0.2	0.007	0.05	<0.02	<0.01	>1200	< 2.66	< 0.033	5.8	28	7.6	<0.0074
<b>Manhole # 1</b>																	
2:30 PM	97	417.5	2.52	201	153	<0.2	0.006	2.51	<0.02	<0.01	118,800	16.4	0.047	7.2	27	4.09	<0.0072
2:45 PM	155	483	1.44	886.5	92	<0.2	0.015	2.24	<0.02	<0.01	118,800	5.7	0.033	6.5	27	5.3	<0.007
3:00 PM	109	794	1.02	580	70.3	0.21	0.021	1.03	<0.02	<0.01	118,800	17	0.188	6.4	27	5.7	<0.0069
3:20 PM	54	336	0.39	290	139	<0.2	0.006	0.003	<0.02	<0.01	118,800	14.2	0.5	6.8	26.1	5.64	<0.0066

**Table 3.9:** Chemical analyte concentrations (mg/L) and fecal coliform counts (CFU/100 ml) for wet weather #2 (7 October 2005).

	BOD	TSS	NH3	COD	HARD	FL	Cu	Zn	Pb	Ni	Fecal	O&G	Phenols	Ph	Temp	DO	Bis 2
<b>UPSTREAM # 2</b>																	
3:40 AM	< 2	5.5	0.63	19	157	0.2	< 0.006	0.006	< 0.02	< 0.01	210	< 2.66	< 0.033	7.5	23	7.84	< 0.006
3:55 AM	< 2	14.5	0.245	20	165	0.22	0.007	0.014	< 0.02	< 0.01	288	< 2.66	< 0.033	7.7	23	7.95	< 0.006
4:15 AM	< 2	10.5	0.123	21	165	0.22	0.007	0.005	< 0.02	< 0.01	350	< 2.66	< 0.033	7.5	23	7.89	< 0.006
4:30 AM	< 2	3	0.245	29	160	0.24	< 0.006	0.007	< 0.02	< 0.01	356	< 2.66	< 0.033	7.6	23	7.83	< 0.006
<b>DOWNSTREAM #2</b>																	
3:40 AM	< 2	25.5	< 0.106	23	157	0.25	0.006	0.004	< 0.02	< 0.01	460	< 2.66	< 0.033	7.6	23	7.71	< 0.006
3:55 AM	< 2	13	0.455	16	158	0.24	0.007	0.017	< 0.02	< 0.01	2000	< 2.66	< 0.033	7.8	23	7.84	< 0.006
4:15 AM	< 2	18	< 0.123	16	155	0.23	0.008	0.015	< 0.02	< 0.01	1966.7	< 2.66	< 0.033	7.8	23	7.76	< 0.006
4:30 AM	< 2	13	< 0.123	13.7	159	0.23	0.006	0.005	< 0.02	< 0.01	925	< 2.66	< 0.033	7.8	23	7.82	< 0.006
<b>Manhole # 2</b>																	
3:40 AM	124	266	0.53	317	52.3	< 0.2	0.025	0.833	0.022	< 0.01	204600	9.89	< 0.033	7.1	14	7.97	0.228
3:55 AM	39	156	0.39	155	57.1	< 0.2	0.044	0.276	0.024	< 0.01	59400	9.58	< 0.033	7	13	7.8	0.0438
4:15 AM	16	50	0.46	79	68.5	< 0.2	0.021	0.214	0.02	< 0.01	20791	2.4	< 0.033	7.2	14	8	0.006
4:30 AM	15	73	0.56	76	66.2	< 0.2	0.02	0.154	0.02	< 0.01	6600	3.33	< 0.033	7.5	13	7	0.0703

**Table 3.10:** Chemical analyte concentrations (mg/L) and fecal coliform counts (CFU/100 ml) for wet weather #3 (29 November 2005).

	BOD	TSS	NH3	COD	HARD	FL	Cu	Zn	Pb	Ni	Fecal	O&G	Phenols	Ph	Temp	DO	Bis 2	
<b>UPSTREAM # 3</b>																		
2:30 AM	2	15.2	< 0.106	20	121	< 0.2	< 0.006	0.01	< 0.02	< 0.01	74	< 2.66	< 0.033	7.2	10	9.35	< 0.00601	
2:50 AM	2	13.2	< 0.106	28	121	< 0.2	< 0.006	0.007	< 0.02	< 0.01	60	< 2.66	< 0.033	7	11	7.2	< 0.00601	
3:10 AM	2	9.2	< 0.106	26	121	< 0.2	< 0.006	0.005	< 0.02	< 0.01	94	< 2.66	< 0.033	7.4	11	6.94	< 0.00601	
3:25 AM	2	6.4	< 0.106	24	119	< 0.2	< 0.006	0.003	< 0.02	< 0.01	27	< 2.66	< 0.033	7.4	11	7.1	< 0.00601	
<b>DOWNSTREAM #3</b>																		
2:30 AM	3	55	0.42	19	127	< 0.2	< 0.006	0.009	< 0.02	< 0.1		< 2.66	< 0.033	7.2	10	2:30am	< 0.006	
2:50 AM	4	276.5	0.42	46	115	< 0.2	< 0.006	0.013	< 0.02	< 0.1		< 2.66	< 0.033	7.3	10	2:50am	< 0.006	
3:10 AM	3	57.6	0.455	25	123	< 0.2	< 0.006	0.014	< 0.02	< 0.1		< 2.66	< 0.033	7.2	11	3:10am	< 0.006	
3:25 AM	3	46.3	0.175	25	122	< 0.2	< 0.006	0.011	< 0.02	< 0.1		2.81	< 0.033	7.4	10	3:25am	< 0.006	
<b>Manhole # 3</b>																		
2:30 AM	124	266	0.53	317	52.3	< 0.2	0.025	0.833	0.022	< 0.01	204600	9.89	< 0.033	7.1	14	7.97	0.228	
2:50 AM	39	156	0.39	155	57.1	< 0.2	0.044	0.276	0.024	0.01	59400	9.58	< 0.033	7	13	7.8	0.0438	
3:10 AM	16	50	0.46	79	68.5	< 0.2	0.021	0.214	0.02	0.01	20791	2.4	< 0.033	7.2	14	8	0.006	
3:25 AM	15	73	0.56	76	66.2	< 0.2	0.02	0.154	0.02	0.01	6600	3.33	< 0.033	7.5	13	7	0.0703	

## Chapter 4

### Discussion and Conclusions

***Dry Weather Event 1.*** Samples taken during dry weather periods allow us to look for differences between river and manhole water that can be used to detect the impact of CSO releases on the river environment during rain events. The data from the first dry weather sampling event clearly show a greater number of *E. coli* and fecal coliforms in manhole water than in river water. This is not a surprising observation. Interestingly, though, the total coliform counts for these samples were greater in river water (1,986.3 - 1,203.3 per 100 ml) than in manhole water (172.2 - 980.4 per 100 ml). This means that the percentages of coliforms that were *E. coli* were markedly higher in manhole water (61.1 - 79.9%) than in river water (2.1%).

