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Occurrence and Distribution of Multi-Antibiotic Resistant Bacteria from the Great Kanawha River, West Virginia

Thesis submitted to
The Graduate College of
Marshall University

In Partial Fulfillment of the
Requirements for the Degree of
Master of Science
Biological Sciences

By

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May 8, 2006

Abstract

Occurrence and Distribution of Multi-Antibiotic Resistant Bacteria from the Great Kanawha River, West Virginia

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During the spring and summer of 2004 subsurface mid-channel samples were collected from the Kanawha River and its five primary tributaries (New, Gauley, Elk, Coal and Pocatalico Rivers). The first two objectives of this study were to enumerate bacteria resistant to ciprofloxacin, erythromycin or tetracycline, and test them for multiple resistance to seven commonly used antibiotics. The third objective was to determine the Minimum Inhibitory Concentration (MIC) for seven antibiotics starting at concentrations 20 times the published working concentrations for Gram-negative bacteria. The final objective of this study was to determine if a novel Impact Scoring system incorporating a current water quality indicator, fecal coliforms, and new indicators, antibiotic resistant bacteria could be applied to the Kanawha River. All of the isolates (n = 60) were resistant to 3 or more of the 7 antibiotics tested. Ninety-five percent were resistant to 4 or more, 92% were resistant to 5 or more, 88% were resistant to 6 or more and 81% were resistant to all seven antibiotics. One-hundred percent exhibited resistance to tetracycline. Ninety-eight percent exhibited resistance to ampicillin and sulfamethizole. Ninety-five percent exhibited resistance to ciprofloxacin and 93% were resistant to erythromycin, streptomycin, and virginiamycin. Isolates in non-industrialized regions exhibited sensitivity to some of the antibiotics tested. Isolates collected in industrial regions exhibited resistance to all seven antibiotics. These findings suggest that multiple antibiotic resistance (MAR) may be associated with industrialization on the river.

DEDICATION

I would like to dedicate this work to the memory of my Grandfather Donald E. Ward who passed away April 16, 1998. I promised you I would finish my education and not a day goes by that I don’t think about you and that promise I made before you went away. Though you are not here in body, you are here in spirit guiding me day after day. I wish you were here! I love and miss you more each day!

ACKNOWLEDGEMENTS

At this time I would like to acknowledge first and foremost my husband and best friend James who has supported me every step of the way. To my sister Christina and her daughter Sydney (Moody) Johnson for living with Jordan and me for the past two years, you always have my back! I could not have survived without you. Thank you to Andy, my brother-in-law, for supporting Christina's decision to stay with me in Huntington. To my Dad, C. Roger Young, thank you for allowing Alex to stay with you during the school year so he did not have to leave his friends. Thank you to my son Alex for adjusting so well. Thank you to my entire family for your interest in what I have been doing and for the emotional support during such a disruptive time in our lives.

Thank you to everyone in the Environmental Microbiology Research Laboratory at Marshall University. To Andy Johnson and Lisa Smith for going out on the boat and collecting samples. To Kathy Loughman for helping to prepare media and running samples in the lab. To Chuck Somerville for introducing me to the importance of freshwater monitoring, helping to develop my project and introducing me to the people that helped me get it done. Most of all I thank the EMRL staff for being good friends.

Thanks to Steve Foster at the Army Corps of Engineers for allowing me to tag along on the river and collect water samples. I also want to thank Teresa Fogus for the great GPS map she made using the sample site information.

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CHAPTER I

Introduction

Antibiotic Resistance

Since before the discovery of penicillin by Alexander Fleming in 1929 and the implementation of antibiotics for the treatment of bacterial diseases in the 1940s, bacteria have been exhibiting natural mechanisms of antibiotic resistance. However in recent decades increased bacterial resistance to antibiotics has assumed an increasing importance with regard to its impact on both public and environmental health (1). At present, we are faced with a global increase in the incidence of antibiotic resistance, due to wide and often indiscriminate use of antibiotics in medical and veterinary practices, as well as the agricultural and domestic use of pesticides containing antibiotics and related compounds (3, 28, 29). Changes in the occurrence and levels of antibiotic resistance are not confined to particular bacterial populations and may reflect responses to increased exposure of bacteria to antimicrobial compounds over the past several decades (21). Studies by McArthur and Tuckfield suggest evidence that antibiotic resistance selection can also occur in the absence of antibiotic exposure in the environment (33).

The primary problem presented by the emergence of antibiotic resistant bacteria pathogenic to humans and animals is the difficulty in treating some potentially life-threatening diseases (1, 13). Bacteria are resilient organisms with the ability to adapt to the harsh nature of their environment. Introducing antibiotics, metal compounds and other compounds into the environment *via* point source and non-point source contamination has selected for bacteria with many different mechanisms to withstand the toxic effects of antibiotics. These include molecular mechanisms: reduced drug uptake;

active drug efflux; modification of the drug target; increasing the concentration of the drug target and drug deactivation. Other modes of resistance include natural and acquired mechanisms. Acquisition of resistance can occur by horizontal gene transfer, as well as chromosomal mutations or intercellular transfer of resistance genes through conjugation (direct contact), transformation (indirect contact using surrounding medium) or transduction (bacteriophage) vectors (Appendix B – D).

Currently little quantitative data can be found on the extent of the antibiotic resistance problem. The ecological consequences associated with the dissemination of resistant bacteria in the environment have been scarcely investigated (13, 29). Concern is growing about antimicrobials affecting water quality because they may be accelerating the selection for antibiotic resistant bacteria (30). Without a complete picture of the frequency and distribution of antibiotic resistance in the environment we may not be able to determine the quality of freshwater or anticipate and prevent future disease outbreaks associated with consuming contaminated water. Observing pH, Dissolved Oxygen, heavy metals, etc. may not be enough to determine the health of aquatic ecosystems which have the largest impact on all terrestrial communities from humans and animals to plants and insects.

The term antibiotic is used most commonly to refer to a substance produced by, or a semi-synthetic substance derived from, a microorganism, such as a fungus or bacterium, and able in dilute concentrations to inhibit or kill other microorganisms (44). Antibiotics are substances that selectively inhibit the invading pathogenic organism without harming the host. Their selectivity is dependant on the mechanism used by the drug to damage the pathogen. Antibiotics show varying ranges of host toxicity, for

example the most selective drugs affect structures like the cell wall or functions like the production of folic acid which irreversibly and fatally damages the bacterial cell but does not harm the host cell. Less selective antibiotics, which may cause harm to the host cell, affect protein synthesis or nucleic acid synthesis which is essential to both prokaryotic and eukaryotic cells.

According to a survey of commonly used antibiotics by NDC Health, Inc. (53) 234.0 million antibiotic prescriptions were issued in 2003 alone (Appendix F). These antibiotics will not remain in the human or animal body for long and will ultimately be excreted and their residues will find there way into the water-table and ultimately into streams and rivers. According to a Danish survey, antibiotics and antibiotic resistant bacteria can and do survive waste water treatment and have the continued ability to pass on resistance to environmental isolates (13), even after the death of the bacterial cells.

