



**The Application of Multiple Antibiotic Resistance Profiles
of Coliforms to Detect Sources of
Bacterial Contamination of the Anacostia River**

Final Report

Submitted to

DISTRICT OF COLUMBIA WATER RESOURCES RESEARCH INSTITUTE

By:

**Dr. David W. Morris
Associate Professor
Department of Biological Sciences
George Washington University**

May 2011

Abstract

The Anacostia River in Washington, DC has been burdened with the problem of an estimated 2,142 million gallons of sewage overflow per year. Our study is part of a year-long investigation to identify major sources of fecal pollution in the District of Columbia from both combined sewage outlet (CSO) sites and non-point sources (NPS). This project has involved the training and active participation of a least 84 undergraduate students in four classes. Samples were obtained from the outflow of CSOs located in the District from the Northeast Boundary to the Douglass Bridge following precipitation events. The presence of *Escherichia coli* and other coliforms were confirmed and differentiated from other known enteric bacteria using characterization media. Isolates were tested for MAR using a panel of drugs commonly prescribed in clinical and agricultural practice. To determine antibiotic resistance patterns, a two-sided test of binomial proportion and Euclidian metric analysis were used. Isolates from all CSO sources showed significantly greater resistance and higher MAR indices than the NPS sites ($p < 0.05$). The highest MAR indices were obtained from outflows from CSO sites 14, 16, 17, 18 and 19. MAR testing has proved to be a quick and reliable measure of identifying the source of fecal contamination and have clearly shown that multiple drug resistance (MDR) fecal coliforms are associated with CSO overflows. Ultimately, our study will provide a comprehensive “before and after” assessment of fecal contamination in the watershed as projected revitalization continues. The continuation of this study is focused on determining antibiotic resistance transfer to environmental sources of *E. coli*, *Enterobacter* spp. and non-coliform enteric bacteria such as *Salmonella* spp., as well as the impact of MDR organisms and their long-term presence in the watershed. Many isolates were identified as possible Extended-Spectrum β -Lactamase (ESBL)-producing bacteria based on their resistance to cephalosporin drugs and aztreonam. Others were resistant to the new fluoroquinolone antibiotics. These studies point to the critical need to hasten current efforts for the rehabilitation of the river.

Introduction

The Anacostia River is an urban tributary in a highly industrial surrounding, making it a dynamic and unique environment in which to study fecal pollution. It flows approximately 8.5 miles from Prince George’s County, Maryland through Washington, D.C., before finally joining the Washington Canal and emptying into the Potomac River. Its watershed covers 176 square miles and contains 13 sub-watersheds. Although encompassed by parkland, the Anacostia is heavily polluted from sediment, toxins, pathogens, and trash (1,2). Public health risks can originate from sewage drainage directly into the river and is caused by fecal coliform bacteria and other pathogens found in the untreated wastewater. The harmful microbes debilitate water quality and create hypoxic conditions, leading to large-scale fish death and deterioration of the local wild-life (2,3). Water pollution is further compounded by the disrepair of the D. C.

combined sewage outlet (CSO) system, much of which dates back to the early nineteenth century (4).

The CSO system carries water runoff and human waste to treatment facilities; however, problems occur when excessive rainfall overwhelms the internal barrier keeping the water runoff and sewage waste separated. When this occurs, wastewater is directed from sewage lines into the river. CSO's account for an estimated 73% of the average annual increase of fecal coliform bacteria along the D. C. region of the Anacostia River, amounting to 348,000 billion Most Probable Number (MPN) fecal coliforms per year (4,5). Washington Suburban Sanitary Commission (WSSC) estimates 75 overflows occur each year, releasing 2,142 million gallons of untreated water into the environment (6).

Fecal coliforms have frequently been surveyed as indicators of the potential presence of human enteric pathogens. Indeed, standards for the District of Columbia are based upon the detection of fecal coliforms (7), although other indicator bacteria, such as the fecal streptococci (reclassified as *Enterococcus*) are favored by other studies (8). Fecal coliforms are gram-negative bacilli able to ferment lactose at elevated temperatures and include species such as *Escherichia coli* and *Klebsiella pneumoniae* (9). Furthermore, the presence of antibiotic resistant coliforms in water samples is a strong indicator of fecal pollution from animal and/or human sources. Recent studies have shown major sources of fecal water pollution can be determined by conducting a Multiple Antibiotic Resistance (MAR) analysis (8,10,11), or as it is now frequently called Antibiotic Resistance Analysis (ARA). MAR is used to differentiate fecal *E. coli* (and occasionally enterococci) from different loci by assessing the resistance profiles from bacterial isolates using antibiotics employed for human therapy and livestock maintenance (12,13). The underlying principle is that bacteria in the GI tracts of humans and animals are subjected to different types and dosages of antibiotics which select for flora with specific resistance profiles, or "fingerprints" (12). MAR analysis includes both library-dependent and non-library-dependent approaches for studying and tracking the sources of microbial pollution (called Bacterial Source Tracking, or BST). Each strategy has its own advantages and disadvantages. Several studies, for example, have focused on comparing MAR profiles of *Enterococcus* isolates to known source libraries for tracking bacterial pollution (14). Our approach, on the other hand, has been to use the non-library approach which has offered more rapid results which are useful where human

health hazards are suspected (15), Few studies have been carried out to determine the variance of MAR profiles of fecal coliforms in this tributary; therefore, our research links pollution-derived coliform levels, antibiotic resistance in mid-summer water samples, and suggests transference of resistance between human and/or animal-derived and natural-source coliforms. This project, above all, has been designed to train undergraduate students in some of the current methods used to monitor microbial contamination of the nation's waterways and particularly to focus their attention on the remediation efforts for the Anacostia River. Thus, the work described here has been carried out, and in some cases designed, by undergraduate students in our program.

Methods and Materials

The materials and methods used in the research have not differed substantially from the proposal although slight modifications in the proposed procedure have been necessary. While general methods are presented here, a short manual for student instruction was prepared and is presented in Appendix A..

Collection of Samples: CSO sites along the Anacostia River between the 11th Street Bridge and the East Capitol Bridge were chosen for MAR analysis. These sites drain both residential (84%) and mixed commercial areas (16%). In addition, all sampled CSO sites experience overflows during minimal (0.1-0.5 inches) rainfall (6). The non-point source (NPS) samples were collected mid-stream at the M Street Railroad Bridge. Approximately 1 liter of water was collected from each site on June 30th 2010, in duplicate, at each area immediately following a high flow storm event. The samples were stored in sterile plastic collection bottles (Fisher Scientific) at 4°C and were analyzed 24 hours later.

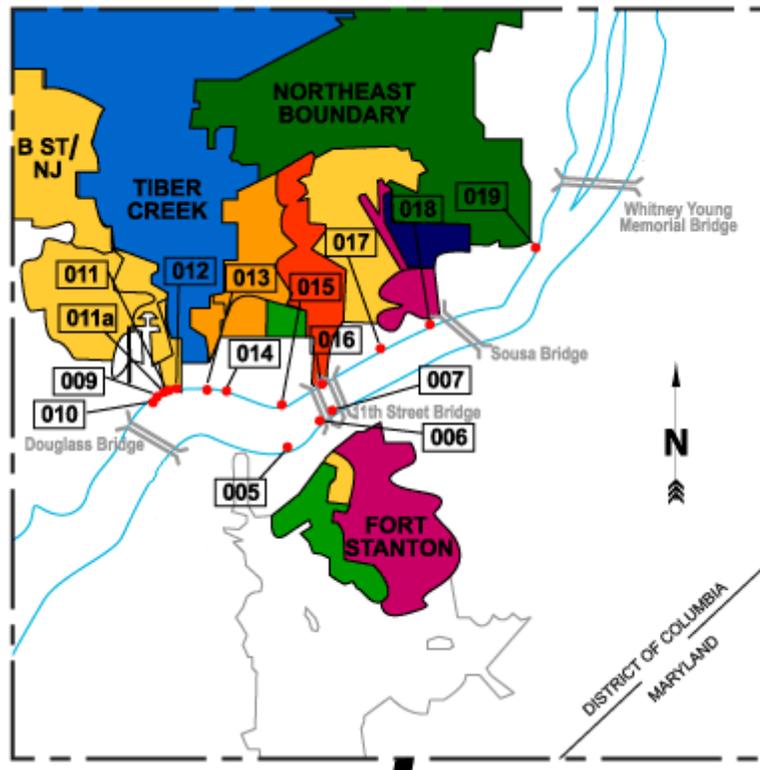


Figure 1: CSO sites on the Anacostia River sampled during this investigation (6)

Isolation, Enumeration, and Identification of Fecal Coliforms: Fecal coliform contamination in each sample was assessed initially by using the Standard Method Analysis recommended by the American Public Health Association (APHA) (16). This method estimates the MPN of fecal coliforms using a standard assay and expressed as MPN /100 mL of water sample. Individual colonies of fecal isolates were obtained based upon sample MPN results: coliform–confirmed water samples were filtered through 0.2 μm pore-sized nitrocellulose filters and the filters incubated on MacConkey Agar plates at 37°C for 48 hours. Lactose-fermenting colonies were further analyzed by replica-plating on Eosin-Methylene Blue, Desoxycholate and Hektoen Enteric agar plates to confirm the isolation of fecal *E. coli*. Fecal coliform isolates were then plated onto antibiotic inoculated LB media and scored according to their resistances to each in order to generate MAR data.