The number of antibiotic resistant coliforms was higher in river water than in manhole water for tetracycline and erythromycin, but not for ciprofloxacin. The number of antibiotic resistant *E. coli* was higher in manhole water than in river water for erythromycin, but not for tetracycline or ciprofloxacin.

Of the chemical analyses that were done, biological oxygen demand (BOD), chemical oxygen demand (COD), the concentration of zinc (Zn), and the concentration of dissolved oxygen (DO) were markedly different between river water and manhole water samples. None of the other analytes were reliably different between river water and manhole water.

***Dry Weather Event 2.*** The data for the second dry weather sampling event clearly show a greater number of *E. coli* and fecal coliforms in manhole water than in river water.



Contrary to the data from the first dry weather samples, the number of total coliforms was higher in manhole water than in river water. However, the percentage of total coliforms that were *E. coli* remained markedly higher in manhole water (41.1 - 82.1%) than in river water (1.1%).

The number of antibiotic resistant coliforms was higher in manhole water than in river water for erythromycin, but the two sample sources were not markedly different with respect to tetracycline or ciprofloxacin resistant coliforms. Similarly, the number of erythromycin resistant *E. coli* was higher in manhole water than in river water, but the counts did not differ by source for tetracycline or ciprofloxacin resistance.

With respect to chemical analyses BOD, total suspended solids (TSS), the concentration of ammonia (NH<sub>3</sub>), COD, Zn, and DO were markedly different between river water and manhole water samples taken during this sampling event. None of the other analytes reliably differentiated river water from manhole water.

***Dry Weather Event 3.*** The data for the third dry weather sampling event again show much greater numbers of *E. coli* and fecal coliforms in manhole water than in river water. The numbers of total coliforms were similar in both manhole water and river water samples. Once again, the percentage of total coliforms that were *E. coli* were markedly higher in manhole water (60.6 - 82.1%) than in river water (2.9 - 4.5%).

The number of antibiotic resistant coliforms was higher in manhole water than in river water for ciprofloxacin, but the two sample sources were not markedly different with respect to erythromycin or tetracycline resistant coliforms. The number of

erythromycin resistant *E. coli* was higher in manhole water than in river water, but the counts were not markedly different by source for tetracycline or ciprofloxacin resistance.

With respect to chemical analyses BOD, Zn, and DO were notably different between river water and manhole water samples taken during this sampling event. None of the other analytes reliably differentiated river water from manhole water.

***Summary of Dry Weather Data.*** Comparison of the data from the three dry weather sampling events reveals some consistent observations. Analyses for *E. coli*, fecal coliforms, BOD, Zn, and DO did consistently differentiate between samples taken from the river and samples taken from the manhole. Therefore, these analytes are most likely to be useful in tracking the impact of CSO releases on Ohio River water quality, and will be the only ones considered in analyzing the wet weather data (below).

All three dry weather sampling events indicated that water taken from the river (hereafter referred to as “environmental” water) had total coliform populations that included low percentages (1.1 - 4.5%) of *E. coli*. By contrast, samples taken from the manhole did not always have larger coliform populations, but they did have consistently higher percentages (41.1 - 82.1%) of the coliform population that were *E. coli*. This metric ( $[E. coli \text{ MPN}/\text{total coliform MPN}] \times 100$ ) looks like a promising marker to differentiate “environmental” coliform populations from “sewage” coliform populations.

The prevalence of antibiotic resistance in the coliform population, at least for the antibiotics used here, does not seem to be a good indicator of sewage contamination. The differences between river water and manhole water were dependent on the specific antibiotic being used but, contrary to what might be expected, manhole water was often

not enriched in antibiotic bacterial populations relative to river water. This observation is consistent with data from other studies from the Environmental Microbiology Research Laboratory (EMRL) at Marshall University, where the distributions of antibiotic resistant bacteria and fecal indicator bacteria have been found to be distinct.

***Wet Weather Event 1.*** Samples taken during rain events should detect the impacts, if any, that the CSO release has upon water quality at the downstream sampling site. These samples were taken during an event that produced 0.64 inches of rain in 1 hour. The rain started at 2:20 pm and ended at 3:20 pm, 16 September 2005.

Data from the first wet weather event clearly show a dramatic increase in *E. coli* and fecal coliform counts within the first 15 minutes after the initial samples were taken. The rainfall during this event was such that the *E. coli* and fecal counts at the downstream sampling site rose to very high levels (beyond the limit of resolution for the IDEXX test), and remained high for the duration of the sample period. *E. coli* counts at the upstream site did increase, indicating some impact of surface runoff on the river but not enough to explain the increased numbers at the downstream site. Clearly, measurement of either *E. coli* or fecal coliforms was detecting the impact of the CSO release at the downstream sampling site. There was a moderate drop in *E. coli* counts in the manhole over the course of the rain event, suggesting that dilution of the manhole water could be detected with the IDEXX method. Fecal coliform numbers did not show the same drop, suggesting that determination of *E. coli* counts is a more informative way to monitor the progress of the CSO release.

Total coliform counts were not sufficient to detect the impact of the CSO release because the total coliform counts at the upstream site were already above the limit of resolution for the assay at the first sampling time and remained above limit for the duration of sampling. Total coliform counts were also above the limit of resolution in the manhole samples at every sampling time. Unfortunately, the lack of resolution with respect to total coliform counts made it impossible to determine the percentage of total coliforms that were *E. coli*. Therefore, we were unable to determine if that metric could be used to detect the impact of the CSO release at the downstream site.

Adding antibiotics to the IDEXX assay did provide the advantage of reducing the number of samples that were beyond the limit of resolution of the method. For example, both tetracycline-and erythromycin-supplemented media were able to resolve the shape of the contamination curve at the downstream site. IDEXX Colilert alone indicated that the downstream site reached maximal contamination at 15 minutes, but Colilert supplemented with tetracycline or erythromycin show that the downstream contamination continued to build throughout the sampling period. Similarly, both tetracycline and erythromycin supplements were able to resolve the rates of *E. coli* washout from the manhole. In these samples, ciprofloxacin was too selective, dropping the *E. coli* counts below the limit of detection for the method (less than one ciprofloxacin-resistant *E. coli* per 100 ml).

None of the chemical assays performed were sensitive enough to detect the effect of the CSO release at the downstream site during this rain event. Only biological measurements (*E. coli*, fecal coliforms, tetracycline-resistant *E. coli*, and erythromycin-

resistant *E. coli*) clearly indicated the impact of the release and, in the cases of the antibiotic-supplemented assays, the timing of the contamination.

A t-test was used to determine if there was a significant difference between the upstream and downstream sites. The initial event was found to have an impact on the river concerning the total *E. coli* and erythromycin resistance. Total *E. coli* samples between the upstream and downstream sites were found to have significant difference ( $P \leq 0.05$ ). Erythromycin resistance was also found to have significant difference between the upstream and downstream sites ( $P \leq 0.05$ ). Tetracycline and ciprofloxacin resistance was determined to have no significant difference between the two sites ( $P > 0.05$ ).

