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Survey of the Great Kanawha River, West Virginia, for Virulence Related Gene Markers stx1, stx2, and eaeA

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SURVEY OF THE GREAT KANAWHA RIVER, WEST VIRGINIA, FOR VIRULENCE RELATED GENE MARKERS *stx*$_1$, *stx*$_2$, AND *eaeA*

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ABSTRACT

SURVEY OF THE GREAT KANAWHA RIVER, WEST VIRGINIA, FOR VIRULENCE RELATED GENE MARKERS *stx*$_1$, *stx*$_2$, AND *eaeA*

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Microbial surveying using antibiotic resistant bacteria, fecal coliforms, and virulence genes is an approach not previously tested on the Great Kanawha River. Research objectives were to test antibiotic resistant and fecal coliform bacteria as bioindicators of water quality, and develop a multiplex- polymerase chain reaction (mPCR) system for identification of *stx*$_1$, *stx*$_2$, and *eaeA* DNA sequences in isolated fecal coliforms (FC). Fecal indicator populations and antibiotic resistant populations were demonstrated to be independent. Bacterial populations were assigned impact score(s) (IS) values for each sample site based on data set percent ranks. Population scores were summed per sample site to generate Relative IS, used to visualize water variability. Relative IS$_{95}$ for both seasons were at about zero between river miles (RM) 95-60 (toward origin), reach highest levels at RM 55-45 and return to levels similar to those toward the origin for RM 35-00 (toward mouth). Trends appear to be occurring in approximately the same locations although not with the same level of impact. Spring versus summer Relative IS$_{95}$ comparisons show no significant correlations ($P \geq 0.05$). A total of 13 (12.0%) of 108 mPCR assayed FC isolates were positive for at least one target gene sequence. Two of the 13 were carriers of multiple target genes. No isolates were carriers of all three target genes. Similar IS seasonal patterns show the use of this impact score index to identify areas of poor water quality is independent of sampling season tested as long as samples are collected during similar flow regimes.

DEDICATION

This research is dedicated to my daughters, Emily and Sydney. Let the anticipation of achievement keep you motivated, and never let the fear of failure hold you back!!!!

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LIST OF SYMBOLS, ABBREVIATIONS, AND NOMENCLATURE

Colony Forming Units (CFU)

eaeA – Intimin gene marker

Enterohemorrhagic *Escherichia coli* (EHEC)

Enteropathogenic *Escherichia coli* (EPEC)

Escherichia coli (*E. coli*)

Great Kanawha River - a.k.a. Kanawha River

Impact Score(s) – (IS)

River Tributaries –
- Coal River (Coal)
- Elk River (Elk)
- Gauley River (Gau)
- New River (New)
- Pocatalico River (Poca)

Shiga toxin producing *Escherichia coli* (STEC) Formerly:
- Shiga-like toxin producing *Escherichia coli* (SLTEC)
- Verotoxin producing *Escherichia coli* (VTEC)
- Verocytotoxin producing *Escherichia coli*
- Shiga toxigenic producing *Escherichia coli*

Sht – Shiga-toxin associated with *Shigella dysenteriae*

Standard Operation Procedure (SOP)

Stx1 - Shiga-like toxin 1: Formerly SLT-1 and VT-1 (Verotoxin 1)

Stx2 – Shiga-like toxin 2: Formerly SLT-2 and VT-2 (Verotoxin 2)

stx_1 - Shiga-like toxin 1 gene marker: Formerly *slt-I*

stx_2 - Shiga-like toxin 2 gene marker: Formerly *slt-II*

CHAPTER I

LITERATURE REVIEW

Introduction

Water quality is a concern both for public and environmental health reasons. Human interactions with riverine systems increase the cause for concern of potential health related risks (8, 10, 67). One way to address these risk factors is to use bacteriological bioindicators to monitor water quality (10). Fecal coliform bacteria are the most commonly used bioindicators of fecal pollution in water and food (21). The presence of fecal coliforms in water is not necessarily indicative of a disease outbreak. Outbreaks of waterborne diseases can occur because water is inadequately treated or disinfected, or because it is re-contaminated during distribution (11). A social stigma associated with fecal contamination presumes illness will occur. Further tests must be done on identified coliforms to determine if they are or are not pathogenic strains. Three relatively recent publicized outbreaks of the fecal coliform *Escherichia coli* serotype O157:H7 occurred in Wyoming (1998), New York (1999), and Canada (2000) have increased the awareness of water safety issues (37).

Antibiotics

An antibiotic is a drug that either inhibits the growth of (bacteriostatic) or kills (bactericidal) bacteria by interfering with normal bacterial cell functions. Antibiotics are a class of antimicrobial compounds, synthetic or natural, derived from certain fungi, bacteria, and other organisms (33, 59). With molecular weights of less than 2,000 Daltons antibiotics are considered small molecules (59). Research has shown that many

compounds including antibiotics can enter the environment, disperse, and persist to a greater degree than originally believed. According to a study conducted by the United States Geological Survey (USGS), little is known about the degree of environmental occurrence, transport, and eventual fate of many commonly used organic chemicals after their intended use (26).

Antibiotics can be classified based on their target specificity (i.e. narrow spectrum or broad spectrum). The antibacterial spectrum is the range of activity an antimicrobial exhibits against susceptible bacteria (33, 59). Ciprofloxacin, erythromycin, and tetracycline are three antibiotics, each from a different class of antibiotics, being tested in this survey for antibiotic resistance in surface water. Each of the three antibiotics being examined were identified as emerging environmental contaminants in freshwater systems throughout the United States by the USGS in their 1999-2000 Toxic Substances Hydrology Program Report (26, 51, 53).

Ciprofloxacin is a synthetic antibiotic belonging to the fluoroquinolone class of antibiotics (64). It is a broad spectrum, bactericidal antibiotic active against both Gram-positive and Gram-negative bacteria (33, 45, 61, 64). The chemical formula for ciprofloxacin is $C_{17}H_{18}FN_3O_3$ (Figure 1). It has a molecular weight of 331.4 Daltons (45, 61). Ciprofloxacin is manufactured and sold by Bayer pharmaceutical as Cipro® and Ciproxin® (61). This synthetic chemotherapeutic agent's mode of action works through inhibition of nucleic acid synthesis. Inhibition of bacterial DNA replication is accomplished by binding to DNA gyrase. The DNA gyrase allows for the untwisting that is required for replication of a DNA double helix. Inhibition of nucleic acid synthesis, in turn, leads to the death of susceptible bacterial cells (33, 45, 50, 61).

Erythromycin is a member of the Macrolide group of antibiotics (46, 62). Macrolides are a group of broad spectrum chemotherapeutic agents characterized by having a macrolide ring (63; Figure 2). The chemical formula for erythromycin is $C_{37}H_{67}NO_{13}$, and its molecular weight is 733.93 Daltons (62). Erythromycin is produced by an actinomycete called *Saccaropolyspora erythraea*, formerly known as *Streptomyces erythraeus* (33, 62). Erythromycin works by preventing susceptible bacteria from growing through inhibition of protein synthesis. Protein synthesis inhibition is accomplished by reversibly binding to the 50 S ribosomal subunit which blocks polypeptide elongation (33, 46, 49, 62). This action is mainly bacteriostatic but can be bactericidal at high concentrations (62). The use of erythromycin as a chemotherapeutic agent began in 1952 under the brand name Ilosone®, after the Philippine region of Iloilo where the soil samples it was originally collected from was located. It was also formally called Ilotycin® (62).

Tetracycline is in the Tetracycline class of antibiotics. Tetracyclines are broad spectrum, bacteriostatic chemotherapeutic agents that inhibit protein synthesis in susceptible bacterial cells by binding reversibly to the 30 S ribosomal subunit (33, 48, 65). The reversible binding blocks aminoacyl-transfer RNA (charged tRNA) from binding to the 30 S ribosome-messenger RNA (mRNA) complex and, in turn, inhibits growth by inhibiting translation (66). Tetracycline is produced as a secondary metabolic product of *Streptomyces rimosus* (33, 47, 54, 65). The chemical formula for tetracycline is $C_{22}H_{24}N_2O_8$ (Figure 3), and it has a molecular weight of 444.44 Daltons. Tetracycline was discovered in the research department of the Pfizer pharmaceutical company by Lloyd Conover. The patent for tetracycline (No. 2,699,054) was first issued in 1955. It

is also sold under the brand names Sumycin®; Tetracyn® ; Tetralysal 300® ; Panmycin® ; Brodspec® ; and Tetracap® (65).

Antibiotic Resistance

Antibiotic resistant bacteria are a natural evolutionary phenomenon (58). Bacteria with intrinsic resistance to antibiotics are found in nature (4). The ability of bacteria to develop or acquire resistance is an example of the Darwinian Principal of "survival of the fittest." Only those bacteria with the ability to adapt are those with the ability to survive (31, 58). The results of the USGS National Reconnaissance study indicated that wastewater treatment techniques are not adequately removing antibiotic compounds prior to treated water being reintroduced in to natural water reservoirs (26, 51).

Hirsch *et al.* (22) and Koplin *et al.* (26) suggest that the rate at which pathogenic bacteria develop resistance to antibiotics is affected by even low-level concentrations of antibiotic residues present in the environment. Thus antibiotic residues could result in serious threats to public health as more bacterial infections become resistant to treatments using presently known antibiotics (22). Repeated and continuous use of antibiotics also creates selection pressures that favor the growth of antibiotic resistant mutants (15, 54). A survey of six freshwater streams in Hong Kong found that multiple antibiotic resistant bacterial species are common in environmental organisms even in the absence of specific antibiotic pressures (20). The increased frequency and spectrum of antibiotic resistance has also been attributed to social and technical changes that increase the transmission rate of resistant organisms. These changes include an increase in the use and accessibility of antimicrobial agents and medically invasive procedures (15).

Antibiotic resistance, to a single antibiotic or to multiple antibiotics, can be acquired through mutational changes or by acquisition of resistance encoding genetic material which has the ability to be transferred from one bacterium to another (15, 58). The acquisition of external genetic material may allow a bacterium to exhibit resistance to an entire class of antibiotics (58). Three lateral gene transfer mechanisms (also known as horizontal gene transfer) have been identified: transformation, conjugation, and transduction (Figures 4, 5, 6; 43, 67). All three mechanisms are believed to occur in aquatic environments (67). Lateral gene transfer is an important evolutionary mechanism for bacterial species. It creates diversity within bacterial species. Mechanisms for transferring genetic material from one bacterium to another are integral to aid in the understanding of how virulence factors and antibiotic resistance spread through bacterial populations (43).

Transformation, when compared to conjugation and transduction, rarely occurs in nature and the degree to which it contributes to genetic diversity is not known (43, 48). Transformation involves the transfer and incorporation of naked DNA (DNA that has been released from a cell) into competent bacterial host cells (Figure 4; 15, 43). The competency of a bacterial host cell (i.e. its ability to transport and express foreign DNA) is dependant upon stages of the cell's life cycle. Competency is usually maximal prior to completion of cell wall synthesis but not all bacteria can become competent (43).

Conjugation is probably the most common form of bacterial gene transfer mechanism. Gene transfer by conjugation is dependent upon cell to cell contact and a specialized appendage, known as the F- pilus (or sex pilus) (Figure 5; 15, 43, 60). Plasmids encoding antibiotic-resistance genes are able to pass throughout populations of

bacteria, and between multiple species of bacteria using conjugation as their means of gene transfer (43).

Transduction occurs when the DNA of a host (donor cell) is encapsulated into a bacteriophage which acts as a vector and injects the acquired DNA material in to a recipient cell (Figure 6; 15, 48). Both Gram-positive and Gram-negative bacterial species are capable of acquiring antibiotic resistance this way (48). The fact that a limited amount of host DNA can be packed into the head of a bacteriophage, and that this process is dependent on specific phages, suggests that this type of gene transfer is probably a minor source for multiple drug resistance.

In a 1976 study (24), done on waterways in Oregon, potentially pathogenic Gram-negative bacteria and fecal coliforms were isolated to determine if transfer of resistance genes could occur in streams, rivers, bays, and other waterways. Of the 2,763 bacterial colonies isolated 2,445 were identified as fecal coliforms, *Pseudomonas, Moraxella, Actinobacter,* or *Flavobacterium-Cytophaga*. Based on antibiotic resistance data, fecal coliforms were found to survive better in surface water environments than other more sensitive organisms. The study concluded that the survival potential of fecal coliforms in the environment is related to their ability to acquire antibiotic resistance (24). Resistance to multiple antibiotics is considered common among fecal coliforms isolated from both humans and animals. The pattern of resistance is related to the gastrointestinal microflora's exposure to antibiotics. Shared patterns of resistance between humans and animals is attributed to the approval for use of most antibiotics by both humans and animals (21).

Resistance genes and mechanisms existed long before antimicrobials were introduced into clinical medicine. It is thought that bacterial species capable of producing antimicrobials (intrinsically resistant organisms) may be one source of resistance genes (4, 58). These bacteria must possess a resistance mechanism to protect themselves from their own antibiotic action and may, in turn, pass the resistance on to other bacteria (58). Intrinsically antibiotic resistant organisms may also acquire additional resistance genes from bacterial species introduced into soil or water (4). The proportion of some bacterial species present at a site could affect the total amount of resistance measured for particular antibiotics or antibiotic classes. For example, *Klebsiella* strains have an intrinsic ability to be resistant to Ampicillin (34).

Toxins of Enteric Bacteria

The coliform group (meaning "coli" like or *E. coli* like) is defined as all aerobic and facultative anaerobic, non-spore forming, Gram-negative, rod-shaped bacteria that ferment lactose with the production of gas within 48 hours at 35° C (95° F; 10). Examples of coliform bacteria include those bacteria in the genera *Escherichia* (e.g. *E. coli*), *Klebsiella* (e.g. *K. pneumoniae*), *Enterobacter* (e.g. *E. cloacai*), and *Citrobacter* (C. *rodentium*; formerly *C. freundii*). Fecal coliforms are a subset of the coliform group. Fecal coliform bacteria are heat tolerant, Oxidase negative, and associated with feces from warm blooded animals (e.g. humans, domestic pets, farm animals, and wildlife). Some are considered part of the normal commensal gastrointestinal flora in warm blooded animals (e.g. *Escherichia coli*). Fecal coliform bacteria, members of the family *Enterobacteriacae*, include *Escherichia, Enterobacter*, *Klebsiella*, and *Citrobacter*

species (Appendix E). *Escherichia coli* is one species within the fecal coliform subset of the coliform group of bacteria (Figure 7**).** The presence of *E. coli* in a water source is considered a specific indicator of recent fecal contamination and increases the likelihood of enteric pathogens (e.g. *Vibrio cholerae*, *Salmonella typhi*, *Shigells sp*., *Salmonella sp*., or *Campylobacter jejuni*) being present (3, 10, 52). There are at least 700 strains of *E. coli* recognized, many of them non-infectious (29). Approximately ten percent of solid human waste is made up of *E. coli* cells and under ideal laboratory conditions *E. coli* cells can divide every twenty minutes (23).

E. coli O157:H7, an enterohemorrhagic *Escherichia coli* (EHEC), is believed to have evolved from an atypical enteropathogenic *E. coli* (EPEC) ancestor of serotype O55:H7 (27, 31, 44). This EPEC ancestor contained the locus of enterocyte effacement (LEE) containing genes for the intimin adhesion protein, but lacked the genes encoding shiga-toxins (27, 44). According to O'Brien (36), epidemiological studies have shown that production of high or moderate levels of shiga-like toxins is associated with many EPEC and most EHEC strains that cause diseases in humans. *Escherichia coli* O157:H7, a shiga-toxin producing *Escherichia* coli (STEC), is related to *Shigella dysenteriae* through a piece of viral DNA that was introduced into each strain's genetic code. The viral genes code for a toxin that is essentially identical in both strains. *Shigella dysenteriae* possesses the genes that encode the toxin that causes dysentery, Shiga toxin (Sht; 9, 23, 32, 36). The toxins produced by the STEC bacteria are termed shiga-like toxins (SLTs; 32). Two main categories of SLTs have been distinguished. *Escherichia coli* shiga-like toxin 1 (Stx1; formerly SLT1 and verotoxin 1, VT1) is almost identical to the Sht of *Shigella dysenteriae*, they can not be distinguished serologically (6, 57).

Escherichia coli shiga-like toxin 2 (Stx2; formerly SLT2 and verotoxin 2, VT2) is less related to the Sht of *Shigella* (6, 9). The term shiga-like toxin is used to describe toxins neutralized by anti-Shiga toxins that are produced by *Shigella* serotypes other than *Shigella dysenteriae* (6, 9). It is speculated that the *E. coli – Shigella* gene transfer occurred by means of lateral gene transfer within the last few decades (23, 30, 31). The transfer could have occurred during a widespread outbreak of toxin-bearing *Shigella* in Central America during the 1970's. Close proximity within the gastrointestinal track and the presence of viruses -Stx1 and Stx2 are phage encoded- provided optimal conditions to facilitate gene transfer (5, 9, 23).

Shiga toxin-producing *Escherichia coli* (STEC; also known as enterohemorrhagic *Escherichia coli* (EHEC*)* and verocytotoxin producing *Escherichia coli* (VTEC)) are well documented human pathogens with the capacity to cause large outbreaks of gastrointestinal illness (27, 28). Primer sets have been designed and used in previously published polymerase chain reaction (PCR) assays to detect *stx*$_1$ (formerly *slt-I*) and *stx*$_2$ (formerly *slt-II*) virulence gene markers (Table 1 and 2; 17, 28, 32, 39, 40, 41, 58). The presence of either *stx*$_1$ or *stx*$_2$ genes indicates the presence of a STEC species (39). Sht, Stx1, and Sxt2 toxins inhibit protein synthesis in host cells causing cell death (19, 25, 36). No single factor is responsible for the virulence of STEC (6). The most common serotype of STEC worldwide is *Escherichia coli* O157:H7 (25). Studies have shown that the prevalence of STEC *stx*$_1$ and *stx*$_2$ genes vary based on the location where assayed isolates were collected. Khan *et al.* (25) found the dominant combination of virulence factors was *stx*1 and *stx*2 during a study in Calcutta, India. In Germany mostly *stx*1 was found and in France only *stx*2 genes were identified (25).

In the United States alone approximately 4,000 confirmed *E. coli* O157:H7 cases are reported each year (29). *Escherichia coli* O157:H7 outbreaks associated with both drinking and recreational water raise concerns about waterborne illness outbreak. Studies have shown that the O157:H7 strain is considered a hardy pathogen that has the ability to survive for extended periods in water, especially cold temperatures (e.g. 8° C; 14). With a low infectious dose of only 10-100 cells and a relatively short incubation period of 1-8 days, *E. coli* O157:H7 is a serious health risk (18, 37).

Of the three aforementioned *E. coli* O157:H7 outbreaks, the outbreak that occurred during May/June 2000 in Walkerton, Ontario, Canada (Figure 8**)** was the most severe (7, 12, 37). This incident represented the first documented outbreak of *E. coli* O157:H7 infection associated with a municipal water supply in Canada and the largest multi-bacterial (*Campylobacter spp.* also identified) waterborne outbreak in Canada as of October, 2000 (7, 55). One thousand and forty six cases of gastroenteritis were reported following exposure to the Walkerton municipal water supply. Heavy rainfall during mid-May caused gross contamination of the municipal water distribution system by manure run-off (7, 12, 29). Six people died and 27 people developed hemolytic uremic syndrome (HUS) a serious kidney complication of *E. coli* infections that can lead to kidney failure (7).

Intimin

Intimin is an outer membrane protein. It is encoded by the *E. coli* attaching and effacing (*eaeA)* gene (16, 28, 30, 39, 40, 41, 42, 56). Intimin proteins are expressed by enteric bacterial pathogens capable of inducing intestinal attachment and effacement

lesions. A bacterium attaches itself to a target intestinal cell by embedding its receptor in the epithelial membrane of the host cell (1, 44). The locus of enterocyte effacement (LEE) is required to produce attaching-effacing lesions. Theses lesions are characteristic of EPEC induced pathology (44). The intimin proteins are required for intimate adherence to intestinal epithelial cells, characteristic of attaching and effacing enteropathogens and for full virulence of EPEC (42). The genes associated with the development of theses lesions are clustered in a pathogenicity island called LEE (16, 28, 30, 39, 40, 41, 42, 56). Pathogenicity islands are discrete segments of DNA that encode virulence traits and are usually acquired from other organisms (e.g. bacteriophage or via conjugation) (31).

The *eaeA* primer set used during Paton and Paton's (39, 41) characterization of shiga-toxigenic *Escherichia coli* (STEC) and Lopez-Saucedo *et al.* studies were selected because it appeared to be conserved among both STEC and EPEC strains examined up until that date (28, 39, 40, 41, 68). Confirmed STEC strains can be negative for the *eaeA* gene and positive for either one or both stx_1 and stx_2 genes. This indicates that the STEC identified lacked the LEE pathogenicity island (39).

The polymerase chain reaction (PCR) is commonly used for the detection of genes associated with virulence factors. PCR techniques produce rapid, sensitive, and specific results. Gel electrophoresis is usually used in conjunction with PCR to aid in the detection of the amplified DNA products (35). This method allows detection of gene sequences in both cultivable and non-cultivable bacterial cells (18). Wang *et al.* (57) and Osek (38) suggests that the scientific community needs to develop more rapid, sensitive, simple, and reproducible procedures for the detection of STEC pathogenic strains and for

characterization of their toxins in not only human specimens but also in water and food sources (38, 57).

Research Objectives

During spring and summer 2004, twenty mainstem and five tributary water samples were collected along the Great Kanawha River, West Virginia. The objectives of this research were to detect *stx*$_1$, *stx*$_2$, and *eaeA* virulence related gene markers, determine if fecal coliform bacteria were present in collected water samples, and to test antibiotic resistant and fecal coliform bacteria as bioindicators of water quality.

CHAPTER II

METHODS

Site Descriptions

During April and July 2004, twenty mainstem and five tributary water samples were collected along the Great Kanawha River, West Virginia. Mainstem samples were collected every five river miles starting at river mile 95 (origin) in Fayette County and ending at river mile 00 (mouth) in Mason County at the confluence of the Kanawha and Ohio Rivers (Appendix C). Detailed mainstem and tributary sample site coordinates and descriptions are listed in Appendices A and B, respectively (see also Figure 9).

Sample Collections

Samples were collected using sterilized glass jars with twist on leak proof lids. Collection jars were labeled with the river mile or tributary being collected (e.g. River mile 00 – 95, Gau, New, Coal, Elk, or Poca), and the time of collection was documented. Samples were collected mid channel in an attempt to ensure homogeneous and representative water samples. Collection jars were placed in the water pointing downwards until fully submerged to avoid collecting the surface film. The jars were then inverted to allow the water to fill the jar. A small amount of water was poured off to allow air to be present in the jar. Collection jars were sealed, placed in a cooler and stored on ice. Each sample was processed within 6 hours of collection.

Water samples from five main tributaries of the Kanawha were also analyzed. Collections were made following the same procedures as mainstem sample collections. The five tributary sites included the Gauley, New, Elk, Coal, and Pocatalico Rivers. To

ensure homogeneous water samples were collected each subsurface tributary sample was collected above the debris line before the mixing zone near the point of discharge.

Sample Processing

***Total Cultivable Bacteria*:** Total cultivable bacteria were enumerated by first taking a water collection jar containing a sample from the cooler and shaking several times to ensure a homogeneous mixture of the sediments within the jar and removing the lid. From the undiluted samples, 100 µl (0.1 ml) was aseptically transferred to a sterile 9.9 ml dilution blank in a sterile screw-cap test tube. Caps were replaced following the transfer and the tubes were vortexed on high speed for at least five seconds. From the diluted samples 100 µl aliquots were aseptically transferred in triplicate to sterile petri dishes (150×15 mm, Becton-Dickinson) containing Difco R2A agar (Becton, Dickinson and Company, Sparks, Maryland) plus fungizone (375 ng/ml; Cambrex Bio Science Walkersville Inc., Walkersville, Maryland). Diluted samples were spread over the surface of the agar using five sterile solid glass beads (5 mm). Petri dishes were shaken in a front to back and side to side motion five to six times and rotated a quarter of a turn. This motion was repeated until the sample was evenly distributed over the surface of the agar and the agar surface was dry. Each plate was inverted to allow the beads to be discarded without completely removing the lid. Beads were colleted in a beaker containing 70% ethanol. To identify plates, each set was marked with river mile or tributary abbreviation (e.g. Poca, New, Elk, Gau, or Coal), date of inoculation, and inoculation condition (e.g. I: total cultivable). Finally, the plates were wrapped in parafilm, inverted, and incubated for seven days at 25° C. Following incubation, each

plate was examined for colony forming units (CFU) and the number of CFU per plate was recorded. MicroSoft Excel was used to determine the CFU per ml of total cultivable bacteria in the original sample. The average CFU value from triplicate counts was multiplied by a dilution factor of 1,000 (10^{-2} for the initial dilution and a 100 µl plating volume) to determine the CFU per ml of total cultivable bacteria in the original sample.

***Antibiotic Resistant Bacteria*:** Antibiotic resistance was analyzed by enumeration on R2A agar plus fungizone and ciprofloxacin (4 mg/L), erythromycin (8 mg/L) or tetracycline (12.5 mg/L) (Appendix D). Water samples were shaken to ensure a homogeneous mixture of the sediments within the jar. From the undiluted sample 100 µl was aseptically transferred in triplicate to sterile petri dishes (150×15 mm, Becton-Dickinson) containing Difco R2A agar plus fungizone (375 ng/ml), plus the appropriate concentration of a single antibiotic (Table 4). The samples were spread on the agar as previously described. To identify plates, each set was marked with river mile or tributary abbreviation, date of inoculation, and inoculation condition (i.e. II: ciprofloxacin, III: erythromycin, or IIII: tetracycline). Finally they were wrapped in parafilm, inverted, and incubated for seven days at 25° C. Following incubation, each plate was examined for colony forming units (CFU) and the number of CFU per plate was recorded. MicroSoft Excel was used to calculate the mean and standard deviations for each set of inoculated plates. Average CFU values were multiplied by a dilution factor of 10 (plating volume of 100 µl) to determine the number of resistant CFU per ml of total cultivable bacteria in the original sample.

Fecal Indicators: Fecal coliform bacteria were enumerated using membrane filtration and cultivation on m-FC medium. Collection jars were shaken to ensure

homogeneous distribution of sediments with in the jar. One, five and ten milliliter sub-samples were filtered to yield a range of 10 to 60 colonies per membrane. Samples were transferred to separate sterile, analytical test filter funnels (Nalge Nunc International, 0.45 MIC, 100 ml) containing 100 ml of sterile tap water. Samples were filtered, and filter membranes were transferred using forceps stored in ethanol. Filter membranes were placed in sterile petri dishes (Millipore, 47 mm) with pads impregnated with 2 ml sterile m-FC media with Rosolic acid (Fisher-Scientific cat no. M00000P2F), and incubated upright at 44.5 ± 0.2°C for 24 hours.

Blue colonies were considered typical fecal coliforms (Figure 10). Grey to cream colored colonies were considered non-fecal coliforms and were not counted. Fecal coliform densities were calculated for each sample by multiplying the number of colony forming units (CFU), cultivated on a membrane filter within the desired countable range of 10 to 60 CFU, by 100 ml and dividing the product by the sample volume (i.e. 1, 5, or 10 ml). Densities were recorded as fecal coliforms per 100 ml.

***Impact Score Determination*:** Data from the enumeration of total cultivable, antibiotic resistant, and fecal indicator bacteria were entered in an Excel (MicroSoft 2002) spreadsheet and used to establish an impact score (IS) for each site. For each population (erythromycin resistant, ciprofloxacin resistant, tetracycline resistant, and fecal coliform bacteria), the average counts for each site were ranked for each population data set using the percentile rank function. The percentile rank output was multiplied by 100 to give a percentile score for each data point with in the total population data set. Boundaries were chosen as a means of determining population impact scores for each site. For example, a boundary of IS_{90} (Impact Score at the 90^{th} percentile boundary)

weights sites with population counts above the 90th percentile and below the 10th percentile. Next, numerical values of 1, 0 or -1 were given to each site population count based on the site percentile value. A population score of 1 was assigned to all data points falling above the upper (i.e. 90th) percentile boundary. Population scores of 0 were assigned to all data points falling between the upper and lower (i.e. between 90th and 10th) percentile boundaries. Population scores of -1 were assigned to all data points falling below the lower (i.e. 10th) percentile boundary. This method of value assignment was repeated for all populations enumerated (erythromycin, ciprofloxacin, and tetracycline resistant, and fecal coliform bacteria). Total impact scores were determined by adding all the population scores for an individual sample site. For this study impact scores ranged from -4 to +4 (3 antibiotics and 1 fecal indicator measured). Higher impact scores (i.e. +4) indicate a sample site as more impacted. Lower impact scores (i.e. -4) indicate a sample site as a less impacted area. To visualize water quality variability the total relative impact score for each site was plotted versus river mile. Relative impact scores were plotted for spring and summer sampling seasons in order to compare the two seasons.

Sample processing methods followed the Standard Operating Procedure (SOP) outlined in the Marshall University, Environmental Microbiology Research Laboratory and prepared by Dr. Charles Somerville. A complete SOP has been included in Appendix F.

Primer Design

Oligonucleotide primer sets were designed for detection of shiga-like toxin 1 (*stx*1), shiga-like toxin 2 (*stx*2), and intimin (*eaeA*) virulence related DNA sequences based on previously published research (Table 1). Primers were synthesized by Marshall University DNA Core Facility in Huntington, West Virginia. Oligonucleotide analysis was calculated by the Core Facility using Oligo 4.0 Primer Analysis Software (NBI). Characteristics of the oligonucleotide primers used for the detection of potentially toxigenic *Escherichia coli* collected from water samples are presented in Table 2.