MAR Analysis: The MAR value for a given organism or source relies upon the specific panel of antibiotics which are used for testing. MAR indices were determined using similar patterns to those employed by Kasper, *et al* (15). Isolates confirmed as fecal coliforms were tested for antibiotic resistance on drug-infused LB agar plates of several different antibiotics used for clinical therapy in humans and prophylactic use in livestock (Table 1). Isolates were replica-plated from master plates to each of the antibiotic plates and incubated at 37°C for 18 to 24 hours. Isolates were recorded as resistant to an antibiotic if $\geq 80\%$ colonial growth was observed. The MAR index for each isolate was calculated using the following relationship: number of antibiotics to which the isolate was resistant / number of antibiotics tested. MAR indices for each sample site were calculated as the number of antibiotics to which all isolates were resistant / (number of antibiotics tested x number of isolates inoculated per site) (15). Significant differences between antibiotic resistance patterns at each site were determined by a two-sided test of binomial proportion ($p < 0.05$). Inter-isolate relationships were examined by converting the data to binary code and analyzed by a Euclidian metric, average linked method (DendroUPGMA Program)(12,17).

	Antibiotic Common Name (abbreviation)	Concentrations Used ($\mu\text{g/mL}$)	Use in Humans	Use in Animals
1.	Nalidixic Acid (Nal)	25.0	Clinical/Disease Control	Restricted
2.	Ampicillin (Ap)	10.0	Clinical/Disease Control	Restricted
3.	Streptomycin (Sm)	12.5	Clinical/Disease Control	Restricted
4.	Ciprofloxacin (Cp)	25.0	Clinical/Disease Control	Restricted
5.	Chloramphenicol (Cml)	25.0	Clinical/Disease Control	Restricted
6.	Chlortetracycline (Ctc)	25.0	Clinical/Disease Control	Disease Control/Feed Efficiency/Growth Promotion
7.	Kanamycin (Kan)	25.0	Clinical/Disease Control	Restricted
8.	Tetracycline (Tc)	25.0	Clinical/Disease Control	Restricted
9.	Oxytetracycline (Otc)	25.0	Clinical/Disease Control	Disease Control/Feed Efficiency/Growth Promotion
10.	Neomycin (Neo)	50.0	Clinical/Disease Control	Disease Control/Feed Efficiency/Growth Promotion

Table 1: Antibiotic Concentrations and Uses. Isolates confirmed as fecal coliforms were tested for antibiotic resistance on drug-infused LB agar plates of several different antibiotics consistent with animal and human treatment

Results and Discussion

Undergraduate involvement in the project (individual and class) commenced in late summer 2010 and carried into spring 2011. Two classes were presented with group projects involving some aspect of the work. The first was: “Do We Need Biotechnology?”, a part of the Dean’s Seminar Series and is attended exclusively by freshmen (science and non-science majors). This year’s class, held in the fall of 2010, constituted 18 students, only two of whom were from the D.C. area. The second class was Introductory Microbiology (BiSc 2237 and BiSc 2237W), designed for upper-level undergraduates (juniors and seniors,), which was held in fall 2010 and spring 2011. Each class contained 32 students. Including all participating undergraduate students conducting individual research projects in the laboratory, a total of 84 undergraduates have been actively involved in the water monitoring project. Class work and laboratory exercises relevant to the project included water sampling and testing, determination of fecal coliforms by APHA-recommended procedures (Most Probable Number (MPN) and water filtration techniques), isolation, enumeration and characterization of enteric bacteria (*Escherichia coli*, *Enterobacter*, *Salmonella* spp., and *Shigella* spp.) from water and fecal samples, MAR analysis and plasmid DNA isolation procedures.

Comparison of Multiple-Antibiotic-Resistance (MAR) Profiles of fecal *Escherichia coli* at CSO Sites 17 and 18 and a Non-Point Source on the Anacostia River

CSO17 and CSO 18 along the Anacostia River were sampled for this study in the early fall of 2010. Both sites lie between the 11th Street Bridge and the Sousa Bridge. Samples of approximately 1 liter of water were collected three times at each area in September of 2011. The non-point source (NPS) samples were obtained mid-stream near the John Philip Sousa Bridge. The water samples were analyzed as described previously. Briefly, water samples were filtered through a 0.2 µm pore-sized nitrocellulose filters, the filters were then placed on Desoxycholate Agar and further differentiated on MacConkey and Hektoen Enteric Agars. Each plate was incubated at 42.5°C. Suspected fecal *E. coli* isolates were plated on a grid for subsequent MAR analysis. MAR indices were determined by the method of Kaspar et al. (15). Isolates were identified as antibiotic-resistant if growth was identical to that on the MH plate without antibiotics. In comparison to the control plate, if the growth of bacterial colonies of an isolate

was reduced by 20% or more, then the sample was marked as sensitive to the antibiotic. MAR indices for each sample site were calculated as the number of antibiotics to which all isolates were resistant / number of antibiotics tested x number of isolates inoculated per site. Antibiotic resistance patterns at each site were determined by a two-sided test of binomial proportion ($p < 0.05$) (15).

The results indicated that isolates from both CSO sites showed significantly greater resistance ($p < 0.001$) and higher MAR indices than the NPS sites, with an average MAR index of 0.36 ± 0.04 . In contrast, NPS isolates exhibited resistance with an average MAR index of 0.07 ± 0.04 (Figures 2 and 3).

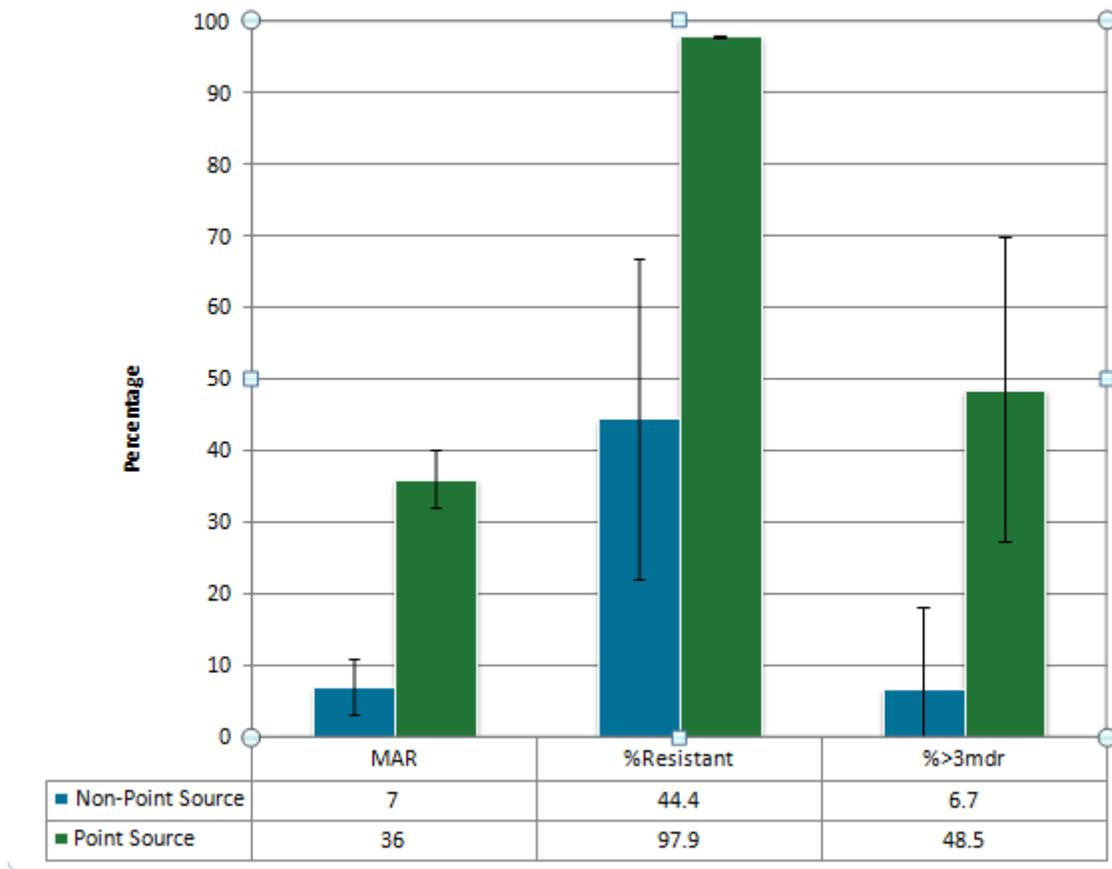


Figure 2: Multiple antibiotic resistance testing for both non-point and point sources. From left to right the bars represent the MAR frequency (number of antibiotic resistant isolates / total number of antibiotics tested), the percent of resistant isolates, and the percent of isolates that had resistances to three or more antibiotics. PS (green bars); NPS (blue bars).