***Wet Weather Event 2.*** These samples were taken during an event that produced 1.62 inches of rain in 5 hours. The rain started at 3:00 am and ended at 8:00 am, 7 October 2005.

Data from the second wet weather event also showed an increase in *E. coli* and fecal coliform counts within the first 15 minutes after the initial samples were taken. The rainfall during this event was such that the *E. coli* counts at the downstream site rose to very high levels (beyond the limit of resolution for the IDEXX test), but they then decreased again during the remainder of the sample period. It is clear from these data that the magnitude and impact of a CSO release can vary significantly with the amount and duration of the rainfall. *E. coli* counts at the upstream site did increase moderately during the rain event, indicating some impact of surface runoff on the river, but not enough to explain the increased numbers at the downstream site. Clearly, measurement

of either *E. coli* or fecal coliforms was detecting the impact of the CSO release at the downstream sampling site. There was a marked drop in *E. coli* counts in the manhole over the course of the rain event, suggesting that dilution of the manhole water could be detected with the IDEXX method. Fecal coliform numbers suggest the same type of drop, but counts were above the limit of resolution of the method and, therefore, offer less information about the time course of the washout effect. As in the first wet weather event, determination of *E. coli* counts was a more informative way to monitor the progress of the CSO release.

Total coliform counts were not sufficient to detect the impact of the CSO release because the total coliform counts at the upstream site were already above the limit of resolution for the assay at the first sampling time and remained above the limit for the duration of sampling. Total coliform counts were also above the limit of resolution in the manhole at the first sampling time but the numbers dropped over the course of the rain event. The total coliform count alone did not help to track the degree or time course of the impact on the river due to the CSO release. Although total coliform and *E. coli* counts in the manhole did support the previous observation that a high percentage of *E. coli* in total coliforms is an indication of sewage contamination, high total coliform counts (beyond the limits of resolution) made it impossible to determine the percentage of total coliforms that were *E. coli* at the downstream site. Therefore, we were again unable to determine if that metric could be used to detect the impact of the CSO release at the downstream site.

As in the first wet weather event, adding antibiotics to the IDEXX assay did provide the advantage of reducing the number of samples that were beyond the limit of resolution

of the method. In this event, only the tetracycline-supplemented media was able to fully resolve the shape of the contamination curve at the downstream site. Both tetracycline and erythromycin supplements were able to resolve the rates of *E. coli* washout from the manhole. Once again, ciprofloxacin was too selective, dropping all but one of the *E. coli* counts below the limit of detection for the method.

Of the chemical assays performed, only TSS detected a slight and transient impact of the CSO release at the downstream site during this rain event. Because TSS was not able to differentiate between river water and manhole water during dry weather, it is not recommended as a method for detecting the impact of a CSO release.

Statistically, the second wet weather event results differed from the first in the case of which analyte showed a significant difference between the upstream and downstream sites. The counts of *E. coli*, tetracycline and ciprofloxacin resistant *E. coli* samples were not significantly different ( $P > 0.05$ ). Erythromycin resistance was determined to have a significant difference between the two sites ( $P \leq 0.05$ ). The lack of significance relative to the *E. coli* counts can be attributed to the transient nature of the downstream impact. Our interpretation is that *E. coli* counts by the IDEXX method were sufficiently sensitive to detect the impact of the CSO release.

**Wet Weather Event 3.** These samples were taken during an event that produced 0.56 inches of rain in 1.25 hours. The rain started at 2:15 am and ended at 3:30 am, 29 November 2005.

Data from the third wet weather event clearly show a sharp increase in *E. coli* counts within the first 15 minutes after the initial samples were taken. The rainfall during this

event was such that the *E. coli* counts at the downstream sampling site rose to very high levels (beyond the limit of resolution for the IDEXX test), and remained near the limit of resolution for the duration of the sample period. *E. coli* counts at the upstream site fluctuated, showing no discernable impact of surface runoff. Clearly, measurement of *E. coli* counts was detecting the impact of the CSO release at the downstream sampling site. There was a marked drop in *E. coli* counts in the manhole over the course of the rain event, suggesting that dilution of the manhole water could be detected with the IDEXX method.

During the third rain event, total coliform counts at the upstream site were low enough to detect the impact of the CSO release at the downstream site. However, the timing of the increase in total coliform counts could not be adequately resolved. Total coliform counts at the downstream site exceeded the limits of the assay by the second sampling time and remained above the limit for the duration of the rain event. Unfortunately, the lack of resolution with respect to total coliform counts made it impossible to determine the percentage of total coliforms that were *E. coli*. Therefore, we were, again, unable to determine if that metric could be used to detect the impact of the CSO release at the downstream site.

Adding antibiotics to the IDEXX assay did provide the advantage of reducing the number of samples that were beyond the limit of resolution of the method. In this event, both tetracycline-and ciprofloxacin-supplemented media were able to resolve the shape of the contamination curve at the downstream site. In this case both Colilert alone, and Colilert supplemented with either tetracycline, erythromycin or ciprofloxacin was able to resolve the rates of *E. coli* washout from the manhole. Overall, erythromycin was not



selective enough, and ciprofloxacin was too selective, to provide full information on contamination rates. Tetracycline-supplemented media performed best in this sampling event.

Of the chemical assays performed, only TSS and COD detected transient effects at the downstream site during this rain event. Biological measurements were more robust in detecting the impact of the release and, in the cases of the antibiotic-supplemented assays, determining the dynamics of the contamination.

Statistically, the final wet weather event results were similar to the initial event. Tetracycline and ciprofloxacin resistant *E. coli* had no significant difference between the upstream and downstream sites ( $P > 0.05$ ). Total *E. coli* did have a significant difference between the two site just as the erythromycin resistant *E. coli* ( $P \leq 0.05$ ).

A correlation coefficient test was calculated on all biological and chemical analytes to determine if any of them could serve as a proxy analyte for one or more of the others. We found that biological oxygen demand (BOD) and *E. coli* were significantly correlated ( $P \leq 0.01$ ). Total suspended solids (TSS) also were found to have a correlation between it and *E. coli* ( $P \leq 0.01$ ). Chemical oxygen demand (COD) was found not only to be correlated to total *E. coli* but also erythromycin resistance ( $P \leq 0.01$ ). Total zinc was similar to COD, having significant correlations with total *E. coli* and erythromycin resistance ( $P \leq 0.01$ ). Fecal coliform was found to have a correlation ( $P \leq 0.01$ ) with total *E. coli*, tetracycline and erythromycin resistance.

Based on the observations made during both the wet and dry weather sampling events, only the biological indicators, particularly fecal coliforms and *E. coli* counts, were reliable in both differentiating river water from manhole water and detecting the impact

of a CSO release at a downstream site. Antibiotic supplements were helpful during wet weather events in order to bring *E. coli* counts within the limits of resolution of the IDEXX method. It should be noted, however, that there is significant evidence that antibiotic resistance cannot be used as a direct marker for fecal contamination, so one must use caution in interpreting the results. Specifically, antibiotic-supplemented cultures can be useful for determining the rate dynamics of a CSO loading to a downstream site or the washout rate from a CSO but they should not be used to infer the magnitude of the fecal contamination. Absolute numbers of resistant *E. coli* are entirely dependent on which antibiotic is used, and none of the three tested herein was appropriate in every circumstance. Finally, we have good reason to believe that the measurement of percent of coliforms that are *E. coli* may be a useful metric in differentiating environmental water from water that has chronic fecal contamination.