Colony Cultivation

A total of 108 fecal coliform colonies were randomly chosen and sub-cultured for analysis from m-FC plates inoculated with water from the summer sample collection. Typical blue colonies were transferred to Luria-Bertani (LB) agar (Appendix G) using sterile wooden applicators. Bacterial samples were assigned identification numbers based on the notebook page on which the original transferred colony was documented, the location of the sample collection site (e.g. K95), and the sequential order in which each isolate was transferred (e.g. 1, 2, 3, etc.). Transferred colonies were incubated 24 to 48 hours at 35°C on LB agar plates before being processed for nucleic acid preparation.

Nucleic Acid Preparation from Pure Cultures

Sterile wooden applicators were used to collect cultivated fecal coliform colonies from the LB agar. Colonies were transferred from the applicator to sterile 2 ml Eppendorf tubes containing 50 µl of sterile distilled water (E-pure;

Barnstead/Thermolyne, model D4631: 15 -16 megohms) by stirring the applicator in the tube for 5 – 10 seconds. The tubes were incubated in a 0.6 amp (75 watt) Thermolyne heating block (Barnstead, model DB17615) for 5 – 7 minutes at 95- 100°F to lyse the cells and free the nucleic acids. Following incubation the tubes were placed in a centrifuge (Eppendorf, model 5415 D) at 13,000 × g for 3 minutes at room temperature. Supernatant fluid was transferred from the centrifuged tube using sterile pipet tips and collected in sterile 2 ml Eppendorf tubes. Tubes were labeled using the same format established for the colony cultivation. This procedure was repeated for all sample colonies and for positive and negative controls. Prepared supernatants were frozen until PCR analyses began.

PCR Detection of Virulence Genes

Supernatant fluids from nucleic acid preparations were used in multiplex polymerase chain reaction (mPCR) assays for the detection of $stx_{1,}$ $stx_{2,}$ and *eaeA* virulence genes. Amplification of bacterial DNA was performed using 30 µl volumes each containing 2 µl of sample supernatant; 1.25 µl each of forward and reverse 100 µM oligonucleotide (primer) stock solutions ($stx_{1,}$ $stx_{2,}$ and *eaeA)*; and 20.5 µl Taq Supermix, ready to use mixture of DNA Polymerase, salts, magnesium, and dNTPs (GenScript Corporation, Cat.No. E00033, www.genscript.com). The sequences and predicted sizes of amplified products for specific oligonucleotide primers are shown in Table 1. Oligonucleotide primers were designed according to published literature (Table 1).

PCR amplifications were performed in a Bio-Rad Gene Cycler™ Version 1.7 (Model No. Gene Cycler). Samples were subjected to the following cycling conditions:

95°C (5 min, 1 cycle); 95°C for 30 seconds, 55°C for 60 seconds, and 72°C for 90 seconds (repeated 30 cycles); and a final cycle of 95°C for 30 seconds, 55°C for 60 seconds, and 72°C for 5 minutes. Products were visualized by ultraviolet transillumination following electrophoresis and ethidium bromide staining.

Escherichia coli , designated EDL933, serotype O157:H7, (ATCC No. 35150, Culti-Loops®, Lot No. 607572, Remel Europe, Ltd.) was used as the positive control (*stx*$_{1,}$ *stx* $_{2,}$ and *eaeA*) for all assays, and *Escherichia coli* (ATCC No. 25922, Bactrol Disks, Lot No. 145103, Difco Laboratories) was the negative control used for all assays (2, 13). Positive and negative control strains were processed using the same protocol designated for sample preparations.

Gel Electrophoresis

Amplified products were resolved by gel electrophoresis using 10 µl of the final reaction mixture and 2 µl Loading dye (Blue/Orange 6x, Promega, Madison, Wisconsin) on a 2% agarose gel in 1x TAE buffer. Gels were stained in 0.1 µl/ml of 1% ethidium bromide (Fisher Biotech, Fair Lawn, New Jersey). PCR reaction mixtures were electrophoresed at 116 - 121 volts for 35-45 minutes or until dye front reached the end of the gel. The Alpha Innotech FluorChem™ 9900 (San Leandro, California), ultraviolet florescence, and ethidium bromide were used to visualize amplified DNA fragments. Figure 25 shows a representative gel for both positive and negative controls used for all assays.

CHAPTER III

RESULTS

Seasonal Bacterial Growth

Average bacterial counts and standard deviations for total cultivable, ciprofloxacin-resistant, erythromycin-resistant, and tetracycline-resistant bacterial data were calculated using MicroSoft Excel for each sample site during both spring and summer sampling seasons (Tables 3 and 4).

Total cultivable bacterial counts for spring (Figure 11a) and summer (Figure 12a) are shown in figures 11 and 12. A Log scale comparison showed average total cultivable counts were 1-3 orders of magnitude greater than the average ciprofloxacin-resistant, erythromycin-resistant or tetracycline-resistant counts in any given sample along the mainstem during the spring sampling season (Figure 11b), and 1-2 orders of magnitude greater for the summer season (Figure 12b) .

Comparison of spring and summer mainstem and tributary ciprofloxacin-resistant cells show average ciprofloxacin resistant cell counts were, in general, greater during the summer sampling season than during the spring season (Figures 13 and 14). Average mainstem and tributary erythromycin-resistant and tetracycline-resistant cellular counts were also shown generally to be higher for summer samples than for spring samples (Figures 15 -18). During, the summer season consistently high counts of resistant bacteria occurred between river miles 40-55 and at the Pocatalico River tributary site (Figures 13-18).

Seasonal comparisons of main stem fecal coliform counts show average counts between river miles 95-55 (the upper river) to be lower during the spring than summer

averages (Figures 19a and 19b). Between river miles 50-00 (the lower river) the opposite was found, average fecal coliform counts were greater during spring sampling than during summer sampling (Figure 19a and 19b). A seasonal comparison using average tributary fecal coliform counts showed that summer counts were generally higher than those for spring (Figure 20).

Mean ciprofloxacin-resistant (566 CFU/ml), erythromycin-resistant (935 CFU/ml), and tetracycline-resistant (160 CFU/ml) counts, were two orders of magnitude higher than mean fecal coliform counts (2 CFU/ml) for spring samples (Figure 21a). Mean ciprofloxacin-resistant (1803 CFU/ml), erythromycin-resistant (2766 CFU/ml), and tetracycline-resistant (1436 CFU/ml) bacterial counts, were three orders of magnitude higher than mean fecal coliform counts (5 CFU/ml) for summer samples (Figure 21b).

Microbiological and Physical Parameter Associations

Water temperature (°C), turbidity (NTU), and pH were measured and included as physical parameter data during spring and summer sampling seasons along the mainstem (K95-K00) of the river (Appendix K). Linear regressions were used to measure the strength of association between any two environmental variables. The variables included were water temperature, turbidity, pH, total cultivable bacteria (Totals), ciprofloxacin-resistant bacteria (Cipro), erythromycin-resistant bacteria (Erythro), tetracycline-resistant bacteria (Tet), and fecal coliform bacteria (FC) for both sampling seasons.

Linear dependencies were found in spring microbiological data. The variables that were statistically significantly correlated at $P \leq 0.05$ (95% confidence interval; Table 5), were Cipro vs. Tet ($P = 0.020$; R = 0.517). Statistically significant linear correlations

were identified between Totals vs. Turbidity ($P = 0.000$; R = -0.927), Totals vs. pH ($P = 0.000$; R = 0.734), and Totals vs. water temperature ($P = 0.000$; R = -0.772). Statistically significant linear correlations were also identified for turbidity vs Tet ($P = 0.012$; R = 0.552), and water temperature vs. Erythro ($P = 0.040$; R = -0.464) for spring data (Table 5).

Summer microbiological data identified one statistically significant linear correlation, variables were significantly statistically correlated at $P \leq 0.05$ (95% confidence interval; Table 6), between Tet vs. Erythro ($P = 0.000$; R = 0.944). No statistically significant linear correlations were identified between any physical parameters and any microbiological counts (Table 6).

The correlation coefficient test identified no significant linear correlations ($P \geq 0.05$; Table 7), between microbiological variables for spring vs. microbiological variables for summer, Totals vs. Totals ($P = 0.120$; R = -0.359), Cipro vs. Cipro ($P = 0.459$; R = 0.176), Erythro vs. Erythro ($P = 0.438$; R = 0.195), Tet vs. Tet ($P = 0.807$; R = -0.058), and FC vs. FC ($P = 0.107$; R = 0.107).

Impact Scores

Impact Scores (IS) for spring and summer data were determined for each sample site using the 95th (IS_{95}) boundary level (Table 9). The 95th percentile boundary gives the clearest visualization of signal-to-noise ratio for these data (Figure 22-24; Appendix T, U, and V show spring and summer IS_{90}, IS_{85}, and IS_{80}). IS_{95} scores were plotted versus sample site river mile to visualize water quality variability (Figures 22 and 23; [Appendix W, X, and Y (IS_{90}); Appendix Z, AA, and AB (IS_{85}); Appendix AC, AD, and AE (IS_{90}).

IS_{95} scores for the spring collection were at or below zero near the origin (river miles 95-55), reached their highest levels between river miles 50-45 and 25-20, and show a trend returning levels to those similar to the upper part of the river for miles 40-30 and 15-00, toward the mouth (Figure 22; Table 8). Relative IS_{95} for summer collections show a general trend at or about zero toward the origin for river miles 90-60 reach highest point at river mile 55 and begins a gradual decline between river miles 45-00 toward the mouth (Figure 23; Table 8).

A comparison of spring and summer relative IS_{95} shows river trends are relatively similar for both sampling seasons. Trends appear to be occurring in approximately the same locations although not with the same level of impact. For spring (river miles 95-50) and summer (river miles 90-60) trends are at or about zero, reach highest levels between river miles 55-45 (industrialized area) and then return to about or below zero for river miles 35-00 (Figure 24; Table 8).

Gene Detection Assays

Gene detection assays were performed on 108 typical fecal coliform colonies (Figure 7) sub-cultured from m-FC impregnated filter pads using summer water samples. Assays were validated by testing positive control (*Escherichia coli ,* designated EDL933, serotype O157:H7; ATCC No. 35150, Culti-Loops®, Lot No. 607572, Remel Europe, Ltd.) and negative control (*Escherichia coli;* ATCC No. 25922 Bactrol Disks, Lot No. 145103, Difco Laboratories) strains. Positive and negative controls were selected on the basis of the presence or absence of specified verocytotoxin genes as described in previous literature and visualized using gel electrophoresis (Figure 25).

The result of the present study indicated that of 108 typical fecal coliform isolates assayed, 13 (12.0%) were positive for at least one of the three gene markers (Tables 9 and 10). A total of 2 (1.85%) isolates were identified as carries of the *stx*$_1$, one isolate each from sites K-65 and the Elk River tributary (Figure 26). Nine (8.33%) isolates were identified as carriers of the *stx*$_2$ gene, 2 isolates each from sample sites K-75, K-70, and the Elk River tributary, and one isolate each from sample sites K-65, K-55, and K-00 (Figure 26; Tables 9 and 10). A total of 4 (3.70%) isolates were identified as carriers of the *eaeA* gene, one isolate each from sites K-55 and K-05, and two from site K-00 (Tables 9 and 10).

Two (1.85%) of the isolates assayed, one each from sites K-65 and K-55, were identified by mPCR as carriers of multiple target genes. Of 6 isolates assayed from site K-65, 1 was identified as a carrier of both *stx*$_1$ and *stx*$_2$ gene markers (Figure 26; Tables 9 and 10). Site K-55 showed 1 isolate out of a total of 10 assayed as positive for both *stx*$_1$ and *eaeA* (Table 9). Multiplex PCR showed no isolates assayed were carriers of all three; *stx*$_1$, *stx*$_2$, and *eaeA* genes, nor were any of the characterized strains carriers of both *stx*$_2$ and *eaeA* genes (Tables 9 and 10).

CHAPTER IV

DISCUSSION

The Kanawha River

The Great Kanawha River is the largest water way wholly contained in West Virginia. It flows in an approximate southeast to northwest direction. The river begins at the confluence of the New and Gauley Rivers in Fayette County (River mile 99.5) and ends 99.5 river miles west as it enters the Ohio River at Point Pleasant in Mason County (River mile 0), West Virginia. Microbial surveying based on antibiotic resistant bacteria, fecal coliform bacteria, and virulence related genes is an approach not previously tested on the Great Kanawha River.

Microbiological and Physical Parameter Data

Antibiotic resistance and fecal coliform data for spring and summer sampling seasons were used to develop an Impact Score assessment. No significant linear correlations were found between spring and summer microbiological variables. These finding were expected due to the river being a lotic system and in constant motion. Different microbiological peaks would be expected to be found at different locations along the river during different seasons. These data do allow us to identify individual areas of concern (hot spots).

Physical parameter data were used to identify linear correlations associated with microbiological data. Statistically significant linear dependencies were identified between total cultivable bacteria and three physical data parameters (pH, turbidity, and water temperature) for spring data. These results suggest that pH, turbidity and water

temperature play a role in the distribution of bacterial populations in this river system. These findings were not substantiated during summer sampling. Additional sampling seasons would have to be tested to definitively substantiate the spring findings.

Seasonal Data

Spring samples were collected, along the Great Kanawha River's mainstem and five major tributaries, over a two day period from April 5 to April 6, 2004. During both days, the river's flow regime was consistent. No rain events occurred during or just prior to the collection of samples. Summer samples were originally collected over a two day period between July 12 and 13, 2004. Due to an incubation error fecal coliform counts from samples collected on July 12 at river locations; K95 –K55 and the Gauley, New and Elk River Tributaries were excluded from further analysis. Antibiotic resistance data acquired during the July 12 and 13 sampling set was used to determine antibiotic resistance patterns along the river during similar flow regimes.

On August 5, 2004 additional summer samples were collected for K90-K55 and Elk River Tributary sample sites. Samples were unable to be collected for mainstem site K95 and for the Gauley and New River Tributaries due to a heavy rain event that occurred during collection. Summer IS analyses could not be performed on these three sites. Antibiotic resistance and fecal coliform data, using water samples collected on July 13 and August 5, were used for summer IS data analyses; sample sites K90-K00 and the Elk, Coal, and Pocatalico River Tributaries.

Fecal Coliforms

One method of addressing water quality is to monitor bacterial populations found in water as bioindicators of water quality (10). Fecal coliform bacteria are the most commonly used bioindicators of fecal pollution in water and food (21). Membrane filtration (MF) is a highly reproducible technique. MF can be used to test relatively large volumes of sample and produces rapid numerical results. For this study MF, in conjunction with m-FC media plus Rosolic acid impregnated filter pads, was used to determine if fecal coliform bacteria were present. And if so, were the significantly different in spring and summer sampling seasons.

Fecal coliform bacteria were enumerated along the mainstem and at tributary sample locations for both spring and summer sampling seasons. Spring samples showed no noticeable mainstem spikes in fecal coliform counts. The relative consistency in counts could have been influenced by similar river flow regime patterns present during the two day sample collections. The spring season is normally associated with increased rain events and run-off introduced in to the river system. This normal weather occurrence possibly accounts for the higher fecal coliform counts found between sample sites K50 and K00 during the spring season as opposed to summer. The counts found between these sites from the July 13, summer collection were visibly lower than those collected on April 6 during the spring collection.

Fecal coliform counts were visibly higher between sites K90-K55 than between K50-K00 for the summer sampling season. This occurrence is possibly due to the heavy rain event occurring during sample collection. Noticeable spikes were visible at sites K75 and K55 for summer mainstem fecal coliform data. Site K75 is located near the

unincorporated town of Shrewsbury in Kanawha County. The sample for K75 was collected within fifty yards of a small river island (Goat Island) inhabited by a small herd of goats. Goat Island is also frequented by campers using out-house type restroom facilities. Site K55 is located behind the Union Carbide Plant in the town of South Charleston in Kanawha County. These spikes could have been exacerbated by the rain event which occurred during sample collections at sites K90-K55 on August 5, 2004.

A comparison of mean ciprofloxacin resistant, erythromycin resistant, tetracycline resistant, and fecal coliform bacterial counts, suggests the fecal coliform bacteria appear to be of different populations of bacteria. Resistant bacterial organisms were found in greater numbers than fecal coliform organisms. This finding is an indication that the isolated resistant organisms are of different bacterial populations than the fecal coliform bacteria present. This data was consistent for both spring and summer sampling seasons. These findings allow us to use the fecal coliform bacteria isolated from processed water samples as a variable to assess water quality in the Impact Score (IS) index tested during this study.

Impact Scores

The Impact Score (IS) tested here was originally developed for use on the Ohio River system, by Dr. Charles Somerville of the Marshall University Environmental Research Laboratory in Huntington, West Virginia. The IS index was not developed as a means of comparing river system's water quality against one another. It was designed to be used as a means of identifying "hot spots" or areas with water quality concerns within a single river system.

The impact assessment range of -4 to +4 is based on the four microbiological variables being tested. Fecal coliform bacteria were chosen as a water quality variable for this IS index because they are the most commonly used bioindicators of fecal pollution in water and food (21). The presence of fecal coliforms (e.g. *E. coli*) in a water source is an indicator of recent fecal contamination (3, 10, 52). Fecal contamination can be considered a major concern when assessing the health of a river system. Ciprofloxacin, erythromycin, and tetracycline are three antibiotics, each from a different class of antibiotics, being tested as bioindicator variables in this research. Each of the three antibiotics examined was identified as an emerging environmental contaminant in freshwater systems in the United States by the USGS in their 1999-2000 Toxic Substances Hydrology Program Report (26, 51, 53). The antibiotics tested were added to the R2A agar medium at minimum inhibitory concentrations (MIC) for Gram-negative bacterial isolates.

The 95^{th} percentile boundary level was used to visualize water quality variability along the river system for both sampling seasons. This boundary level gave the clearest visualization based on the signal-to-noise ratio for areas considered most impacted. Similar IS seasonal patterns show the use of this IS index to identify areas of poor water quality is independent of sampling season tested. One limitation of the index was identified using data collected during the second summer sampling (August 5, 2004). Due to a heavy rain event encountered during sample collections, data produced inflated bacterial counts as a result of increased run off (Appendix H, I and J). This finding suggests that similar flow regimes are required for definitive IS comparisons. A second limitation is based on the predetermined microbiological variables tested. In order for a

river system to be compared against itself on a seasonal basis, the variables tested must be constant. Changing variables could affect the identification of areas of concern along the system.

A potential environmental risk cannot be definitively assessed for the Great Kanawha River because a causal connection between antibiotic resistant bacteria and the presence of antibiotics is the water system has not been established. Future research should attempt to determine the composition of resistant bacteria in order to determine if the bacterial populations present are intrinsically resistant to the antibiotics being tested or if there resistance is environmentally induced.

Gene Detection

Two major categories of *Escherichia coli* shiga-like toxins are Stx1 and Stx2. Stx1 is a relatively homogenous family of toxins that are almost identical to the Shiga-toxins of *Shigella dysenteriae*. Stx2 toxins are a more heterogenous group that are serologically distinct from Stx1 (57). Certain strains of STEC isolates appear to have a greater degree of virulence associated with humans. Previous studies have shown epidemiologically that Stx2 is more critical than Stx1 for developing virulence factors associated with disease development (57). The gene for Stx2 was found more often than the other two genes in this study. PCR primer pairs were synthesized with reference to published sequence data for stx_1, stx_2, and *eaeA* gene markers. Details of the nucleotide sequence, the target gene, and the size of the PCR product for each primer pair are listed in Table 3.

The mPCR based diagnostic described in this study is for the detection of *stx*$_1$ (shiga-like toxin 1), *stx*$_2$ (shiga-like toxin 2), and *eaeA* (Intimin) virulence genes. The mPCR assay described is a direct and effective method for the simultaneous detection of three DNA sequences found in strains of STEC serogroup O157, known to be associated with causing human disease. Due to time and funding constraints, mPCR assays were only performed using water samples collected during the summer sampling season.

For each of the 108 mPCR assays, the expected results were obtained for the positive and negative control strains. The result of the present study indicated that of 108 typical fecal coliform isolates assayed, 13 (12.0%) were positive for at least one of the three gene markers. Two (1.85%) were positive for at least two gene markers, and none were positive for all three. These results were expected due to little agricultural activity being located within close proximity to the river along the entire span of the river system. Higher gene detection rates would have been expected along a river system with increased ruminant activity or areas with little or no sanitation or water treatment facilities present. Identification of virulence genes using this method is important for assessing potential sources of human shiga-like toxin producing *Escherichia coli* infections

There is a necessity for reliable methods of detection and isolation of virulent bacterial strains considered potentially pathogenic for humans. A need also exists for the identification of potential reservoirs of these bacteria, such as food, feces, and water sources. Ruminants are known to harbor shiga-like toxin producing *Escherichia coli* isolates, but not enough research has been performed to allow an understanding of the virulence potentials of these isolates. *Escherichia coli* are normally commensal

organisms associated with the gastrointestinal tract flora of warm blooded animals. These organisms have the potential of becoming pathogenic and causing opportunistic infections when they acquire virulence genes located on plasmids, bacteriophages or pathogenicity islands (e.g. *stx*$_1$, *stx*$_2$ or *eaeA*) (33).

CHAPTER V

CONCLUSION

The objectives of this research were, first to determine if fecal coliform bacteria were present in collected water samples. The second objective was to test antibiotic resistant and fecal coliform bacteria as bioindicators of water quality. The final objective was to use a multiplex- polymerase chain reaction (mPCR) method to detect *stx*$_1$, *stx*$_2$, and *eaeA* virulence genes in fecal coliform bacteria isolated from summer water samples.

Fecal coliform bacteria were identified as present in water samples collected along the mainstem and at tributary sample locations for both spring and summer sample seasons. Fecal indicator populations and antibiotic resistant populations were demonstrated to be independent populations. This finding allowed the fecal indicator population to be included as part of the Impact Score (IS) index being tested here.

The second objective was accomplished by testing the IS index as a measure of water quality variability. The index tested was found to be a useful tool for identifying areas of concern along the Great Kanawha River system in West Virginia. Similar IS seasonal patterns show the use of the impact score to identify areas of poor water quality is independent of sampling season tested. Two index limitations were identified. First, similar flow regimes are required for IS comparison. And second, test variables must remain constant for seasonal comparisons (e.g. antibiotics, antibiotic concentrations, fungicide, fungicide concentration, inoculation volumes, media etc.).

A potential environmental risk cannot be definitively assessed for the Great Kanawha River yet as the causal connection between antibiotic resistant bacteria and the

presence of antibiotics is the water system has not been established. Future research should attempt to determine the composition of resistant bacteria in order to determine if the bacterial populations present are intrinsically resistant to the antibiotics being tested or if there resistance is environmentally induced.

There is a necessity for reliable methods of detection and isolation of virulent bacterial strains considered potentially pathogenic for humans. A need also exists for the identification of potential reservoirs of these bacteria, such as food, feces, and water sources. The final research objective was accomplished by performing a mPCR based diagnostic on 108 fecal coliform isolates collected using summer water samples. The mPCR assay described is a direct and effective method for the simultaneous detection of three DNA sequences found in strains of STEC serogroup O157, known to be associated with causing human disease. Identification of virulence genes using this method is important for assessing potential sources of human shiga-like toxin producing *Escherichia coli* infections

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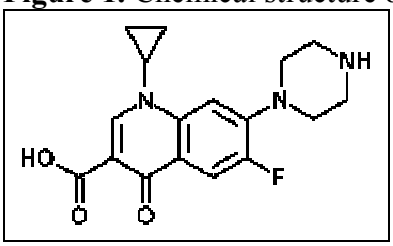
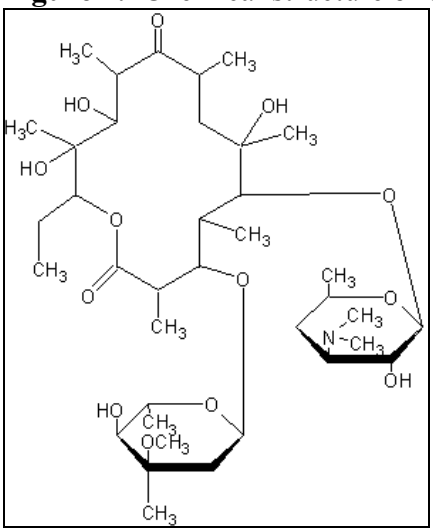
Figure 1. Chemical structure of ciprofloxacin



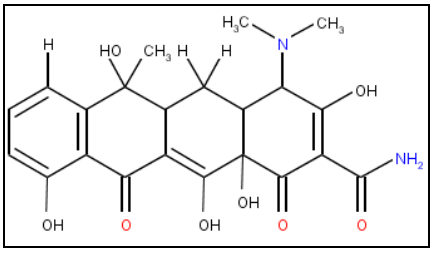
1, 4-dihydro-1-cyclopropyl-6-fluoro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid
Image from http://en.wikipedia.org/wiki/Image:Ciprofloxacin.png

Figure 2. Chemical structure of erythromycin



(3R*, 4S*, 5S*, 6R*, 7R*, 9R*, 11R*, 12R*, 13S*, 14R*)-4-((2,6-Dideoxy-3-C-methyl-3-O-methyl-a-L- ribo- hexopyranosyl) -oxy) -14- ethyl-7,12,13- trihydroxy - 3,5,7,9,11,13-hexa methyl-6- ((3,4,6-trideoxy-3-(dimethylamino)-b-D-xylo-hexopyranosyl)oxy)oxacyclotetradecane-2,10-dione
Image from http://en.wikipedia.org/wiki/Image:Erythromycin.png

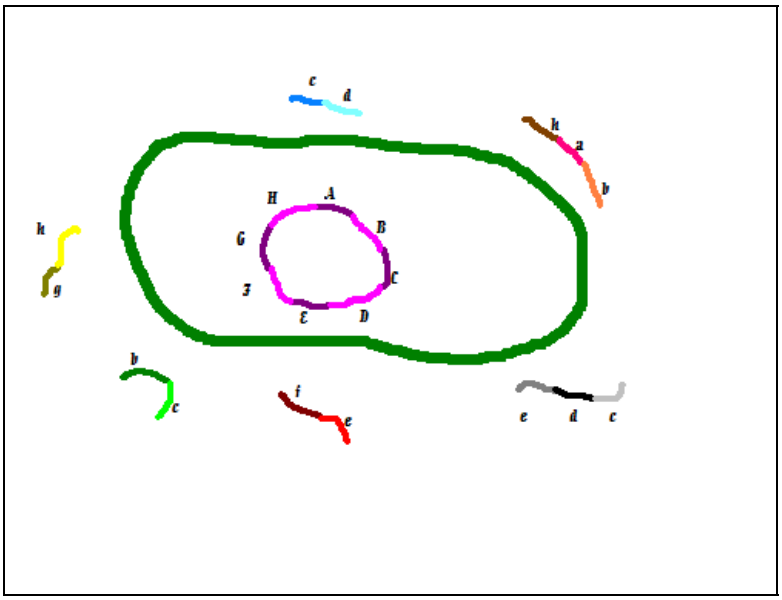
Figure 3. Chemical structure of tetracycline.



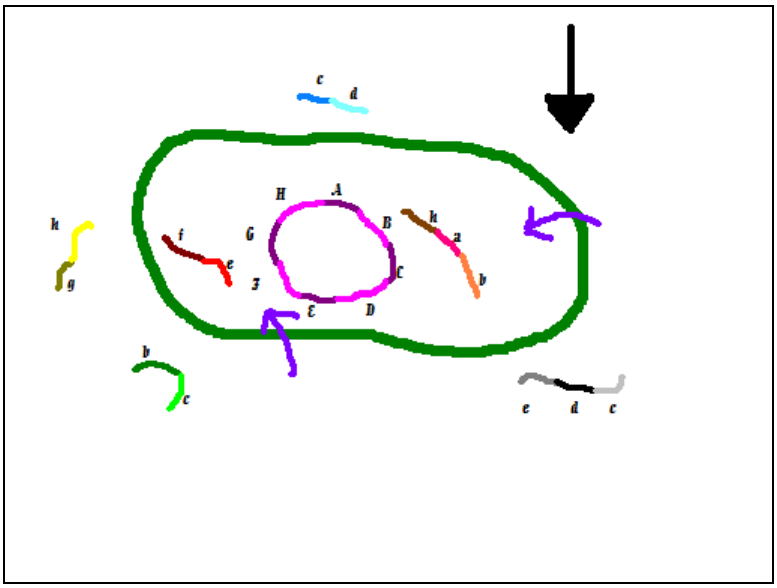
2-(amino-hydroxy-methylidene)-4-dimethylamino-6, 10, 11,12a-tetrahydroxy-6-methyl-4,4a,5, 5a-tetrahydrotetracene-1,3,12-trione
Public domain image from http://manske.virtualave.net/nupedia/tetracycline.png

Figure 4. Mechanism of Transformation. The steps of bacterial transformation are shown in a cartoon depiction.

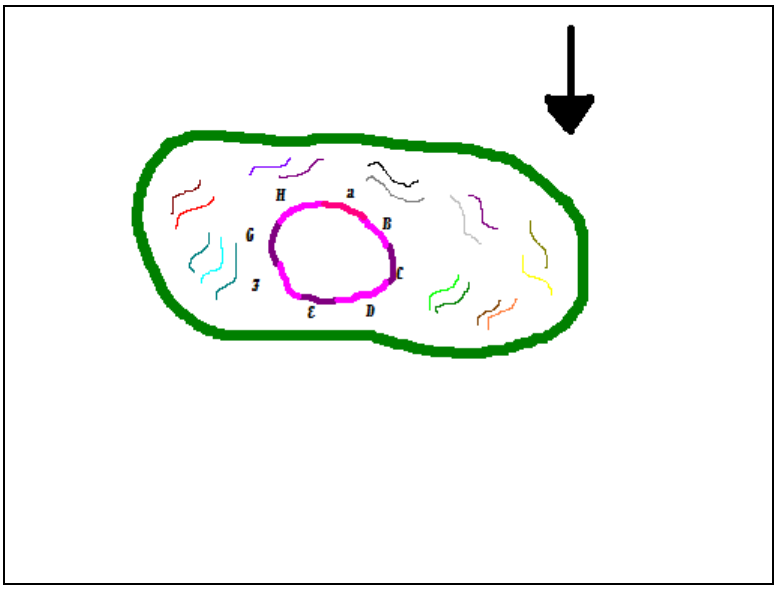
1. Naked DNA fragments from disintegrated cells (DNA not incorporated into structures such as chromosomes) in the area of a potential recipient cell. This cell must be of the correct genus and be in a state of competence (capable of taking up DNA), allowing the entry of the DNA fragments.