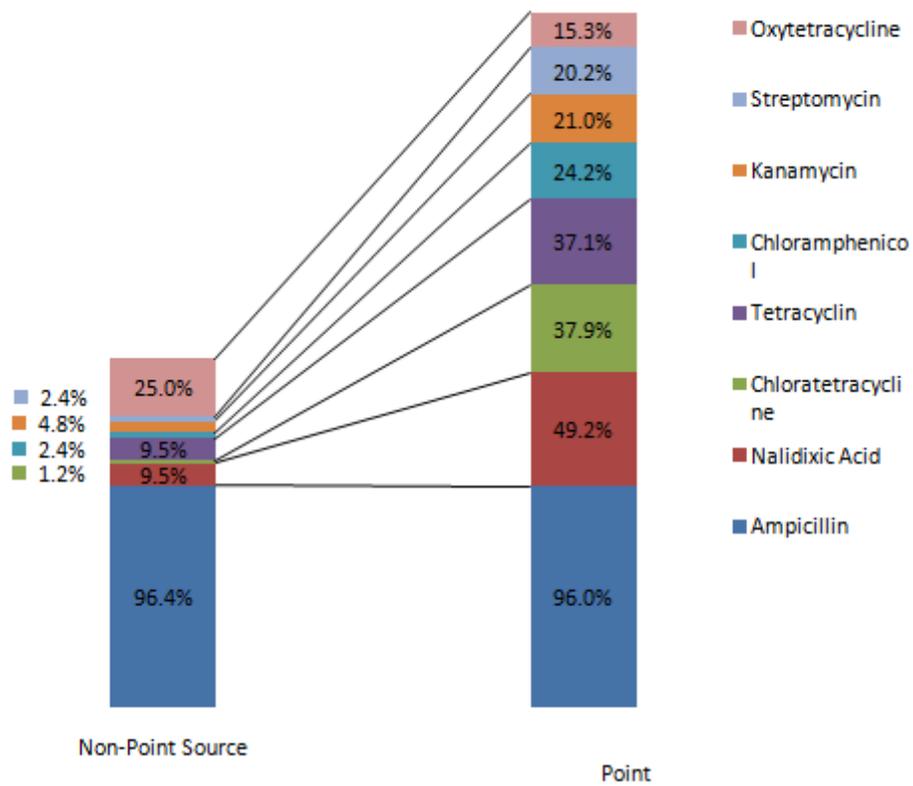


Figure 3: Antibiotic resistance profiles of coliform isolates from PS and NPS sources.

Multiple drug resistance tests also revealed that 81.5% of point source samples showed resistance to multiple drugs compared to 46.3% of nonpoint sources. Point source isolates also expressed resistance to 8 or more different drugs in 7.8% of the samples, an astounding number. Nonpoint source isolates showed varied resistance to no more than 6 drugs in any sampling and only 2.8% were resistant to more than 3. Point source samples showed significantly higher levels of widespread antibiotic resistance than non-point source samples (Figure 4).

Number of Resistances in PS and NPS Isolates

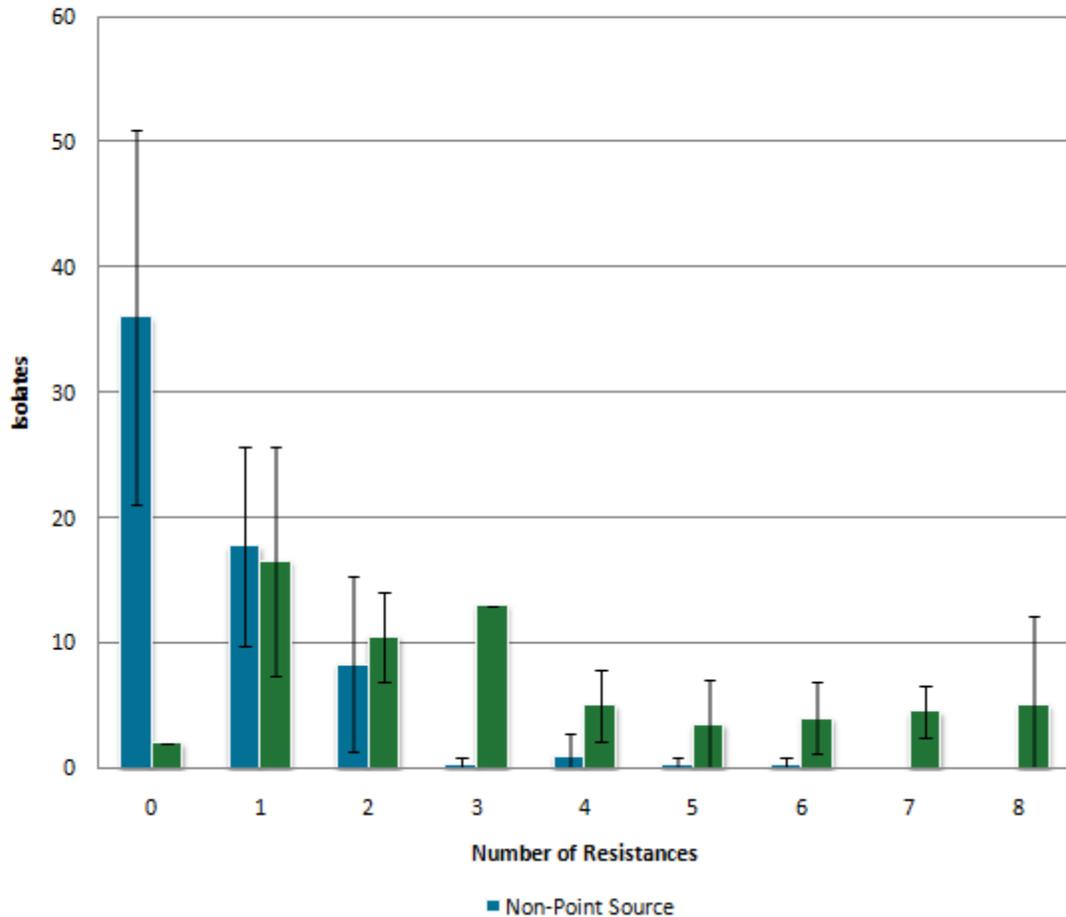


Figure 4: A comparison of the number of antibiotics each isolate was resistant to. Numbers on the x-axis represent the number resistances that each isolate had and the y-axis represents the number of isolates.

The MAR values and patterns of the PS isolates were similar to fecal *E. coli* isolates recovered from raw sewage samples in the D.C. metropolitan area, strongly indicating that multiple drug resistant (MDR) *E. coli* are being directly deposited into the river from these CSO overflows. This portion of the study was carried out by students of the Dean's Seminar Class (Do We need Biotechnology? It was presented as a poster for the George Washington Research Day in March 2011 and later at the Undergraduate Research Symposium in April where it won first prize. The full poster is shown in Appendix B.