***Overall Conclusions.*** This study confirms that total coliform counts alone are not a useful indicator of water quality. Total coliform counts can be high when *E. coli* or fecal coliform counts are low. We have also seen that total coliform counts can be higher in river water samples than in manhole water samples. However, there does seem to be utility in determining the percentage of total coliforms that are *E. coli*. In this study that percentage could be used to reliably discriminate between river water and manhole water samples regardless of the total magnitude of either the total coliform or *E. coli* counts.

Antibiotic supplements can be very useful for studying the dynamics of a CSO release, specifically by reducing bacterial counts to a range where loading and washout curves can be resolved. However, real care must be taken in the selection of antibiotics

and in the interpretation of the resulting data. The general rule is that antibiotic supplemented media can be used to infer the rates and dynamics but not the magnitude, of fecal loading. The percentage of total coliforms that are *E. coli* metric described above may not be useful when antibiotic resistant sub-populations of total coliforms and *E. coli* are used. This probably derives from the observation that there is not a direct relationship between fecal contamination and antibiotic resistance in environmental samples.

It is clear that CSO releases caused by rain events have a detectable impact on the water quality at downstream sites. The magnitude and duration of the impact varies dramatically in response to the magnitude and duration of the rainfall. Our data strongly suggest that only biological indicators, specifically *E. coli* and fecal coliform counts, adequately and reliably detect the impact of a CSO release.

This study does not fully illuminate the duration of impact caused by a CSO release, or how the detected impacts will mix into, and therefore impact, the river further downstream. This study does suggest, however, that biological testing, specifically enumeration of *E. coli*, is the preferred method for studying CSO impacts.

## Chapter 5

### Summary

The three objectives of this study were to determine the level of occurrence of antibiotic resistance in the environment, if a small quantity CSO has an impact on the Ohio River banks during a storm event and if antibiotic resistance could be correlated with any other pollutant in a CSO.

The data collected from all three dry weather sample events revealed the occurrence of erythromycin resistant *E. coli*. It was found that erythromycin resistant *E. coli* made up approximately 60% of the total *E. coli* population sampled from the Ohio River. Tetracycline resistant *E. coli* made up a small portion of the total *E. coli* counts while ciprofloxacin resistant *E. coli* was minimal.

The second objective was to determine if a CSO could have an impact on the Ohio River. The 25<sup>th</sup> Street CSO was found to have an impact on the Ohio River during storm events. Total *E. coli* counts were significantly different ( $P < 0.05$ ) two-thirds of the time between the upstream and downstream sites of the CSO. Only the transient nature of the second rain event kept the *E. coli* counts from being significantly different during all three rain events. Erythromycin resistant *E. coli* were found to be significantly different ( $P < 0.05$ ) during every storm event that was sampled. These numbers indicate the Ohio River banks can be impacted, even if just for a short distance, by a small quantity CSO.

The third objective of this study was to find correlations between any of the bacterial pollutants with any of the other pollutants associated with a CSO. It was found that *E. coli* can be correlated ( $P < 0.01$ ) with BOD, TSS, COD, and zinc. Tetracycline

resistance was correlated ( $P < 0.01$ ) with only fecal. Erythromycin resistance was found to have correlations ( $P < 0.01$ ) with COD and zinc.

## Literature Cited

1. Alvero, C.C. (1987) Antibiotic Resistance of Heterotrophic Bacterial Flora of Two Lakes. *System. Appl. Microbiol.* 9:169-72
2. Arvanitidou, M., Tsakris, A., Constantinidis, T.C., and Katsouyannopoulos, V.C. (1997) Transferable antibiotic resistance among *Salmonella* strains isolated from surface waters. *Water Research* 31:1112-1116
3. Bauger, A.J., Jensen, J., and Krogh, P.H. (2000) Effects of antibiotics oxytetracycline and tylosin on soil fauna. *Chemosphere* 40:751-757
4. Boon, P.I., and Cattnach, M. (1999) Antibiotic resistance of native and faecal bacteria isolated from rivers, reservoirs and sewage treatment facilities in Victoria, south-eastern Australia. *Applied Microbiology* 28:164-168
5. Chee-Sanford, J.C., Aminov, R.I., Krapac, I. J., Garrigues-Jean, N., and Mackie, R.I. (2001) Occurrence and Diversity of Tetracycline Resistance Genes in Lagoons and Groundwater Underlying Two Swine Production Facilities. *Appl Env, Microbiology* 67:1494-1502
6. Dhakephalkar, P.K. and Chopade B.A. (1994) High levels of multiple metal resistance and its correlation to antibiotic resistance in environmental isolates of *Acinetobacter*. *BioMetals* 7:67-74
7. Dotson, T., G.N. Chou, and C.C. Somerville. 2006. Antibiotic Resistant Bacteria are not a Subset of Fecal Indicator Bacteria in the Mud River, West Virginia. 106<sup>th</sup> General Meeting of the American Society for Microbiology, 21-25 May, Orlando, FL, USA
8. Drugbank Website. 3 March 2006, last modification date. PharmaSearch. Sulfamethizole. [Online.] <http://redpoll.pharmacy.ualberta.ca/drugbank/cgibin/getCard.cgi?CARD=APRD00684.txt> [2 February 2007, last date accessed.]
9. DuPont, H.L., and Steele, J.H. (1987) Use of Antimicrobial Agents in Animal Feeds: Implications for Human Health. *Reviews of Infectious Diseases* 9:447-460
10. El-Zanfaly, H.T. (1991) The Need for New Microbiological Water Quality Criteria. *Water Science Technology* 24:43-48
11. Esiobu, N., Armenta, L., and Ike, J. (2002) Antibiotic resistance in soil and water environments. *International Journal of Environmental Health Research* 12:133134