2. Naked DNA enters into the competent cell.

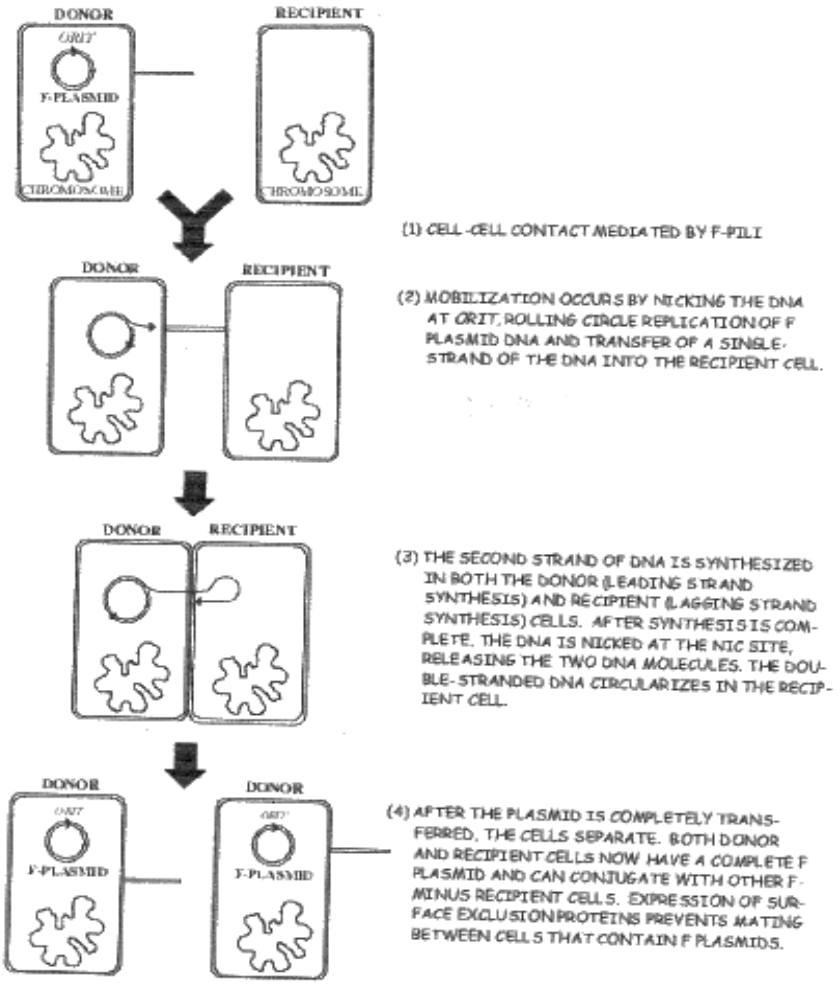


3. Recombination of DNA fragments occurs and replaces some of the original host cell DNA. The resultant recombinant cell will now express the foreign genes and pass them on to future offspring. The DNA that does not recombine during this process is broken down into enzymes.



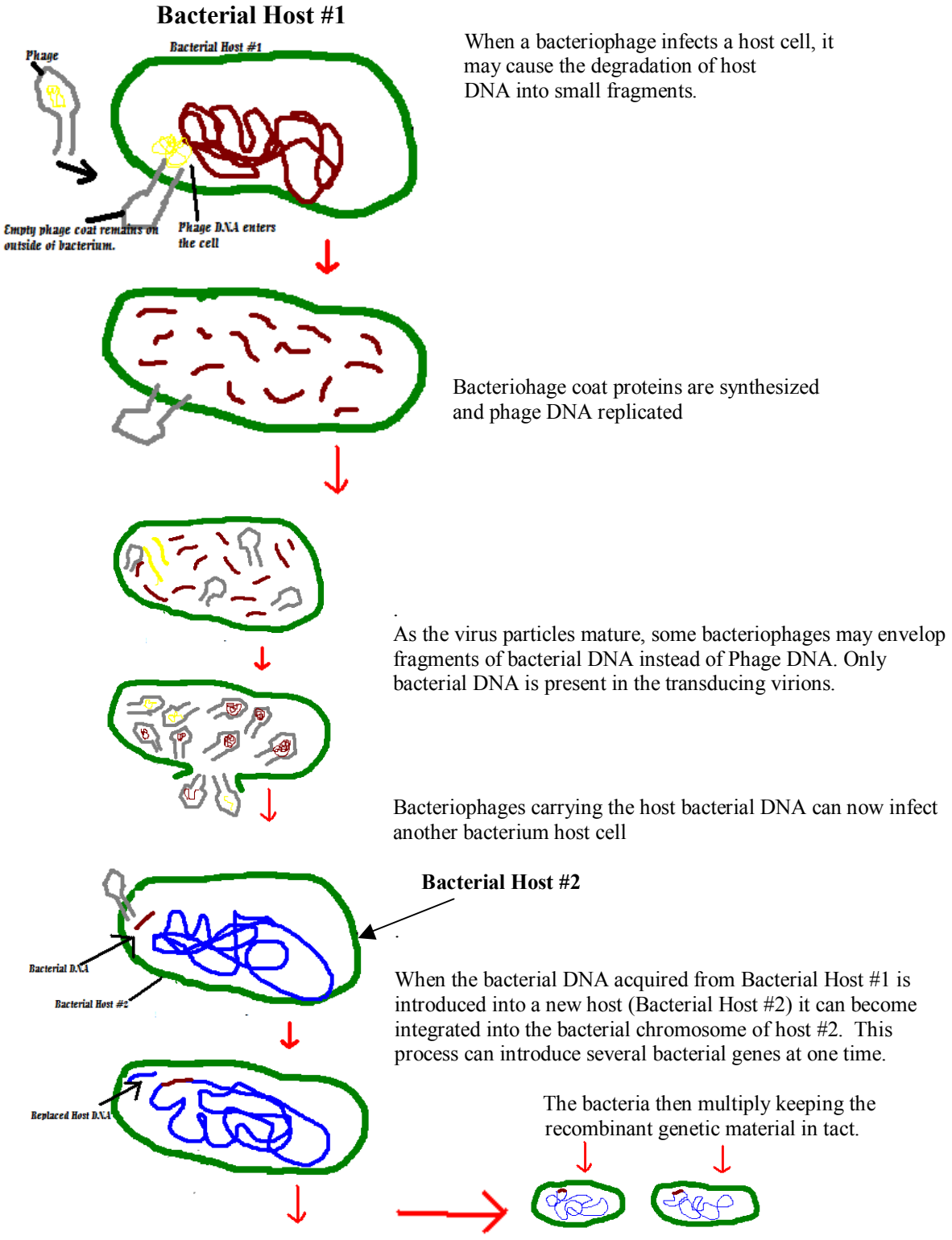
Steps for bacterial transformation adapted from the Rediscovering Biology Online Textbook Website. http://www.learner.org/channel/courses/biology/archive/images/1854_d.html

Figure 5. Mechanism of Conjugation. The steps of F-plasmid conjugation are shown in a cartoon depiction.



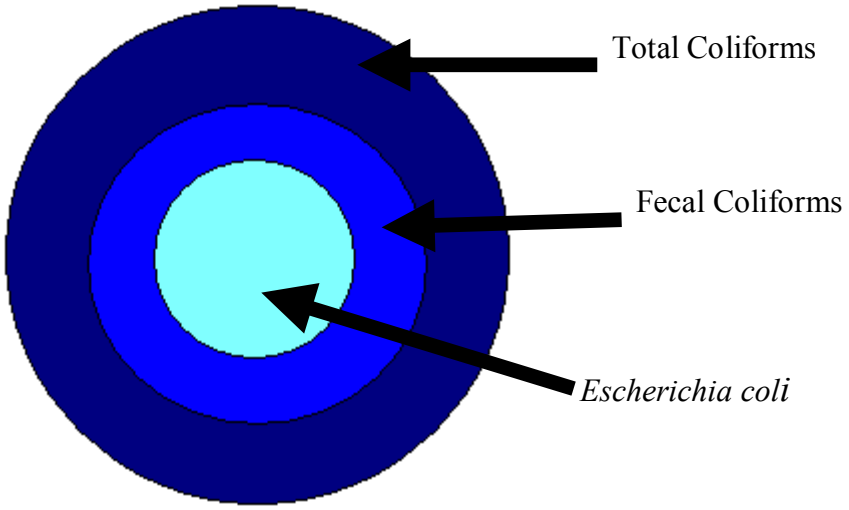
Copied from the website maintained by Stanley Maloy. (17 October 2000, last date modified)
http://www.sci.sdsu.edu/~smaloy/MicrobialGenetics/topics/plasmids/conjugation-mech.html

Figure 6. Mechanism of Transduction. The steps of bacterial transduction is depicted in a cartoon.



Steps for bacterial transformation adapted from the Rediscovering Biology Online Textbook Website. http://www.learner.org/channel/courses/biology/archive/images/1854_d.html

Figure 7. Total coliforms and fecal coliforms.

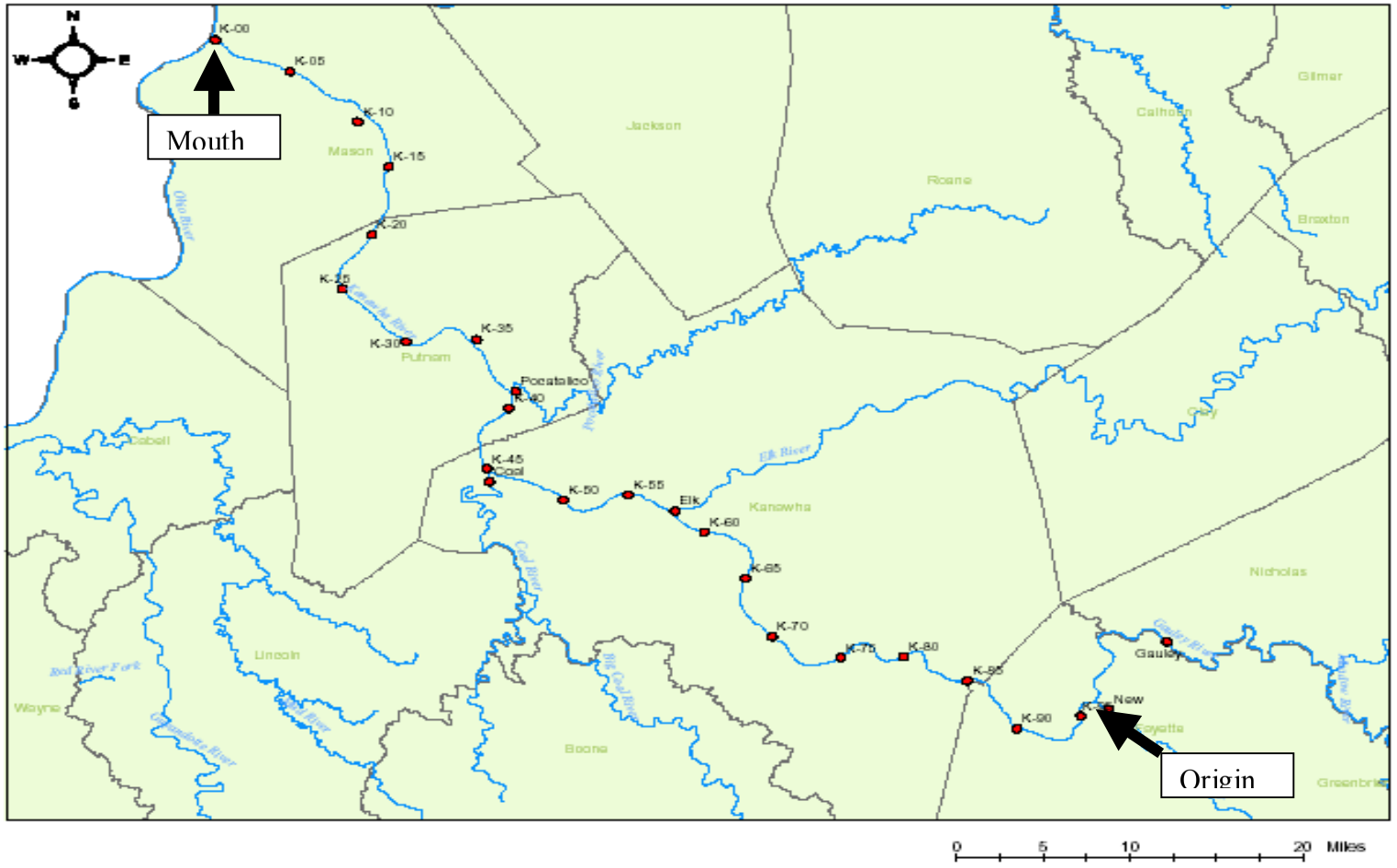


The total coliform group encompasses both fecal coliforms and *E. coli*. *E. coli* is a subset of the fecal coliform group and fecal coliforms are a subset of the total coliform group.

Figure 8. Map showing location of Walkerton, Ontario, Canada.

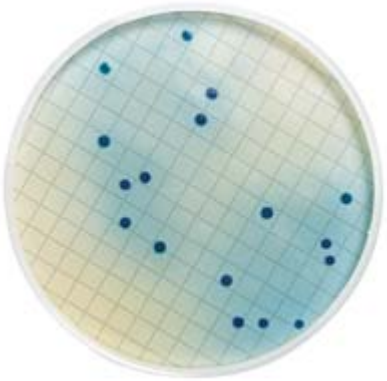
Walkerton, located in Ontario, Canada was the location of a waterborne outbreak of *E. coli* O157:H7 during May/June 2000 that resulted in 6 deaths.
http://uk.multimap.com/map/browse.cgi?client=public&X=-9000000.2612631&Y=5500000.68730376&gride=-9033710.2612631&gridn=5456082.68730376&scale=10000000&coordsys=mercator&db=CA&lang=&inmap=&table=&ovtype=&localinfosel=&local=&kw=&srec=0&mapsize=big&db=CA&rt=

Figure 9. Sample Site Locations.



[a] Twenty mainstem and five tributary sites along the Kanawha River in West Virginia were sampled during Spring and Summer, 2004. River mile K-95 is located at the origin and River mile K-00 is located at the mouth of the river. The five tributaries included the Gauley, New, Elk, Coal, and Pocatalico Rivers. Sample site locations are base of GPS coordinates.

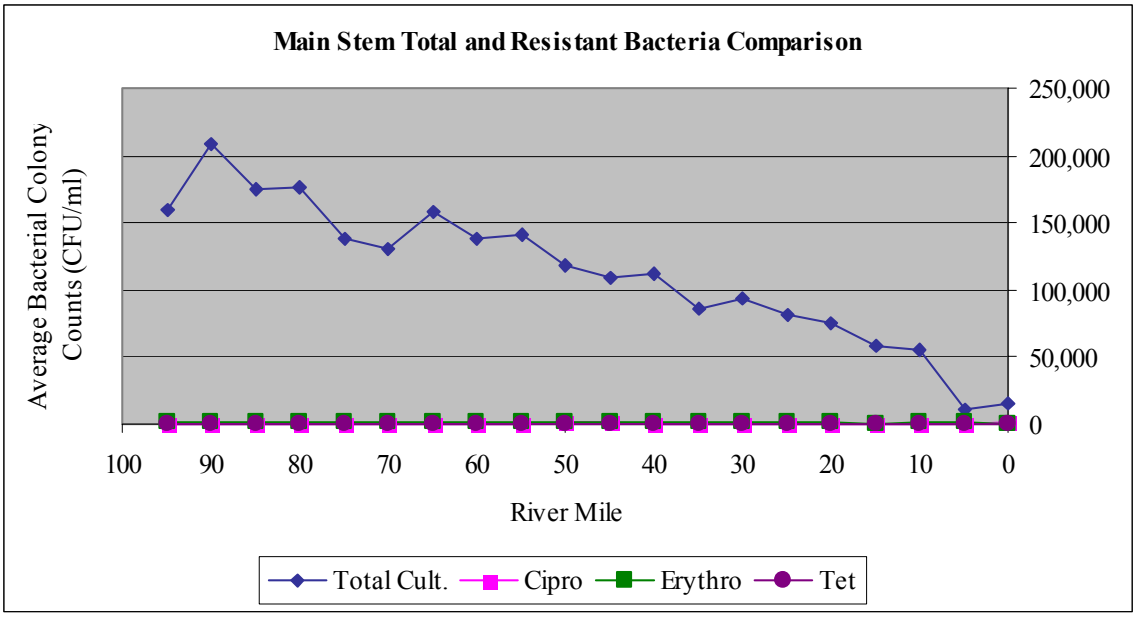
Figure 10. Typical Fecal Coliforms.



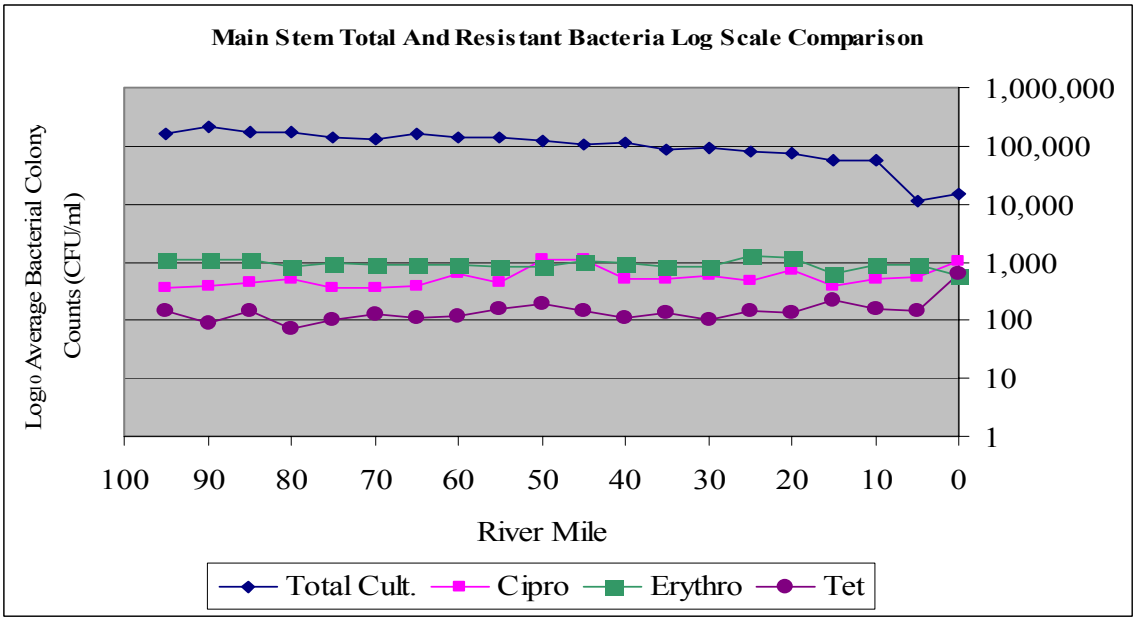
Blue colonies produced using m-FC medium with Rosolic acid are considered typical fecal coliforms, following incubation at 44.5 ± 0.2°C for 24h.
http://www.millipore.com/catalogue.nsf/docs/M00000P2F

Figures 11a and 11b. Comparison of main stem total cultivable and antibiotic resistant bacteria for spring data

a.



b.

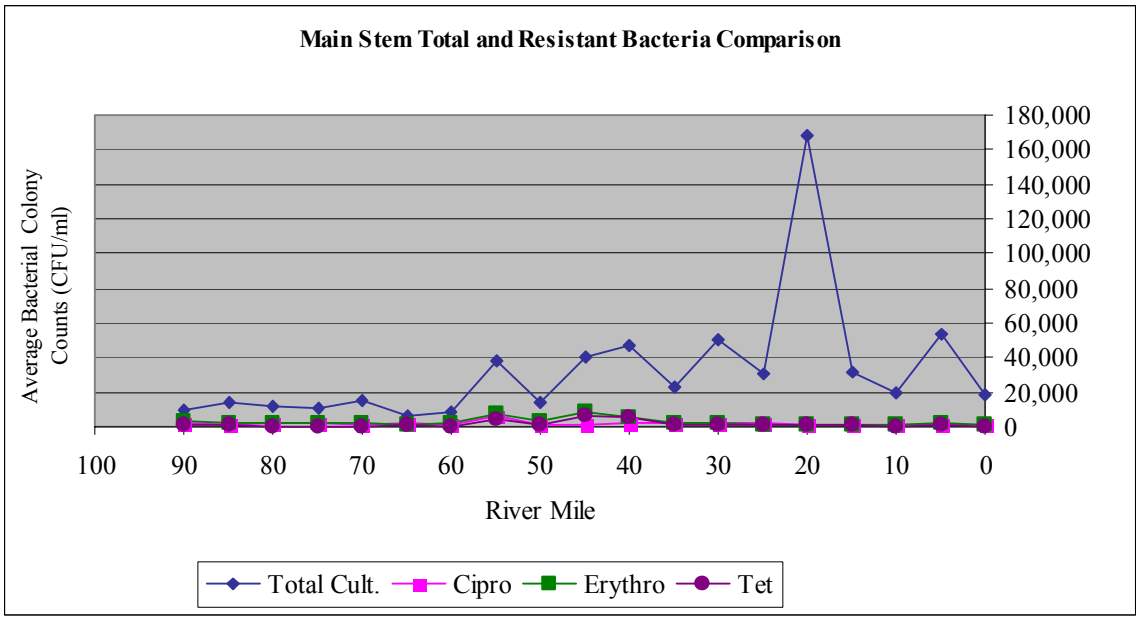


[a.] In Figure 11a is suggestive of total cultivable bacterial growth from spring samples, appears not to be inhibited by R2A agar plus fungizone (375 ng/ml) media when compared to resistant bacterial growth present on R2A agar plus fungizone with either; ciprofloxacin (4 µg/ml), erythromycin (8 µg/ml), or tetracycline (12.5 µg/ml) antibiotics added to the media.

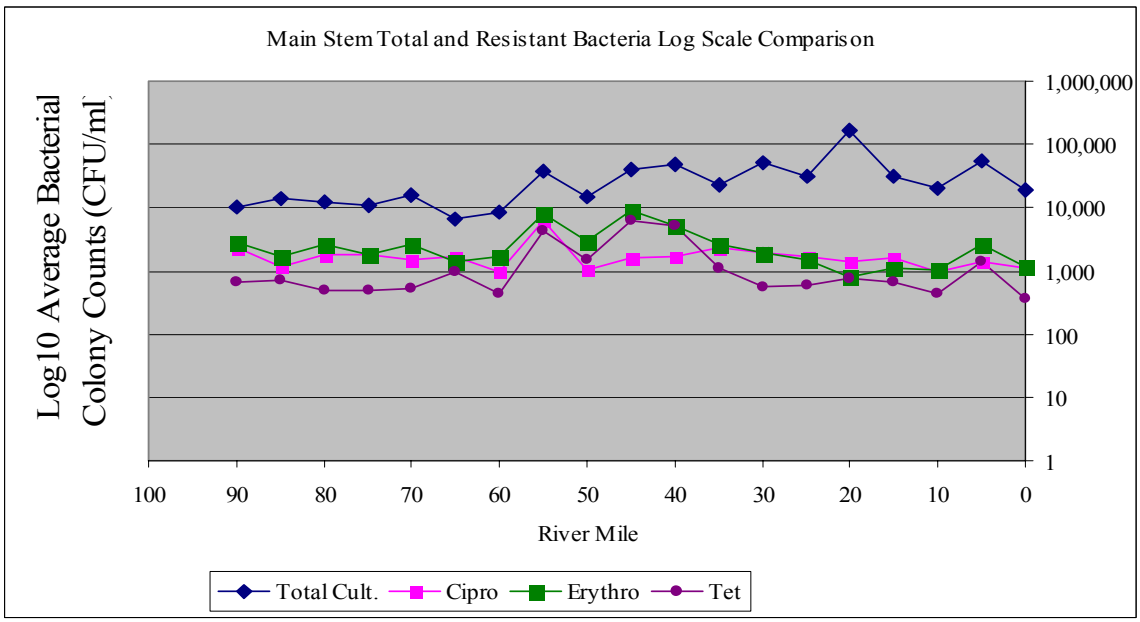
[b.] Figure 11b represents the Log scale comparison of main stem total cultivable and antibiotic resistant bacteria for spring data. Figure 11.2 is a graphical representation suggestive of total cultivable bacterial growth from spring samples, appears not to be inhibited by R2A agar plus fungizone (375 ng/ml) media when compared to resistant bacterial growth present on R2A agar plus fungizone with either; ciprofloxacin (4 µg/ml), erythromycin (8 µg/ml), or tetracycline (12.5 µg/ml) antibiotics added to the media.

Figures 12a and 12b. Comparison of main stem total cultivable and antibiotic resistant bacteria for summer data.

a.



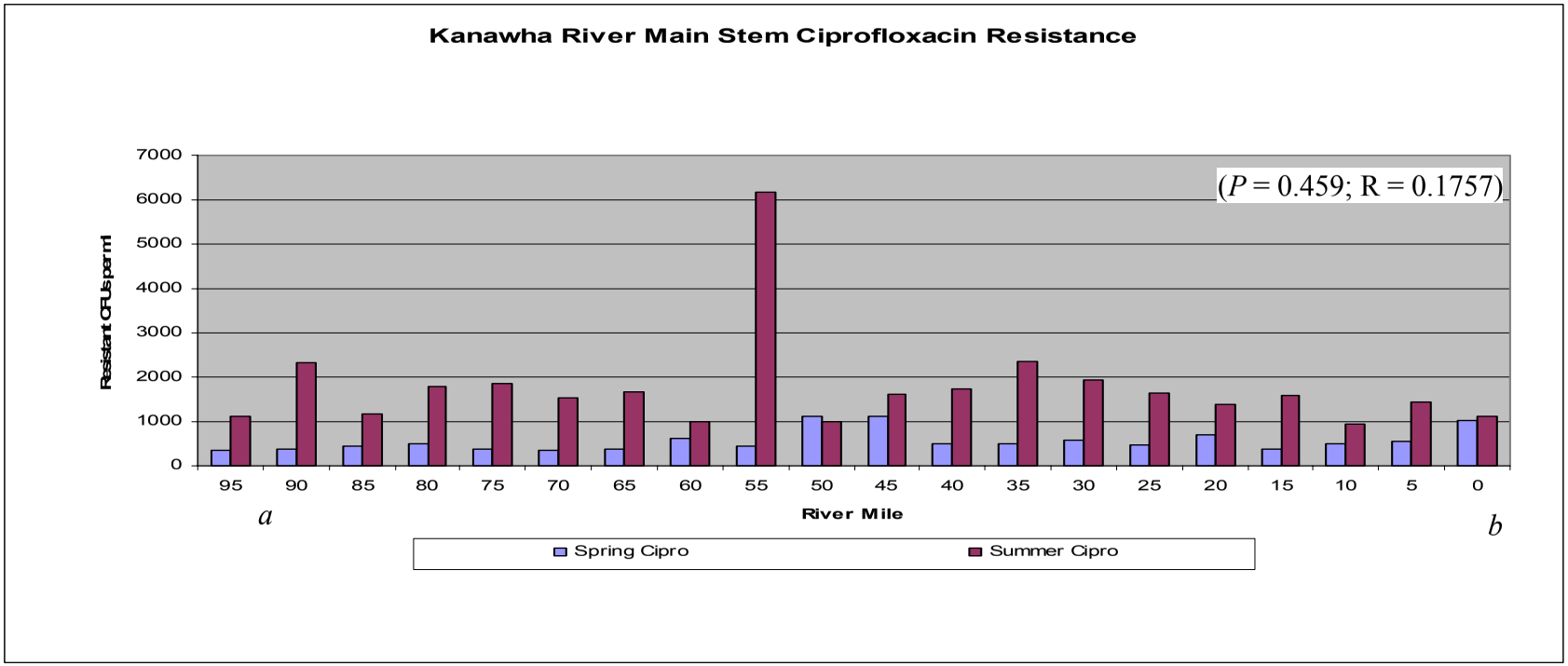
b.



[a] Figure 12a is suggestive of total cultivable bacterial growth from summer samples, appears not to be inhibited by R2A agar plus fungizone(375 ng/ml) media when compared to resistant bacterial growth present on R2A agar plus fungizone with either; ciprofloxacin (4 µg/ml), erythromycin (8 µg/ml), or tetracycline (12.5 µg/ml) antibiotics added to the media.

[b] Figure 12b is a Log scale comparison of main stem total cultivable and antibiotic resistant bacterial counts for summer data. . Figure 12.2 is a graphical representation suggestive of total cultivable bacterial growth from spring samples, appears not to be inhibited by R2A agar plus fungizone (375 ng/ml) media when compared to resistant bacterial growth present on R2A agar plus fungizone with either; ciprofloxacin (4 µg/ml), erythromycin (8 µg/ml), or tetracycline (12.5 µg/ml) antibiotics added to the media.