Using Multiple Antibiotic Resistance (MAR) Analysis to Identify CSOs as Sources of Fecal *Escherichia coli* Contamination on the Anacostia River

More extensive studies were carried out by undergraduate students engaged in individual research projects in the laboratory throughout the summer and fall of 2010, and continued into the new year. Five CSO sites along the Anacostia River between the 11th Street Bridge and the East Capitol Bridge (CSO's 14, 16, 17, 18 and 19) were chosen for MAR analysis. All sites drain both residential (84%) and mixed commercial areas (16%). In addition, all sampled CSO sites experience overflows during minimal (0.1-0.5 inches) rainfall. The non-point source (NPS) samples were collected mid-stream at the M Street Railroad Bridge. The MAR Index values for all CSO's calculated an average of 52%, significantly higher than the NPS samples calculated at 16% ($p < 0.05$). This indicates that the CSO's are major point sources for contamination by fecal *E. coli*. In addition, CSO isolates showed much higher resistance to combinations of antibiotics than NPS isolates. No significant differences in MAR indexes were calculated between each of the CSO sites ($p = 0.85$); however, all CSO sites showed a significant difference with the NPS site ($p < 0.05$). 98% of isolates from CSO sites were resistant to one or more antibiotics. 83% of isolates from mid-stream samples were resistant to one or more antibiotics. Most importantly, the majority of *E. coli* isolates from CSO sources were resistant to three or more antibiotics.

Site	CSO 14	CSO 16	CSO 17	CSO 18	CSO 19	NPS
MDR	65.7	61.4	74	70.1	82.5	4.2
MAR	52	42	52	46	70	16
n	67	98	115	172	172	154

Table 2 MAR and MDR Index Percent Values for CSO Sites. No significant differences in MAR indexes were calculated between each of the CSO sites ($p = 0.85$); however, all CSO sites showed a significant difference with the NPS site ($p < 0.05$). 98% of isolates from CSO sites had resistance to one or more antibiotics. 83% of isolates from mid-stream samples were resistant to one or more antibiotics.

Comparative antibiotic resistance profiles of CSO sites showed no significant differences for ampicillin, ciprofloxacin, streptomycin and chlortetracycline resistances. However, isolates from the downstream CSO14, CSO16 and CSO17 sites showed significantly more resistance to naladixic acid, tetracycline and oxytetracycline. Upstream CSO18 and CSO19 isolates showed

significantly more resistance to chloramphenicol. Profiles of isolates from mid-stream (NPS) samples showed similar levels for ciprofloxacin and ampicillin resistances to CSO isolates only. More diverse patterns of antibiotic resistance were seen in the CSO isolates compared to the NPS source. 43 different resistance patterns were seen collectively in the CSO isolates compared to 8 patterns seen in the NPS isolates. These results are shown in detail on the poster display in Appendix C. This presentation was made at the Maryland Water Monitoring Council Conference in Baltimore, Maryland, in November 2010.

Extensive studies were also undertaken on samples obtained from CSO sites 5, 6 and 7 (at the Fort Stanton area on the south-eastern bank of the Anacostia River). MAR and MDR index percent values for each of these CSO sites (27.8%) was significantly lower than for CSO sites 14, 16, 17, 18 and 19 ($p < 0.05$) but significantly higher than NPS values ($p < 0.05$). Low MAR percent index values were also obtained for CSO 8 (20.5%) and CSO 9 (20.7%).

Overall, our studies indicated that CSO sites, 14, 16, 17, 18 and 19 contribute a significantly greater load of fecal *E. coli* contaminants to the Anacostia River during CSO overflows than the other CSO sites examined in this study. The *E. coli* isolates from the Navy Yard and North-East Boundary sites also show a wider variation in antibiotic resistance patterns. One explanation for this wide variation may be due to the exchange of R-factors carried on conjugative R-plasmids. It has been shown that plasmid transference readily occurs among fecal coliforms in the microbial milieu of mammalian GI systems and in stagnant bodies of wastewater (18,19). WSSC has reported that the D. C. region of the Anacostia River is a stagnant water body with a long resting time in these urbanized areas that favors such exchange (5). Consequently, the sluggish flow of the river does not allow for effective aeration of the water. Low O₂ saturation levels as well as high water temperatures likely favor the survival of facultatively anaerobic coliforms, resulting in genetic exchange between particularly virulent microbes and those occurring naturally within the environment (20,21). Future work will focus on these and other CSO sites this summer to confirm the consistency of our results.

There is an effort to remediate the Anacostia River and the watershed that supplies it. However, an aging city sewage system is likely to maintain the current high levels of fecal coliform contamination in the river. Any serious effort to improve the condition of the Anacostia

must be accompanied by careful monitoring of bacterial populations. We believe that using MAR profiles of selected sites on the Anacostia River (CSO and NPS), as we have described here, may be a useful and simple tool for monitoring the rehabilitation of the CSO system.

Studying the Antibiotic “Resistome” of the Anacostia Watershed

It is generally understood that, due to the overwhelming proliferation of antibiotic resistant bacteria, we are now living in a “Post-Antibiotic Era” (22). Microbial resistance to antibiotics now spans all known classes of both naturally-produced substances as well as chemically-synthesized compounds. D’ Costa and others (23,24) have argued that studying reservoirs of antibiotic resistant bacteria (in biotic and abiotic sources) could provide an early warning system for the potential transfer of antibiotic resistance genes to clinical isolates. Equally possible is the transfer of resistance genes from clinical pathogens to naturally occurring bacterial populations. It seemed logical, therefore, to extend our work into investigating the MAR spectra of fecal bacteria found in the human and animal sources which contribute to the contamination of the Anacostia River and its watershed.

In these studies, we extended the range of antibiotics used for antibiotic resistance analysis to take into account recent studies which point to new patterns of antibiotic resistance acquisition by enteric bacteria which constitute a public health threat. The additional antibiotics included cefoxitin, aztreonam, piperacillin, ofloxacin and nitrofurantoin. The emergence of resistance to expanded-spectrum cephalosporins has been a major concern and is due to the production of Extended-Spectrum Beta-Lactamases (ESBLs)(25). ESBLs confer resistance to many cephalosporin antibiotics, such as cefoxitin, and related oxyimino- β lactams, such as aztreonam (26). This latter antibiotic is primarily administered intramuscularly due to its inability to pass through the digestive tract unaltered. Resistance to piperacillin, another extended spectrum β -lactam antibiotic, has also raised concerns (27). Dug resistant *E. coli* have been identified in sewage and sludge specimens in Austria and Spain, and recently seen in enteric bacteria isolated from avian sources in Spain and South Africa (28). ESBLs are frequently encoded by plasmid-borne genes. These plasmids responsible for ESBL production frequently carry genes encoding resistance to other drug classes (i.e., aminoglycosides)(29). Resistance to ofloxacin, a second-generation fluoroquinolone, which has been associated with clinical strains of *Mycobacterium tuberculosis*, and has now been noted in among enteric isolates, including *E. coli* (27), In addition, we incorporated nitrofurantoin which is often used to combat urinary tract infections caused by *E. coli*. Rates of resistance to nitrofurantoin in the United States have, until

recently, remained low (0.4 to 0.8%) but clinical isolates resistant to the antibiotic have increasingly appeared over the past few years (30).

MAR Profiles of Coliform and Non-Coliform Bacteria from the Anacostia River and some of its Tributaries in the Anacostia Watershed. Initial results suggest that resistances to some of these “second generation” antibiotics in fecal coliform isolates from some CSO sources are high. For example, in a survey of *E. coli* isolates from CSO 5, 6 and 7, out of a total of 384 individual isolates, 63% were resistant to cefoxitin, 63% to nitrofurantoin and 38% to aztreonam. In a similar study on *Enterobacter* isolates from CSO 19, 91% were resistant to aztreonam, and 39% to nitrofurantoin; all isolates were resistant to cefoxitin and streptomycin (total 128 isolates). Major patterns of antibiotic resistance are shown in Table 4 below: over half of the isolates (54%) were resistant to the combination aztreonam-cefoxitin-streptomycin.

RESISTANCE PATTERN	PERCENT ISOLATES
Az-Ce-St	54
Az-Cef-St-Nf	26.5
Ce-St	15.6
Ce-Sm-Nf	3.1

Table 4: Major resistance patterns seen in *Enterobacter* isolates from CSO 19. Az: aztreonam; Ce: cefoxitin; St: streptomycin; Nf: nitrofurantoin.(n=128.)

MAR Profiles of Enteric Bacteria Isolated from Human Sources. *E. coli*, *Enterobacter* and *Salmonella* spp. were isolated from individual human fecal samples and raw sewage obtained in the vicinity of CSO 19. Isolates were characterized and identified by standard microbiological procedures before being plated onto a grid for subsequent MAR analysis. MAR indices and resistance patterns from individual samples varied considerably; however, sewage isolates exhibited very high MAR indices (>90%) and a wide range of multiple drug resistance patterns. Although this part of the project is still in the preliminary stages, one study on *Salmonella* spp. isolates from raw sewage taken from a sampling point near CSO 19, found that the major resistance pattern was Az-Ce-St, which has also been found in *Enterobacter* isolates from CSO 19 (see Table 4 above). Over 15% of the resistant isolates carried resistances to 9 of the 11 antibiotics tested.