12. Goñi-Urriza, M., Capdepuy, M., Arpin, C., Raymond, N., Caumette, P. and Quentin C. (2000) Impact of an urban effluent on antibiotic resistance of riverine *Enterobacteriaceae* and *Aeromonas* spp. *Applied and Environmental Microbiology* 66:125-132
13. González, R.G., Taylor, M.L., and Alfaro G. (1982) Bacteriologic Study of a community water supply; detection of enterotoxigenic activity and resistance to antimicrobial drugs. *Bulletin of the Pan American Health Organization* 16:341-355.
14. Grabow, W.O.K., Prozesky, O.W., and Burger, J.S. (1974) Behaviour in a River and Dam of Coliform Bacteria with Transferable or Non-Transferable Drug Resistance. *Water Research* 9:777-782
15. Greenberg, A.E., L.S. Clesceri, A.D. Eaton. (1992) Standard Methods for the Examination of Water and Wastewater, 18<sup>th</sup> Edition
16. Halling-Sorensen, B. Nielsen, S.N., Lanzky, P.F., Ingerslev, F., Lützholtz, H.C., and Jørgensen, S.E. (1998) Occurrence, Fate and Effects of Pharmaceutical Substances in the Environment- A Review. *Chemosphere* 30:357-393
17. Huber, W.G. (1971) Antibacterial drugs as environmental contaminants. In *Advances in Environmental Science and Technology*, Vol.2 ed. Pitts, J.N., Jr and Metcalf, R. L. pp. 289-320. New York: Wiley-Interscience.
18. Iwane, T., Urase, T., and Yamamoto, K. (2001) Possible impact of treated wastewater discharge on incidence of antibiotic resistant bacteria in river water. *Water Science and Technology* 43:91-99
19. Kolpin, D.W. *et al.* (2002) Pharmaceutical, Hormones, and other Organic Wastewater Contaminants in U. S. Streams, 1999-2000: A National Reconnaissance *Environ. Sci. Technol.*, 36:1202-1211
20. Lipsitch, M., and Samore, H., (2002) Antimicrobial Use and Antimicrobial Resistance: A Population Perspective. *Emerging Infectious Diseases* 8:347-354
21. Jones, J.G., Gardener, S., Simon, B.M., and Pickup, R.W. (1986) Antibiotic Resistant Bacteria in Windermere And Two Remote Upland Tarns In The English Lake District. *Journal of Applied Bacteriology* 60:443-53
22. Kelch, W.J. and Lee, J.S. (1978) Antibiotic resistance patterns of gram-negative bacteria isolated from environmental sources. *Applied and Environmental Microbiology* 36:450-456

23. Kentucky Administrative Regulations 401:5
24. Levy, S.B. (1998) Multidrug Resistance: A Sign of the Times. *The New England Journal of Medicine* 338:1376-1378
25. McArthur, J.V. and Tuckfield, R.C. (2000) Spatial patterns in antibiotic resistance among stream bacteria: effects of industrial pollution. *Applied and Environmental Microbiology* 66:3722-3726
26. McKeon, D.M., Calabrese, J.P., and Bissonnette, G.K. (1995) Antibiotic Resistant Gram-Negative Bacteria In Rural Groundwater Supplies. *Water Research* 29:1902-08
27. Niemi, M., Sibakov, M., Niemela, S. (1983) Antibiotic resistance among different species of fecal coliforms isolated from water samples. *Applied and Environmental Microbiology* 45:79-83
28. Ohio Administrative Code 3745:1-32
29. ORSANCO (Ohio River Valley Water Sanitation Commission) (1994) Ohio River Fact Book. ORSANCO, Cincinnati, OH
30. Rooklidge, S.J. (2003) Environmental antimicrobial contamination from terraccumulaation and diffuse pollution pathways. *Science of the Total Environment* 325:1-13
31. Solo-Gabriele, H.M., Wolfert, M.A., Desmarais, T.R., and Palmer, C.J. (2000). Sources of *Escherichia coli* in a Costal Subtropical Environment. *Applied and Environmental Microbiology* 66:230-237
32. Somerville, C.C., Saunders, S. van Meter, and J. Wells. 2002. Frequency and distribution of antibiotic resistant bacteria in the Ohio River: August 2001. 102<sup>nd</sup> General Meeting of the American Society for Microbiology. 19-23 May, Salt Lake City, UT
33. Somerville, C.C., A.P. Sweeney, and S.L. Chadwick, 2007. Antibiotic Resistant Bacteria in the Ohio River are not a Subset of Fecal Indicator Bacteria. 107<sup>th</sup> General Meeting of the American Society for Microbiological, 21-25 May, Toronto, Ontario, Canada
34. Stephenson, S. (1997) Evaluation of Impact to Surface Water from On-Site Wastewater Treatment Systems n the Park Hills Area of Huntington, West Virginia. West Virginia Graduate College.



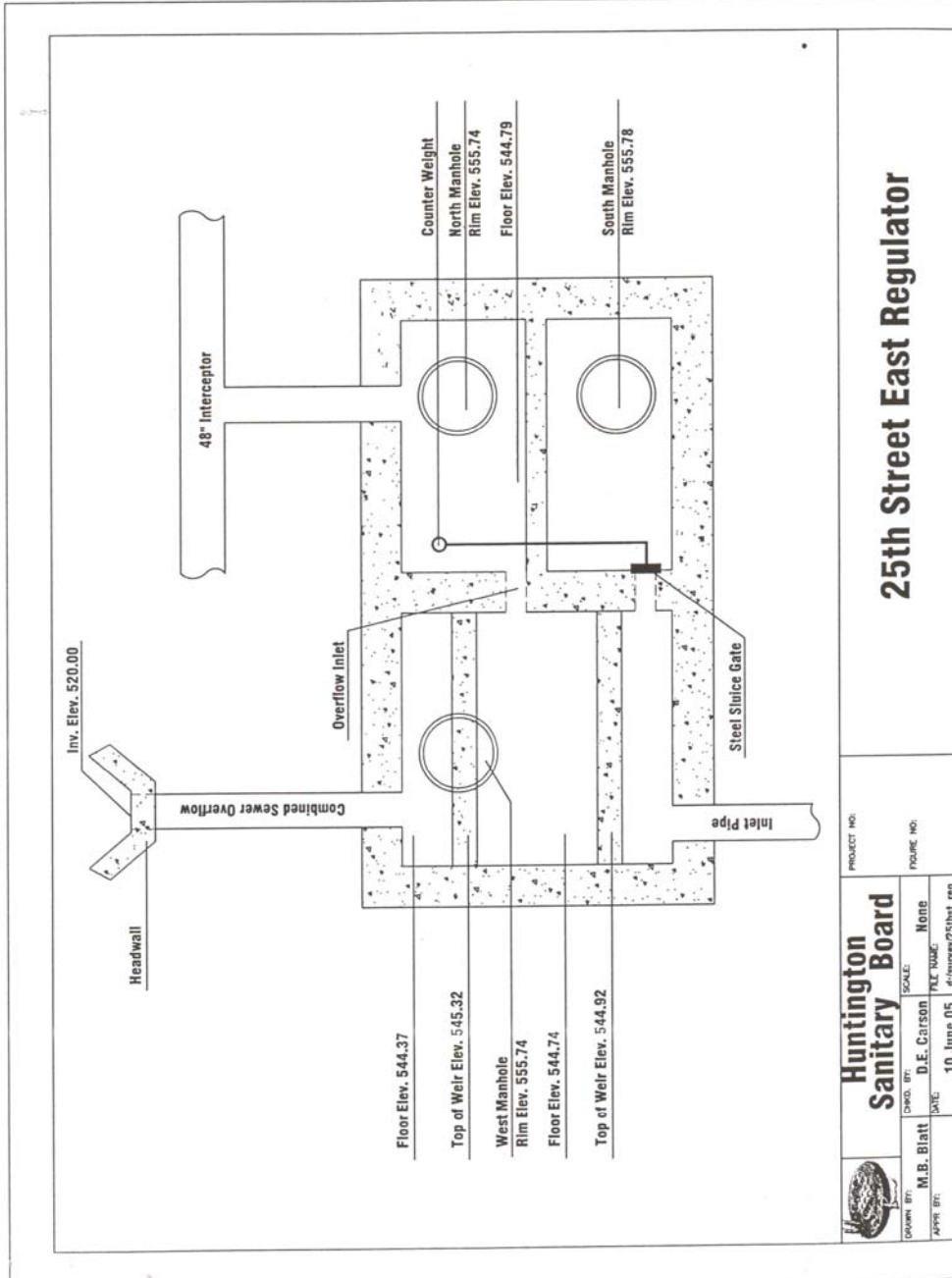
35. Tenover, F.C. (1996) Reasons for the Emergence of Antibiotic Resistance. *American Journal of the Medical Sciences* 311:9-16
36. Tran, J.H., and Jacoby, G.A. (2002) Mechanism of Plasmid-mediated quinolone resistance. *Proc Natl Acad Sci USA* 99:5638-42
37. United States Environmental Protection Agency. (2000) Membrane Filter Method for the Simultaneous Detection of Total Coliforms and *Escherichia coli* in Drinking Water. Office of Research and Development. Cincinnati, OH EPA 600-R-00-013
38. West Virginia 47 CSR2
39. Wolf, H.W. (1985) The Coliform Count As a Measure Of Water Quality. In *Water Pollution Microbiology*. ed. R. Mitchell. Wiley-Interscience, New York, pp 333-345
40. Young, H.K. (1993) Antimicrobial resistance spread in aquatic environments. *Journal of Antimicrobial Chemotherapy* 31:627-635