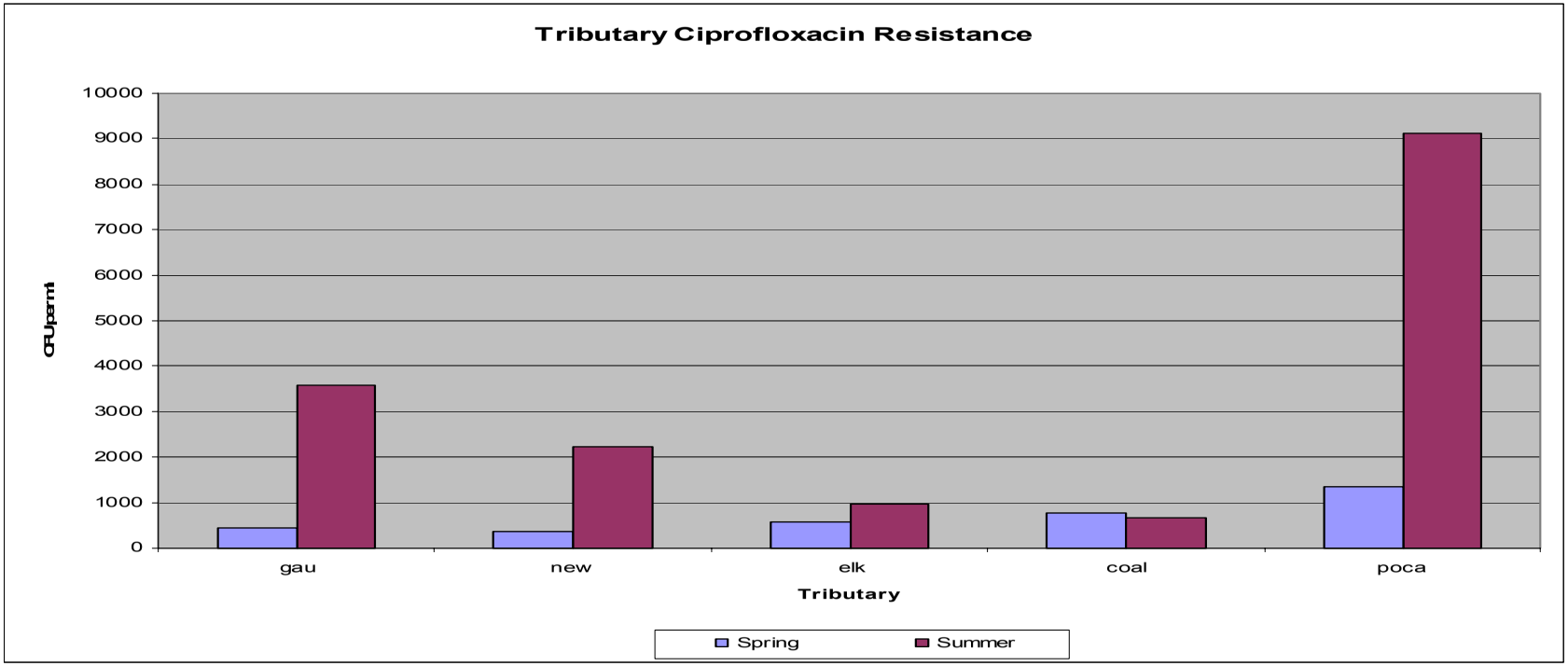
Figure 13. Ciprofloxacin (4 µg/ml) resistance comparison between the means of main stem sample site counts in spring vs. summer.



Average ciprofloxacin resistant cells were greater during the summer sampling season than during the spring season. With the exception of sample site K-50. The correlation coefficient test was used to measure the strength of association between spring and summer variables. A *P*-value of 0.459 suggests that the variables are not correlated and no linear dependences were identified.

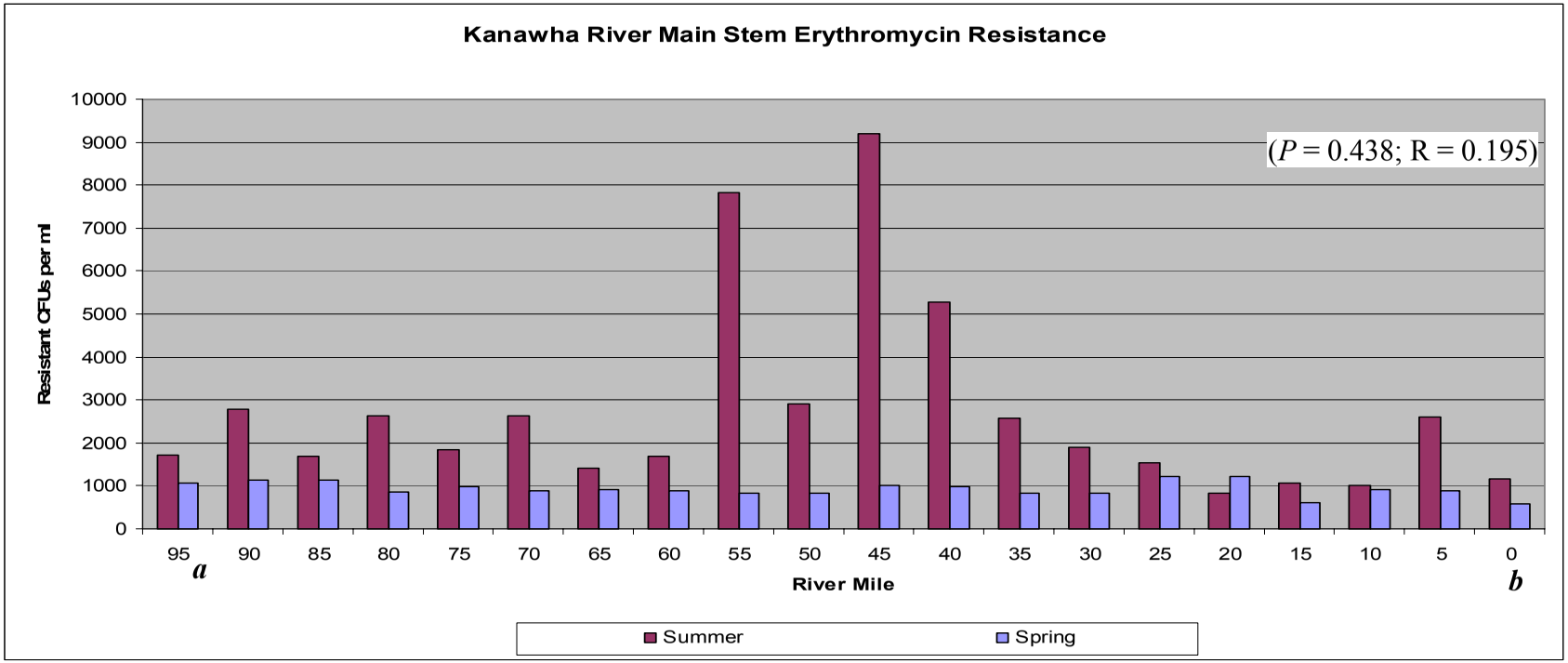
a Indicates the origin at the confluence of the New and Gauley Rivers.
b Indicates the mouth at the confluence of the Ohio River.

Figure 14. Ciprofloxacin (4 µg/ml) resistance comparison between the means of tributary sample site counts in spring vs. counts for summer.



Average ciprofloxacin resistant cells were greater during the summer sampling season than during the spring season, with the exception of the Coal River, tributary sample site.

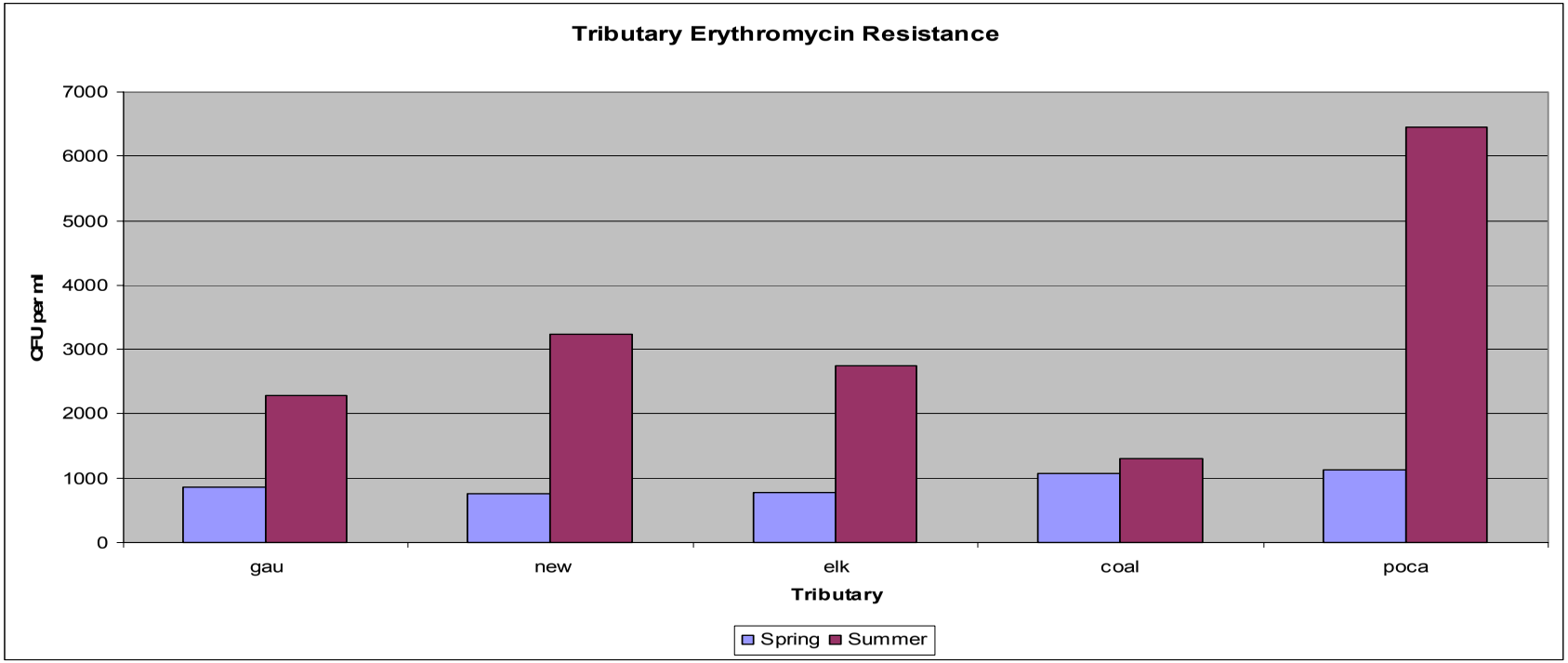
Figure 15. Comparison of erythromycin (8µg/ml) resistance counts for main stem sample sites during spring vs. summer.



Average erythromycin resistant cells counts were greater during the summer sampling season than during the spring season, with the exception of sample site K-20. The correlation coefficient test was used to measure the strength of association between spring and summer variables. A *P*-value of 0.0.438 suggests that the variables are not correlated and no linear dependences were identified.

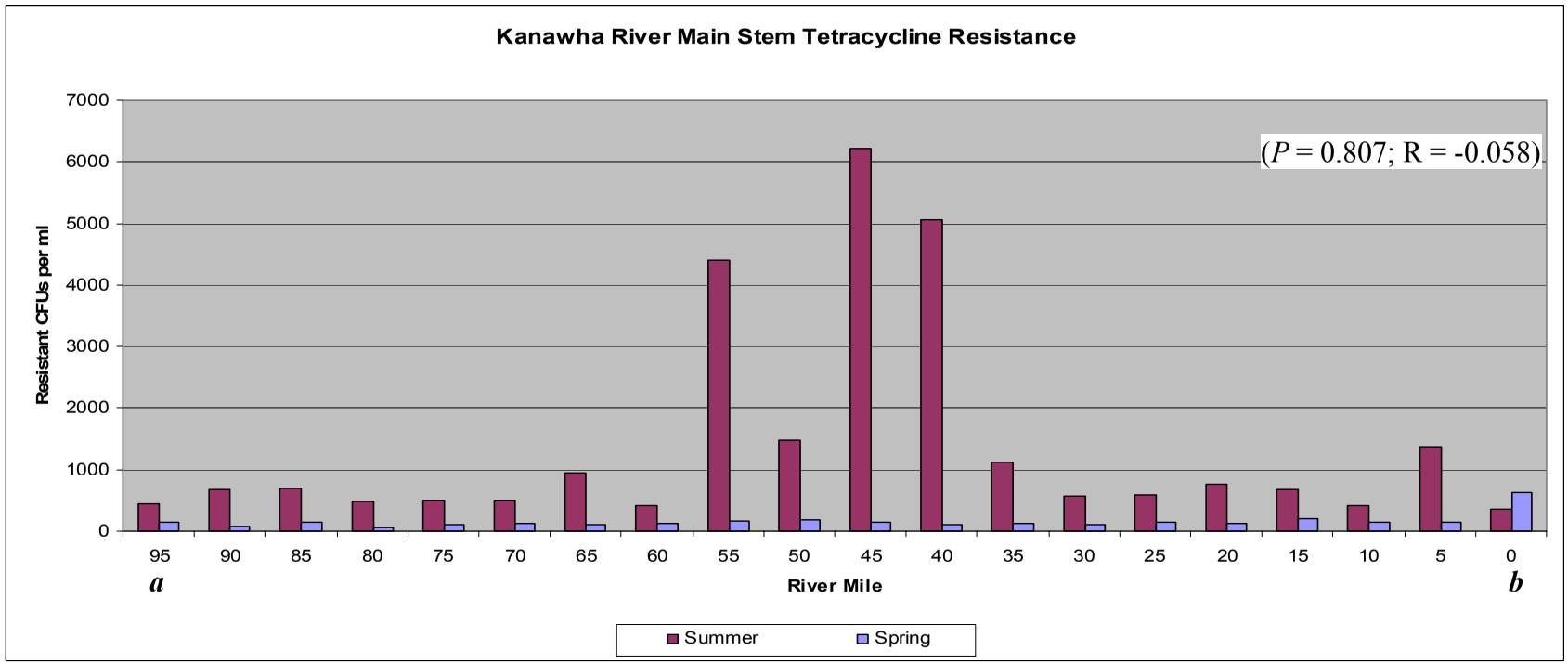
a Indicates the origin at the confluence of the New and Gauley Rivers.
b Indicates the mouth at the confluence of the Ohio River.

Figure 16. Comparison of erythromycin (8µg/ml) resistance counts for tributary sample sites during spring vs. summer.



Average erythromycin resistant cells counts were greater during the summer sampling season than during the spring season.

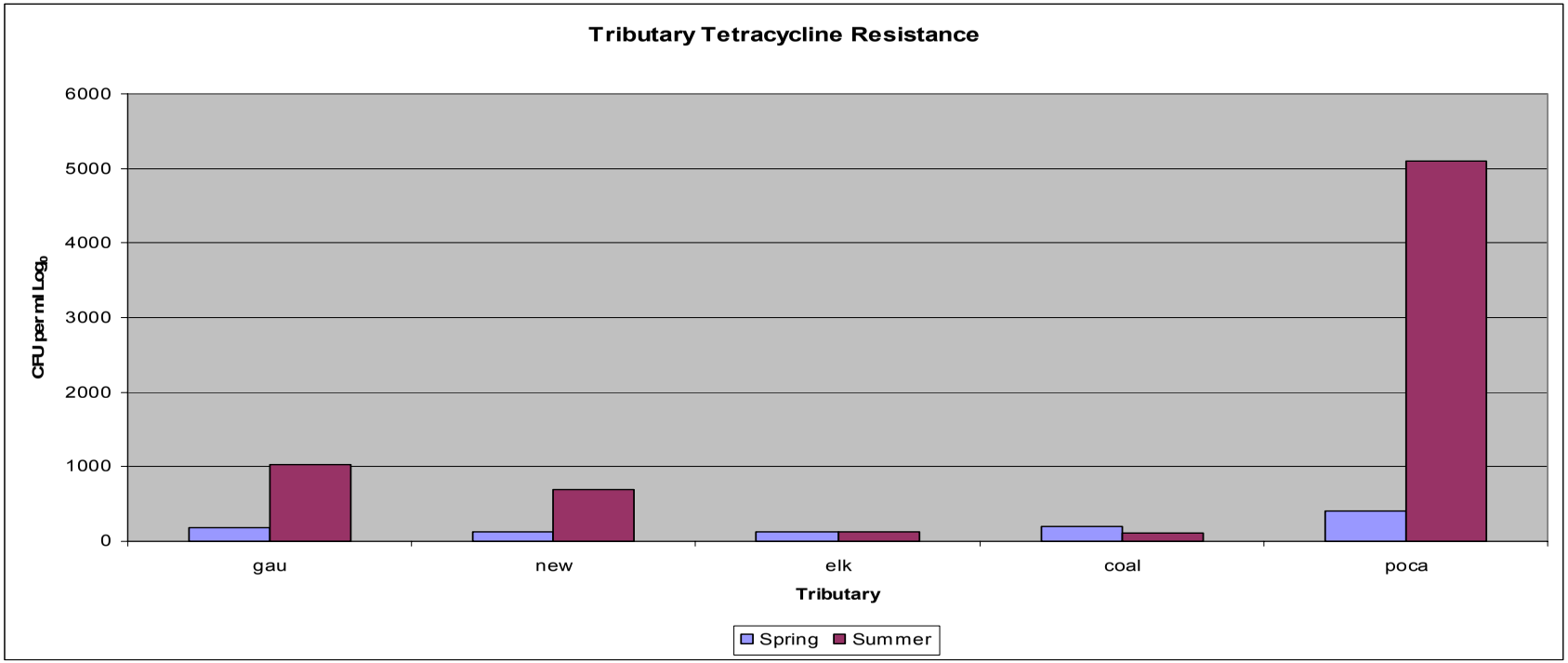
Figure 17. Comparison of tetracycline (12.5 µg/ml) resistance counts for main stem sample sites during spring vs. summer.



Average tetracycline resistant cell counts were greater during the summer sampling season than during the spring season. The correlation coefficient test was used to measure the strength of association between spring and summer variables. A *P*-value of 0.807 suggests that the variables are not correlated and no linear dependences were identified.

a Indicates the origin at the confluence of the New and Gauley Rivers.
b Indicates the mouth at the confluence of the Ohio River.

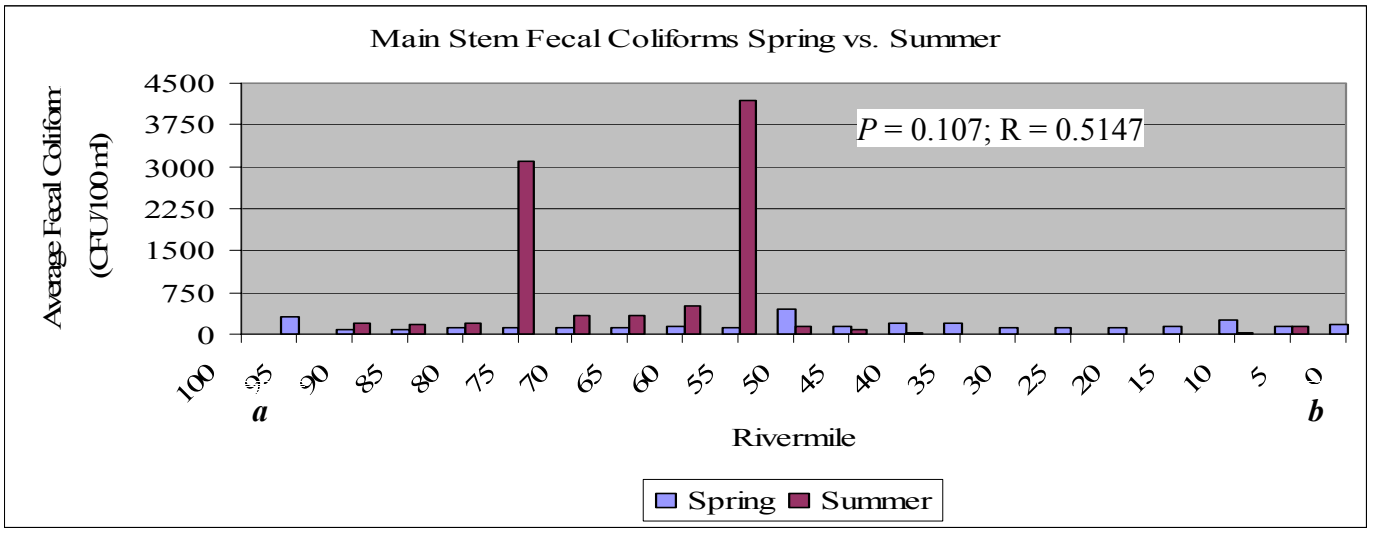
Figure 18. Comparison of tetracycline (12.5 µ/ml) resistance counts for tributary sample sites during spring vs. summer.



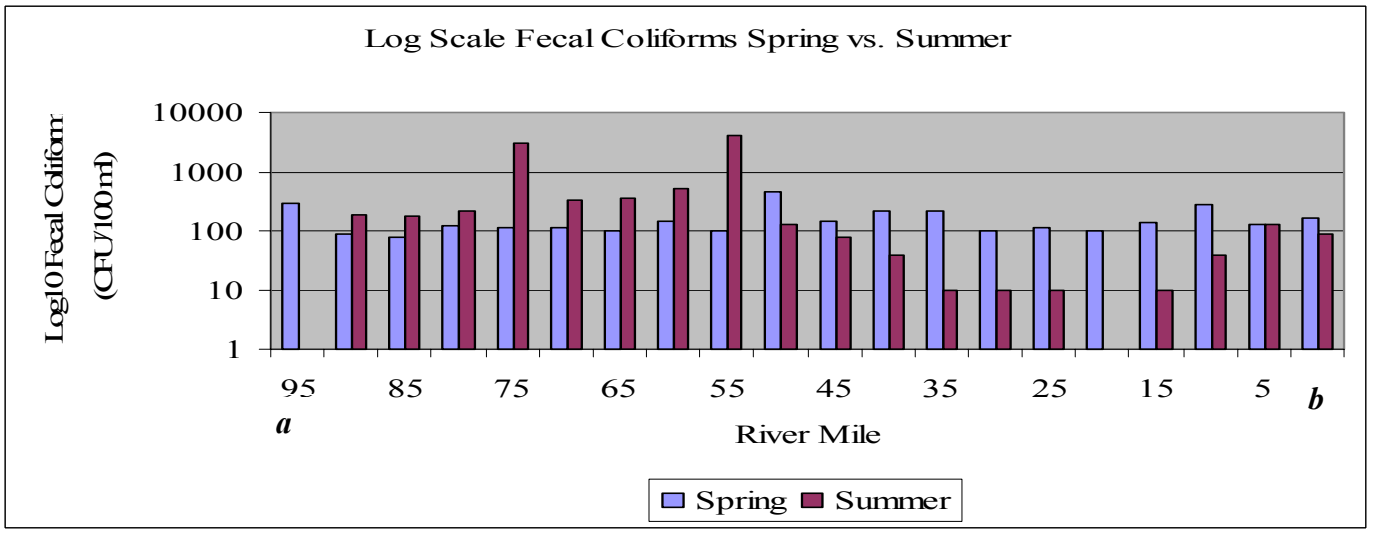
Average tetracycline resistant cell counts were greater during the summer sampling season than during the spring season, including the Elk River tributary in which counts were 123.33 CFU/100 ml (spring) and 190.00 CFU/100 ml (summer).

Figures 19a and 19b. Comparison of main stem fecal coliform bacteria for spring and summer seasonal data.

a.



b.

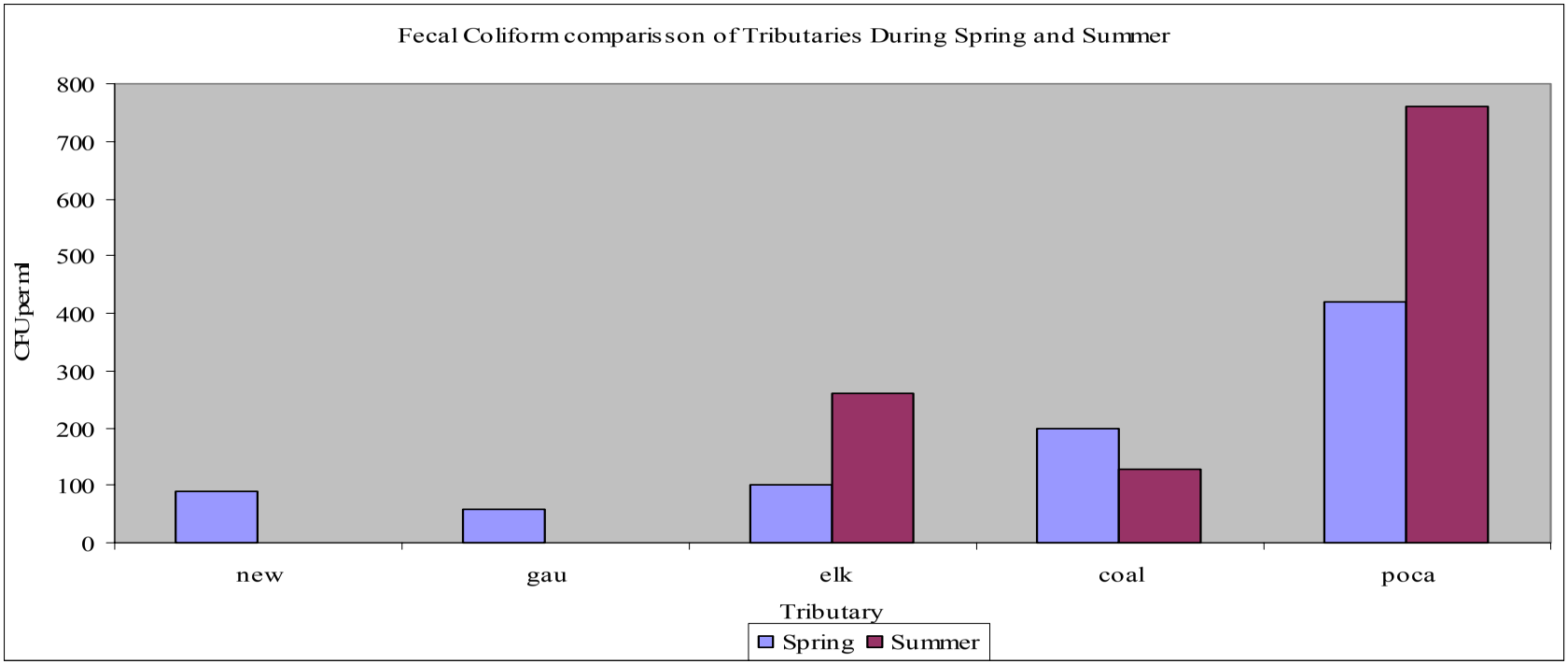


Figures 19a and 19b show two spikes in fecal coliform growth were present during summer sampling at river miles 75 (3100 CFU/100 ml) and 55 (4200 CFU/100 ml). River mile 55 is located behind the Union Carbide Plant in South Charleston (Kanawha County), West Virginia, and is in a heavily industrialized area. River mile 75 is located near the unincorporated town of Shrewsbury in Kanawha County. The sample for river mile 75 was collected within fifty yards of a river island (Goat Island) inhabited by a small herd of goats. Goat Island is also frequented by campers using out-house type facilities as restrooms. Figure 19b is shown using a Log scale to give better visualization of fecal coliform counts for seasonal data.

Average fecal coliform counts were greater at KR50-KR00 during the spring compared to summer.
Average fecal coliform counts were lower at KR95-KR55 during the spring compared to summer. This occurrence is possibly attributed to a rain event that occurred during summer sample collections for site K90-K55.

a Indicates the origin at the confluence of the New and Gauley Rivers.
b Indicates the mouth at the confluence of the Ohio River.

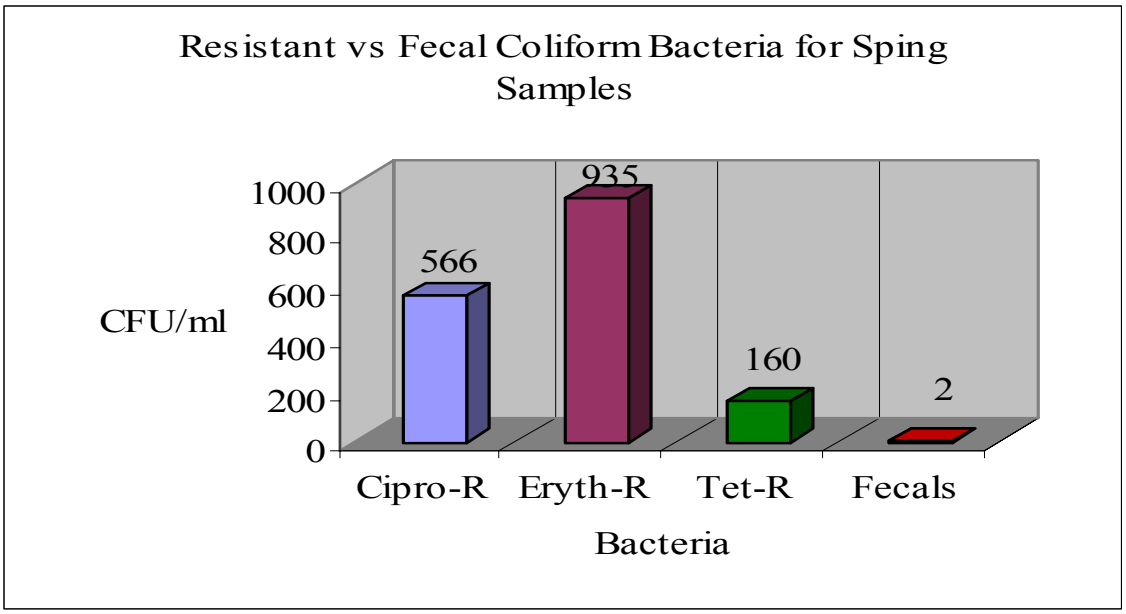
Figure 20. Comparison of tributary fecal coliform bacteria for spring and summer seasonal data.