Multiple Antibiotic Resistance

Another problem that is arising in the environment is the presence of bacteria with resistance to multiple antibiotics. Guardabassi and Dalsgaard (13) discovered that antibiotic resistant bacteria occurring in raw sewage could survive treatment and reach natural aquatic environments *via* municipal sewage treatment effluents. They also found that the resistant bacteria could survive for relatively long periods and maintain their resistance properties in the natural aquatic habitats, and that resistant strains originating from sewage are able to transfer their resistance genes to bacteria living in non-polluted habitats. Improperly operating septic systems, poor well maintenance, surface application of waste waters and direct injection have led to contamination of ground water (6) which

will, over time, seep into the streams and river systems. Previous studies have found correlations between the occurrence and distribution of antibiotic resistant bacteria in the surface waters of Australia (4), urban waste water discharge (15) and heavy metal pollution (33). These findings suggest that antibiotic resistant bacteria could provide an important indicator of water quality (47).

Even in the absence of antibiotics in the environment bacteria can exhibit resistance to antibiotics. In two independent studies it was discovered that genes encoding for antibiotic resistance were carried on the same plasmid encoded for metal resistance (51, 52). Another study suggests Multiple Antibiotic Resistance (MAR) may be the result of a single *mar* plasmid instead of multiple plasmids exhibiting resistance (12). However increased global usage of antibiotics may also be a contributing factor in the ever increasing resistance being observed in the environment.

Antibiotics Selected in the Kanawha River Study

In previous surveys on emerging contaminants in US streams five of the seven antibiotics tested in this study were found in freshwater systems along with other prescription and non-prescription drugs, hormones, wastewater products, etc (2, 20, 52). Ciprofloxacin was found in aquatic environments at ranges from 0.02 µg/L to 0.03 µg/L. Erythromycin was also found in aquatic environments ranging form 0.05 µg/L to 1.7 µg/L. Tetracycline and sulfamethizole were found in aquatic environments at ranges from 0.05 µg/L to 0.13 µg/L, and virginiamycin was found at 0.10 µg/L (20). With this knowledge data was collected to determine resistance of bacteria to ciprofloxacin, erythromycin and tetracycline from 25 predetermined sites (Figure 1, Table 1). This

information was used to determine multiple antibiotic resistance (MAR) and the spatial distribution of MAR on the Kanawha River. The information was also used to test a novel water quality index developed for the Ohio River that incorporates enumeration of antibiotic resistant and fecal coliform bacteria.

Fecal Coliforms as Water Quality Indicators

Current water quality testing uses fecal coliform counts and water chemistry analyses as a means of determining the health of aquatic systems. Microbial pollution of water in the United States is a growing crisis in environmental and public health (34) and needs to be studied extensively to determine its current and future impact on human health. According to Mara and Haran (32), the role of fecal indicator organisms is central to the reduction of this crisis which is occurring in all parts of the world. Fecal coliforms do not occur naturally in aquatic and terrestrial environments and are only found inhabiting the guts of warm-blooded animals. Due to their inability to survive in the environment for long periods of time, when found in the environment, fecal coliforms are indicative of recent fecal contamination. Sources of fecal contamination include domestic sewage, point source and non-point source runoff, containing the excretions (10^7 cells per gram of fecal matter (46)) of humans and animals. Coliforms are not the most abundant gut flora of humans and animals but they are easily cultivated and are useful indicators of recent fecal contamination (8, 46-48). Common factors contributing to fecal contamination include leaking of overflowing sewage collection systems, illegal homeowner sewage discharge by straight pipes or failing septic systems, and runoff from urban areas and agricultural lands. With knowledge of fecal coliforms as a documented

water quality indicator, samples were analyzed to determine if correlations could be found between the presence of fecal coliforms in the Kanawha River and antibiotic resistant bacteria.

Study Area

The Great Kanawha River is the 10th most commercially traveled river in the United States and, at 99.5 river miles in length, is the largest river to be wholly contained within the borders of West Virginia. The flow of the Kanawha takes it through industrialized and agricultural areas that have major impacts on its aquatic microbial communities. The Kanawha provides for both domestic and industrial use, and is an important recreation resource in the region. The Kanawha River and its tributaries supply an estimated 360,000 West Virginians (20% of the state's population) with drinking water.

Antibiotic resistance studies have been conducted on other aquatic habitats such as the Ohio River, but, prior to this study, had never been studied in the Kanawha River. Previous studies of the river primarily focused on benthic species, fish, mollusks and potentially hazardous vegetation (5, 9, 14, 19, 23, 25, 31, 40-42, 45, 49, 50). Antibiotic resistance data from this study will provide valuable information to aid in future studies to determine the contributing agent(s) for antibiotic selectivity on the Kanawha River.

Study Objectives

Objectives one and two of this study were to enumerate bacteria resistant to ciprofloxacin, erythromycin or tetracycline, and to test those isolates for multiple

resistance to commonly used antibiotics, including ampicillin, streptomycin, sulfamethizole, virginiamycin, ciprofloxacin, erythromycin and tetracycline. This information will be used to determine the spatial distribution of Multiple Antibiotic Resistance (MAR) on the mainstem of the Kanawha River. Spatial distribution information will be used to identify areas more susceptible to multiple antibiotic resistance. In this survey we are trying to determine if industrialized areas are more susceptible to MAR than the less industrialized areas.

Objective three of this study was to determine the Minimum Inhibitory Concentration (MIC) for the seven antibiotics tested starting at concentrations 20 times the published working concentrations for Gram-negative bacteria (46-48). This information will be useful in determining if antibiotic concentrations that are used in health care applications are relevant to resistance characteristics of environmental isolates.

The final objective of this study was to determine if a novel Impact Scoring system originally developed for the Ohio River could be applied the Kanawha River. The Impact Scoring system includes a current water quality indicator, fecal coliforms, and new indicators, antibiotic resistant bacteria. The Impact Scoring system will be described in detail in Chapter 2, Materials and Methods.

CHAPTER II

Materials and Methods

Water Sample Collections

On April 5–6, 2004 subsurface, mid-channel water samples were collected in pre-sterilized mason jars from the confluence of the New and Gauley Rivers, located in Fayette County, to Point Pleasant in Mason County every 5 river miles and from 5 tributaries (99.5 river miles, 25 samples) (Figure 1). Samples were placed on ice and transported to the environmental microbiology lab at Marshall University for microbiological analyses. A complete description, including longitude and latitude, for each sample site can be found in Tables 1-2. Summer samples were collected July 12-13, 2004 and August 5, 2004 following the same protocol as previously described.

Enumeration of Total Cultivable Bacteria

A sample bottle, stored on ice, was removed and mixed by inversion to re-suspend any sediment that may have settled out during transit to the laboratory. Aliquots (0.1 ml) of the sample were aseptically transferred to a sterile 9.9 ml dilution blank in a screw-cap test tube and mixed full speed on a vortex mixer for a minimum of 5 seconds. Aliquots (0.1 ml) of diluted sample were then aseptically transferred to each of three plates of Difco (Becton Dickinson, Sparks, MD) R2A agar plus 375 ng/ml fungizone. The diluted water sample was spread 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) until all of the liquid had been absorbed. The plates were then wrapped in parafilm, inverted and incubated at room temperature for one week prior to counting. After incubation the number of colony forming units (CFU) were counted on each plate and recorded. The

mean and standard deviation of CFU counts were determined and used to establish 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).

Enumeration of Antibiotic Resistant Bacteria

A sample bottle, stored on ice, was removed and mixed by inversion to re-suspend any sediment that may have settled out during transit to the laboratory. Aliquots (0.1 ml) of undiluted sample were aseptically transferred to each of three plates of Difco (Becton Dickinson, Sparks, MD) R2A agar plus 375 ng/ml fungizone, and ciprofloxacin (4 µg/ml), erythromycin (8 µg/ml), or tetracycline (12.5 µg/ml). The undiluted water sample was spread 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) until all of the liquid had been absorbed. Plates were clearly marked with sample number and date of inoculation. Each set of three plates were wrapped with parafilm and incubated inverted at room temperature for one week. After incubation the number of colony forming units (CFU) were counted on each of the replicate plates and recorded. The mean and standard deviation of CFU counts were determined and used to establish 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).