**Table 1:  
Pollutants Sampled for Dry and Wet Weather Events**

<b>Analysis</b>	<b>Preserve</b>	<b>Hold Times</b>	<b>Method</b>
<b>Biological Oxygen Demand (BOD<sub>5</sub>)</b>	<b>NONE</b>	<b>48 Hours</b>	<b>SM 18<sup>th</sup> 5210B</b>
<b>Total Suspended Solids (TSS)</b>	<b>NONE</b>	<b>7 Days</b>	<b>SM 18<sup>th</sup> 2540</b>
<b>Chemical Oxygen Demand (COD)</b>	<b>H<sub>2</sub>SO<sub>4</sub></b>	<b>28 Days</b>	<b>SM 18<sup>th</sup> 5220D</b>
<b>Hardness</b>	<b>HNO<sub>3</sub></b>	<b>6 Months</b>	<b>SM 18<sup>th</sup> 2340 B</b>
<b>Ammonia-Nitrogen</b>	<b>H<sub>2</sub>SO<sub>4</sub></b>	<b>28 Days</b>	<b>SM 18<sup>th</sup> 4500 B&amp;E</b>
<b>Fluoride</b>	<b>NONE</b>	<b>7 Days</b>	<b>EPA 300.0</b>
<b>Oil &amp; Grease</b>	<b>HCl</b>	<b>28 Days</b>	<b>EPA 1664A</b>
<b>Phenols</b>	<b>H<sub>2</sub>SO<sub>4</sub></b>	<b>28 Days</b>	<b>SM 18<sup>th</sup> 5530 A&amp;C</b>
<b>Ph</b>	<b>NONE</b>	<b>Immediately</b>	<b>EPA 150.1</b>
<b>Temperature</b>	<b>NONE</b>	<b>Immediately</b>	<b>EPA 170.1</b>
<b>Dissolved Oxygen (DO)</b>	<b>NONE</b>	<b>Immediately</b>	<b>SM 18<sup>th</sup> 4500O&amp;G</b>
<b>Copper</b>	<b>HNO<sub>3</sub></b>	<b>6 Months</b>	<b>EPA 200.7</b>
<b>Zinc</b>	<b>HNO<sub>3</sub></b>	<b>6 Months</b>	<b>EPA 200.7</b>
<b>Lead</b>	<b>HNO<sub>3</sub></b>	<b>6 Months</b>	<b>EPA 200.7</b>
<b>Nickel</b>	<b>HNO<sub>3</sub></b>	<b>6 Months</b>	<b>EPA 200.7</b>
<b>Bis (2-Ethylexyl) Phthalate</b>	<b>NONE</b>	<b>7 Days</b>	<b>EPA 625</b>
<b>Fecal Coliform</b>	<b>Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub></b>	<b>6 Hours</b>	<b>SM 18<sup>th</sup> 9222D</b>
<b>Total Coliform</b>	<b>Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub></b>	<b>6 Hours</b>	<b>Idexx Quanti- Tray/ 2000©</b>
<b>E. Coli</b>	<b>Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub></b>	<b>6 Hours</b>	<b>Idexx Quanti- Tray/ 2000©</b>

# Figure 1

## 25<sup>th</sup> Street Regulator



## **Appendix A**

### **CSO SAMPLING PROTOCOL FOR OUTFALL 014**

#### **EAST 25<sup>TH</sup> STREET DIVERSION CHAMBER**

##### ***DRY WEATHER SAMPLING***

Dry weather samples will be collected to define background characteristics of the sewer system and East 25<sup>th</sup> Street Diversion Chamber. Up to three 24-hour grab and composite samples will be collected from the manhole prior to the CSO. One set of grab samples will be collected 100 feet upstream (in residential area of Huntington), 100 feet downstream, and at the West Virginia American Water intake.

##### ***WET WEATHER SAMPLING STRATEGY***

The Sanitary Board will consider a valid wet weather event to be 0.3 inches of rain or greater to the Huntington area within a six hour period following a 48 hour dry period. The National Weather Service will be used when possible via the internet, local TV forecasts, etc. to aid in the predictions. The Board will place and monitor a portable rain gage at the WVAW sampling site. If more than six hours elapses without an overflow, the rain event will not be sampled.

The sampling event will begin once the CSO becomes active and will be sampled every 15 minutes after the initial purge for the first hour. Grab samples will be collected with an automated sampler inside the CSO outfall. Upstream and downstream sampling grabs will be done by samplers and the WVAW samples will be collected as hand grabs from the intake sample port. After the first hour of sampling the CSO, if still active, it will continue to be sampled every hour afterwards for three additional samples. A flow meter will be placed by the tide gate to determine the amount of water discharged to the river.

- Total suspended solids
- 5-Day Biochemical Oxygen Demand
- Chemical Oxygen Demand
- Hardness
- Ammonia-Nitrogen
- Fluoride
- Fecal Coliform
- Oil and Grease
- Phenols

pH  
Temperature  
Dissolved Oxygen  
Copper  
Zinc  
Lead  
Nickel  
Bromodichloromethane\*  
Chlorodibromomethane\*  
Chloroform\*  
Bis (2-Ethylexyl) Phthalate\*  
Diethyl Phthalate\*

\*(After inspection of local industries these analytes may be present. These will be done only on initial dry weather and if they are not present then will no longer be sampled for)

The goal of this sampling program is to generate 3-5 sets of data resulting from 0.3 inches or more of rain.