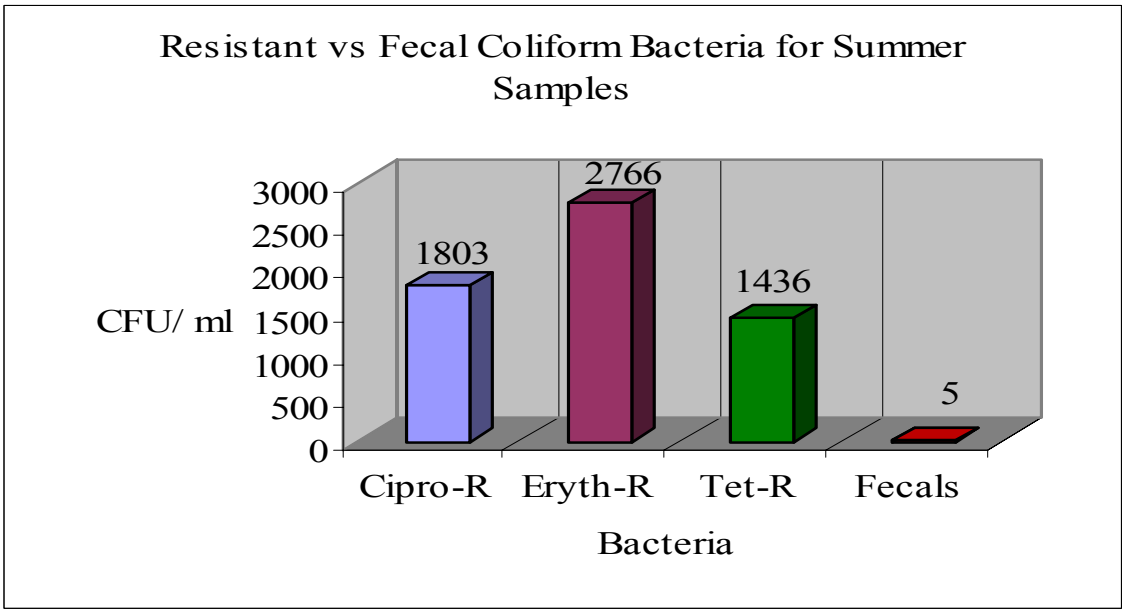
Fecal coliform counts were generally greater during the summer sampling season than during the spring season, with the exception of the Coal River tributary sample site. No summer data collected for the New and Gauley River tributaries.

Figure 21a and 21b. Mean main stem antibiotic resistant and fecal coliform bacterial data from spring and summer sampling seasons.

a.



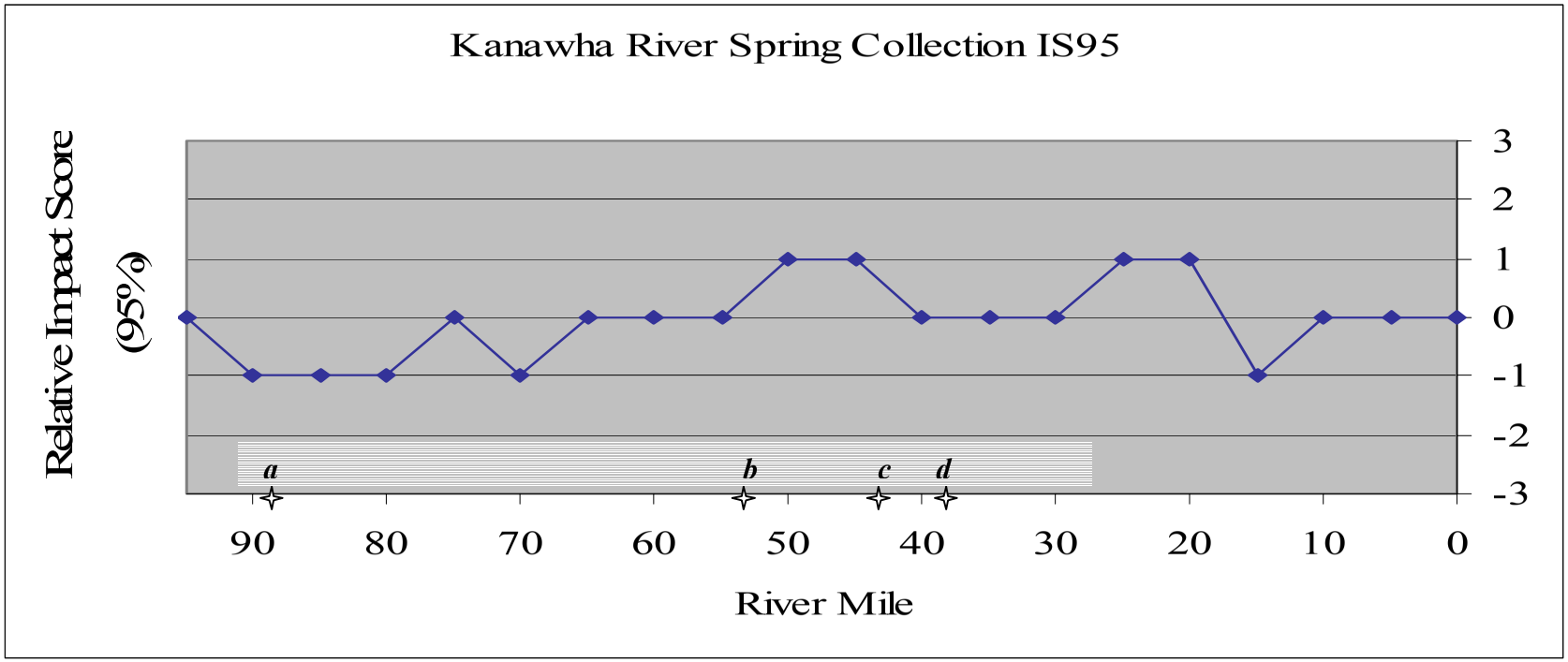
b.



[a] Mean Ciprofloxacin-resistant (Cipro-R), mean Erythromycin-resistant (Eryth-R), mean Tetracycline-resistant (Tet-R), and mean Fecal Coliform (Fecals) bacteria appear to be of different populations for both spring (Figure 21a) and summer (Figure 21b) sampling seasons.

[b] All bacterial counts were reported as per 1 ml. Fecal coliforms were converted from per 100 ml to per 1 ml of sample.

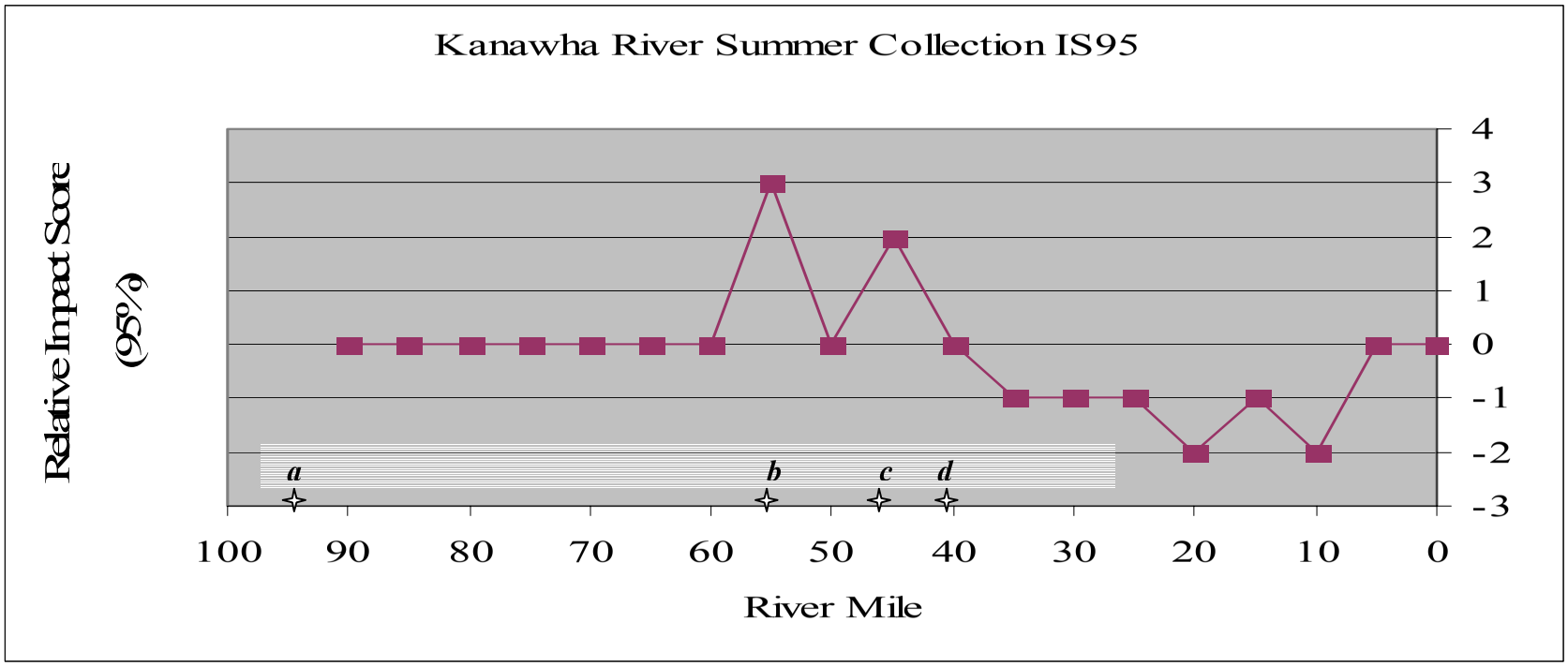
Figure 22. Kanawha River spring relative impact scores for the main stem using the 95th percentile boundary.



✧Indicates entry point of a tributary
[a] New and Gauley River enters the main stem at the headwaters KR95
[b] Elk River enters the main stem at KR57.5
[c] Coal River enters the main stem at KR45
[d] Pocatalico River enters the main stem at KR41

Relative IS_{95} for the spring collection are at or below zero toward the origin between river miles 95-55, reach highest levels between river miles 50-45 and 25-20, and show a trend returning levels to those similar to the upper part of the river for miles 40-30 and 15-00, toward the mouth.

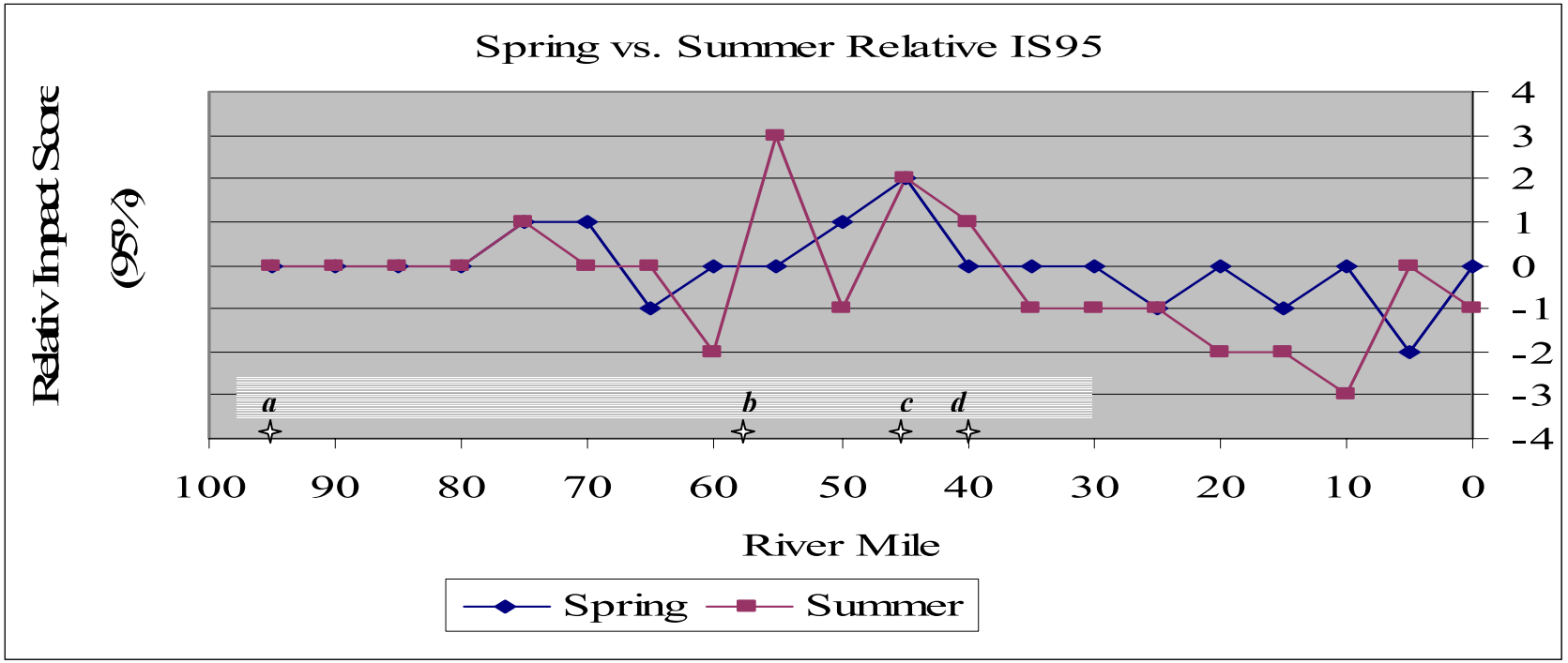
Figure 23. Kanawha River summer relative impact scores for the main stem using the 95th percentile boundary.



✧Indicates entry point of a tributary
[a] New and Gauley River enters the main stem at the headwaters KR95
[b] Elk River enters the main stem at KR57.5
[c] Coal River enters the main stem at KR45
[d] Pocatalico River enters the main stem at KR41

Relative impact scores at the 95th percentile boundary for the summer collections show a general trend at or about zero toward the origin for river miles 90-60 reach highest point at river mile 55 and begins a gradual decline between river miles 45-00 toward the mouth.

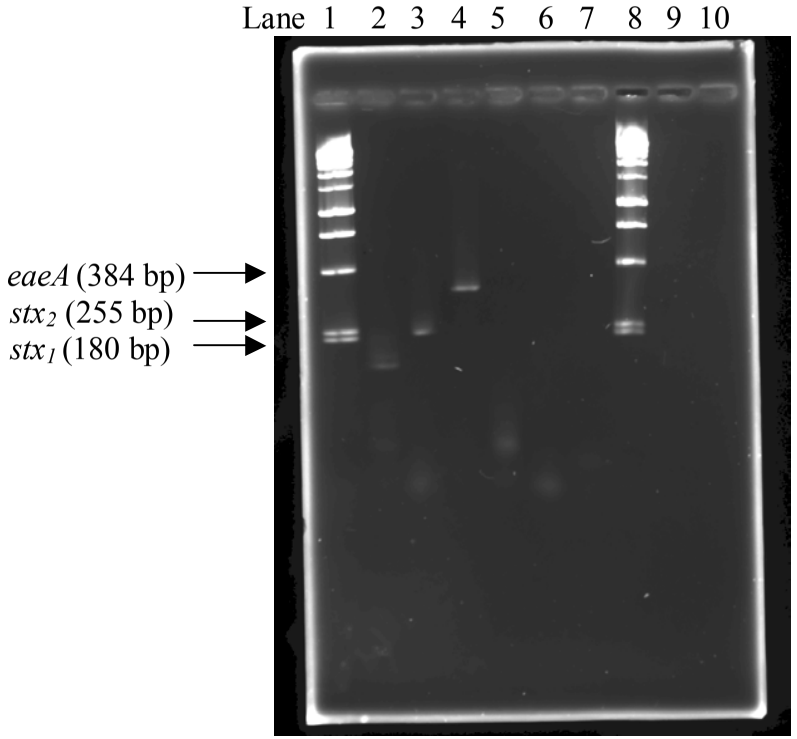
Figure 24. Kanawha River relative impact score spring and summer comparison for the main stem using the 95th percentile boundary.



✧Indicates entry point of a tributary
[a] New and Gauley River enters the main stem at the headwaters KR95
[b] Elk River enters the main stem at KR57.5
[c] Coal River enters the main stem at KR45
[d] Pocatalico River enters the main stem at KR41

A comparison of spring and summer relative impact scores at the 95th percentile boundary shows river trends are relatively similar for both sampling seasons. Trends appear to be occurring in approximately the same locations although not with the same level of impact. For spring (river miles 95-50) and summer (river miles 90-60) trends are at or about zero, reach highest levels between river miles 55-45 (industrialized area) and then return to about or below zero for river miles 35-00. Both seasons show an increase at river mile 75 (Goat Island).

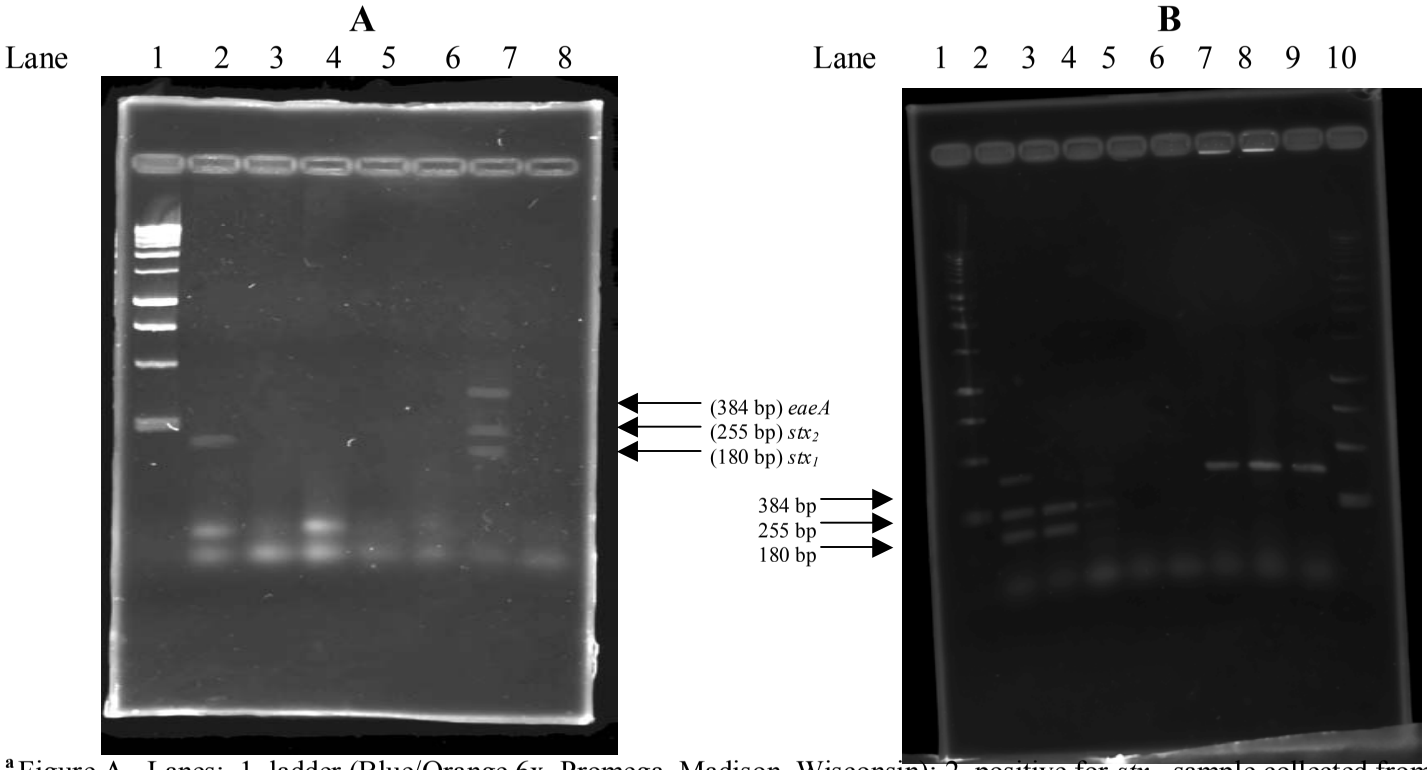
Figure 25. Representative gel of positive and negative controls used for multiplex polymerase chain reaction (mPCR) assays.



[a] *Escherichia coli* , designated EDL933, serotype O157:H7, (ATCC No. 35150, Culti-Loops®, Lot No. 607572, Remel Europe, Ltd.) was used as the positive control (*stx*$_1$, *stx* $_2$, and *eaeA*) for all assays, and *Escherichia coli*, designated FDA strain Seattle 1946, (ATCC No. 25922, Bactrol Disks, Lot No. 145103, Difco Laboratories) was the negative control for all assays.

[b] Lanes 1 and 8: ladder. Lanes 2-4 are visualizing *Escherichia coli,* serotype O157:H7, positive control. Lane 2: positive for *stx1* (shiga-like toxin 1). Lane 3: positive for *stx2* (shiga-like toxin 2). Lane 4: positive for *eaeA*. Lanes 5-8 are visualizing *Escherichia coli*, designated FDA strain Seattle 1946, negative control. Lane 5: negative for *stx*2. Lane 6: negative for *stx*1. Lane 7: negative for *eaeA*. Lanes 9 and 10 were not used for control visualization.

Figure 26. Multiplex PCR analysis of fecal coliform isolates from the summer sampling season.



[a] Figure A. Lanes: 1, ladder (Blue/Orange 6x, Promega, Madison, Wisconsin); 2, positive for stx_1, sample collected from the Elk River tributary; 3 to 6, negative for stx_1 (shiga-like toxin 2), stx_2 (shiga-like toxin 2), and *eaeA*; 7 is visualizing *Escherichia coli,* serotype O157:H7, positive control;. positive for stx_1 (shiga-like toxin 2), stx_2 (shiga-like toxin 2), and *eaeA*; 8 is visualizing *Escherichia coli* negative control.

[b] Figure B. Lanes: 1, ladder (Blue/Orange 6x, Promega, Madison, Wisconsin); 2, is visualizing *Escherichia coli,* serotype O157:H7, positive control;. positive for stx_1 (shiga-like toxin 2), stx_2 (shiga-like toxin 2), and *eaeA*; 3, positive for stx_1 *and* stx_2, sample collected from sample site K-65; 4, positive for stx_2, collected from sample site K-75; 5 to 6, negative for all target genes; 7, positive for *eaeA*, collected from sample site K-05 (toward mouth); 8 to 9, positive for *eaeA*, collected from sample site K-00 (at mouth); and 10, ladder (Blue/Orange 6x, Promega, Madison, Wisconsin).

Table 1. Oligonucleotide Primers. Oligonucleotide forward and reverse primer sequences used for polymerase chain reaction (PCR).

Primer	Target gene or virulence	Primer Sequence (5' to 3')	Amplicon size (bp)	Reference
stx 1 F	*stx* 1	ATA AAT CGC CAT TCG TTG ACT AC	180	Robins-Browne *et al.* 2004
stx 1 R		AGA ACG CCC ACT GAG ATC ATC		López-Saucedo *et al.* 2003
				Paton and Paton 2002
				Paton and Paton 1999
				Paton and Paton 1998
stx 2 F	*stx 2*	GGC ACT GTC TGA AAC TGC TCC	255	Robins-Browne *et al.* 2004
stx 2 R		TCG CCA GTT ATC TGA CAT TCT G		Paton and Paton 2002
				Paton and Paton 1999
				Paton and Paton 1998
eae A F	*eaeA*	GAC CCG GCA CAA GCA TAA GC	384	Robins-Browne *et al.* 2004
eae A R		CCA CCT GCA GCA ACA AGA GG		López-Saucedo *et al.* 2003
				Paton and Paton 2002
				Paton and Paton 1999
				Paton and Paton 1998

Table 2. Oligonucleotide Characteristics**.** Characteristics of the oligonucleotide primers tested to detect pathogenic *Escherichia coli* collected from water samples along the Great Kanawha River. Oligonucleotide analysis data calculated by Marshall University DNA Core Facility using OLIGO 4.0 Primer Analysis Software (NBI).

Primer	Primer Sequence (5' to 3')	Oligonucleotide Analysis
stx 1 F	ATA AAT CGC CAT TCG TTG ACT AC	Td = 64.8°C (nearest neighbor method) Tm = 68.2°C (%GC method) Tm = 64.0°C [2*(A+T) + (G+C)] nmol/OD = 4.5 (nearest neighbor extinction coefficient) µg/OD = 31.8 Composition: A + T 14 60.9% C + G 9 39.1%
stx 1 R	AGA ACG CCC ACT GAG ATC ATC	Td = 65.6°C (nearest neighbor method) Tm = 70.8°C (%GC method) Tm = 64.0°C [2*(A+T) + (G+C)] nmol/OD = 4.88 (nearest neighbor extinction coefficient) µg/OD = 31.5 Composition: A + T 10 47.6% C + G 11 52.4%
stx 2 F	GGC ACT GTC TGA AAC TGC TCC	Td = 66.3°C (nearest neighbor method) Tm = 62.8°C (%GC method) Tm = 66.0°C [2*(A+T) + (G+C)] nmol/OD = 5.28 (nearest neighbor extinction coefficient) µg/OD = 34.1 Composition: A + T 9 42.9% C + G 12 57.1%
stx 2 R	TCG CCA GTT ATC TGA CAT TCT G	Td = 64.6°C (nearest neighbor method) Tm = 69.6°C (%GC method) Tm = 64.0°C [2*(A+T) + (G+C)] nmol/OD = 4.96 (nearest neighbor extinction coefficient) µg/OD = 33.5 Composition: A + T 12 54.5% C + G 10 45.5%
eae A F	GAC CCG GCA CAA GCA TAA GC	Td = 69.5°C (nearest neighbor method) Tm = 72.3°C (%GC method) Tm = 64.0°C [2*(A+T) + (G+C)] nmol/OD = 5.1 (nearest neighbor extinction coefficient) µg/OD = 31.5 Composition: A + T 8 40.0% C + G 12 60.0%
eae A R	CCA CCT GCA GCA ACA AGA GG	Td = 68.1°C (nearest neighbor method) Tm = 72.3°C (%GC method) Tm = 64.0°C [2*(A+T) + (G+C)] nmol/OD = 5.13 (nearest neighbor extinction coefficient) µg/OD = 31.7 Composition: A + T 8 40.0% C + G 12 60.0%

Table 3. Spring data average bacterial counts and standard deviations.

River Mile/ Site ID	Totals	S.D. Totals	Cipro	S.D. Cipro	Erythro	S.D. Erythro	Tet	S.D. Tet	* FC	S.D. * FC
K-95	159666.67	38.40	356.67	21.08	1086.67	13.61	146.67	5.13	300.00	N.A
K-90	208000.00	13.00	386.67	21.13	1126.67	15.95	86.67	2.89	90.00	N.A
K-85	175000.00	12.29	443.33	10.41	1140.00	17.35	146.67	2.89	80.00	N.A
K-80	175666.67	19.40	496.67	10.07	853.33	3.21	70.00	4.00	120.00	N.A
K-75	138000.00	67.58	373.33	3.79	990.00	23.30	103.33	1.15	110.00	N.A
K-70	131000.00	74.05	353.33	14.74	876.67	25.93	126.67	9.07	110.00	N.A
K-65	157333.33	17.93	376.67	5.51	923.33	20.82	113.33	2.31	100.00	N.A
K-60	137333.33	10.02	616.67	25.38	893.33	20.55	116.67	3.79	150.00	N.A
K-55	141666.67	28.73	455.00	21.92	816.67	14.84	160.00	4.58	100.00	N.A
K-50	118666.67	19.86	1103.33	23.12	840.00	15.13	196.67	9.29	460.00	N.A
K-45	109000.00	2.00	1106.67	41.10	1026.67	19.14	150.00	4.00	150.00	N.A
K-40	112333.33	10.02	510.00	12.77	993.33	14.01	106.67	0.58	210.00	N.A
K-35	86333.33	12.66	496.67	14.43	823.33	19.14	133.33	3.21	200.00	N.A
K-30	93666.67	16.26	576.67	2.52	826.67	32.32	100.00	2.65	100.00	N.A
K-25	81000.00	4.36	483.33	6.81	1236.67	22.37	150.00	4.36	110.00	N.A
K-20	75500.00	17.68	706.67	4.16	1226.67	49.74	136.67	4.62	100.00	N.A
K-15	57666.67	20.31	393.33	26.95	620.00	8.89	220.00	9.17	140.00	N.A
K-10	55000.00	12.29	496.67	17.21	920.00	22.72	153.33	1.53	270.00	N.A
K-05	11500.00	21.92	553.33	3.51	890.00	6.24	150.00	4.58	130.00	N.A
K-00	15000.00	3.61	1033.33	12.58	596.67	5.13	640.00	7.07	170.00	N.A
coal	37000.00	12.73	763.33	19.66	1066.67	10.21	196.67	6.35	200.00	N.A
poca	95000.00	16.52	1353.33	7.77	1133.33	12.42	406.67	6.11	420.00	N.A
gau	32333.33	6.66	440.00	8.89	850.00	30.12	183.33	3.79	60.00	N.A
new	162666.67	56.32	346.67	11.72	760.00	7.21	116.67	4.73	90.00	N.A
elk	53666.67	18.77	563.33	26.58	780.00	24.06	123.33	3.21	100.00	N.A

*Fecal coliforms reported as per 100 ml: All others reported as per 1 ml.

N.A. = sample locations where only one plate was countable and standard deviations were not applicable.

Table 4. Summer data average bacterial counts and standard deviations.