Enumeration of Fecal Coliform Bacteria

Fecal coliforms were enumerated using the membrane filtration technique. Aliquots (1 ml, 5 ml, and 10 ml) were transferred into 100 ml of sterile distilled water and suspended cells were trapped on 0.45 µm pore size membrane filters (Fisher Scientific, cat. No. 09-740-30D) by vacuum filtration. The filters were then transferred to plates containing m-FC medium (Gelman Sciences, Ann Arbor, MI) and incubated for 24 hours at 44.5˚ C. The typical blue colonies were counted (30-60) and the dilution (1 ml etc.) documented to estimate the number of CFU's per 100 ml.

Determination of Multiple Antibiotic Resistance

MAR (Multiple Antibiotic Resistance) was determined using samples from each site during summer collections. One colony from the most predominant colony morphology on R2A plus antibiotic from each sample site was transferred into Mueller-Hinton Broth (Difco) containing the antibiotic on which the strain was isolated. These isolates were then maintained by sub-culturing bi-weekly. The stock cultures were then transferred into Mueller-Hinton Broth (Difco) plus ampicillin (50 µg/ml), ciprofloxacin (4 µg/ml), erythromycin (8 µg/ml), streptomycin (25 µg/ml), sulfamethizole (128 µg/ml), tetracycline (12.5 µg/ml), or virginiamycin (16 µg/ml) and incubated 24 hours at 34.5 ± 2.5˚ C. Each isolate was tested in triplicate against 6 antibiotics in addition to the one on which it was isolated. The NCCLS (National Committee for Clinical Laboratory Standards) recommends the use of Mueller-Hinton Broth for antibiotic sensitivity testing due to its reproducibility (36).

Determination of Minimum Inhibitory Concentrations

The Microdilution broth technique (37) using plain Mueller-Hinton broth (PMHB) was used to determine the Minimum Inhibitory Concentration (MIC) for ampicillin (max conc. 990 µg/ml), ciprofloxacin (max conc. 70 µg/ml), erythromycin (max conc. 150 µg/ml), streptomycin (max conc. 490 µg/ml), sulfamethizole (max conc. 2550 µg/ml), tetracycline (max conc. 240 µg/ml) and virginiamycin (max conc. 310 µg/ml). Antibiotics were prepared using the Standard Operating Procedure (SOP) in Appendix G. The antibiotics were diluted in 2-fold serial dilutions from the maximum concentrations in sterile 96 well round bottom microtiter plates (Falcon) in 100µl aliquots (listed above concentration ranges are shown in Table 3). An inoculum of each isolate was prepared in plain Mueller Hinton broth, prepared according to manufacturer's suggestions, and transferred in 10 µl aliquots into each of the wells containing the antibiotic. Antibiotic concentration ranges were then adjusted to reflect the addition of the inoculum. Microtiter plates were covered and wrapped in parafilm and incubated at 34.5 ± 2.5° C for 48 hours. MICs were determined visually by the development of turbidity compared to the control (no antibiotic). Each MIC range was tested in triplicate for each culture.

Determination of Impact Scores

The Somerville method (46) using percentile ranks was used to determine the relative water quality (Impact Score) of the Kanawha River at each sample site and at the mouths of 5 major tributaries. Data from the enumeration for fecal indicators and antibiotic resistant bacteria were entered into an Excel spreadsheet. For each population

(e.g.. fecal coliforms or ciprofloxacin resistant cells), the average count for a site within the entire population data set of all sites was ranked using the PERCENTRANK function. The PERCENTRANK output was multiplied by 100 to achieve a percentile score for each data point within the entire population data set. Boundaries were then chosen for the data. For example, an IS_{90} score weights sites with population counts above the 90th percentile and below the 10th percentile. An IS_{80} score weights sites with population counts above the 80th percentile and below the 20th percentile. IS_{85} to IS_{90} scores provide a useful signal to noise ratio in the index (C. Somerville, Personal Communication). A population score of 1 was assigned to all data points that fell above the upper percentile boundary. A population score of -1 was assigned to all data points that fell below the lower percentile boundary, and a population score of 0 was assigned to all data points that fell between the chosen boundaries. The determination of population scores was repeated for all microbial populations enumerated, i.e. for each antibiotic resistant population measured and for the fecal indicator population. The total impact score (IS) was determined 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. Impact Score versus river mile is then plotted to get a visual representation of water quality variability relative to position.

Data Analyses

All data were analyzed using Microsoft® Office XP program Microsoft® Excel® version 2002.

CHAPTER III

Results

Seasonal Variation in Antibiotic Resistance

Antibiotic resistance comparisons were made for each of the three antibiotics tested (ciprofloxacin, erythromycin and tetracycline) between seasons using the Students *t*-test with unequal variances. A comparison of ciprofloxacin resistance between spring and summer seasons suggests a significant increase ($P < 0.01$) in resistant cells during the summer sampling season (Figure 2). In the tributaries the same trend occurred with mean ciprofloxacin resistance counts exhibiting an increase during the summer within four of the five tributaries (Figure 3). The Coal River was the exception exhibiting an increase in resistance to ciprofloxacin during the spring sample season.

Erythromycin resistance counts exhibited the same trend as ciprofloxacin resistance during the summer season on the mainstem and within the tributaries. Analysis indicates erythromycin resistant cells were significantly higher ($P < 0.01$) during the summer season when compared to samples collected during the spring season (Figure 4). In the five tributaries (Figure 5) all sites exhibited increased resistance to erythromycin during the summer compared to samples analyzed from the spring season (Table 4).

Tetracycline resistance counts on the mainstem exhibited the same trend as ciprofloxacin and erythromycin resistance counts being significantly higher ($P < 0.01$) during the summer season compared to the spring (Figure 6). However only three of the five tributaries exhibited increased mean resistance during the summer season (Figure 7, Table 4). The Coal River continued to follow the same trend as ciprofloxacin resistance with a mean increase in tetracycline resistance during the spring season. The Elk River

also exhibited an increase in tetracycline resistance during the spring season not exhibited with erythromycin and ciprofloxacin resistance.

Comparison of Fecal Coliform Counts to Seasonal Antibiotic Resistance

Due to a previously mentioned fecal coliform incubation error comparative analysis of spring fecal coliforms to summer fecal coliforms could not be performed on the mainstem in its entirety. The mainstem of the Kanawha River was divided into Upper Kanawha, including KR95 to KR55 sites, and Lower Kanawha, which includes sites KR50 to KR00, the confluence of the Kanawha and Ohio Rivers. Statistical comparisons were made using a Students *t*-test with unequal variances on the mainstem of the river for each of the river divisions. The Upper Kanawha exhibited a significant increase in the presence of fecal coliforms enumerated during the summer season ($P < 0.01$). The same increase in fecal coliforms was also observed in the Lower Kanawha during the summer sample season ($P = 0.01$, Figure 8). Tributary data indicated mean increases in fecal coliforms counts in three of the five tributaries during the summer season compared to mean counts during the spring. Analysis indicated increases in mean fecal coliform counts during the spring sample season in the Gauley and Coal River tributaries (Figure 9).