MAR Profiles of Enteric Bacteria Isolated from Animal Sources. Antibiotic resistance analysis was also undertaken on enteric isolates from domestic and wild animals. These included cats, dogs, ferrets, horses and geese.. It has been suggested that wild birds, particularly migratory fowl, may harbor a reservoir of antibiotic resistant bacteria and have the potential to disseminate them over very long distances (32,33). One of our student studies focused on geese because these are common denizens of the Anacostia River and its watershed. Fresh goose fecal samples were collected from the Roosevelt Island area earlier this spring and *Enterobacteria* spp. isolates examined by MAR analysis. Our studies showed that goose fecal isolates were far more variable in antibiotic resistance patterns than any other animals tested in this project. The MAR index of the source was 41% (n=64). Most isolates were resistant to the β -lactams, aztreonam, piperacillin and ceftiofur (Figure 5) and carried resistances to multiple antibiotics (Figure 6).

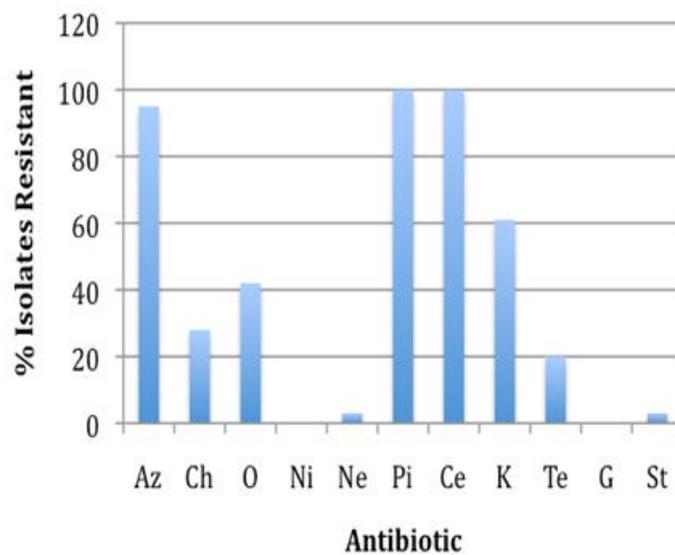


Figure 5: Percentages of individual goose *Enterobacter* isolates resistant to 11 antibiotics. Az: aztreonam; C: chloramphenicol; O: ofloxacin; Ni: nitrofurantoin; Ne: neomycin; Pi: piperacillin; Ce: ceftiofur; K: kanamycin; Te: tetracycline, G: gentamicin; S: streptomycin.

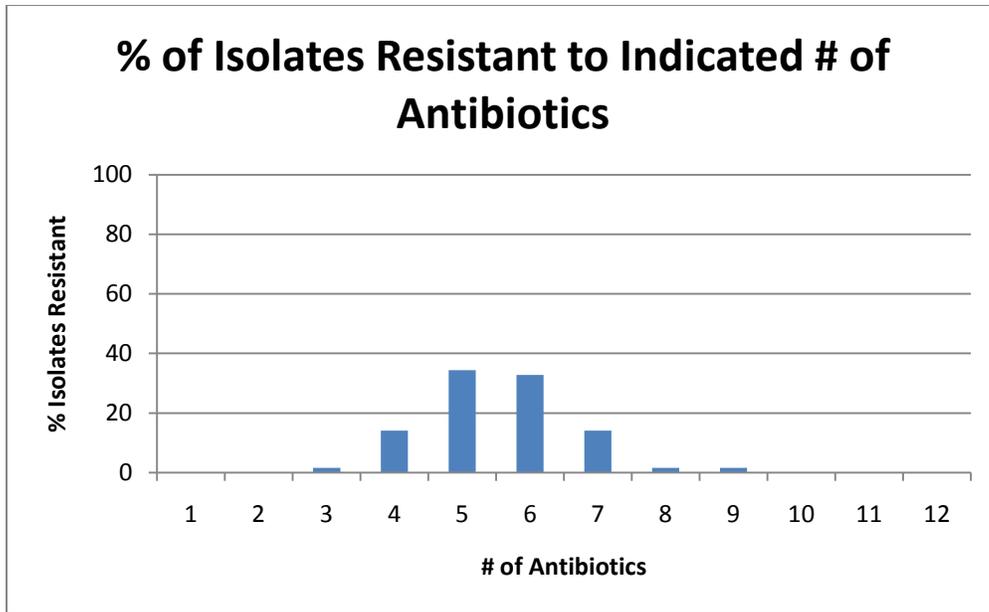


Figure 6: Percentage of individual goose *Enterobacter* isolates resistant to multiple antibiotics

19 different antibiotic resistance patterns were seen among the isolates, the most common depicted in Table 5.

Major Resistance Pattern	% Isolates
<u>Az-Pi-Ce-K</u>	17.2%
<u>O-Pi-Ce</u>	4.7%
<u>Az-Ch-Pi-Ce-K</u>	15.6%
<u>Az-O-Pi-Ce-K</u>	14.1%
<u>Az-Pi-Ce</u>	14.1%
<u>Az-O-Pi-Ce</u>	10.9%

Table 5: Predominant antibiotic resistance patterns in Goose *Enterobacteria*. Antibiotic abbreviations are the same as those in Figure 5.

These studies may be important for identifying sources of fecal coliform contamination of the Anacostia River and its watershed. Geese defecate freely in and around ponds and coastal waterways within their migration paths. Indeed, recent studies have shown that geese and gulls carry more antibiotic resistant coliforms when they nest in urban areas, especially near waste water, or agricultural water. Many of these isolates were found to have antibiotic resistance profiles similar to clinical isolates (31,34), suggesting that they may be potent disseminators of antibiotic resistance determinants (35).

Plasmid DNA Profiles of Multiple Drug Resistant Bacteria. In addition to changes in the selection of antibiotics used for MAR analysis, plasmid DNA isolation is underway to determine the molecular basis of multiple drug resistance seen in many bacterial isolates from CSO sites as well as animal and human sources. It has been well-established that the wide variation seen in antibiotic resistance, especially among Gram-negative bacteria, is due to the exchange of R-factors carried on conjugative R-plasmids (22,24). It has been shown that plasmid transfer readily occurs among fecal coliforms in the microbial milieu of mammalian gastro-intestinal systems and in stagnant bodies of wastewater (18,36). Thus, it is reasonable to hypothesize that the multiple-drug resistance profiles (particularly those which recur frequently) we see in our isolates are a direct cause of fecal contamination by the CSO system throughout the D.C. metropolitan area linked to shared R-plasmids. Furthermore, plasmids are easily transferred between species of bacteria. For this reason, this study was expanded to include other bacteria known to inhabit the intestinal tract such as *Enterococcus faecalis* (another indicator, in addition to *E. coli*, of recent human fecal contamination [8]), *E. coli* O157:H7, and *Enterobacter* spp. (in which the percentages of ESBL producers have risen sharply over the past few years [37]). Additional media and other assays have allowed our students to confirm the source of fecal contaminations, catalogue each isolate by source, and compare singular and multiple drug resistance profiles of the various water samples (NPS and CSO) to raw fecal samples.