## ***RECORD KEEPING***

A notebook will be used to record all field notes, and immediate testing parameters. All sample points will have appropriate Field Monitoring Reports (FMR) with the sampling data. The notebook will contain important information about the rainfall event including, but not limited to the amount and duration of the precipitation. The rain gage at the Treatment Plant will be used to track amounts and durations of precipitation after a sampling event.

All samples will be properly logged in at the plant's laboratory and tracked with Chains of Custody. Upon receiving all results of analyses, a summary will be prepared for the dry weather and wet weather studies of all parameters.

## Appendix B Letter from State Approving SOP



---

west virginia department of environmental protection

---

Division of Water and Waste Management  
601 57<sup>th</sup> Street SE  
Charleston, WV 25304  
Phone: (304) 926-0495  
FAX: (304) 926-0496

Joe Manchin III, Governor  
Stephanie R. Timmermeyer, Cabinet Secretary  
[www.wvdep.org](http://www.wvdep.org)

June 1, 2005

Mr. Mike Copley  
Operations Superintendent  
Huntington Sanitary Board  
P.O. Box 1659  
Huntington, WV 25704

Re: Impact of CSO Outfalls on  
Downstream Drinking Water Intakes

Dear Mr. Copley:

This letter is in response to the Huntington Sanitary Board's (HSB) May 26, 2005 correspondence addressing the sampling plan for CSO Outfall 014 to evaluate the impact the outfall has on the downstream drinking water intake. We concur with HSB's proposed sampling plan for CSO Outfall 014, and will review the sampling plan for CSO Outfall 015 when it is submitted at a later date.

If you have any questions or comments, you can call me at (304) 926-0499, extension 1595.

Sincerely,

Donald W. Lewis  
Engineering Section

DWL/l

cc: Charlene DeBord, Industrial Pretreatment Coordinator, HSB  
Cynthia Musser, Environmental Enforcement Supervisor

Promoting a healthy environment.

## Appendix C

### Antibiotic Stock Solutions

1. The antibiotics, solvents, and concentrations used are shown in Table 1.

**Table 1.** Antibiotics used and recommended concentrations.

Antibiotic	Catalog No.	Solvent <sup>a</sup>	Stock Conc.	Working Conc.
Fungizone	BioWhitaker 17-836R	N/A	250 µg/ml	375 ng/ml
Ampicillin Sodium Salt	Fisher BP1760-25	H <sub>2</sub> O	50 mg/ml	50 µg/ml
Ciprofloxacin	Cellgro 61- 277-RF	DMSO	4 mg/ml	4 µg/ml
Erythromycin	Fisher BP920-25	EtOH:H <sub>2</sub> O	8 mg/ml	8 µg/ml
Streptomycin Sulfate	Fisher BP910-50	Water	25 mg/ml	25 µg/ml
Sulfamethizole	Fisher ICN15671125	DMSO	128 mg/ml	128 µg/ml
Tetracycline Hydrochloride	Fisher BP912-100	EtOH:H <sub>2</sub> O	12.5 mg/ml	12.5 µg/ml
Virginiamycin	Fisher 50- 213-730	DMSO	16 mg/ml	16 µg/ml

<sup>a</sup> Fungizone is purchased as a stock solution, it is stored frozen and thawed before use. DMSO = dimethylsulfoxide (Certified ACS). EtOH:H<sub>2</sub>O = a mixture of equal parts ethanol (100% USP) and reagent grade water (18 MΩ).

2. Using an analytical balance, weigh out sufficient antibiotic to make a 10 ml stock (see Table 1 and note below) and transfer the antibiotic powder to a sterile 15 ml plastic centrifuge tube (Falcon 2095; Becton Dickinson, Sparks, MD or equivalent).

### Note – for determining amount of antibiotic powder to use

- a. Be sure to account for the purity of the antibiotic powder by dividing the weight of pure antibiotic required by the purity. For example, ciprofloxacin may be provided as a powder that contains 803 mg ciprofloxacin per gram. To achieve a stock concentration of 4 mg ciprofloxacin per ml, it is necessary to add 4.98 [or  $4.0 \text{ mg cipro} \times (1000 \text{ mg powder} / 803 \text{ mg cipro})$ ] mg powder per ml of stock solution.
3. Add 10 ml of the appropriate solvent (see Table 1) to the tube, and vortex to mix.
4. In some cases (e.g. when making stock solutions of ciprofloxacin) the tube can be placed in a bath sonicator to facilitate dissolution of the solute. Take care to be certain that all of the antibiotic has gone into solution.
5. Draw the antibiotic solution into a sterile 10 ml syringe, and sterilize by forcing the solution through a sterile, 0.2  $\mu\text{m}$  syringe filter (Fisher Scientific cat. no. 09-719C or equivalent) into a second sterile plastic centrifuge tube. **Do not filter sterilize antibiotics dissolved in DMSO.**
6. Store the antibiotic stocks at  $-20^{\circ}\text{C}$  until used. Replace antibiotic stocks each month.

### Media Preparation

1. Suspend 9.1 grams Difco R2A agar (Becton Dickinson, Sparks, MD; cat no. 218263) in 500 ml of purified water in a 1,000 ml capacity glass Erlenmeyer flask.
2. Add a magnetic stir bar, cover the flask with aluminum foil, place and piece of autoclave tape on the foil, and mark the name of the antibiotic to be added (if appropriate) on the foil.
3. Swirl the flask to evenly hydrate the suspended powder, and autoclave at  $121^{\circ}\text{C}$  and 15 psi for 20 minutes on a slow exhaust cycle.
4. Move the medium from the autoclave to a  $48^{\circ}\text{C}$  water bath, and hold for at least 30 minutes but not more than 4 hours.
5. While the medium is cooling, remove the appropriate antibiotic stock solutions from the freezer and thaw on ice (all solvents except DMSO) or at room temperature (antibiotics in DMSO).



6. Place the flask on a magnetic stir plate and stir gently until the medium is well mixed. Be careful not to introduce bubbles. Test the temperature of the medium by touching the side of the flask briefly with your bare hand. It should be warm, but not hot. If the flask is hot to the touch, return it to the water bath until it has cooled enough to be handled comfortably. Do not allow the medium to cool below 48°C.
7. Wear disposable latex gloves for the remaining steps of media preparation. When properly tempered, again move the medium to the magnetic stirrer. While stirring gently, **aseptically** add 750 µl of fungizone stock.
8. Continue stirring for 15 to 30 seconds after the addition of the fungizone to the medium. Tilt the flask to insure that all the fungizone stock solution is transferred to the medium.
9. If you are preparing R2A plus fungizone for the enumeration of total cultivable bacteria, aseptically pour 25 ml per plate into pre-sterilized 100 x 15 mm Petri dishes (Falcon 1029, Becton Dickinson, Sparks, MD or equivalent).
10. If you are preparing R2A plus fungizone and an additional antibiotic for the enumeration of a particular resistant population, **aseptically** add 500 µl of the appropriate antibiotic stock to the flask. Stir gently for an additional 15 seconds and tilt the flask to insure that all the antibiotic stock is transferred to the medium.
11. Pour the plates as described in step 9.
12. Clearly mark the plates to indicate media content. E.g. "R2Af " can be used to indicate R2A agar plus fungizone, and "R2Afc" to indicate R2A agar plus fungizone and ciprofloxacin, etc.
13. Allow plates to cure at room temperature for at least 48 hours before use. Plates should be inoculated no later than seven days after pouring.