Statistical comparisons were also performed on fecal coliform counts to seasonal antibiotic resistance counts using the Students *t*-test with unequal variances. Fecal coliforms enumerated during the spring were compared to ciprofloxacin resistant cells enumerated during the same sample season. The analysis found a significant difference ($P < 0.01$) in fecal coliform cells versus ciprofloxacin resistant cells (Figure 10). During

the spring season mean fecal coliform counts (1.6 CFU/ml) were lower for the spring than ciprofloxacin resistance counts (5.66×10^2 CFU/ml) for the same season. During the summer the same trend occurred between fecal coliforms and ciprofloxacin resistance; however due to the previously mentioned fecal coliform incubation error the statistical analysis between summer fecal coliforms and summer ciprofloxacin resistance could only be performed on the Lower Kanawha (KR50-KR00). For the summer season the mean fecal coliform count (0.5 CFU/ml) was significantly lower ($P < 0.01$) in the Lower Kanawha compared to ciprofloxacin resistance counts (2.07×10^3 CFU/ml) for the same season (Figure 11). The same trend was observed during the spring and summer comparison; however, statistical analysis was not performed on the individual tributary sites (Figures 12-13).

The same analytical methods used to compare seasonal fecal coliforms counts to ciprofloxacin resistance counts was used for the comparisons of seasonal fecal coliform counts to erythromycin and seasonal fecal coliform counts to tetracycline resistance. During the spring ($P < 0.01$) and summer ($P < 0.01$) fecal coliforms vs. erythromycin resistance followed the same trend as ciprofloxacin (Figure 14-15). The mean fecal coliform count (1..6 CFU/ ml) was significantly lower than mean the erythromycin resistant count (8.68×10^2 CFU/ml) during both seasons. This same trend was also observed in the five tributaries during both the spring and summer seasons (Figure 16-17). Tetracycline resistance compared to fecal coliforms followed the same trend as ciprofloxacin and erythromycin during the summer but behaved differently during the spring season. During the spring season analysis indicated that the mean fecal coliform count (1.6 CFU/ml) was not significantly lower ($P = 0.49$) than the mean tetracycline

resistance count (1.60×10^2 CFU/ml) (Figure 18). This was not the case for the summer sample season. During the summer fecal coliforms were significantly lower ($P < 0.01$) than tetracycline resistant cells collected concurrently on the mainstem (Figure 19). In the tributaries fecal coliform counts and tetracycline resistance counts were observed to be higher during the spring season decreasing during the summer season (Figure 20-21) with the exception of the Elk River during the summer which indicated an increase in tetracycline resistance.

Multiple Antibiotic Resistance Distribution

Multiple antibiotic resistance distributions were estimated on mainstem bacterial isolates by testing seven antibiotics (ampicillin, ciprofloxacin, erythromycin, streptomycin, sulfamethizole, tetracycline and virginiamycin at minimum inhibitory concentrations (MICs) appropriate for Gram-negative cells. Multiple resistance was not limited to one section of the mainstem but was distributed over the entire length of the river. Areas showing the most frequent sensitivity to antibiotics occurred in the Upper Kanawha and the most resistant sites occurring in the Lower Kanawha (Figure 22). Isolates from the most resistant sites were resistant to all seven antibiotics tested. In the tributaries, the Pocatalico River was the only tributary exhibiting resistance to all seven antibiotics (Figure 23).

Mainstem Cumulative Multiple Antibiotic Resistance Percentages

Tributary data were not included in determining the percent of isolates that were resistant to the seven antibiotics tested. Cumulative data (n = 60) from the mainstem

cultures indicate that 100% of the isolates were resistant to 3 or more of the seven antibiotics tested. Ninety-five percent of the isolates were resistant to 4 or more, 92% were resistant to 5 or more, 88% were resistant to 6 or more and 81% were resistant to all 7 antibiotics tested.

On the mainstem (n = 60) 100% of the isolates were resistant to tetracycline. Ninety-eight percent of the isolates were resistant to ampicillin and sulfamethizole, 93% were resistant to erythromycin, streptomycin and virginiamycin and 95% were resistant to ciprofloxacin (Figure 24).

Mainstem Minimum Inhibitory Concentrations

The 48-h MICs of the seven antibiotics tested at each of the mainstem sites are shown in Tables 5 through 28. One-hundred percent of the cultures isolated on ciprofloxacin (4 μg/ml) were resistant to ampicillin at concentrations ranging from 0.9667 μg/ml through 247.5 μg/ml and 95% grew in the presence of ampicillin at concentrations from 495 μg/ml to 990 μg/ml (Table 5). One-hundred percent of the cultures isolated on erythromycin (12.5 μg/ml) were resistant to ampicillin at concentrations ranging from 0.9667 μg/ml through 30.94 μg/ml and 95% were resistant at concentrations from 61.88 μg/ml through 990 μg/ml (Table 6). One-hundred percent of the cultures isolated on tetracycline (12.5 μg/ml) were resistant to ampicillin (Table 7) at all concentrations.

Ninety-five percent of the cultures isolated on ciprofloxacin (4 μg/ml) were resistant to ciprofloxacin at concentrations ranging from 8.75 μg/ml through 70 μg/ml and 100% of isolates were resistant at ciprofloxacin concentrations less than 8.75 μg/l

(Table 8). One-hundred percent of the isolates initially resistant to erythromycin (8 µg/ml) and tetracycline (12.5 µg/ml) were resistant to ciprofloxacin (Tables 9-10) at all concentrations.

Ninety-five percent of cultures isolated on ciprofloxacin were resistant to erythromycin at concentrations ranging from 0.1465 µg/ml through 0.5859 µg/ml, 85% at 2.344 µg/ml, 80% at 4.688 µg/ml through 18.75 µg/ml and 75% were resistant at 37.5 µg/ml through 150 µg/ml (Table 11). Ninety-five percent of cultures isolated on erythromycin (8 µg/ml) were resistant to erythromycin at 9.375 µg/ml through 150 µg/ml (Table 12). Isolates initially resistant to tetracycline (12.5 µg/ml) were resistant to erythromycin (Table 13) at all concentrations.

One-hundred percent of cultures isolated on ciprofloxacin (4 µg/ml) grew in the presence of streptomycin at concentrations ranging from 0.4785 µg/ml through 30.625 µg/ml, 90% grew at 61.25 µg/ml and 80 % grew at 122.5 µg/ml through 490 µg/ml (Table 14). One-hundred percent of cultures isolated on erythromycin grew at streptomycin concentrations ranging from 0.4875 µg/ml through 15.313 µg/ml, 90% grew at 30.625 µg/ml and 85% grew at ranges 245 µg/ml through 490 µg/ml (Table 15). The cultures isolated on tetracycline (12.5 µg/ml) were resistant to streptomycin at all concentrations (Table 16).

One-hundred percent of cultures isolated on ciprofloxacin (4µg/ml) were resistant to sulfamethizole at concentration ranges from 2.492 µg/ml through 39.844 µg/ml, 95% grew at 79.688 µg/ml through 159.375µg/ml, 90% grew at 318.75 through 1275 µg/ml and 85% grew at 2550 µg/ml (Table 17). All (100%) cultures isolated on erythromycin

(8 μg/ml), and tetracycline (12.5 μg/ml) grew in the presence of sulfamethizole at all concentrations (Tables 18-19).