River Mile/ Site ID	Totals	S.D. Totals	Cipro	S.D. Cipro	Erythro	S.D. Erythro	Tet	S.D. Tet	* FC	S.D. * FC
K-95	20000.00	5.57	1123	7.23	1710.00	46.18	433.30	15.28	N.D.	N.D
K-90	10000.00	4.36	2330	22.63	2785.00	0.71	666.67	24.54	190.00	N.A
K-85	14333.33	8.08	1180	21.66	1673.33	37.17	690.00	2.65	180.00	N.A
K-80	12333.33	4.04	1795	48.79	2633.33	23.50	485.00	23.33	210.00	N.A
K-75	11000.00	13.86	1850	12.12	1850.00	11.31	503.33	21.55	3100.00	N.A
K-70	15666.67	7.51	1520	N.D.	2653.33	52.20	510.00	22.63	340.00	N.A
K-65	6666.67	0.58	1667	48.76	1403.33	56.15	946.67	7.64	350.00	N.A
K-60	8666.67	4.73	990	23.30	1690.00	72.51	423.33	19.55	520.00	N.A
K-55	38333.33	5.03	6167	275.25	7826.67	109.28	4400.00	28.28	4200.00	N.A
K-50	14666.67	1.53	1010	10.54	2923.33	49.60	1480.00	51.64	130.00	N.A
K-45	40666.67	7.57	1617	8.96	9200.00	101.12	6226.67	46.72	80.00	N.A
K-40	47333.33	19.55	1737	68.72	5275.00	169.00	5046.67	85.70	40.00	N.A
K-35	23333.33	8.02	2353	66.01	2576.67	30.01	1123.33	21.50	10.00	N.A
K-30	50333.33	37.00	1937	34.50	1886.67	54.08	563.33	3.21	10.00	N.A
K-25	30666.67	22.14	1660	15.72	1525.00	13.44	600.00	9.90	10.00	N.A
K-20	168000.00	36.37	1370	47.32	813.33	27.75	763.33	14.64	0.00	N.A
K-15	31666.67	12.66	1590	36.77	1070.00	7.00	676.67	8.08	10.00	N.A
K-10	20000.00	4.00	940	28.00	1013.33	6.51	423.33	4.73	40.00	N.A
K-05	53000.00	16.09	1433	35.22	2593.33	33.08	1376.67	11.50	130.00	N.A
K-00	19000.00	4.00	1107	23.63	1170.00	8.00	370.00	8.89	90.00	N.A
coal	77000.00	19.47	3560	139.53	2273.33	22.85	1015.00	21.92	100.00	N.A
poca	64000.00	30.64	2213	68.39	3236.67	11.24	690.00	20.42	30.00	N.A
gau	28000.00	4.00	963	20.11	2750.00	0.00	116.67	0.58	N.D.	N.D.
new	13333.33	3.06	670	10.82	1300.00	26.91	170.00	1.41	N.D.	N.A
elk	52333.33	2.08	9120	94.57	6460.00	100.54	5096.67	269.02	4300.00	N.A

*Fecal coliforms reported as per 100 ml: All others reported as per 1 ml.

N.A. = sample locations where only one plate was countable and standard deviations were not applicable. **N.D.** = No Data Reported

Table 5. Spring correlations for microbiological and physical parameter data.

(*P* - value; R - value)

Totals	Totals					
Cipro	0.112	Cipro				
Erythro	0.089	0.408	Erythro			
Tet	0.013	0.020	0.022	Tet		
Fecal	0.590	0.062	0.476	0.520	Fecal	
Turbidity	0.000	0.197	0.144	0.012	0.487	Turbidity
pH	0.000	0.166	0.717	0.150	0.862	pH
W °C	0.000	0.145	0.040	0.063	0.609	W °C

[a] Measure of the strength of association between two variables using *P*-values based on the correlation coefficient statistical test.

[b] Variables included are Total cultivable (Totals), Ciprofloxacin-resistant (Cipro), Erythromycin-resistant (Erythro), and Tetracycline-resistant (Tet) bacteria and turbidity, pH, and water temperature (W°C).

[c] Values in red are considered correlated at $P \leq 0.05$, suggesting the variables are correlated and linear dependences were identified; values in blue are uncorrelated at $P \geq 0.05$, suggesting the variables are not correlated and no linear dependences were identified.

Table 6. Summer correlations for microbiological and physical parameter data.

Totals	Totals					
Cipro	0.130	Cipro				
Erythro	0.737	0.177	Erythro			
Tet	0.385	0.057	0.000	Tet		
Fecal	0.312	0.098	0.326	0.513	Fecal	
Turbidity	0.759	0.837	0.526	0.766	0.923	Turbidity
pH	0.935	0.933	0.952	0.728	0.712	pH
W °C	0.207	0.507	0.642	0.331	0.730	W °C

[a] Measure of the strength of association between two variables using *P*-values based on the correlation coefficient statistical test.

[b] Variables included are Total cultivable (Totals), Ciprofloxacin-resistant (Cipro), Erythromycin-resistant (Erythro), and Tetracycline-resistant (Tet) bacteria and turbidity, pH, and water temperature (W°C).

[c] Values in red are considered correlated at $P \leq 0.05$, suggesting the variables are correlated and linear dependences were identified; values in blue are uncorrelated at $P \geq 0.05$, suggesting the variables are not correlated and no linear dependences were identified.

Table 7. Spring vs. summer correlations for microbiological data.

	Totals Summer		Cipro Summer		Erythro Summer		Tet Summer		Fecals Summer
Totals Spring	0.120	Cipro Spring	0.459	Erythro Spring	0.438	Tet Spring	0.807	Fecals Spring	0.107
R-Values	-0.359		0.176		0.195		-0.058		0.515

[a] Measure of the strength of association between two variables using *P*-values based on the correlation coefficient statistical test. A *P*-value ≥ 0.05 suggests the variables are not correlated and no linear dependences were identified.

[b] Variables included are Total cultivable (Totals), Ciprofloxacin-resistant (Cipro), Erythromycin-resistant (Erythro), and Tetracycline-resistant (Tet) bacteria and turbidity, pH, and water temperature (W°C).

[c] Values in red are considered correlated at $P \leq 0.05$; values in blue are uncorrelated at $P \geq 0.05$.

Data used for figures 13, 15, 17, and 19

Table 8 . Relative Impact Scores (range -4 to 4) for Spring and Summer using the 95th Percentile (IS_{95}).

River Mile or Tributary	[a]Site Designation	[b c]Spring IS_{95}	[b c] Summer IS_{95}
New River	T	-1	N.D.
Gauley	T	-1	N.D.
95	U	0	N.D.
90	U	-1	0
85	U	-1	0
80	U	-1	0
75	U	0	0
70	U	-1	0
65	U	0	0
60	U	0	0
Elk	T	0	3
55	U	0	3
50	L	1	0
Coal	T	0	0
45	L	1	2
Pocatalico	T	3	0
40	L	0	0
35	L	0	-1
30	L	0	-1
25	L	1	-1
20	L	1	-2
15	L	-1	-1
10	L	0	-2
5	L	0	0
0	L	0	0

[a] Designation of U (Upper Kanawha between river miles KR95-KR55), L (Lower Kanawha between river miles KR50-KR00), or T (Tributary) indicates the region of the River or Tributary entering the river.
[b] Fields highlighted in red indicates an impacted area.
[c] Fields highlighted in blue indicates less impact.
[d] "N.D." = area where impact score determination was not applicable.

Data used for figures 22, 23, and 24.

Table 9. Target gene identification per sample site location.

ID No.	River Mile/ Tributary	Isolate No.	*stx*$_1$	*stx*$_2$	*eaeA*
*CJ1-39	K00	1	-	+	-
CJ1-39	K00	2	-	-	-
CJ1-39	K00	3	-	-	-
*CJ1-39	K00	4	-	-	+
*CJ1-39	K00	5	-	-	+
*CJ1-39	K05	1	-	-	+
CJ1-39	K05	2	-	-	-
CJ1-39	K05	3	-	-	-
CJ1-39	K05	4	-	-	-
CJ1-39	K05	5	-	-	-
CJ1-39	K10	1	-	-	-
CJ1-39	K10	2	-	-	-
CJ1-39	K15	1	-	-	-
CJ1-39	K25	1	-	-	-
CJ1-39	K30	1	-	-	-
CJ1-39	K35	1	-	-	-
CJ1-39	K40	1	-	-	-
CJ1-39	K40	2	-	-	-
CJ1-39	K40	3	-	-	-
CJ1-39	K40	4	-	-	-
CJ1-39	K45	1	-	-	-
CJ1-39	K45	2	-	-	-
CJ1-39	K45	3	-	-	-
CJ1-39	K45	4	-	-	-
CJ1-40	K50	1	-	-	-
CJ1-40	K50	2	-	-	-
CJ1-40	K50	3	-	-	-
CJ1-40	K50	4	-	-	-
CJ1-40	K50	5	-	-	-
CJ1-40	K50	6	-	-	-
CJ1-40	K50	7	-	-	-
CJ1-40	K50	8	-	-	-
CJ1-40	K50	9	-	-	-

+ = isolate positive for target gene
- = isolate negative for target gene
"*" indicates an isolate was positive for at least one target gene sequence
"^" indicates an isolate was positive for at least two target gene sequences

Table 9 (continued)

ID No.	River Mile/ Tributary	Isolate No.	*stx*$_1$	*stx*$_2$	*eaeA*
CJ1-67	K55	1	-	-	-
CJ1-67	K55	2	-	-	-
CJ1-67	K55	3	-	-	-
CJ1-67	K55	4	-	-	-
CJ1-67	K55	5	-	-	-
CJ1-67	K55	6	-	-	-
CJ1-69	K55	7	-	-	-
^*CJ1-69	K55	8	-	+	+
CJ1-69	K55	9	-	-	-
CJ1-69	K55	10	-	-	-
CJ1-67	K60	1	-	-	-
CJ1-67	K60	2	-	-	-
CJ1-67	K60	3	-	-	-
CJ1-67	K60	4	-	-	-
CJ1-67	K60	5	-	-	-
CJ1-67	K60	6	-	-	-
CJ1-67	K65	1	-	-	-
^*CJ1-67	K65	2	+	+	-
CJ1-67	K65	3	-	-	-
CJ1-67	K65	4	-	-	-
CJ1-67	K65	5	-	-	-
CJ1-67	K65	6	-	-	-
CJ1-68	K70	1	-	-	-
CJ1-68	K70	2	-	-	-
CJ1-68	K70	3	-	-	-
*CJ1-68	K70	4	-	+	-
*CJ1-68	K70	5	-	+	-
CJ1-68	K70	6	-	-	-
*CJ1-68	K75	1	-	+	-
*CJ1-68	K75	2	-	+	-
CJ1-68	K75	3	-	-	-
CJ1-68	K75	4	-	-	-
CJ1-68	K75	5	-	-	-

+ = isolate positive for target gene
- = isolate negative for target gene
"*" indicates an isolate was positive for at least one target gene sequence
"^" indicates an isolate was positive for at least two target gene sequences

Table 9 (continued)

ID No.	River Mile/ Tributary	Isolate No.	*stx*$_1$	*stx*$_2$	*eaeA*
CJ1-68	K75	6	-	-	-
CJ1-69	K75	7	-	-	-
CJ1-69	K75	8	-	-	-
CJ1-69	K75	9	-	-	-
CJ1-69	K75	10	-	-	-
CJ1-68	K80	1	-	-	-
CJ1-68	K80	2	-	-	-
CJ1-68	K80	3	-	-	-
CJ1-68	K80	4	-	-	-
CJ1-68	K80	5	-	-	-
CJ1-68	K80	6	-	-	-
CJ1-68	K85	1	-	-	-
CJ1-68	K85	2	-	-	-
CJ1-68	K85	3	-	-	-
CJ1-68	K85	4	-	-	-
CJ1-68	K85	5	-	-	-
CJ1-68	K85	6	-	-	-
CJ1-69	K90	1	-	-	-
CJ1-69	K90	2	-	-	-
CJ1-69	K90	3	-	-	-
CJ1-69	K90	4	-	-	-
CJ1-69	K90	5	-	-	-
CJ1-69	K90	6	-	-	-
N.D.	NEW	N.D.	N.D.	N.D.	N.D.
N.D.	GAU	N.D.	N.D.	N.D.	N.D.
CJ1-67	ELK	1	-	-	-
CJ1-67	ELK	2	-	-	-
*CJ1-67	ELK	3	-	+	-
CJ1-67	ELK	4	-	-	-
CJ1-67	ELK	5	-	-	-
*CJ1-67	ELK	6	-	+	-
CJ1-69	ELK	7	-	-	-
CJ1-69	ELK	8	-	-	-

+ = isolate positive for target gene
- = isolate negative for target gene
"*" indicates an isolate was positive for at least one target gene sequence
"^" indicates an isolate was positive for at least two target gene sequences

Table 9 (continued)

ID No.	River Mile/ Tributary	Isolate No.	*stx*$_1$	*stx*$_2$	*eaeA*
CJ1-69	ELK	9	-	-	-
*CJ1-69	ELK	10	+	-	-
CJ1-40	COAL	1	-	-	-
CJ1-40	COAL	2	-	-	-
CJ1-40	COAL	3	-	-	-
CJ1-40	COAL	4	-	-	-
CJ1-40	COAL	5	-	-	-
CJ1-40	COAL	6	-	-	-
CJ1-40	POCA	1	-	-	-
CJ1-40	POCA	2	-	-	-
CJ1-40	POCA	3	-	-	-

+ = isolate positive for target gene
- = isolate negative for target gene
"*" indicates an isolate was positive for at least one target gene sequence
"^" indicates an isolate was positive for at least two target gene sequences
"N.D." designates sites where no data were collected.

[a.] Thirteen (12.0%) of 108 isolates assayed were positive for at least on target gene sequence; two (1.85%) isolates were positive for *stx*$_1$, nine (8.33%) were positive for *stx*$_2$, and four (3.70%) were positive for *eaeA* target gene sequences. See Table 10 for a target gene identification summary.

[b.] Two (1.85%) of the 108 isolates assayed were positive for at least two target gene sequences, and no isolates assayed were positive for all three target gene sequences.

Table 10. Summery of target gene identification per sample site location. The proportion of genes identified at each river mile.

River Mile / Tributary		*stx*$_1$	*stx*$_2$	*eaeA*		Isolates Assayed
95		N.D	N.D.	N.D.		0
90		0	0	0		6
85		0	0	0		6
80		0	0	0		6
75		0	2	0		10
70		0	2	0		6
*65		1	1	0		6
60		0	0	0		6
*55		0	1	1		10
50		0	0	0		9
45		0	0	0		4
40		0	0	0		4
35		0	0	0		1
30		0	0	0		1
25		0	0	0		1
20		0	0	0		0
15		0	0	0		1
10		0	0	0		2
5		0	0	1		5
0		0	1	2		5
New		N.D	N.D	N.D		0
Gau		N.D	N.D	N.D		0
Elk		1	2	0		10
Coal		0	0	0		6
Poca		0	0	0		3
	Total	2(1.85%)	9(8.33%)	4(3.70%)	Total Isolates	108 (100%)

***** = sites identified as positive for multiple target genes.
"N.D." designates sites where no data were collected.

Appendix A. The Great Kanawha River's Mainstem Sample Site Coordinates and Descriptions for River Miles K-95 – K-00.

River Mile	Latitude	Longitude	Site Description
K-95	38°8'48" N	- 81°12'39.7" W	Right descending bank below island.
K-90	38°8'0" N	- 81°16'43" W	Mid-channel, 90.5 river miles from mouth. Near the town of Alloy in Fayette County.
K-85	38°10'56" N	- 81°.19'53" W	Mid-channel. Less than one mile down stream of Montgomery Bridge, in Montgomery. Upstream of London Locks.
K-80	38°12'25" N	- 81°23'57" W	Mid-channel. Downstream of London Locks. The town of Riverside in Kanawha County located on Right descending bank.
K-75	38°12'20" N	- 81°27'56.22" W	Mid-channel, 1.6 river miles upstream of Chelyan Bridge. The town of Shrewsbury located on right descending bank.
K-70	38°13'38" N	- 81°32'19" W	Mid-channel, 2.5 river miles up stream of Marmet Locks, before DuPont Plant. The town of Belle located on right descending bank.
K-65	38°17'13" N	- 81°34'3" W	Mid-channel, 2.5 river miles down stream of Marmet Locks. Near the town of Malden.
K-60	38°20'3" N	- 81°36'41" W	Mid-channel. In Charleston. The state capital complex is visible on the right descending bank.

Appendix A. (continued) The Great Kanawha River's Mainstem Sample Site Coordinates and Descriptions for River Miles K-95 – K-00.

River Mile	Latitude	Longitude	Site Description
K-55	38°22'20" N	- 81°41'33" W	Mid-channel. Down stream side of Union Carbide Island, South Charleston. Three miles down stream of the Elk River tributary.
K-50	38°21'59" N	- 81°45'41" W	Mid-channel. Near Dunbar.
K-45	38°23'53" N	- 81°50'34" W	Mid-channel. Near Nitro, 400 meters down stream of Coal River.
K-40	38°27'37" N	- 81°49'13" W	Mid-channel. 1 river mile up stream of Pocatalico River, Putnam County.
K-35	38°31'52" N	- 81°51'20" W	Mid-channel. 4 miles down stream of Pocatalico River and 3.6 river miles up stream of Winfield Locks.
K-30	38°31'42" N	- 81°55'52" W	Mid-channel. 1.2 river miles down steam of Winfield Locks.
K-25	38°34'57" N	- 81°59'58" W	Mid-channel. Near Fraziers Bottom.
K-20	38°38'17" N	- 81°58'7" W	Mid-channel. 1.5 river miles down stream of Buffalo boat launch.
K-15	38°42'30" N	- 81°57'6" W	Mid-channel. Near Arbuckle, Mason County.
K-10	38°456'15" N	- 81°59'7" W	Mid-channel. Near confluence with Ten Mile Creek.
K-05	38°48'18" N	- 82°3'31" W	Mid-channel. Near Ambrosia near Rockcastle Creek.
K-00	38°50'14" N	- 82°.8'21" W	Mid-channel. Point Pleasant, at mouth of river. Before Kanawha River empties into Ohio River. Up stream of Rt. 2 bridge.

Appendix B . The Great Kanawha River's Tributary Sample Site Coordinates and Descriptions.

River Tributary	Latitude	Longitude	Site Description
Gauley	38°13"21.43" N	- 81°7'12" W	Turn off Route 39 at Swiss. Travel 1.2 miles out road. Crossover railroad tracks to right and drive down to river. Site is a National Park Service site.
New	38°9'12.3" N	- 81°10'53.1" W	On New River, 2 miles upstream of Kanawha Falls on right bank. Pull off on right side of road after campground, follow path to river bank.
Elk	38°21'21.5" N	- 81°38'35.4" W	On Elk River, one tenth of a mile from the mouth, on the left descending bank.
Coal	38°23'4.38" N	- 81°50'24.7" W	On the Coal River, sample collected at station site 0.70 miles from mouth of stream and 7/10 of the way across the stream from the left descending bank.
Pocatalico	38°28'40.9" N	- 81°48'48.1" W	On Pocatalico River, left bank on other side of WV-62 bridge.

Appendix C. Sample site locations by river mile and county.

River Mile/Tributary	County Location
New	Fayette
Gauley	Fayette
K-95	Fayette
K-90	Kanawha
K-85	Kanawha
K-80	Kanawha
K-75	Kanawha
K-70	Kanawha
K-65	Kanawha
K-60	Kanawha
Elk	Kanawha
K-55	Kanawha
K-50	Kanawha
Coal	Kanawha
K-45	Kanawha
K-40	Putnam
Pocatalico	Putnam
K-35	Putnam
K-30	Putnam
K-25	Putnam
K-20	Putnam
K-15	Mason
K-10	Mason
K-05	Mason
K-00	Mason

[a] The Great Kanawha River spans across four West Virginia Counties: Fayette, Kanawha, Putnam, and Mason.

Appendix D. Antibiotics and concentrations tested.

Antibiotic	Catalog No.	Solvent[a]	Stock Conc.	Working Conc.
Ciprofloxacin	Fisher 61-277-RF	DMSO	4 mg/ml	4 µg/ml
Erythromycin	Fisher BP920-25	EtOH:H_2O	8 mg/ml	8 µg/ml
Tetracycline	Fisher BP912-100	EtOH:H_2O	12.5 mg/ml	12.5 µg/ml

[a] DMSO = dimethylsulfoxide (Certified ACS). EtOH:H_2O = a mixture of equal parts ethanol (100% USP) and reagent grade water (18 MΩ).

Appendix E. Proteobacteria.

1. Proteobacteria

a. The gram-negatives:

i. The *Proteobacteria* are the gram-negatives, all of which are thought to have derived from an ancestral purple photosynthetic bacterium.

ii. Of those bacterial groups which are actively studied by humans, the *Proteobacteria* form the most diverse taxon.

b. The *Proteobacteria* group includes:

i. the *alpha subdivision* (which includes *Agrobacterium spp.*, *Brucella spp.*, *Zea mays* mitochondria, the purple non-sulfur bacteria, and the rickettsias)

ii. the beta subdivision (which includes Bordetella spp., Neisseria spp., and some Pseudomonas spp.)

iii. the gamma subdivision (which includes Family Enterobacteriaceae, Haemophilus spp., Legionella spp., Pasteurella spp., Vibrio spp., the purple sulfur bacteria, and some Pseudomonas spp.)

iv. the delta subdivision (which includes the Bdellovibrio)

v. the epsilon subdivision (which include Helicobacter spp.).

c. **Facultatively anaerobic gram-negative rods**

d. *Gram-negative , facultatively anaerobic bacilli* are a very medically relevant group of bacteria .

e. There are three families making up the bulk of *facultative anaerobic gram-negative rods* (39 of 46 genera):

i. Enterobacteriaceae

ii. Vibrionaceae

iii. Pasteurellaceae

f. Enterobacteriaceae:

i. See section below.

g. Family Pasteuellaceae includes the genera :

i. *Actinobacillus*

ii. *Haemophilus*

iii. *Pasteurella*

h. Family Vibrionaceae includes the genera :

i. Aeromonas

ii. Enhydrobacter

iii. Phobacterium

iv. Plesiomonas

v. *Vibrio*

2. Enterobacteriaceae [enterics]

a. The enterics:

i. Members of family *Enterobacteriaceae* tend to inhabit the intestinal tracts of animals.

ii. Members of family *Enterobacteriaceae* are found in the intestines:
    1. some are found in nearly all members of a given species
    2. some in a smaller fraction
    3. some only during (and causing) a disease state

b. Glucose fermenters:

i. *Enterics* tend to be glucose fermenters .
ii. Some are fermenters of other carbohydrates .
iii. Lactose non-fermeters tend to be pathogenic.

c. Additional characteristics:

i. If motile they have peritrichous flagella .
ii. May have fimbriae which help them to adhere to mucous membranes .

d. Family *Enterobacteriaceae* includes the genera:

i. *Citrobacter*
ii. *Enterobacter*
iii. *Erwinia*
iv. *Escherichia*
v. *Klebsiella*
vi. *Proteus*
vii. *Providencia*
viii. *Salmonella*
ix. *Serratia*
x. *Shigella*
xi. *Yersinia*

The above Appendix E information was copied form the website http://www.mansfield.ohio-state.edu/~sabedon/biol3018.htm which is maintained by Stephen T Abedon Ph. D. 12 May 1998, last modification date.

Appendix F. Environmental Microbiology Research Laboratory Standard Operation Procedure for Impact Score Determination. Prepared by Charles Somerville, PhD.

Antibiotic Stock Solutions

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

Table 1. Antibiotics tested and recommended concentrations.

Antibiotic	Catalog No.	Solvent[a]	Stock Conc.	Working Conc.
Fungizone	BioWhitaker 17-836R	N/A	250 µg/ml	375 ng/ml
Ampicillin Sodium Salt	Fisher BP1760-25	H_2O	50 mg/ml	50 µg/ml
Ciprofloxacin	Cellgro 61-277-RF	DMSO	4 mg/ml	4 µg/ml
Erythromycin	Fisher BP920-25	EtOH:H_2O	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_2O	12.5 mg/ml	12.5 µg/ml
Virginiamycin	Fisher 50-213-730	DMSO	16 mg/ml	16 µg/ml

[a] Fungizone is purchased as a stock solution, it is stored frozen and thawed before use. DMSO = dimethylsulfoxide (Certified ACS). EtOH:H_2O = 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 mg cipro x (1000 mg powder / 803 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 □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°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°C and 15 psi for 20 minutes on a slow exhaust cycle.

4. Move the medium from the autoclave to a 48°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 antibiotics except ciprofloxacin) or at room temperature (ciprofloxacin).

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 □l 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 □l 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.

Enumeration of Fecal Coliform Bacteria

1. Label the 47 mm Petri dishes with absorbent pads (Millipore, cat. no. PD1004705) and the prepared m-E plates with media type (i.e. mFC), date, sample ID, and aliquot amount to be sampled.

2. Place the m-FC Medium with Rosolic Acid, 2 ml plastic ampules (Cat. No. M00000P2F, Millipore) on ice and set aside until step 6

3. Pour sterile tap water into a 100 ml capacity analytical test filter funnel with 47mm cellulose nitrate membrane, 0.45µm pore size (Fisher Scientific, cat. no. 09-740-30D or equivalent) until the membrane is covered to an approximate depth of 5-10 mm.

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

5. Aseptically transfer 0.1 to 50 ml (see note) of undiluted sample to the sterile tap water in the analytical filter funnel, swirl gently to evenly distribute the sample, and filter the water through the funnel. Rinse the sides of the funnel with sterile tap water at least two times and filter through membrane.

   Note – for selection of plating volume

   a. Preliminary tests to determine the volume of sample to be plated are recommended. Plating volumes of 0.1 ml, 0.5 ml, and 1.0 ml are the default volumes for triplicate sampling. However, if the number of colony forming units does not consistently fall within the 20-60 colonies per membrane standard, the volume should be adjusted accordingly.

6. Open m-FC Medium with Rosolic Acid, 2 ml ampule and squeeze contents onto the absorbent pad in the pre-labeled corresponding 47 mm Petri dish with absorbent pad.

7. Remove the disposable funnel wall and aseptically transfer the membrane (using 95% ethyl alcohol flame-sterilized flat forceps) to the pre-labeled corresponding 47 mm Petri dish with absorbent pad soaked with the appropriate medium.

8. Incubate the plates as follows: m-FC (44.5 ± 0.2°C for 24 hours.

9. After incubation, count the number of colony forming units (CFU) on each plate and record in a laboratory notebook. For the m-FC plates, count only the blue colonies.

10. Determine the mean and standard deviation of CFU counts on replicate plates and record in a laboratory notebook.

11. Determine the CFU per 100 ml of fecal coliform and total coliform bacteria in the original sample by multiplying the average CFU value by a dilution factor (i.e. DF of 10 for a plating volume of 0.1 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^{th} percentile and below the 10^{th} percentile. An IS_{80} score weights sites with population counts above the 80^{th} percentile and below the 20^{th} 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.

Appendix G. Luria Bertani media preparation Standard Operating Procedure. Prepared by Christina Johnson.

Media Preparation

1. Suspend 12.5 grams Difco Luria-Bertani Broth (Becton Dickinson, Sparks, MD: cat no. 221970) and 7.5 grams of Difco granulated (Becton Dickinson, Sparks, MD: cat no. 3171850) in 500 ml of purified water in a 1,000 ml capacity glass Erlenmeyer flask.

2. Cover flask with aluminum foil, place a piece of autoclave tape on the foil, and mark the media name of the foil.

3. Swirl the flask to evenly hydrate the suspended powder, and autoclave at 121º C and 15 psi for 20 minutes on a slow exhaust cycle.

4. Move the medium from the autoclave to a 48º C water bath, and hold for at least 30 minutes but not more than 4 hours to temper the medium.

5. 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 48º C.

6. Wear latex gloves for the remaining steps of media preparation.

7. Aseptically pour 100 ml per plate into pre-sterilized 150 × 15 mm Petri dishes (Falcon 1058, Becton Dickinson, Lincoln Park, NJ or equivalent) or 25 ml per plate in to pre-sterilized 100 × 15 mm Petri dishes (Falcon 1029, Becton Dickinson, Sparks MD or equivalent).

8. 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.

Appendix H. Total cultivable and ciprofloxacin resistant average bacterial counts reported.

	Total Cultivable			Ciprofloxacin		
River Mile/ Tributary	Spring April 5-6, 2004	Summer A July 12-13, 2004	Summer B August 5, 2004	Spring April 5-6, 2004	Summer A July 12-13, 2004	Summer B August 5, 2004
95	159666.67	20000	N.D.	356.67	1123	N.D.
90	208000.00	18000	10000.00	386.67	783	2330.00
85	175000.00	9333.33	14333.00	443.33	400	1180.00
80	175666.67	18666.67	12333.00	496.67	883	1795.00
75	138000.00	20000	11000.00	373.33	623	1850.00
70	131000.00	4666.67	15667.00	353.33	410	1520.00
65	157333.33	6666.67	6667.00	376.67	397	1666.67
60	137333.33	8333.33	8667.00	616.67	363	990.00
55	141666.67	7000	38333.00	455.00	1643	5830.00
50	118666.67	14666.67	N.D.	1103.33	1010	N.D.
45	109000.00	40666.67	N.D.	1106.67	1617	N.D.
40	112333.33	47333.33	N.D.	510.00	1737	N.D.
35	86333.33	23333.33	N.D.	496.67	2353	N.D.
30	93666.67	50333.33	N.D.	576.67	1937	N.D.
25	81000.00	30666.67	N.D.	483.33	1660	N.D.
20	75500.00	168000	N.D.	706.67	1370	N.D.
15	57666.67	31666.67	N.D.	393.33	1590	N.D.
10	55000.00	20000	N.D.	496.67	940	N.D.
5	11500.00	53000	N.D.	553.33	1433	N.D.
0	15000.00	19000.00	N.D.	1033.33	1107	N.D.
New	162666.67	13333.33	N.D.	346.67	670	N.D.
Gau	32333.33	84000.00	N.D.	440.00	963	N.D.
Elk	53666.67	20333.33	5233.00	563.33	1813	9120.00
Coal	37000.00	77000.00	N.D.	763.33	3560	N.D.
Poca	95000	64000	N.D.	1353.33	2213	N.D.