### Sample Collection

1. Whole water samples must be collected in sterile containers with secure, leak-proof lids. Containers must be clearly labeled with a sample number, and the sample number must be recorded in a notebook in which the location, date and time of sampling are clearly and fully described. If available, include additional information such as: latitude and longitude, air temperature, water temperature, weather conditions, turbidity, level of boating activity, land use patterns, etc.

2. The container should be opened so that the opening is pointing downward, and the inside of the lid does not come into contact with any non-sterile surfaces.
3. Continue holding the opening downward while passing the container through the surface tension layer.
4. When the container is fully submerged, invert it so that it fills with water.
5. Pour off enough water to leave approximately a 10% air headspace.
6. Seal the container and place on ice. Samples should be cultivated within 6 hours of collection.

### **Enumeration of Total Cultivable Bacteria**

1. Remove a sample bottle from the ice chest and mix by inversion to re-suspend any sediment that may have settled out during transit.
2. Aseptically transfer 0.1 ml of sample to a sterile 9.9 ml dilution blank in a screw-cap test tube.
3. Tightly cap the tube and mix at full speed on a vortex mixer for at least 5 seconds.
4. Aseptically transfer 0.1 ml of diluted sample to each of three plates of Difco R2A agar plus 375 ng/ml fungizone.
5. Spread the diluted water sample on the surface of the agar plates using a sterile glass spreading rod, a pre-sterilized inoculating loop, or five sterile glass beads (5 mm; see note) until all of the liquid has been absorbed.

#### **Note – for use of sterile glass beads**

- a. Place six glass beads (Fisher Scientific cat no. 11-312C) into a 1000 ml pipette tip (Biolog cat no. 3001; other tips should be tested for suitability). One set of beads is required for each plate inoculated.
- b. Place the tip with beads into the original pipette box, cover all the tips with a sheet of aluminum foil, place the cap on the box, place a piece of autoclave tape on the box, and autoclave at 121°C and 15 psi for 15 minutes.

- c. When plating – open the pipette tip box, roll back the aluminum foil to expose a single row of pipette tips, remove one tip at a time, lift the lid of an inoculated plate, and pour the sterile beads onto the agar surface. Normally, one bead remains stuck in the bottom of the tip.
  - d. Repeat step c for all replicate plates.
  - e. Cover the plates and stack them. Then shake the plates by moving them in a quick back and forth motion while keeping the bottom plate in contact with the bench top - *it is important to avoid allowing the beads to run in a circular motion around the outer edge of the plate*. Shake five times, then rotate the plates by one-quarter turn and shake again five times. Repeat shaking and turning the plates a total of five times.
  - f. Invert the plates and collect the used beads in a beaker containing 70% ethanol.
6. Plates must be clearly marked with sample number and date of inoculation.
  7. Wrap each set of three plates with parafilm and incubate inverted at 25°C for one week (see note)

**Note – for incubation of R2A plates**

- a. R2A agar plates inoculated with river or lake water will continue to develop new microcolonies for 5 to 6 days after inoculation. Therefore, incubation for at least seven days is recommended. Incubation at temperatures above 25°C is not recommended as it may reduce the number of colony forming units.
8. After incubation, count the number of colony forming units (CFU) on each plate and record in a laboratory notebook.
  9. Determine the mean and standard deviation of CFU counts on replicate plates and record in a laboratory notebook.
  10. Determine the CFU per ml of total cultivable bacteria in the original sample by multiplying the average CFU value by a dilution factor of 1,000 (accounts for the initial  $10^{-2}$  dilution and the plating volume of 0.1 ml). Record this value in the laboratory notebook.

**Enumeration of Antibiotic Resistant Bacteria**

1. Remove a sample bottle from the ice chest and mix by inversion to re-suspend any sediment that may have settled out during transit.

2. Aseptically transfer 0.1 to 0.2 ml (see note) of undiluted sample to each of three plates of Difco R2A agar plus 375 ng/ml fungizone, plus the appropriate concentration of a single antibiotic (see Table 1).

**Note – for selection of plating volume**

- a. Preliminary tests to determine the volume of sample to be plated are recommended. A plating volume of 0.1 ml is the default volume, but if the number of antibiotic resistant colony forming units is consistently less than 30 per plate, the volume should be increased to 0.2 ml
3. Spread the undiluted water sample on the surface of the agar plates using a sterile glass spreading rod, a pre-sterilized inoculating loop, or five sterile glass beads (5 mm; see note above) until all of the liquid has been absorbed.
4. Plates must be clearly marked with sample number and date of inoculation.
5. Wrap each set of three plates with parafilm and incubate inverted at 25°C for one week (see note above).
6. After incubation, count the number of colony forming units (CFU) on each plate and record in a laboratory notebook.
7. Determine the mean and standard deviation of CFU counts on replicate plates and record in a laboratory notebook.
8. Determine the CFU per ml of total cultivable bacteria in the original sample by multiplying the average CFU value by a dilution factor of 10 (for a plating volume of 0.1 ml) or 5 (for a plating volume of 0.2 ml). Record this value in the laboratory notebook.

**Determination of Impact Scores**

1. Enter enumeration data for fecal indicators and antibiotic resistant bacteria into an Excel spreadsheet.
2. For each population (i.e. fecal coliforms or ciprofloxacin resistant cells), rank the average count for a site within the population data set of all sites using the PERCENTRANK function. Multiply the PERCENTRANK output by 100 to achieve a percentile score for each data point within the entire population data set (see note).

**Note – on determining percentile scores**

- a. The PERCENTRANK function in Excel can not simply be copied and pasted from cell to cell. If the function is transferred it will carry the original array size, but the array will be offset and the function will calculate an inappropriate rank. *Therefore, you must set the array to contain the entire population data set for each individual data point.*
3. Choose the boundaries that you wish to apply to the data. For example, an  $IS_{90}$  score weights sites with population counts above the 90<sup>th</sup> percentile and below the 10<sup>th</sup> percentile. An  $IS_{80}$  score weights sites with population counts above the 80<sup>th</sup> percentile and below the 20<sup>th</sup> percentile. In our hands,  $IS_{85}$  to  $IS_{90}$  scores provide a useful signal to noise ratio in the index.
  4. Assign a population score of 1 to all data points that fall above the upper percentile boundary.
  5. Assign a population score of -1 to all data points that fall below the lower percentile boundary.
  6. Assign a population score of 0 to all data points that fall between the chosen boundaries.
  7. Repeat the determination of population scores for all microbial populations enumerated, i.e. for each antibiotic resistant population measured and for the fecal indicator population.
  8. Determine the total impact score (IS) by adding the population scores. For studies that include three antibiotics and one fecal indicator, impact scores can range from -4 to +4. Higher impact scores are indicative of a more impacted water source.
  9. Plot IS versus river mile to get a visual representation of water quality variability.