One-hundred percent of the cultures isolated on ciprofloxacin (4 μg/ml) were resistant to tetracycline at concentrations ranging from 0.2344 μg/ml through 1.875 μg/ml, 90% grew at 3.75 μg/ml through 7.5 μg/ml, 85% at 15 μg/ml through 30 μg/ml, and 80% at 60 μg/ml through 240 μg/ml (Table 20). One-hundred percent of cultures isolated on erythromycin (8 μg/ml) were resistant to tetracycline at 0.2344 μg/ml through 30 μg/ml, and 90% grew at 60 μg/ml through 240 μg/ml (Table 21). One-hundred percent of the cultures isolated on tetracycline (12.5 μg/ml) also grew in the presence of tetracycline (Table 22) at all concentrations.

One-hundred percent of cultures isolated on ciprofloxacin (4μg/ml) were resistant to virginiamycin at concentration ranges 0.3027 through 1.2109, 85% were resistant at 2.422 μg/ml, 80% at 4.844 μg/ml through 9.688 μg/ml, 75% at 19.375 μg/ml, and 65% 38.75 μg/ml through 310 μg/ml (Table 23). One-hundred percent of the isolates initially resistant to erythromycin were resistant at 0.3027 μg/ml through 2.422, 95% grew at 4.844 μg/ml through 19.375 μg/ml, and 90% from 38.75 μg/ml through 310 μg/ml (Table 24). All isolates (100%) initially cultivated on tetracycline (12.5 μg/ml) were resistant to virginiamycin (Table 25) at all concentrations.

MIC values were different for each of the cultures isolated on ciprofloxacin, erythromycin and tetracycline. MIC values were determined at each river mile when the value was within the minimum and maximum concentration ranges for that site. Site specific MIC values for the mainstem are shown in Tables 26-28.

An individual MIC value could not be determined for ampicillin using the cultures isolated on ciprofloxacin, erythromycin or tetracycline. MIC values were different for each isolate and were determined by site when a value was established. The MIC for ampicillin (Table 26) cultivated from the ciprofloxacin resistant isolate could only be determined using the isolate from KR85. At KR85 the MIC for ampicillin was determined to be 495 µg/ml and for the erythromycin resistant isolate the MIC value could only be determined from the isolate collected from KR05 (Table 27). At KR05 the MIC value for ampicillin was 61.88 µg/ml. MICs for ampicillin could not be determined for the other sample sites. The other sample site values for ampicillin were greater than the highest antibiotic concentration tested (>990 µg/ml) (Tables 26-28).

Ciprofloxacin MIC values could not be determined using the cultures isolated on ciprofloxacin (4 µg/ml) or erythromycin (8 µg/ml). All of these cultures (100%) were resistant to ciprofloxacin at all concentrations (Table 26-27).

Using the cultures isolated on ciprofloxacin (4 µg/ml) MICs were determined at sites KR95-90, KR80 and KR60 for erythromycin. At KR95 the MIC value for erythromycin was determined to be 2.344 µg/ml and at KR95 the MIC value was determined to be 0.2930 µg/ml. At KR80 the MIC for erythromycin was determined to be 4.688 µg/ml and at KR60 the MIC was 37.5 µg/ml (Table 26). Using the cultures isolated on erythromycin only KR80 sample site developed a MIC value. The MIC value of erythromycin at KR80 was determined to be 9.375 µg/ml. All other isolates tested had values greater than the highest concentration of erythromycin tested (> 150 µg/ml) (Table 27).

Cultures collected from KR90-80 and KR35 were the only isolates that produced MIC values for streptomycin tested with the cultures isolated on ciprofloxacin. At KR90 and KR80 the MIC value was determined to be 61.25 μg/ml and at sites KR85 and KR35 the MIC value was determined to be 30.625 μg/ml for streptomycin (Table 26). Using the cultures isolated on erythromycin sites KR95, KR85 and KR70 produced the only MIC values for streptomycin. At KR95 and KR85 the MIC values were determined to be 30.625 and at KR70 the MIC value was determined to be 245 μg/ml for streptomycin (Table 27).

Sulfamethizole developed MIC values at KR60 and KR35 using the cultures isolated on ciprofloxacin. At KR60 the MIC value for sulfamethizole was determined to be 637.5 μg/ml and at KR35 was 79.688 μg/ml (Table 26). A MIC value was not developed at any site using the cultures isolated on erythromycin (Table 27) all isolates (100%) were resistant to sulfamethizole at the highest concentration (> 2550) tested.

MIC values were developed for tetracycline using the cultures isolated on ciprofloxacin and erythromycin. The ciprofloxacin isolate produced MIC values at KR95-KR90, KR80 and KR60. At KR95 the MIC value was determined to be 3.75 μg/ml, at sites KR90 and KR80 the value was determined to be 15 μg/ml and at KR60 the value was determined to be 60 μg/ml for tetracycline (Table 26). Using the cultures isolated on erythromycin MIC values were developed at sites KR85 and KR75. At both KR85 and KR75 the MIC values were determined to be 60 μg/ml. MIC values could not be developed for the other sample sites, all isolates were resistant to tetracycline at the highest concentration ((> 240 μg/ml) tested.

Virginiamycin expressed the largest number of MIC values using the cultures isolated on ciprofloxacin. MIC values were developed at KR95-80, KR65-KR60 and at KR35. At KR95 and KR60 the MIC values were determined to be 38.75 µg/ml, at KR90 and KR80 the value was 2.422 µg/ml, at KR85 the value was 310 µg/ml, at KR65 the value was 4.844 µg/ml and at KR35 the value was determined to be 19.375 µg/ml for virginiamycin (Table 26). Two sample sites, KR95 and KR85, produced MIC values for virginiamycin using the cultures isolated on erythromycin. At KR95 the MIC value was determined to be 38.75 µg/ml and at KR85 the value was 4.844 µg/ml for virginiamycin (Table 27).

MIC values could not be determined for the seven antibiotics tested using the cultures isolated on tetracycline (12.5 µg/ml). All isolates (100%) grew in the presence of all seven antibiotics at there highest concentrations (Table 28).

Tributary Minimum Inhibitory Concentrations

Following the same format as with the mainstem, MIC values were developed using each of the five tributaries samples. The 48-h MIC values of the seven antibiotics tested at each of the tributaries are shown in Tables 5 through 25 and Tables 29 through 31. One-hundred percent of the cultures isolated on ciprofloxacin (4 µg/ml) grew in the presence of ampicillin at concentration ranges 0.9667 µg/ml through 3.867 µg/ml, and 80% grew in the presence of ampicillin at ranges 7.734 µg/ml through 990 µg/ml (Table 5). One-hundred percent of the cultures isolated on erythromycin (8 µg/ml) and tetracycline (12.5 µg/ml) grew in the presence of ampicillin at all concentrations (Tables 6-7).

All (100%) of the cultures isolated on ciprofloxacin (4 µg/ml), erythromycin (8 µg/ml) and tetracycline (12.5 µg/ml) were resistant to ciprofloxacin at all concentrations tested (Tables 8-10).

Eighty percent of the cultures isolated on ciprofloxacin (4 µg/ml) grew in the presence of erythromycin at concentrations of 0.1465 µg/ml through 0.2930 µg/ml and 60% grew at concentrations ranging from 0.5859 µg/ml through 150 µg/ml (Table 11). One-hundred percent of the cultures isolated on erythromycin (8 µg/ml) grew in the presence of erythromycin at concentrations ranging from 0.1465 µg/ml through 4.688 µg/ml and 80% grew in the presence of erythromycin at concentrations ranging from 9.375 µg/ml through 150 µg/ml (Table 12). Cultures isolated on tetracycline (12.5 µg/ml) were resistant to erythromycin at all concentrations (Table 13).