[a] Data used for figures 11-18, and 21

[b] Total cultivable and ciprofloxacin resistant average bacterial counts were reported as CFU per 1 ml of sample.

[c] "N.D." designates sites were not data were reported.

Appendix I. Erythromycin and tetracycline average bacterial counts reported.

	Erythromycin			Tetracycline		
River Mile/ Tributary	Spring April 5-6, 2004	Summer A July 12-13, 2004	Summer B August 5, 2004	Spring April 5-6, 2004	Summer A July 12-13, 2004	Summer B August 5, 2004
95	1086.67	1710	N.D.	146.67	433.3	N.D.
90	1126.67	N.D.	2785.00	86.67	106.7	666.67
85	1140.00	1406.7	1673.33	146.67	96.7	690.00
80	853.33	1300	2633.33	70.00	116.7	485.00
75	990.00	1280	1850.00	103.33	110	503.33
70	876.67	456.7	2653.33	126.67	73.3	510.00
65	923.33	873.3	1403.33	113.33	196.7	946.67
60	893.33	N.D.	1690.00	116.67	73.3	423.33
55	816.67	170	7826.67	160.00	313.3	4400.00
50	840.00	2923.3	N.D.	196.67	1480.00	N.D.
45	1026.67	9200	N.D.	150.00	6226.67	N.D.
40	993.33	5275	N.D.	106.67	5046.67	N.D.
35	823.33	2576	N.D.	133.33	1123.33	N.D.
30	826.67	1886.7	N.D.	100.00	563.33	N.D.
25	1236.67	1525	N.D.	150.00	600.00	N.D.
20	1226.67	813.3	N.D.	136.67	763.33	N.D.
15	620.00	100	N.D.	220.00	676.67	N.D.
10	920.00	1013.3	N.D.	153.33	423.33	N.D.
5	890.00	2593.3	N.D.	150.00	1376.67	N.D.
0	596.67	1170	N.D.	640.00	370.00	N.D.
New	760.00	1300	N.D.	116.67	170	N.D.
Gau	850.00	2750	N.D.	183.33	116.7	N.D.
Elk	780.00	1480	6460.00	123.33	190	5096.67
Coal	1066.67	2273.3	N.D.	196.67	101.5	N.D.
Poca	1133.33	3236.7	N.D.	406.67	690	N.D.

[a] Data used for figures 11-18, and 21

[b] Erythromycin and tetracycline resistant average bacterial counts were reported as CFU per 1 ml of sample.

[c] "N.D." designates sites were not data were reported.

Appendix J. Fecal coliform counts reported per 100 ml.

	Fecal Coliforms		
River Mile/ Tributary	Spring April 5-6, 2004	Summer A July 12-13, 2004	Summer B August 5, 2004
95	300	N.D.	N.D.
90	90	N.D.	190.00
85	80	N.D.	180.00
80	120	N.D.	210.00
75	110	N.D.	3100.00
70	110	N.D.	340.00
65	100	N.D.	350.00
60	150	N.D.	520.00
55	100	N.D.	4200.00
50	460	130.00	N.D.
45	150	80.00	N.D.
40	210	40.00	N.D.
35	200	10.00	N.D.
30	100	10.00	N.D.
25	110	10.00	N.D.
20	100	0.00	N.D.
15	140	10.00	N.D.
10	270	40.00	N.D.
5	130	130.00	N.D.
0	170	90.00	N.D.
New	90	N.D.	N.D.
Gau	60	N.D.	N.D.
Elk	100	N.D.	4300.00
Coal	200	100.00	N.D.
Poca	420	30.00	N.D.

[a] Data used for figures 19-21.
[b] Fecal coliform average bacterial counts were reported as CFU per 100 ml of sample.
[c] "N.D." designates sites were not data were reported.

Appendix K . Physical parameter data collected at time of sampling.

	Spring April 5-6, 2004			Summer A July 12-13, 2004			Summer B August 5, 2004		
River Mile	Water Temp. °C Spring	Turbidity (NTU) Spring	pH (units) Spring	Water Temp. °C Summer A	Turbidity (NTU) Summer A	pH (units) Summer A	Water Temp. °C Summer B	Turbidity (NTU) Summer B	pH (units) Summer B
95	6.90	20.00	8.30	27	5	8.1	N.D.	N.D.	N.D.
90	7.40	14.00	8.10	27.2	3	8.3	25.4	16	8.1
85	7.50	14.00	8.10	27.8	N.D.	8.6	25.9	20	8.1
80	7.80	15.00	8.30	27.4	6	8.3	25.9	23	8.1
75	8.00	15.00	8.30	28.8	6	8.4	25.6	24	8
70	8.10	16.00	8.40	29	N.D.	8.6	26.5	21	8
65	8.30	17.00	8.40	28.7	8	8.5	26.1	28	8
60	8.40	18.00	8.30	28.6	8	8.4	26.2	23	8
55	8.40	18.00	8.00	28.8	8	8.4	26.3	19	8
50	8.10	22.00	8.10	28.7	8	8.4	N.D.	N.D.	N.D.
45	8.10	18.00	8.00	29	8	8.5	N.D.	N.D.	N.D.
40	8.20	19.00	8.00	28.4	6	8.4	N.D.	N.D.	N.D.
35	8.30	21.00	7.90	28.9	8	8.7	N.D.	N.D.	N.D.
30	8.40	25.00	8.00	28.3	11	8.2	N.D.	N.D.	N.D.
25	8.50	26.00	7.90	28.2	7	8.4	N.D.	N.D.	N.D.
20	8.40	26.00	7.90	29.3	6	8.5	N.D.	N.D.	N.D.
15	8.40	28.00	7.90	28.4	13	8.6	N.D.	N.D.	N.D.
10	8.50	31.00	7.90	28.8	23	8.5	N.D.	N.D.	N.D.
5	8.60	40.00	7.80	28.2	80	8.1	N.D.	N.D.	N.D.
0	8.90	36.00	7.90	28.5	30	8.2	N.D.	N.D.	N.D.

[a] "N.D." designates sites where no data were reported.
[b] Data used for Table 5.

Appendix L. Spring Ciprofloxacin resistant bacterial impact score (range -1 to +1) determinations.

River Mile / Tributary	Average Cipro Resistant per 1 ml	Percent Rank	IS95	IS90	IS85	IS80
95	356.67	0.083	0	-1	-1	-1
90	386.67	0.208	0	0	0	0
85	443.33	0.333	0	0	0	0
80	496.67	0.458	0	0	0	0
75	373.33	0.125	0	0	-1	-1
70	353.33	0.041	-1	-1	-1	-1
65	376.67	0.166	0	0	0	-1
60	616.67	0.75	0	0	0	0
55	455.00	0.375	0	0	0	0
50	1103.33	0.916	0	1	1	1
45	1106.67	0.958	1	1	1	1
40	510.00	0.583	0	0	0	0
35	496.67	0.458	0	0	0	0
30	576.67	0.708	0	0	0	0
25	483.33	0.416	0	0	0	0
20	706.67	0.791	0	0	0	0
15	393.33	0.25	0	0	0	0
10	496.67	0.458	0	0	0	0
05	553.33	0.625	0	0	0	0
00	1033.33	0.875	0	0	1	1
coal	763.33	0.833	0	0	0	1
poca	1353.33	1	1	1	1	1
gau	440.00	0.291	0	0	0	0
new	346.67	0	-1	-1	-1	-1
elk	563.33	0.666	0	0	0	0

[a] Individual site Impact Scores (IS) were determined for the 95th (IS95), 90th (IS90), 85th (IS85), and 80th (IS80) percentile boundaries.
[b] Impact scores were interpreted as "-1" = less impacted, "0" = impacted, and "1" = more impacted.
[c] Data used for Appendix T-Z, AA, and AE

Appendix M. Spring Erythromycin resistant bacterial impact score (range -1 to +1) determinations.

River Mile / Tributary	Average Erythro Resistant per 1 ml	Percent Rank	IS95	IS90	IS85	IS80
95	1086.67	0.791	0	0	0	0
90	1126.67	0.833	0	0	0	1
85	1140.00	0.916	0	1	1	1
80	853.33	0.375	0	0	0	0
75	990.00	0.625	0	0	0	0
70	876.67	0.416	0	0	0	0
65	923.33	0.583	0	0	0	0
60	893.33	0.5	0	0	0	0
55	816.67	0.166	0	0	0	-1
50	840.00	0.291	0	0	0	0
45	1026.67	0.708	0	0	0	0
40	993.33	0.666	0	0	0	0
35	823.33	0.208	0	0	0	0
30	826.67	0.25	0	0	0	0
25	1236.67	1	1	1	1	1
20	1226.67	0.958	1	1	1	1
15	620.00	0.041	-1	-1	-1	-1
10	920.00	0.541	0	0	0	0
05	890.00	0.458	0	0	0	0
00	596.67	0	-1	-1	-1	-1
coal	1066.67	0.75	0	0	0	0
poca	1133.33	0.875	0	0	1	1
gau	850.00	0.333	0	0	0	0
new	760.00	0.083	0	-1	-1	-1
elk	780.00	0.125	0	0	-1	-1

[a] Individual site Impact Scores (IS) were determined for the 95th (IS95), 90th (IS90), 85th (IS85), and 80th (IS80) percentile boundaries.
[b] Impact scores were interpreted as "-1" = less impacted, "0" = impacted, and "1" = more impacted.
[c] Data used for Appendix T-Z, AA, and AE

Appendix N. Spring Tetracycline resistant bacterial impact score (range -1 to +1) determinations.

River Mile / Tributary	Average Tet Resistant per 1 ml	Percent Rank	IS95	IS90	IS85	IS80
95	146.67	0.5	0	0	0	0
90	86.67	0.041	-1	-1	-1	-1
85	146.67	0.5	0	0	0	0
80	70.00	0	-1	-1	-1	-1
75	103.33	0.125	0	0	-1	-1
70	126.67	0.375	0	0	0	0
65	113.33	0.208	0	0	0	0
60	116.67	0.25	0	0	0	0
55	160.00	0.75	0	0	0	0
50	196.67	0.833	0	0	0	1
45	150.00	0.583	0	0	0	0
40	106.67	0.166	0	0	0	-1
35	133.33	0.416	0	0	0	0
30	100.00	0.083	0	-1	-1	-1
25	150.00	0.583	0	0	0	0
20	136.67	0.458	0	0	0	0
15	220.00	0.916	0	1	1	1
10	153.33	0.708	0	0	0	0
05	150.00	0.583	0	0	0	0
00	640.00	1	1	1	1	1
coal	196.67	0.833	0	0	0	1
poca	406.67	0.958	1	1	1	1
gau	183.33	0.791	0	0	0	0
new	116.67	0.25	0	0	0	0
elk	123.33	0.333	0	0	0	0

[a] Individual site Impact Scores (IS) were determined for the 95th (IS95), 90th (IS90), 85th (IS85), and 80th (IS80) percentile boundaries.
[b] Impact scores were interpreted as "-1" = less impacted, "0" = impacted, and "1" = more impacted.
[c] Data used for Appendix T-Z, AA, and AE

Appendix O. Spring Fecal Coliforms impact score (range -1 to +1) determinations.

River Mile / Tributary	Average Fecal Coliforms/ 100 ml	Percent Rank	IS95	IS90	IS85	IS80
95	300.00	0.916	0	1	1	1
90	90.00	0.083	0	-1	-1	-1
85	80.00	0.041	-1	-1	-1	-1
80	120.00	0.5	0	0	0	0
75	110.00	0.375	0	0	0	0
70	110.00	0.375	0	0	0	0
65	100.00	0.166	0	0	0	-1
60	150.00	0.625	0	0	0	0
55	100.00	0.166	0	0	0	-1
50	460.00	1	1	1	1	1
45	150.00	0.625	0	0	0	0
40	210.00	0.833	0	0	0	1
35	200.00	0.75	0	0	0	0
30	100.00	0.166	0	0	0	-1
25	110.00	0.375	0	0	0	0
20	100.00	0.166	0	0	0	-1
15	140.00	0.583	0	0	0	0
10	270.00	0.875	0	0	1	1
05	130.00	0.541	0	0	0	0
00	170.00	0.708	0	0	0	0
coal	200.00	0.75	0	0	0	0
poca	420.00	0.958	1	1	1	1
gau	60.00	0	-1	-1	-1	-1
new	90.00	0.083	0	-1	-1	-1
elk	100.00	0.166	0	0	0	-1

[a] Individual site Impact Scores (IS) were determined for the 95th (IS95), 90th (IS90), 85th (IS85), and 80th (IS80) percentile boundaries.
[b] Impact scores were interpreted as "-1" = less impacted, "0" = impacted, and "1" = more impacted.
[c] Data used for Appendix T-Z, AA, and AE

Appendix P. Summer Ciprofloxacin resistant bacterial impact score (range -1 to +1) determinations.

River Mile / Tributary	Average Cipro Resistant per 1 ml	Percent Rank	IS95	IS90	IS85	IS80
95	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
90	2330	0.809	0	0	0	1
85	1180	0.19	0	0	0	0
80	1795	0.619	0	0	0	0
75	1850	0.666	0	0	0	0
70	1520	0.333	0	0	0	0
65	1667	0.523	0	0	0	0
60	990	0.047	-1	-1	-1	-1
55	6167	0.952	1	1	1	1
50	1010	0.095	0	-1	-1	-1
45	1617	0.428	0	0	0	0
40	1737	0.571	0	0	0	0
35	2353	0.857	0	0	1	1
30	1937	0.714	0	0	0	0
25	1660	0.476	0	0	0	0
20	1370	0.238	0	0	0	0
15	1590	0.38	0	0	0	0
10	940	0	-1	-1	-1	-1
05	1433	0.285	0	0	0	0
00	1107	0.142	0	0	-1	-1
coal	3560	0.904	0	1	1	1
poca	2213	0.761	0	0	0	0
gau	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
new	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
elk	9120	1	1	1	1	1

[a] Individual site Impact Scores (IS) were determined for the 95th (IS95), 90th (IS90), 85th (IS85), and 80th (IS80) percentile boundaries.
[b] Impact scores were interpreted as "-1" = less impacted, "0" = impacted, and "1" = more impacted.
[c] Data used for Appendix T-Z, AA, and AE .
[d] "N.D." = No Data Reported.

Appendix Q. Summer Erythromycin resistant bacterial impact score (range -1 to +1) determinations.

River Mile / Tributary	Average Erythro Resistant per 1 ml	Percent Rank	IS95	IS90	IS85	IS80
95	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
90	2785.00	0.714	0	0	0	0
85	1673.33	0.285	0	0	0	0
80	2633.33	0.619	0	0	0	0
75	1850.00	0.38	0	0	0	0
70	2653.33	0.666	0	0	0	0
65	1403.33	0.19	0	0	0	-1
60	1690.00	0.333	0	0	0	0
55	7826.67	0.952	1	1	1	1
50	2923.33	0.761	0	0	0	0
45	9200.00	1	1	1	1	1
40	5275.00	0.857	0	0	1	1
35	2576.67	0.523	0	0	0	0
30	1886.67	0.428	0	0	0	0
25	1525.00	0.238	0	0	0	0
20	813.33	0	-1	-1	-1	-1
15	1070.00	0.095	0	-1	-1	-1
10	1013.33	0.047	-1	-1	-1	-1
05	2593.33	0.571	0	0	0	0
00	1170.00	0.142	0	0	-1	-1
coal	2273.33	0.476	0	0	0	0
poca	3236.67	0.809	0	0	0	1
gau	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
new	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
elk	6460.00	0.904	0	1	1	1

[a] Individual site Impact Scores (IS) were determined for the 95th (IS95), 90th (IS90), 85th (IS85), and 80th (IS80) percentile boundaries.
[b] Impact scores were interpreted as "-1" = less impacted, "0" = impacted, and "1" = more impacted.
[c] Data used for Appendix T-Z, AA, and AE. [d] "N.D." = No Data Reported

Appendix R. Summer Tetracycline resistant bacterial impact score (range -1 to +1) determinations.

River Mile / Tributary	Average Tet Resistant per 1 ml	Percent Rank	IS95	IS90	IS85	IS80
95	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
90	666.67	0.38	0	0	0	0
85	690.00	0.476	0	0	0	0
80	485.00	0.142	0	0	-1	-1
75	503.33	0.19	0	0	0	-1
70	510.00	0.238	0	0	0	0
65	946.67	0.619	0	0	0	0
60	423.33	0.047	-1	-1	-1	-1
55	4400.00	0.857	0	0	1	1
50	1480.00	0.809	0	0	0	1
45	6226.67	1	1	1	1	1
40	5046.67	0.904	0	1	1	1
35	1123.33	0.714	0	0	0	0
30	563.33	0.285	0	0	0	0
25	600.00	0.333	0	0	0	0
20	763.33	0.571	0	0	0	0
15	676.67	0.428	0	0	0	0
10	423.33	0.047	-1	-1	-1	-1
05	1376.67	0.761	0	0	0	0
00	370.00	0	-1	-1	-1	-1
coal	1015.00	0.666	0	0	0	0
poca	690.00	0.476	0	0	0	0
gau	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
new	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
elk	5096.67	0.952	1	1	1	1

[a] Individual site Impact Scores (IS) were determined for the 95th (IS95), 90th (IS90), 85th (IS85), and 80th (IS80) percentile boundaries.
[b] Impact scores were interpreted as "-1" = less impacted, "0" = impacted, and "1" = more impacted.
[c] Data used for Appendix T-Z, AA, and AE. [d] "N.D." = No Data Reported.

Appendix S. Summer Fecal Coliform impact score (range -1 to +1) determinations.

River Mile / Tributary	Average Fecal Coliforms per 100 ml	Percent Rank	IS95	IS90	IS85	IS80
95	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
90	190.00	0.666	0	0	0	0
85	180.00	0.619	0	0	0	0
80	210.00	0.714	0	0	0	0
75	3100.00	0.904	0	1	1	1
70	340.00	0.761	0	0	0	0
65	350.00	0.809	0	0	0	1
60	520.00	0.857	0	0	1	1
55	4200.00	0.952	1	1	1	1
50	130.00	0.523	0	0	0	0
45	80.00	0.38	0	0	0	0
40	40.00	0.285	0	0	0	0
35	10.00	0.047	-1	-1	-1	-1
30	10.00	0.047	-1	-1	-1	-1
25	10.00	0.047	-1	-1	-1	-1
20	0.00	0	-1	-1	-1	-1
15	10.00	0.047	-1	-1	-1	-1
10	40.00	0.285	0	0	0	0
05	130.00	0.523	0	0	0	0
00	90.00	0.428	0	0	0	0
coal	100.00	0.476	0	0	0	0
poca	30.00	0.238	0	0	0	0
gau	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
new	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
elk	4300.00	1	1	1	1	1

[a] Individual site Impact Scores (IS) were determined for the 95th (IS95), 90th (IS90), 85th (IS85), and 80th (IS80) percentile boundaries.
[b] Impact scores were interpreted as "-1" = less impacted, "0" = impacted, and "1" = more impacted.
[c] Data used for Appendix T-Z, AA, and AE. [d] "N.D." = No Data Reported

Appendix T. Relative Impact Scores (range -4 to 4) for Spring and Summer using the 90th percentile boundary (IS_{90}).

River Mile or Tributary	[a] Site Designation	[b c] Spring IS_{90}	[b c] Summer IS_{90}
New River	T	-3	N.D.
Gauley	T	-1	N.D.
95	U	0	N.D.
90	U	-2	0
85	U	0	0
80	U	-1	0
75	U	0	1
70	U	-1	0
65	U	0	0
60	U	0	-2
Elk	T	0	4
55	U	0	3
50	L	2	-1
Coal	T	0	1
45	L	1	2
Pocatalico	T	3	0
40	L	0	1
35	L	0	-1
30	L	-1	-1
25	L	1	-1
20	L	1	-2
15	L	0	-2
10	L	0	-3
5	L	0	0
0	L	0	-1

Data used for Appendix W, X, and Y figures.

Individual impact scores (IS) for each sampling season (spring or summer); ciprofloxacin-resistant (Appendix L or P), erythromycin-resistant (Appendix M or Q), tetracycline-resistant (Appendix N or R), and fecal coliform bacteria (Appendix O or S), were combined to give a Relative IS. The range of -4 to +4 is based on the 4 variables being tested (3 antibiotics, and fecal coliforms).

[a] Designation of U (Upper Kanawha between river miles KR95-KR55), L (Lower Kanawha between river miles KR50-KR00), or T (Tributary) indicates the region of the River or Tributary entering the river.

[b] Fields highlighted in red indicates an impacted area.

[c] Fields highlighted in blue indicates less impact.

Appendix U. Relative Impact Scores (range -4 to 4) for Spring and Summer using the 85th percentile boundary (IS_{85}).

River Mile or Tributary	[a] Site Designation	[b c] Spring IS_{85}	[b c] Summer IS_{85}
New River	T	-3	N.D.
Gauley	T	-1	N.D.
95	U	0	N.D.
90	U	-2	0
85	U	0	0
80	U	-1	-1
75	U	-2	1
70	U	-1	0
65	U	0	0
60	U	0	-1
Elk	T	-1	4
55	U	0	4
50	L	2	-1
Coal	T	0	1
45	L	1	2
Pocatalico	T	4	0
40	L	0	2
35	L	0	0
30	L	-1	-1
25	L	1	-1
20	L	1	-2
15	L	0	-2
10	L	1	-3
5	L	0	0
0	L	1	-3

Data used for Appendix Z, AA, and AE figures.

Individual impact scores (IS) for each sampling season (spring or summer); ciprofloxacin-resistant (Appendix L or P), erythromycin-resistant (Appendix M or Q), tetracycline-resistant (Appendix N or R), and fecal coliform bacteria (Appendix O or S), were combined to give a Relative IS. The range of -4 to +4 is based on the 4 variables being tested (3 antibiotics, and fecal coliforms).

[a] Designation of U (Upper Kanawha between river miles KR95-KR55), L (Lower Kanawha between river miles KR50-KR00), or T (Tributary) indicates the region of the River or Tributary entering the river.

[b] Fields highlighted in red indicates an impacted area.

[c] Fields highlighted in blue indicates less impact.

Appendix V. Relative Impact Scores (range -4 to 4) for Spring and Summer using the 80th percentile boundary (IS_{80}).

River Mile or Tributary	[a] Site Designation	[b c] Spring IS_{80}	[b c] Summer IS_{80}
New River	T	-3	N.D.
Gauley	T	-1	N.D.
95	U	0	N.D.
90	U	-2	1
85	U	0	0
80	U	-1	-1
75	U	-2	0
70	U	-1	0
65	U	0	0
60	U	0	-1
Elk	T	-1	4
55	U	0	4
50	L	2	0
Coal	T	0	1
45	L	1	2
Pocatalico	T	4	1
40	L	0	2
35	L	0	0
30	L	-1	-1
25	L	1	-1
20	L	1	-2
15	L	0	-2
10	L	1	-3
5	L	0	0
0	L	1	-3

Data used for Appendix AC, AD, and AE figures.

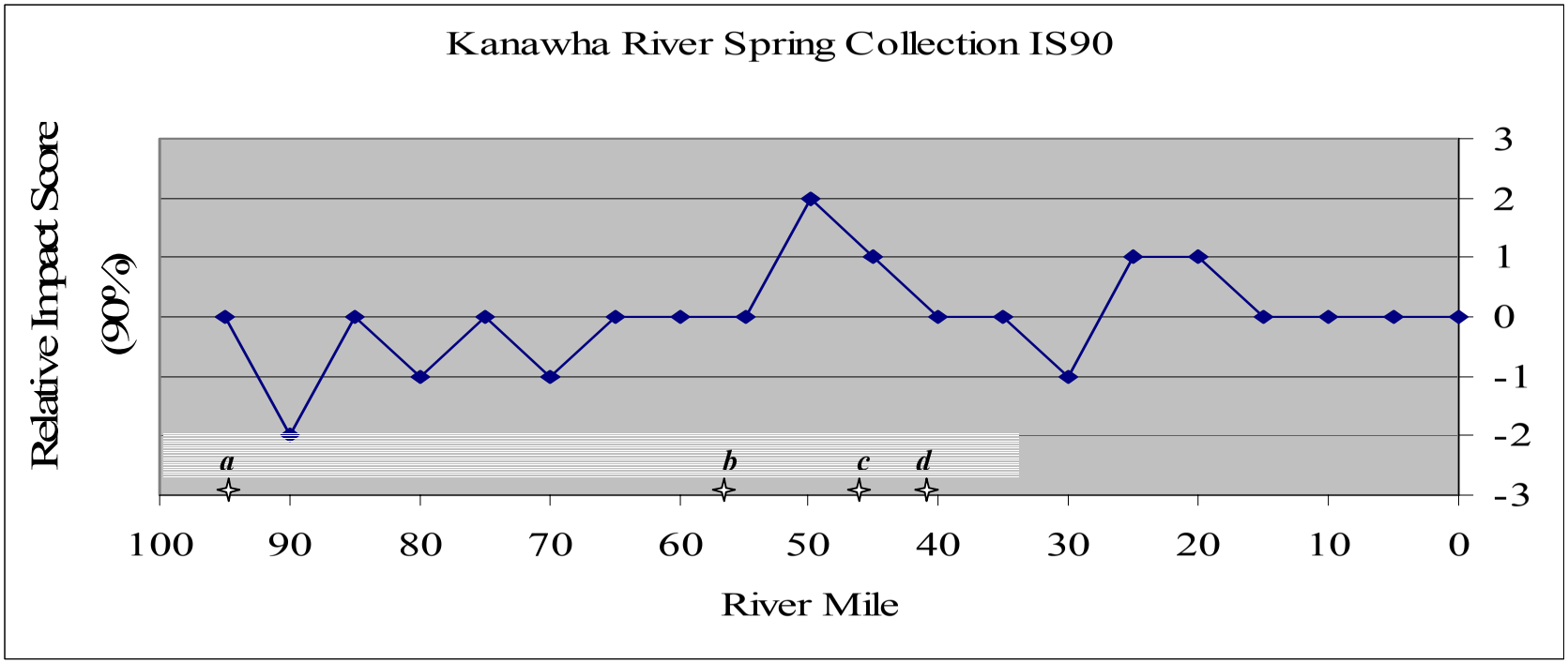
Individual impact scores (IS) for each sampling season (spring or summer); ciprofloxacin-resistant (Appendix L or P), erythromycin-resistant (Appendix M or Q), tetracycline-resistant (Appendix N or R), and fecal coliform bacteria (Appendix O or S), were combined to give a Relative IS. The range of -4 to +4 is based on the 4 variables being tested (3 antibiotics, and fecal coliforms).

[a] Designation of U (Upper Kanawha between river miles KR95-KR55), L (Lower Kanawha between river miles KR50-KR00), or T (Tributary) indicates the region of the River or Tributary entering the river.

[b] Fields highlighted in red indicates an impacted area.

[c] Fields highlighted in blue indicates less impact.

Appendix W. Kanawha River spring relative impact scores for the main stem using the 90th percentile boundary.



✧Indicates entry point of a tributary

[a] New and Gauley River enters the main stem at the headwaters KR95

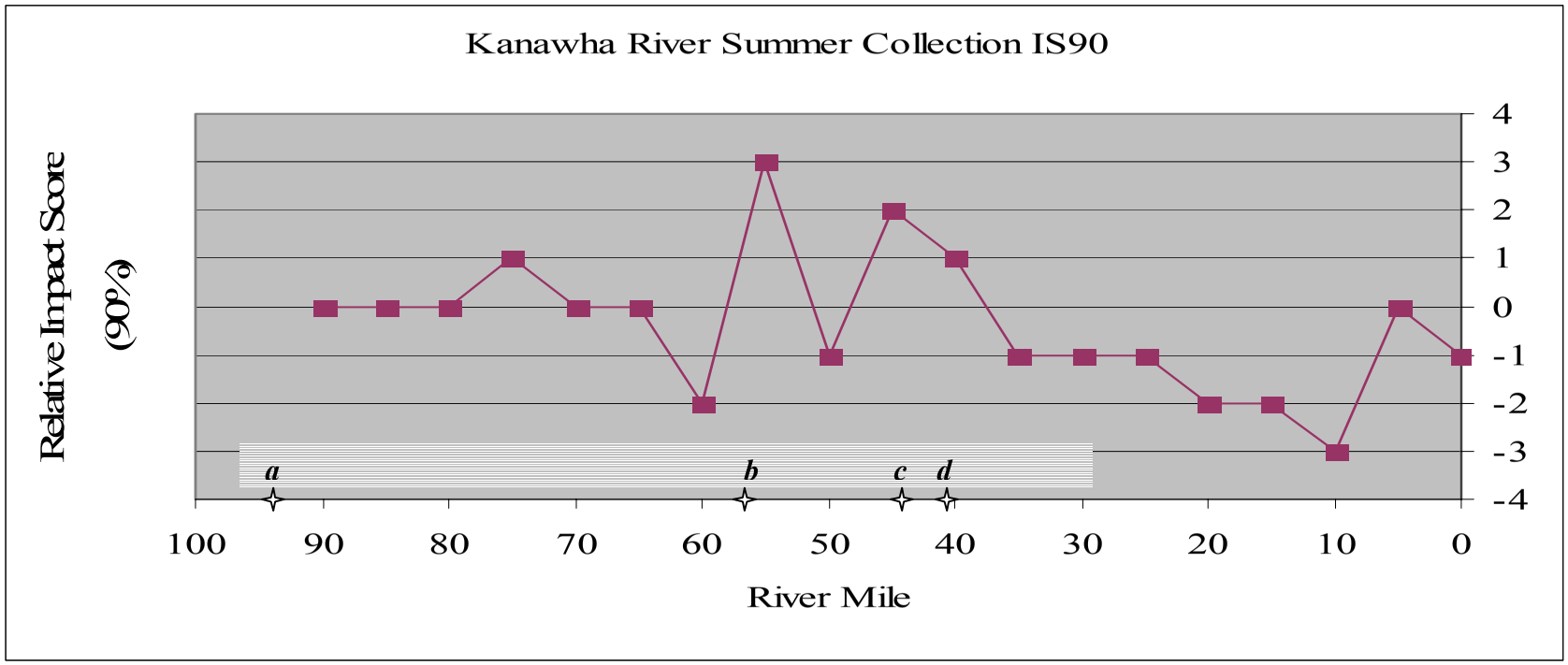
[b] Elk River enters the main stem at KR57.5

[c] Coal River enters the main stem at KR45

[d] Pocatalico River enters the main stem at KR41

Relative impact scores for the 90th percentile boundary for the spring collection are at or below zero toward the origin between river miles 95-55, reach highest levels at river mile 50 and show a trend returning levels to those similar to the upper part of the river for miles 40-00 toward the mouth. One exception for the noticeable trend occurred between river miles 20-25 which showed an increase in the relative impact score.