The first attempts at plasmid DNA isolation has begun with individual isolates of MDR *E. coli* and *Enterobacter* spp. from water sources, and human and animal fecal samples taken in spring 2011. Plasmid isolation was conducted as outlined by Takahashi and Nagano (38) and the samples electrophoresed on 0.8% agarose gel for 120 minutes at 90 volts. Figure 7 shows some of the initial results. Figure 7 shows profiles of isolates from horses (lanes 6-9) and geese fecal samples (lanes 11-16) and human fecal samples (lanes 18-22) taken from Children's National Medical Center in Washington, D.C. All the horse isolates were confirmed to be cefoxitin resistant isolates. The goose fecal samples were inconsistently drug resistant to all antibiotics tested with no significant resistance pattern observed. Human fecal samples were unanimously resistant to cefoxitin, piperacillin, streptomycin and tetracycline. Similar patterns of plasmid banding (with a predominant band at ~30kbp) was observed

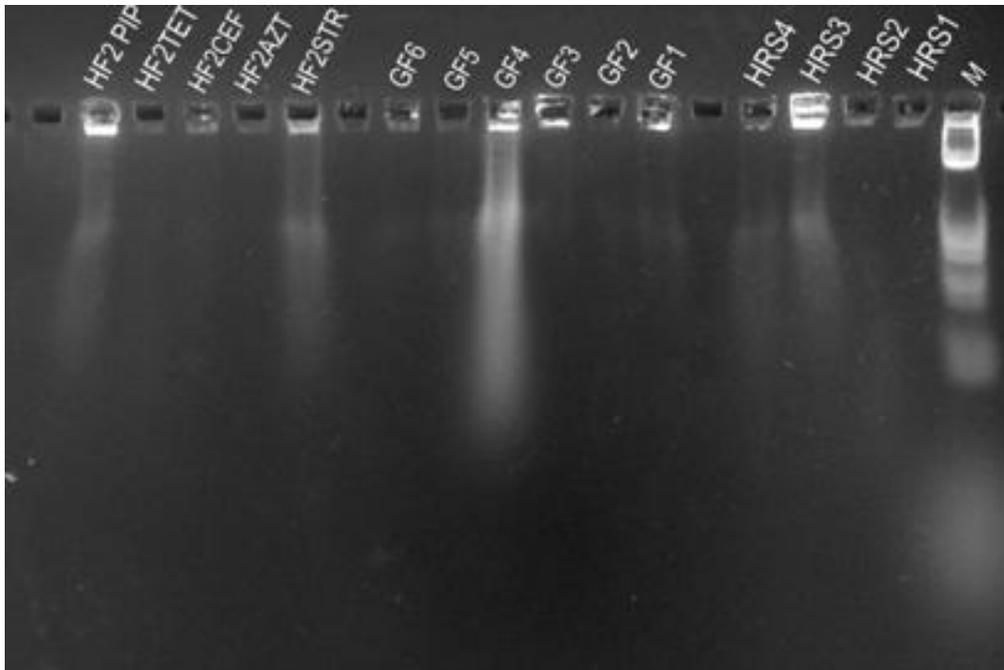


Figure 7. Plasmid profiles of multiple-drug resistant fecal coliform isolates from (L to R) humans (HR), geese (GF) and horse (HR). The single band seen in most profiles was calculated to be about 30 kb in size compared to the standards in lane M.

These investigations will continue this summer. Furthermore, as planned in the Proposal, isolates confirmed for plasmid content will be investigated by resistance transfer testing and plasmid curing (36) to assign resistance genes to plasmid DNA.

Acknowledgments

D.W.M. and E.N.B. would like to thank Stephen Goldstein for help in preparing and editing this manuscript and Nina Sabzevari for preparing preliminary reports and manuals.

References

1. The Anacostia Project. 1997. NOAA Damage Assessment, Remediation, and Restoration. Retrieved 10 May 2011 *from Anacostia River Watershed Database and Mapping Project*. Web Site: <http://mapping2.orr.noaa.gov/portal/AnacostiaRiver/>
2. "Cleaning up the Anacostia River." *National Resources Defense Council*. 10 May 2011. Web Site: www.nrdc.org/water/pollution/anacost.asp.
3. Stoddard, R. et al. 2008. Risk Factors for Infection with Pathogenic and Antimicrobial-Resistant Fecal Bacteria in Northern Elephant Seals in California. *Public Health report*. 123: 360-370
4. Combined Sewer System Overview. 2001. DC Water and Sewer Authority. Retrieved 10 May 2011. Web Site: www.dcwasa.com/education/css/combined_sewer.cfm.
5. Guillozet, P. et al. 2001. DC-WASA Combined Sewer Overflow, Anacostia River. United States Department of Environmental Programs.
6. CSO Overflow Predictions for Average Year. 2004. DC Water and Sewer Authority. Retrieved 10 May 2011. Web Site: www.dcwasa.com/education/css/CSO%20Overflow%20Predictions%20Average%20Year.pdf
7. Environmental Protection Agency. 2003. 1998 Section 303(d) List Fact Sheet for the District of Columbia. Retrieved 10 May 2011. Web Site: http://oaspub.epa.gov/waters/state_rept.control?p_state=DC#TPOL
8. Hagedorn, C. et al. 1999. Determining Sources of Fecal Pollution in a Rural Virginia Watershed with Antibiotic Resistance Analysis Patterns in Fecal Streptococci. *Appl. Environ Microbiol*. 65: 5522-5531.
9. Holt, J.G. et al.. 1994. *Bergey's Manual of Determinative Bacteriology*, 9th Edition. Williams & Wilkins, Baltimore.
10. Scott, T.M., et al. 2002. Microbial Source Tracking: Current Methodology and Future Directions. *Applied and Environmental Microbiology*. 68 (12): 5796-5803.
11. Simpson, J.M. et al. 2002. Microbial Source Tracking: State of the Science. *Environmental Science and Technology*. 36: 5279-5288.
12. Parveen, S., et al. 1997. "Association of Multiple-Antibiotic-Resistance Profiles with Point and Non-Point Sources of *Escherichia coli* in Apalachicola Bay." *Appl. Environ. Microbiol*. 63:2607-2612.
13. Whitlock, J.E., D.T. Jones, V.J. Harwood. 2002. Identification of the Sources of Fecal Coliforms in and Urban Watershed Using Antibiotic Resistance Analysis. *Water*

- Research. 36: 4273-4282.
14. Wiggins, B. A. 1996. Discriminate Analysis of antibiotic resistance patterns in fecal streptococci, a method to differentiate human and animals sources of fecal pollution in natural waters. *Appl. Environ. Microbiol.* 62: 3997-4002.
 15. Kaspar, C.W., et al., 1990. Antibiotic Resistance Indexing of *Escherichia coli* to Identify Sources of Fecal Contamination in Water. *Can. J. Microbiol.* 36: 891-894.
 16. American Public Health Association. 1989. Standard methods for the examination of water and wastewater, 17th ed., p. 9.99–9.107. American Public Health Association. Washington, D.C.
 17. DendroUPGMA: A Dedrogram Construction Utility. 2002. Web Site: <http://genomes.urv.cat/UPGMA/index.php?entrada=Example3>
 18. Goyal, S. et al.. 1979. Transferable drug-resistance in bacteria of coastal canal water and sediment. *Water Res.* 13: 349–356.
 19. Gonzalez A.M. et al. 2001. Relationships between fecal indicators and pathogenic microorganisms in a tropical lagoon in Rio de Janeiro, Brazil. Department of Biological Sciences, Institute of Biology, University Federal of Rio de Janeiro, Prédio, Brazil. 944: 97-112.
 20. Grabow, W et al.. 1973. Survival in maturation ponds of coliform bacteria with transferable drug-resistance. *Water Res.* 7:1589–1597.
 21. Grabow, W et al. 1976. Behavior in conventional sewage purification processes of coliform bacteria with transferable or non-transferable drug-resistance. *Water Res.* 10: 717–723.
 22. Neu, H.C. 1994. The Crisis in Antibiotic Resistance. *Science* 257: 1064-1073.
 23. D’Costa, V.M et al. 2006. Sampling the Antibiotic Resistome. *Science* 2006: 374-377.
 24. Allen, H.K. et al. 2010. Call of the Wild: Antibiotic Resistance Genes in Natural Environments. *Nature Rev. Microbiol.* 8: 250-259.
 25. Bradford PA. 2001. Extended-spectrum β -lactamases in the 21st Century characterization, epidemiology, and detection of this important resistance threat." *Clinic. Microbiol Rev*; 48:933-51.
 26. Jacoby, G.A and P. Han. 1996. Detection of Extended Spectrum Beta-lactamases in Clinical Isolates of *Klebsiella pneumonia* and *Escherichia coli*. *J. Clinic. Microbiol.* 4: 908-911.
 27. Fridkin, S at al. 1999. Surveillance of Antimicrobial Use and Antimicrobial Resistance in United States Hospitals: Project ICARE, Phase 2. *Clinic. Infect. Dis.* 29: 245-252.