One-hundred percent of the cultures isolated on ciprofloxacin (4 µg/ml) were resistant to streptomycin a concentration ranges from 0.4785 µg/ml through 3.828 µg/ml, 60% grew in the presence of ciprofloxacin at concentration ranges 7.656 µg/ml through 245 µg/ml and 40% grew at 490 µg/ml (Table 14). One-hundred percent of the cultures isolated on erythromycin (8 µg/ml) and tetracycline (12.5 µg/ml) were resistant to streptomycin at all concentrations (Tables 15-16).

One-hundred percent of the cultures isolated on ciprofloxacin (4 µg/ml), erythromycin (8 µg/ml) and tetracycline (12.5 µg/ml) were resistant to sulfamethizole at all concentrations (Tables 17-19).

The cultures isolated on ciprofloxacin (4 µg/ml) grew in the presence of tetracycline at concentration ranges 0.2344 µg/ml through 7.5 µg/ml. Eighty percent grew in the presence of tetracycline at range 15 µg/ml, 60% at 30 µg/ml through 60 µg/ml and

40% grew at 120 µg/ml through 240 µg/ml (Table 20). One-hundred percent of the cultures isolated on erythromycin (8 µg/ml) grew in the presence of tetracycline at ranges 0.2344 µg/ml through 60 µg/ml and 80% grew at 120 µg/ml through 240 µg/ml (Table 21). All cultures isolated on tetracycline at 12.5 µg/ml were resistant to tetracycline at all concentrations (Table 22).

In the presence of virginiamycin the cultures isolated on ciprofloxacin (4 µg/ml) were resistant at concentration ranges 0.3027 µg/ml through 2.422 µg/ml. Sixty percent grew in the presence of virginiamycin at 4.844 µg/ml through 38.75 µg/ml and 40% grew at 77.5 µg/ml through 310 µg/ml (Table 23).The cultures isolated on erythromycin at 8 µg/ml and tetracycline at 12.5 µg/ml were resistant to virginiamycin at all concentrations (Table 24).

MIC values were not developed in the tributaries for sulfamethizole and ciprofloxacin using the cultures isolated on ciprofloxacin at 4 µg/ml. All isolates grew in the presence of sulfamethizole (> 2550 µg/ml) and ciprofloxacin (> 70 µg/ml) at the highest concentrations testes. An MIC value (0.5859 µg/ml) was developed for erythromycin using the isolate, recovered from the Coal River, cultivated from ciprofloxacin (4 µg/ml). The MIC values for tetracycline were developed from the isolates recovered from the New, Elk and Coal Rivers. The MIC of tetracycline from the Elk River was determined to be 120 µg/ml, from the Elk River 30 µg/ml and from the Coal River 7.5 µg/ml. Ampicillin produced one MIC value developed from the isolate recovered from the New River. The MIC of ampicillin from the New River was determined to be 7.5 µg/ml. In the presence of streptomycin three values were developed from the New, Elk and Coal Rivers. The MIC value of streptomycin was determined to

be 7.5 µg/ml for both the New and Coal Rivers and 490 µg/ml for the Elk River. In the presence of virginiamycin the isolate also produced MIC values from the New, Elk and Coal Rivers. In the New River the MIC value was determined to be 77.5 µg/ml and in the Elk and Coal Rivers the MIC value for both was determined to be 4.844 µg/ml (Table 29).

Using the cultures isolated on erythromycin (8 µg/ml) MIC values could only be developed for erythromycin, tetracycline and streptomycin from the Gauley River. The MIC for erythromycin from the Gauley River was determined to be 9.375 µg/ml, for tetracycline 120 µg/ml and for streptomycin 61.25 µg/ml (Table 30).

MIC values were not developed for the seven antibiotics tested using the cultures isolated on tetracycline at 12.5 µg/ml. All isolates grew in the presence of the seven antibiotics at concentrations greater than the highest concentration tested for each antibiotic (Table 31).

Impact Scores

Due to an incubation error, samples collected during the spring sampling could not be compared over the entire river against the summer data. Summer samples were collected during July (Lower Kanawha, KR55-00) and August (Upper Kanawha, KR95-50). Sampling must be done consistently during the same day and under the same flow regime. Only KR50 – KR00 River miles were used to compare the water quality of the main stem during the spring to the summer samples collected concurrently in July. However an assessment of water quality for individual seasons, without comparison, was made for each sample season for the entire mainstem. (Figures 25, 26).

Average counts for fecal coliforms, ciprofloxacin resistant, erythromycin resistant and tetracycline resistant bacteria were calculated for each river mile and for each tributary using Microsoft Excel for each season (Appendices O-P). Using the average counts for the fecal coliform and antibiotic resistant bacteria a site impact score (IS) was determined for each site and tributary. An impact score was determined for the spring and summer at three boundary levels: IS_{85} (Appendices L-N), IS_{90} (Appendices H-K), and IS_{95} (Table 32, Figures 25-28), The IS_{95} provides the best signal to noise ratio for these data.

A comparison of all main stem sites (n = 20) from the Kanawha River was made during the spring (Table 32, Figure 25) sample season and for the summer (Table 32, Figure 26) sample season using IS_{95}. Spring Impact Scores (ranged -1 to +1) using the 95th percentile boundary the most impacted areas (IS_{95} = +1) occurred in the more industrial regions of the river (Lower Kanawha). The less impacted area of the river occurs in the Upper Kanawha (range -1 to 0) were there is little or no industrialization. The Upper Kanawha is a predominantly rural area with few industrial facilities, with the exception of Alloy Plant near KR90 (IS_{95} = -1) and an Appalachian Power facility (between KR80 (IS_{95} = -1) and KR75 (IS_{95} = 0).

Summer Impact Scores using the 95th percentile boundary (range -3 to +3) indicate that the most impacted areas occurred in the more industrial regions of the Lower Kanawha (Table 32, Figure 26). The most impacted area (IS_{95} = +3) during this season occurred at KR55 downstream of Union Carbide Island. Comparison of the Upper Kanawha's water quality to the Lower Kanawha indicates the Lower portion of the river

has generally larger populations of the tested water quality indicators, antibiotic resistance and fecal coliforms, than the Upper Kanawha.

Analysis of Impact Score comparison between seasons could only be done in the Lower Kanawha, which has the most industrial plants. Impact Scores (range -3 to +3) using the 95th percentile boundary indicate that KR50-40 are the most impacted areas of the river for both spring and summer (Table 32, Figure 27). During the spring KR25 – KR20 indicated impact that leveled off during the summer season.

Comparison of summer and spring Impact Scores (range -4 to +4) using the 95th percentile boundary for the tributaries indicate that the Pocatalico was more impacted during the spring (IS_{95} = +3) improving (IS_{95} = -3) during the summer sample season (Table 32, Figure 28). During both sample seasons the New and Gauley Rivers, primarily recreational waters, had the least impacted water (IS_{95} = -1 for spring and 0 for summer in both rivers). The Elk (IS_{95} = 0) and Coal Rivers (IS_{95} = 0) remained consistent during both sample seasons.