Appendix X. Kanawha River summer relative impact scores for the main stem using the 90th percentile boundary.



✧Indicates entry point of a tributary

[a] New and Gauley River enters the main stem at the headwaters KR95

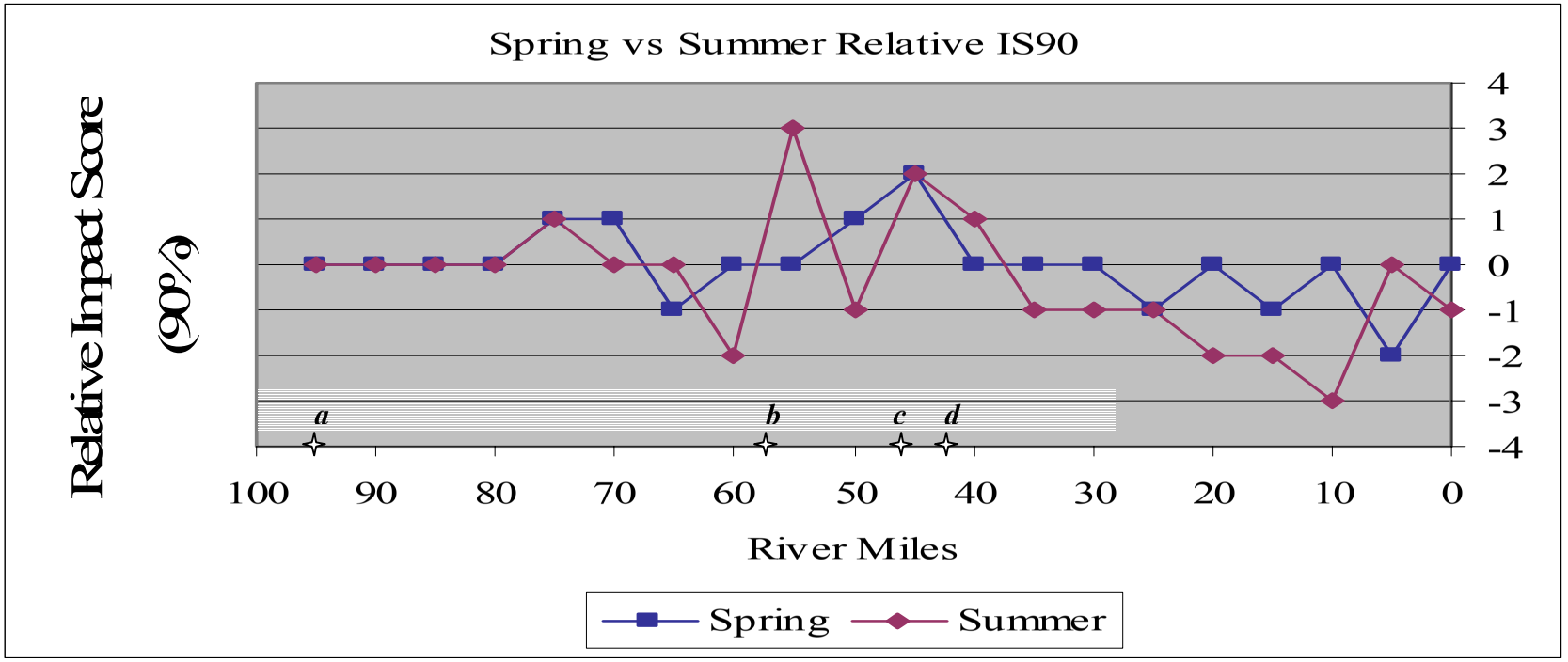
[b] Elk River enters the main stem at KR57.5

[c] Coal River enters the main stem at KR45

[d] Pocatalico River enters the main stem at KR41

Relative impact scores at the 90th percentile boundary for the summer collections show a general trend at or about zero toward the origin for river miles 90-60 reach highest point at river mile 55 and begins a gradual decline between river miles 45-00 toward the mouth.

Appendix Y. Kanawha River relative impact score spring and summer comparison for the main stem using the 90th percentile boundary.



✧Indicates entry point of a tributary

[a] New and Gauley River enters the main stem at the headwaters KR95

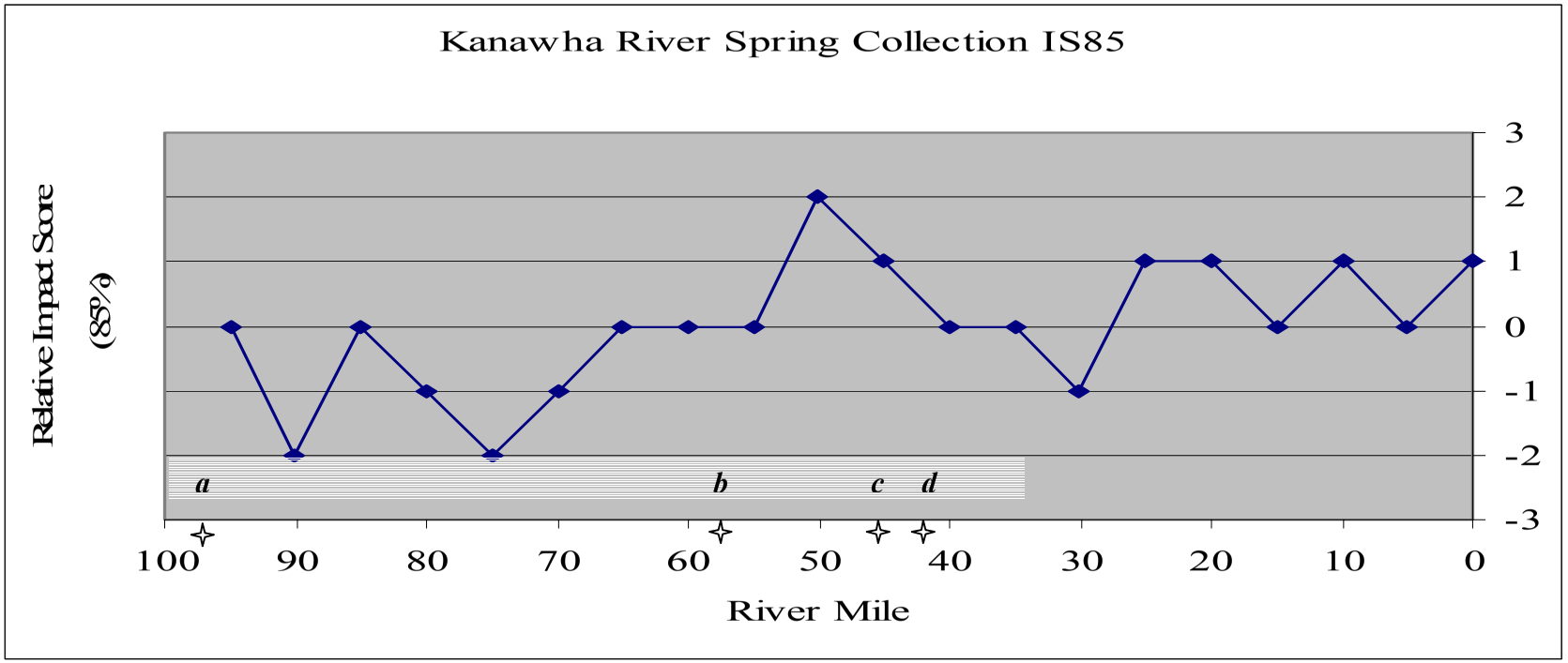
[b] Elk River enters the main stem at KR57.5

[c] Coal River enters the main stem at KR45

[d] Pocatalico River enters the main stem at KR41

A comparison of spring and summer relative impact scores at the 90th percentile boundary shows river trends are relatively similar for both sampling seasons. Trends appear to be occurring in approximately the same locations although not with the same level of impact. For spring (river miles 95-50) and summer (river miles 90-60) trends are at or about zero, reach highest levels between river miles 55-45 (industrialized area) and then return to about or below zero for river miles 35-00. Both seasons show an increase at river mile 75 (Goat Island).

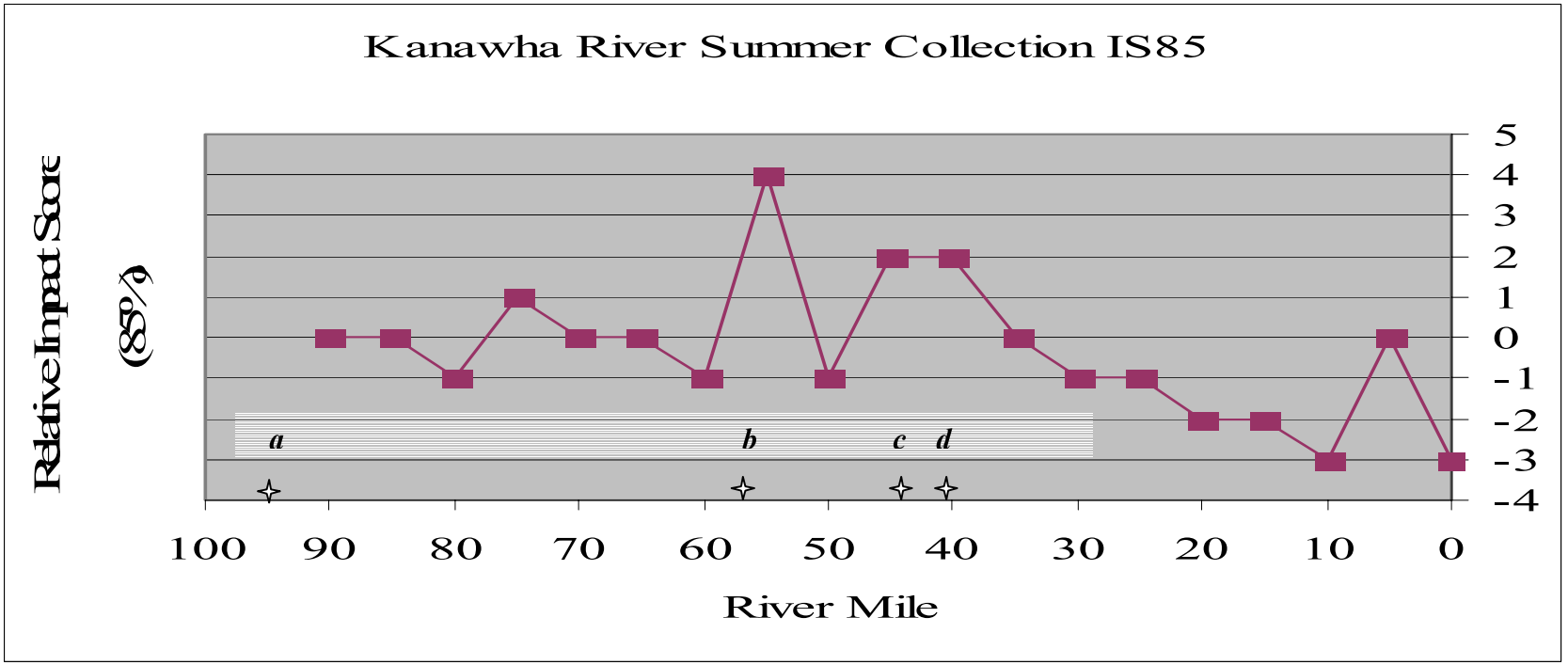
Appendix Z. Kanawha River spring relative impact scores for the main stem using the 85th percentile boundary.



✧Indicates entry point of a tributary
[a] New and Gauley River enters the main stem at the headwaters KR95
[b] Elk River enters the main stem at KR57.5
[c] Coal River enters the main stem at KR45
[d] Pocatalico River enters the main stem at KR41

Relative impact scores for the 85th percentile boundary for the spring collection are at or below zero toward the origin between river miles 95-55, reach highest levels at river mile 50 (Industrialized area) and show a trend returning levels to at or about zero for the lower part of the river, for miles 40-00 toward the mouth.

Appendix AA. Kanawha River summer relative impact scores for the main stem using the 85th percentile boundary.



✧Indicates entry point of a tributary

[a] New and Gauley River enters the main stem at the headwaters KR95

[b] Elk River enters the main stem at KR57.5

[c] Coal River enters the main stem at KR45

[d] Pocatalico River enters the main stem at KR41

Relative impact scores at the 85th percentile boundary for the summer collections show a general trend at or about zero toward the origin for river miles 90-60 reach highest point at river mile 55 (Industrialized area) and begins a gradual decline in impact between river miles 45-00 toward the mouth.

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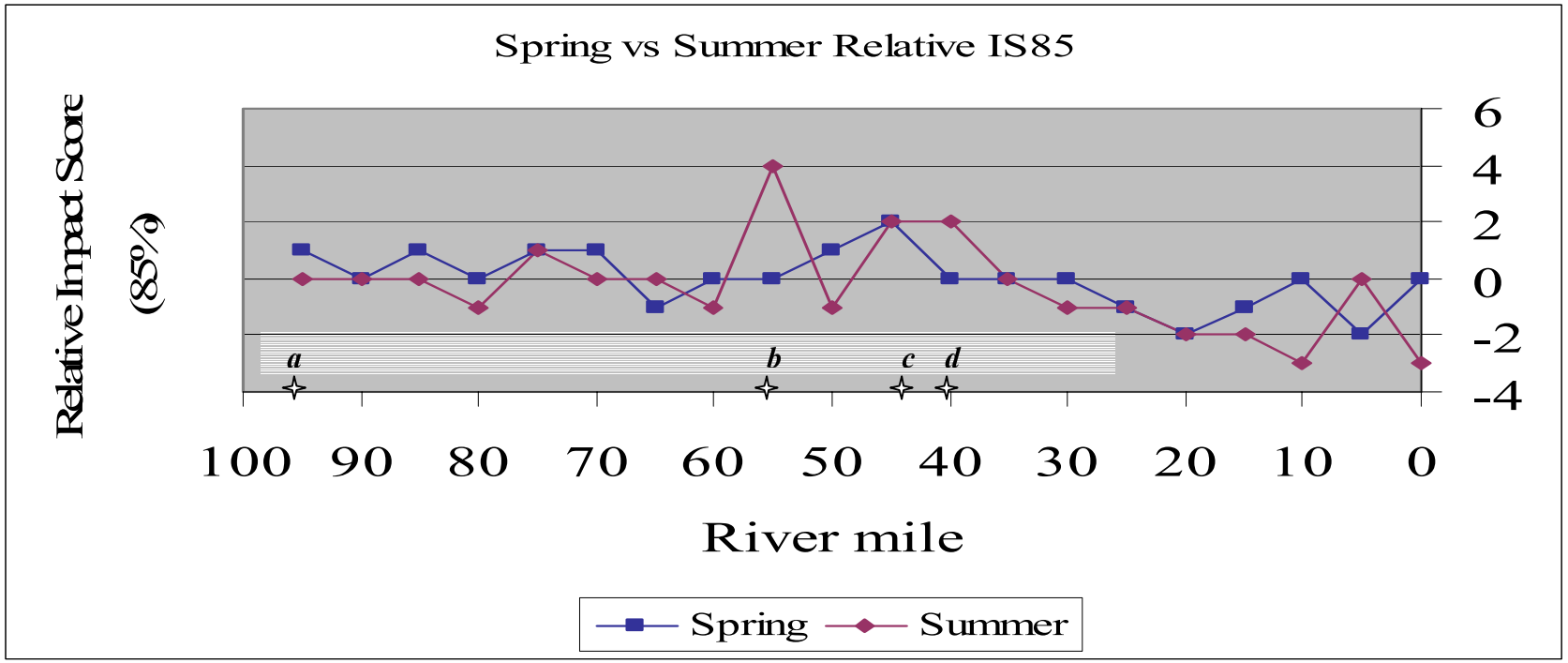
Appendix AB. Kanawha River relative impact score spring and summer comparison for the main stem using the 85th percentile boundary.



✧Indicates entry point of a tributary

[a] New and Gauley River enters the main stem at the headwaters KR95

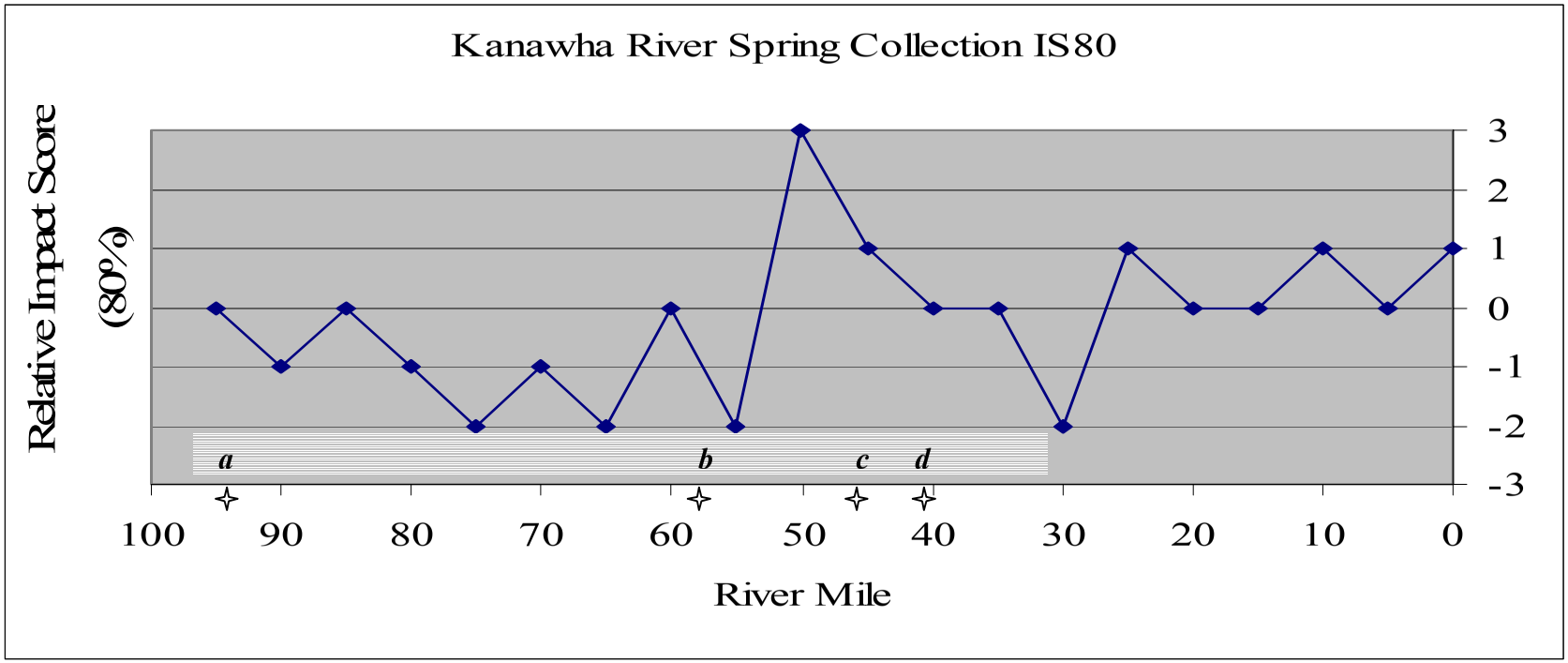
[b] Elk River enters the main stem at KR57.5

[c] Coal River enters the main stem at KR45

[d] Pocatalico River enters the main stem at KR41

A comparison of spring and summer relative impact scores at the 85th percentile boundary shows river trends are relatively similar for both sampling seasons. Trends appear to be occurring in approximately the same locations although not with the same level of impact. For spring (river miles 95-50) and summer (river miles 90-60) trends are at or about zero, reach highest levels between river miles 55-40 (industrialized area) and then return to about or below zero for river miles 35-00. Both seasons show an increase at river mile 75 (Goat Island).

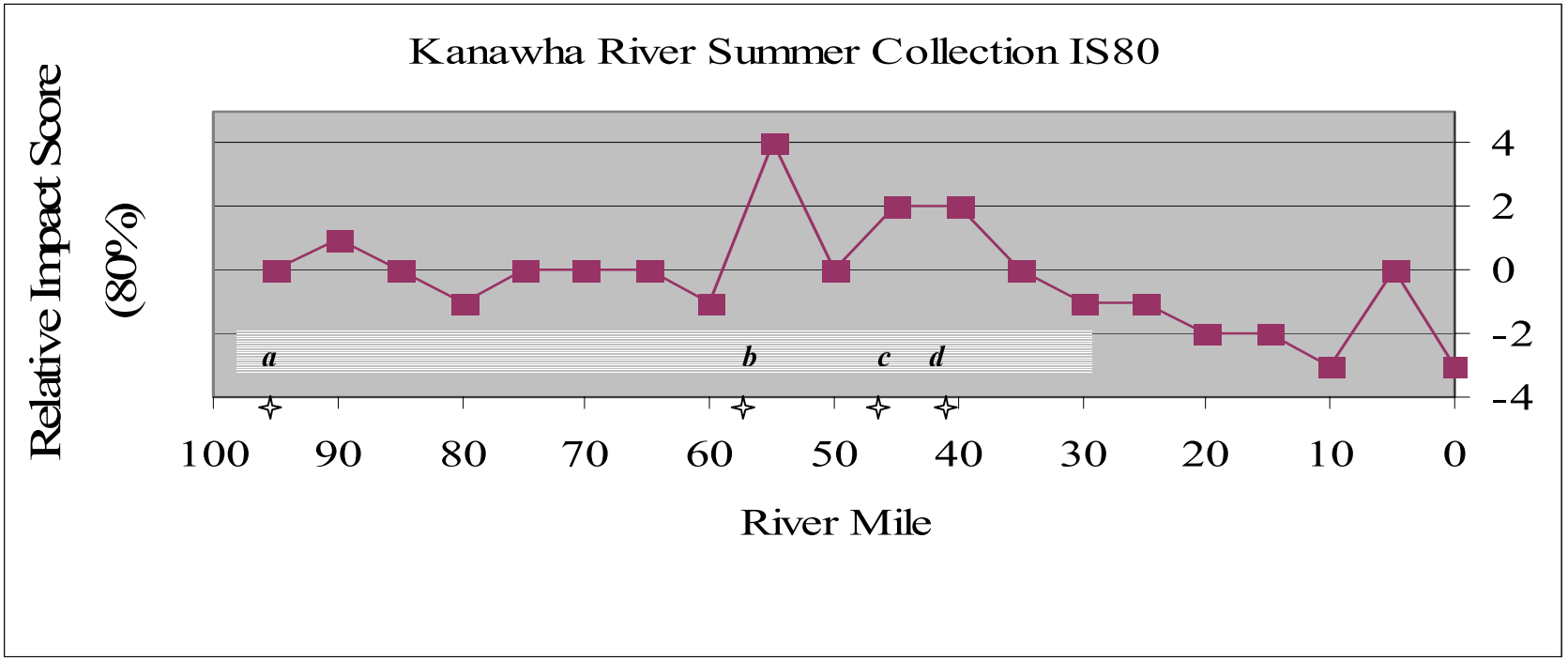
Appendix AC. Kanawha River spring relative impact scores for the main stem using the 80th percentile boundary.



✧Indicates entry point of a tributary
[a] New and Gauley River enters the main stem at the headwaters KR95
[b] Elk River enters the main stem at KR57.5
[c] Coal River enters the main stem at KR45
[d] Pocatalico River enters the main stem at KR41

Relative impact scores for the 80th percentile boundary for the spring collection are at or below zero toward the origin between river miles 95-55, reach highest levels at river mile 50 (Industrialized area) and show a trend returning levels to at or about zero for the lower part of the river, for miles 45-00 toward the mouth.

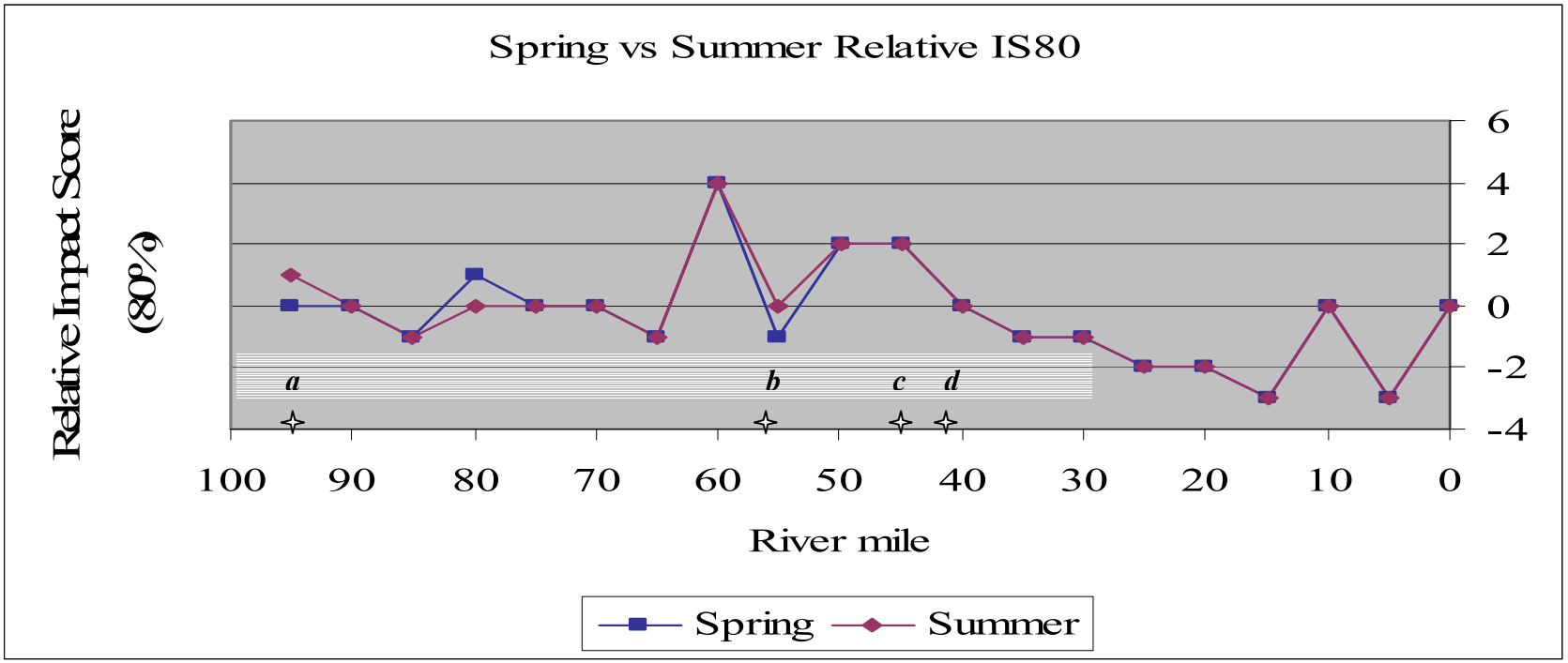
Appendix AD. Kanawha River summer relative impact scores for the main stem using the 80th percentile boundary.



✧Indicates entry point of a tributary
[a] New and Gauley River enters the main stem at the headwaters KR95
[b] Elk River enters the main stem at KR57.5
[c] Coal River enters the main stem at KR45
[d] Pocatalico River enters the main stem at KR41

Relative impact scores at the 80th percentile boundary for the summer collections show a general trend at or about zero toward the origin for river miles 90-60 reach highest point at river mile 55 (Industrialized area) and begins a gradual decline in impact between river miles 45-00 toward the mouth.

Appendix AE. Kanawha River relative impact score spring and summer comparison for the main stem using the 80th percentile boundary.



✧Indicates entry point of a tributary

[a] New and Gauley River enters the main stem at the headwaters KR95

[b] Elk River enters the main stem at KR57.5

[c] Coal River enters the main stem at KR45

[d] Pocatalico River enters the main stem at KR41

A comparison of spring and summer relative impact scores at the 80th percentile boundary shows river trends are relatively similar for both sampling seasons. Trends appear to be occurring in approximately the same locations. For spring (river miles 95-65) and summer (river miles 90-65) trends are at or about zero, reach highest levels between river miles 60-45 (industrialized area) and then return to about or below zero for river miles 40-00.

VITA

Christina Carole (Young) Johnson was born on December 23, 1975, in Montgomery, West Virginia. She was educated in local public schools and graduated from DuPont High School in 1994. She entered West Virginia University Institute of Technology (Formerly West Virginia Institute of Technology), Montgomery, West Virginia during the fall of 1994. She graduated earning two degrees, a Bachelor of Science in Biology and an Associate in Science in Business Technology with an Accounting Emphasis, in December, 2000.

After unsuccessfully seeking full time employment within the scientific community and working part time as a laboratory instructor for West Virginia University Institute of Technology, Montgomery, West Virginia, Mrs. Johnson began a Master's program in Biological Sciences at Marshall University. During the duration of her studies at Marshall University, she worked as a graduate teaching assistant for Marshall University.

Mrs. Johnson is a member of the American Society for Microbiology and the Association of Southeastern Biologists.