28. Blanco, J.S. et al. 1997. Prevalence of Bacterial resistance to Quinolones and Other Antimicrobials Among Avian *Escherichia coli* Strains isolated from Septicemic and healthy Chickens in Spain. *J. Clin. Microbiol.* 35: 2184-2185.
29. Levenstein-van Hall, M.A. et al. 2011. Identical Resistance Genes and Plasmids in *Escherichia coli* from Dutch patients, Poultry and Retail Chicken Meat. *Ned, Tdsch. Geneesk.* 155: A3401.
30. Sahm, D.F. et al. 2001. Multidrug Resistant Urinary Tract Isolates of *Escherichia coli*: Prevalence and Patient Demographics in the United States in 2000. *Antimicrob. Agents Chemother.* 45: 1402-1406.
31. Cole, D. et al. 2005. Free-Living Canada Geese and Antimicrobial Resistance. *Emerg. Infect. Dis.* 11: 935-938.
32. Dojeska, M. et al. 2007. High Prevalence of Antimicrobial-Resistance Genes and Integrons in *Escherichia coli* Isolates from Black-Headed Gullis in the Czech Republic. *J. Appl. Microbiol.* 103: 11-19.
33. Sjolund, M. et al. 2008. Dissemination of Multidrug-Resistant Bacteria in the Arctic. *Emerg. Infect. Dis.* 14: 70-72.
34. Kullas, H. et al. 2002. Prevalence of *Escherichia coli* Serogroups and Human Virulence Factors in Feces of Urban Canadian Geese. *Int. J. Environ. Health Res.* 12: 153-162.
35. Middleton, J.H and A. Ambrose. 2005. Enumeration and Antibiotic Resistance Patterns of Fecal Indicator Organisms Isolated from Migratory Canada Geese (*Banta Canadensis*). *J. Wildlife Dis.* 41: 334-341.
36. Daini O. A. and Adesemowo A. 2008. Antimicrobial Susceptibility Patterns and R-Plasmids of Clinical Strains of *Escherichia coli*. *American Journal of Basic and Applied Sciences.* 3: 397-400.
37. Sidjabat, H. et al. 2007. Identification of Plasmid-Mediated Extended Spectrum and AmpC β -lactamases in *Enterobacter* spp. Isolated from Dogs. *J. Med. Microbiol.* 56: 426-434.
- 38.

Appendix A: Student Manual for the isolation, characterization and MAR analysis of fecal coliforms.

Day 1—Colony Collection

- 1) Water samples should be processed within 48 hours of collection. Shake acquired water samples vigorously to mix contents that may have settled. Organic material caught in the sample is normal and inconsequential.
- 2) Use vacuum filtration located next to the fume hood to filter the samples.
 - a. Turn on the vacuum in the hood and attach a filtration cup to the filter flask.
 - b. Remove the lid of the filtration cup and add desired water volumes
 - i. Late Spring—Early Fall: 5-10mL water or one of each
 - ii. Late Fall—Early Spring: 20-30mL water or one of each
 - c. Filter an additional 10-20 mL of distilled water after the contaminated water.
 - d. Dip tweezers in alcohol and remove the filter paper in the cup and place onto plate consisting of MacConkey's agar, grid-side up.
 - i. Place it down at an angle to avoid air bubbles and ensure the most contact between the paper and the agar. Using the sterilized tweezers, gently press out any remaining air bubbles.
- 3) Place topside down in 37C incubator overnight.
**NOTE: Plates must be read within 24 hours to ensure late sugar fermenting organisms do not influence original results.

Day 2—Master Plates

- 1) Remove plates from incubator to ensure sufficient growth. If sufficient growth is not observed, it may be necessary to filter a larger volume of water to obtain a higher quantity of colony forming organisms.
- 2) Using pre-formed grid papers align fresh MacConkey's plates on top of the grid. Mark the bottom of the plates to orient them. This differentiates the first colony to the last colony when you read/compare them at a later date.
 - a. Label fresh plates with sample name/number from the filtration plates and [current] date.
 - b. Square plates = square grid; round plates = round grid papers
- 3) Using sterile toothpicks, collect fermenting (red) colonies individually from the filter plates and transfer by pricking gently into the new MacConkey's plates. One colony per square.
- 4) Place topside down in 37C incubator overnight. These plates will be referred to as the "master plates" and must be read within 24 hours to ensure late fermenting organisms do not contaminate original results.

Day 3—Classification/Characterization by Differentiation Media

- 1) When you read the previous days plates, note/record the color, shape and other unusual physical characteristics of each colony. Your colonies should be primarily [Lac+] pink→brick red, round formations, and may have a bile precipitate present.

- 2) Align 8 grid papers one after another. The first placement is your master plate followed by one each of your differentiation media:
 - a. Desoxycholate (Desoxy)
 - b. Eosin Methylene Blue (EMB; or // label)
 - c. Hektoen Enteric (HE)
 - d. Simmon's Citrate
 - e. MacConkey's (MAC; or / label)
 - f. Xylose Lysine Desoxycholate (XLD)
 - g. Sorbitol MacConkey's (S. MAC)

Expected Results for *E. coli* (Refer to the Difco Manual):

Desoxy	Red colonies with a surrounding bile precipitate
EMB	Deep red—purple colored colonies with a greenish metal tinge
HE	Large yellow—salmon-pink colonies with bile precipitate
Citrate	No growth: an important test because it distinguishes <i>E. coli</i> from <i>Enterobacter</i> (which does grow and elicits media color change from green to blue). Change in color should be recorded as positive for <i>Enterobacter</i> .
MAC	Pinkish—brick-red colonies and bile precipitate
XLD	Large, flat yellow colonies
S. MAC	Pink—red colonies with bile precipitate; sorbitol non-fermentation is indicative of O157:H7 Enterohemorrhagic <i>E. coli</i> [EHEC]

You may also be recommended to carry out the following confirmatory tests on selected isolates:

- a. Gram staining;
 - b. IMVic tests;
 - c. API test strips for Salmonella/Shigella;
 - d. TSI agar stabs;
 - e. LB Salts (6.5%)

**This test attempts to isolate *Streptococcus faecalis* and growth of mucoidal, round opaque colonies indicates a positive result. Gram stain sample organisms to confirm.
- 3) Label each plate with the corresponding Master Plate information and [current] date. All plates should be marked and oriented the same direction. Using a sterile toothpick, transfer one colony at a time to each of the plates. Complete one master plate at a time and remain consistently centered for ease of replica plating later.
 - a. You only need to use a new toothpick when going from one master plate to another; however you can use one toothpick per master plate across all media.
 - b. You will be transferring one colony at a time so that the grids align across ALL media plates. Colony 1 on the mater plate corresponds with colony 1 on the subsequent media plates. It is okay for the same toothpick to go from HE to citrate, etc.
 - 4) Incubate topside down at 37C overnight.

Day 4—Antibiotic Assay

- 1) Record the results from these plates by noting the appearance of the plates, color changes, any significant patterns and how much growth has occurred. If there are a few colonies, be sure to count them and note this number.
 - a. Observe all physical characteristics of each colony individually—color, shape, aggregation, surrounding media, etc. This is important and informative when we consider source contamination of the water sample in addition to the further classification of each colony. You will use the provided media descriptions to organize and clarify or results.
- 2) Using the original master plates, transfer one colony at a time to antibiotic plates in the same replication pattern used for differentiation. The antibiotics and concentration used are:
 - a. Aztreonam: 0.05 µg/mL
 - b. Geneticin: 25 µg/mL
 - c. Nitrofurantoin: 0.64 µg/mL
 - d. Kanamycin: 50 µg/mL
 - e. Ofloxacin: 10 µg/mL
 - f. Cefoxitin: 0.4 µg/mL
 - g. Piperacillin: 25 µg/mL
 - h. Streptomycin: 12.5 µg/mL
 - i. Neomycin: 50 µg/mL
 - j. Tetracycline: 25 µg/mL
 - k. Chloramphenicol: 25µg/mL
- 3) Using the same technique and setup as above transfer one colony at a time from the MacConkey's master plates onto the antibiotic plates. You should have 11 antibiotic plates per water sample.
- 4) Incubate the plates topside down at 37C overnight.

A. Isolation of *E. coli* from anal swabs, animal feces and raw sewage

- 1) For anal swabs (human and animal).
 - a. Take a sample immediately after defecation and swipe directly onto an EMB plate and incubate for 48 hours.
 - b. Alternatively, the swab may be placed in 1 mL of EC broth, incubated for 8 to 16 hours at 44.5°C (in a water bath) before plating onto EMB plates.
- 2) For fecal specimens,
 - a. Place approximately 1 gm of material in 10 ml of 1% tryptone broth and shake vigorously to disperse the material. Use sterile glass rod if necessary.
 - b. Transfer 1 mL amounts to 5 ml of EC broth and incubate at 44.5°C for 8 to 16 hours. Plate samples (streaking is sufficient) onto EMB plates and incubate at 37°C for 24 to 48 hours.

- 3) For raw sewage, pipette 1 mL amounts into 5 ml of EC broth and process as described above.