CHAPTER IV

Discussion

Seasonal Antibiotic Resistance

Analysis of mainstem and tributary antibiotic resistance using the Students *t*-test indicates significant increases in resistance to the three antibiotics tested during summer. The difference was most noticeable at sites on the mainstem (KR55-KR30) flowing through the industrial portion of the river, which showed an observable increase in mean resistance to erythromycin and tetracycline. However, ciprofloxacin only exhibited an observable increase at KR55 located directly behind an industrial plant. Although there were no significant differences in the spatial distribution of antibiotic resistance in this study, isolates from KR55-KR30, which were within close proximity to industrial activities, exhibited high levels of antibiotic resistance. According to previous studies, high levels of antibiotic resistance have been discovered in heavy metal polluted waters (3, 33). The level and frequency of antibiotic resistance in the Kanawha River suggests that heavy metals present in the river may be impacting the bacterial communities. Biyel (3) speculates there may be a link between heavy metal polluted waters and antibiotic resistance as a result of genes that may be linked resulting in co-selection of linked genetic markers. Genes that code for metal resistance are often carried on the same plasmids or mobile genetic elements (33). This leads researchers to believe that the link in genetic markers may have led to the selection and spread of antibiotic resistance among bacterial communities, even without exposure to antibiotics in the environment. According to McArthur and Tuckfield (33) metal tolerance and antibiotic resistance

increases proportionally along industrial contaminated gradients. Future studies of the Kanawha river should incorporate water chemistry analyses with antibiotic resistance analyses to determine if a link can be found between heavy metal pollution and antibiotic resistance in river water sampled.

Seasonal Fecal Coliforms vs. Seasonal Antibiotic Resistance

During the summer sampling season fecal coliform samples and antibiotic resistance samples were collected on different days under different environmental conditions and flow regimes. Upper Kanawha samples were collected during July and Lower Kanawha samples were collected during August due to a fecal coliform incubation error. Fecal coliforms must be incubated at 44.5 ± 2° C; however the original samples were incubated at 35.5 ± 2° C. When conducting multi-seasonal analyses it is important that samples are collected under the same flow regime and environmental conditions. If samples are not collected during the same environmental conditions statistical analysis can not be performed. Any variability in environment, such as heavy rain or drought conditions during sampling days, can skew analytical results. One advantage of sampling from the Kanawha River is its relatively small size compared to rivers like the Ohio and Mississippi. The Kanawha River is 99.5 river miles long and the size makes it possible to sample the entire river during one full day, or over two-consecutive days if necessary, unlike the Ohio River which must be sampled over several days due to its large size.

The data indicate that fecal coliform levels during the spring were lower than fecal coliform counts in the summer samples. The use of fecal coliforms as a water quality indicator assumes that a majority of fecal coliforms do not occur naturally in

aquatic and terrestrial environments. Fecal coliforms are only found inhabiting the guts of warm-blooded animals and, when found in the environment, are indicative of fecal contamination due to their inability to survive in the environment for long periods of time. The presence of fecal coliforms in the environment is taken to indicate recent input from an unknown source. Some sources of fecal contamination include domestic sewage, point source and non-point source runoff. Our data indicate significant increases in fecal coliform counts during the summer in the Upper Kanawha River, however in the Lower Kanawha there was no observable increase in the presence of fecal coliforms with the exception of two sample sites (KR75 and KR55) both located on the downstream side of river islands (Figure 8). During August a light rain event occurred during sample collections indicating runoff probably contributed to the observed increases at these sites. In the tributaries (Figure 9) mean fecal coliform counts were higher during the summer in 3 of the 5 tributaries sampled. Statistical analysis was not performed on the tributaries due the low number of isolates. Visual observation and mean values were used to assess the presence of fecal coliforms during the two seasons. The Pocatalico and Elk Rivers both exhibited apparent increases in fecal coliform cells during the summer.

Comparisons of fecal coliforms to antibiotic resistance indicated mean fecal coliform counts were consistently lower during both seasons in the mainstem and within the tributaries than mean antibiotic resistance counts (Figures 10 – 21). This suggests that antibiotic resistant bacteria are not subsets of fecal coliform populations. According to this study the enumerated bacterial cells resistant to ciprofloxacin (4 μg/ml), erythromycin (8 μg/ml) and tetracycline (12.5 μg/ml) are independent bacterial populations and were not found to be influenced by increases or decreases in fecal

coliform cells, providing further evidence that the distribution of antibiotic resistance is not determined by antibiotic selection in human and animal guts, and that another source is controlling selection on the Kanawha River and its 5 tributaries.

Minimum Inhibitory Concentrations and Antibiotic Susceptibility

The behavior of environmental isolates and their selectivity for antibiotic resistance is scarcely understood. Due to limitations on the ability to cultivate environmental isolates it is difficult to study their reaction when in the presence or absence of antibiotics. These data further confirm these statements. No single MIC could be determined for the seven antibiotics surveyed on the mainstem or from within its tributaries. This may be attributed to many different factors effecting selectivity for resistance. Do environmental isolates behave *in vivo* as they do in their natural environments? What components in their environments allow them to express resistance? Unless extensive research is performed on the aquatic habitat prior to sampling it will be difficult to duplicate an "optimal environment" that will induce isolates to grow. It is possible that the uncultivable isolates may hold all the answers.

As bacteria exhibit naturally occurring mechanisms of resistance it was expected that resistance would occur within the isolates, however the extent of resistance and spatial distribution on the Kanawha River was not expected. All 75 isolates (100%) from the mainstem and its tributaries were resistant to 3 or more of the seven antibiotics tested (chosen from a list of emerging contaminants (26)). Isolates exhibiting the most resistance, resistance to all 7 antibiotics, occurred in the more industrial regions of the river. This suggests that industry may be playing a role in the dissemination and

acquisition of resistance. Previous studies have also indicated industrialization may be playing a role in antibiotic resistance (4, 6, 15, and 22). Industrial plants, waste water treatment facilities, etc. are permitted by the Environmental Protection Agency under the Clean Water Act to pump treated effluents into surface waters. These treated and untreated (not being monitored) effluents may hold components that provide the conditions that select for resistance. The long-term impacts of effluents may compromise the intended uses of aquatic habitats for many generations.

The isolates tested were selected based on the most abundant colony morphology growing on the R2A based media. Due to lack of funding the isolates could not be identified to genus and species. Without knowing the identity of the isolates tested there is no way of knowing whether the same isolates were being tested at each site. Future studies need to address this issue and incorporate species identification with MIC determination. This information will be useful to determine if the same species are showing resistance at equivalent concentration ranges, and may also determine the spatial distribution of the most resistant bacteria.

Impact Scores

The Impact Scoring system used in this survey was first developed for use on the Ohio River. The system was developed by Dr. Charles Somerville in the Environmental Microbiology Research Laboratory at Marshall University. Part of this study was to determine if this novel Impact Scoring system could be used on a smaller river, compared to the Ohio Rivers size, and its tributaries. The system incorporates a traditional water quality indicator, fecal coliforms, along with potentially new indicators, antibiotic

resistant bacteria. The antibiotics used were chosen based on a previous survey of emerging contaminants in U.S. waters (26).

Impact was determined for the spring and summer samples from the Kanawha River using the 95th percentile boundary which provides a good signal to noise ratio for this data set. Due to an incubation error of fecal coliforms spring and summer impact comparisons could not be made for the entire mainstem, however assessments were made of individual seasons and a comparison of spring to summer impact was made for the Lower Kanawha.

Impact scores ranged -4 to +4 for both sample seasons. The data indicate impact occurring in areas with industrialization beginning near KR55, Union Carbide Island, and leveling off in the lower portion of the river. Increased Impact Scores at these sites may be associated with spikes in fecal coliforms that resulted from a prior rain event. During the spring a heavy rain event had occurred prior to sampling and at the time of the second summer samples light rain fall had occurred during sampling. Weather conditions have a major effect on sampling and runoff as a result of rain has an effect on bacterial populations from point source and nonpoint source runoff. Impact throughout both seasons remained localized in the mid-portion of the river. Conditions in the Upper region and Lower regions appeared to be less impacted compared to the middle region. This indicates an effect is occurring near industry; however the definitive source is still unknown.