Transfer fermentative colonies (deep red in coloration) to fresh EMB plates and screen for *E. coli* as above.

B. Data Calculations and other Schematics

Susceptible: 0→15% growth; Sensitive: 15→79% growth; Resistant: ≥80%

- 1) For each ISOLATE
 - a. Multiple Antibiotic Resistance Index for each isolate calculated as follows:
MAR Index = (# of colonies grown/total # of AB's tested) x 100 =
 - b. Present this information as a bar graph
 - c.
- 2) For each CSO/NPS
 - a. MAR Index for each AB calculated as follows:
AB MAR Index = (total # resistant isolates/total # isolates) x 100 =
 - b. Present this information as a bar graph.
 - c. Make a chart of the samples/antibiotics and write down the number of colonies that showed growth per total number of colonies poked onto the antibiotic plates.
 - a. Note if any plates have turned a greenish color.
 - b. In addition, your record will also need to note growth (as +)/no growth (as -) for each colony inoculated onto the plate. This means, for each square AB plate, you will have a series of 64 +/- notations.
 - c. You will use the above information later to develop analytical tools such as comparative matrices and isolate "fingerprints" called Dendograms.

Appendix B: Fecal Contamination Analysis of the Anacostia, by Gaurav Dhiman and Mark Mallozzi (“Do We Need Biotechnology?” Dean’s Seminar Class).

Presented at the George Washington University Undergraduate Research Symposium. April 25th 2011.

Fecal Contamination Analysis of the Anacostia

Gaurav Dhiman, Mark Mallozzi
Department of Biological Sciences, The George Washington University, Washington, D.C. 20052

ABSTRACT

The Anacostia River in Washington, DC has been burdened with the problem of an estimated 2,142 million gallons of sewage overflow per year [1]. Studies have shown that significant sources of fecal contamination can be determined by conducting Multiple Antibiotic Resistance (MAR) analysis. The hypothesis is that if the bacteria exhibit resistance, they are likely to be derived from human and animals exposed to antibiotics. Therefore, this project seeks to apply similar studies to non-point source (NPS) and combined sewer overflow (CSO) areas along the Anacostia River. Of the 240 samples collected, isolates from the CSO sources showed significantly greater resistance and higher MAR indices than the NPS. MAR testing is proving to be a reliable measure of identifying the source of fecal contamination in rivers and waterways. Our studies on the Anacostia River using this approach may clearly show fecal coliforms are associated with CSO overflows and may prove to be useful in monitoring the current efforts to rehabilitate the river. The implementation of this method in the remediation of the Anacostia River has the ability to serve as a model for future rehabilitation of similar waterways across the nation.

BACKGROUND

The Anacostia is an urban estuary that drains for 8.5 miles from Prince George County in Maryland and empties into the Potomac River at Buzzard Point [Figure 1]. The combined sewer overflow system (CSO) of DC remains as an outdated and inefficient system, with problems occurring when excessive rainfall overwhelms the internal barrier, causing wastewater from sewage lines into the river [1] (Figure 2). The presence of coliform bacteria indicates that disease-causing organisms (pathogens) could be in the water system. However, testing for all pathogens is inefficient, the presence of fecal coliform bacteria can be tested for, which has been surveyed as an indicator for the potential presence of human enteric pathogens [2]. The presence of antibiotic resistant coliforms in water samples is a strong indicator of fecal pollution from animal and/or human sources [3]. Recent studies have shown major sources of fecal water pollution can be determined by conducting a Multiple Antibiotic Resistance (MAR) analysis [4, 5, 6]. The underlying principle is bacteria in the GI tracts of humans and animals are subjected to different types and dosages of antibiotics which select for flora with specific resistance profiles, or “fingerprints”, few studies have been carried out to determine the variance of MAR profiles of fecal coliforms in the Anacostia watershed area. Therefore, our research links pollution-derived coliforms levels and antibiotic resistance in water samples to provide insight in fecal coliform contamination of the Anacostia River.



Figure 1. Map of the CSO and NPS sites along the Anacostia River. The map shows the river's course through Washington, DC, with CSO sites marked in red and NPS sites in blue. A legend identifies the symbols used.

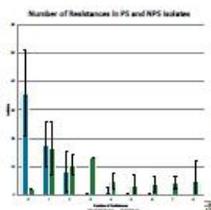


Figure 3. A comparison of the number of antibiotic resistances observed in combined sewer overflow (CSO) and non-point source (NPS) isolates.

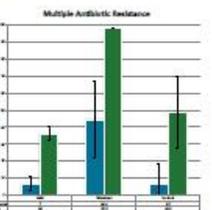


Figure 4. Mean MAR index for CSO and NPS isolates. CSO isolates show a significantly higher MAR index compared to NPS isolates.

METHODS

Isolation, Enumeration, and Identification of Fecal Coliforms
Isolates from the CSO and NPS sites along the Anacostia River were sampled in this study. Both sites lie between the 11th Street Bridge and the Sousa Bridge [Figure 1]. The non-point source (NPS) samples were obtained near the John Philip Sousa Bridge. The water samples were filtered through a 0.2 µm pore-sized nitrocellulose filter, the filters were then placed on Desoxycholate Agar and Hektoen-Enteric Agar. The Desoxycholate and Hektoen-Enteric Agar's were used because they selective and differential medium for the isolation, cultivation and differentiation of gram-negative enteric bacilli [8]. Plates which contained confirmed fecal *E. coli* were used in the characterization and MAR analysis. Sterile toothpicks were used to transfer the colonies in a grid fashion [Figure 3] from the plates to a master plate containing MacConkey Agar, which selectively isolates for the identification of Enterobacteriaceae from feces, urine, wastewater, and foods [9].

MAR Analysis
MAR indices were determined by the method of Kasper et al. [7]. Stock solutions of antibiotics were filtered, sterilized, and prepared on Mueller-Hinton (MH) agar plates to specific concentrations [Figure 6]. Isolates were identified as antibiotic-resistant if growth was identical to that on the MH plate without antibiotics [Figure 4]. In comparison to the control plate, if the growth of bacterial colonies of an isolate was reduced by 20% or more, then the sample was marked as sensitive to the antibiotic [Figure 7]. MAR indices for each sample site were calculated as the number of antibiotics to which all isolates were resistant / number of antibiotics tested x number of isolates inoculated per site [5] [Figure 6]. Antibiotic resistance patterns at each site were determined by a two-sided test of binomial proportion ($p < 0.05$) [1].



Figure 5. Isolates from CSO sites were highly resistant to all antibiotics tested. The plates show varying degrees of bacterial growth inhibition, with some showing almost complete lack of growth.

RESULTS

Isolates from the CSO sources showed significantly greater resistance ($P < 0.001$) and higher MAR indices than the NPS sites, with an average MAR index of 0.3640.04. In contrast, NPS isolates exhibited resistance with an average MAR index of 0.0710.04 [Figure 6]. Multiple drug resistance tests also revealed that a staggering 61.2% of point source samples showed resistance to multiple drugs compared to 46.3% of nonpoint sources. These point sources also expressed resistance to 8 or more different drugs in 7.8% of the samples, an astounding number. Nonpoint samples showed resistance to no more than 6 drugs in any sampling and only 2.8% were resistant to more than 3 [Figure 4]. Point source samples showed significantly higher levels of widespread antibiotic resistance than non-point samples which suggests the misuse of antibiotics and their disposal are a direct cause of the recent surge in antibiotic resistant bacteria.

CONCLUSION

E. coli and other bacteria pose an increasingly dangerous threat due to antibiotics declining effect on disease such as meningitis and urinary tract infections. MAR analysis is essential in the effort to restore the Anacostia and other waterways due to its ability to identify major pollution sites. The remediation procedure of the Anacostia and Chesapeake Bay will not only prove to benefit the DC area but, located in our nation's capital, will also serve as a model for waterways across the country.



Antibiotic Concentration Name	Concentration (µg/ml)	This Is Known	This Is Anticlinic
Amoxicillin	10	Sensitive	Sensitive
Chloramphenicol	10	Sensitive	Sensitive
Clindamycin	10	Sensitive	Sensitive
Clotrimazole	10	Sensitive	Sensitive
Colistin	10	Sensitive	Sensitive
Fluconazole	10	Sensitive	Sensitive
Vancomycin	10	Sensitive	Sensitive

Acknowledgements
To our advisor, Dr. Mark Mallozzi, for his guidance and support throughout this project. We also thank the members of the Department of Biological Sciences for their assistance and support.