CHAPTER V

Conclusions

The objectives of this study were to determine the occurrence and distribution of multi-antibiotic resistant bacteria, determine Minimum Inhibitory Concentrations (MICs) of seven antibiotics identified from a USGS survey (26) and to determine if a novel Impact Scoring system originally developed for the Ohio River could be applied to a smaller body of water such as the Kanawha River.

The first two objectives were accomplished by analyzing microbiological data from 20 main stem sites and from 5 primary tributaries from the Great Kanawha River. Isolates exhibiting resistance to ciprofloxacin, erythromycin, and tetracycline were tested against seven antibiotics: ampicillin, streptomycin, sulfamethizole, virginiamycin, ciprofloxacin, erythromycin, and tetracycline. Analysis confirmed multiple antibiotic resistance was occurring at every sample site on the river's mainstem and from its tributaries. From each of the 75 samples, isolates exhibited resistance to 3 or more antibiotics. Multiple antibiotic resistance is defined as resistance to more than one antibiotic (44). None of the sample isolates from the mainstem or tributaries exhibited resistance to only one antibiotic. The occurrence of resistance to all seven antibiotics was more prevalent in areas known for industrialization, leading to the conclusion that industrial sites are affecting the selective pressure for antibiotic resistance. Samples collected near industrial sites exhibited a higher prevalence of resistance to 5 or more of the seven antibiotics used in this survey. These data provide evidence that industrialization is having an effect on the occurrence of antibiotic resistance as well as MAR (Multiple Antibiotic Resistance) within the Kanawha River.

The third objective was accomplished by increasing the concentrations of the seven antibiotics (ampicillin, streptomycin, sulfamethizole, virginiamycin, ciprofloxacin, erythromycin, and tetracycline) to 20 times their known working concentration (Appendix A) for Gram negative bacteria based on the knowledge that cultivable environmental isolates are predominantly Gram negatives. MICs could not be determined for the entire mainstem or for all five tributaries. MICs were only developed for areas of the river where little or no industrialization had occurred. Isolates sampled from areas that are heavily industrialized exhibited resistance greater than the highest concentration of each of the seven antibiotics. This may be another indication that industrial practices are affecting the occurrence of resistance on the Kanawha River; however original antibiotic concentrations were based on information from clinical settings. Antibiotic concentrations used in clinical settings may not be applicable for use on environmental isolates. Further studies to determine MIC values for environmental isolates need to be conducted in order to eliminate question of relevance with regard to antibiotic concentrations.

A final objective was to determine if an Impact Scoring system originally developed for the Ohio River could be applied to the Kanawha River. This was accomplished by analyzing the site impact scores for each of the 20 mainstem sites. The Impact Scoring system includes a current water quality indicator, fecal coliforms, and new indicators, antibiotic resistant bacteria. The Impact Scoring system results supported previously discussed microbiological analysis indicating industry is affecting water quality in the form of antibiotic resistance.

In conclusion, the spatial distribution of multiple antibiotic resistance is found at each of the 20 mainstem and from each of the 5 tributary sites sampled. The prevalence of resistance to 5 or more of the seven antibiotics was found most frequently in the industrial regions of the river. According to this study industry may be having an adverse affect on the occurrence and distribution of MAR bacteria in the Kanawha River. Therefore; industrial rivers may be an important environmental reservoir for MAR resistant bacteria.

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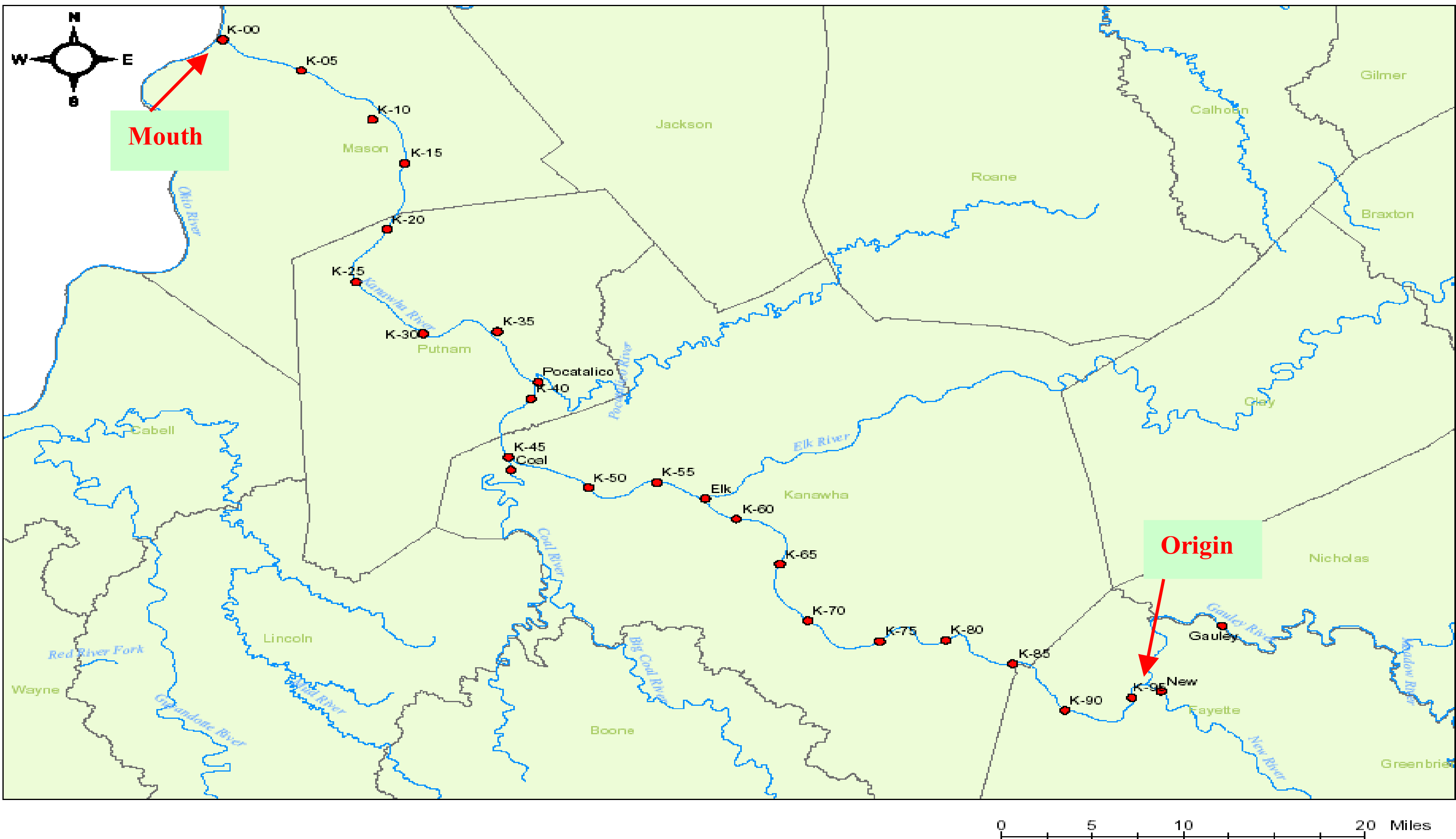


Figure 1. Kanawha River and primary tributary sample site locations map based on GPS latitude and longitude coordinates.

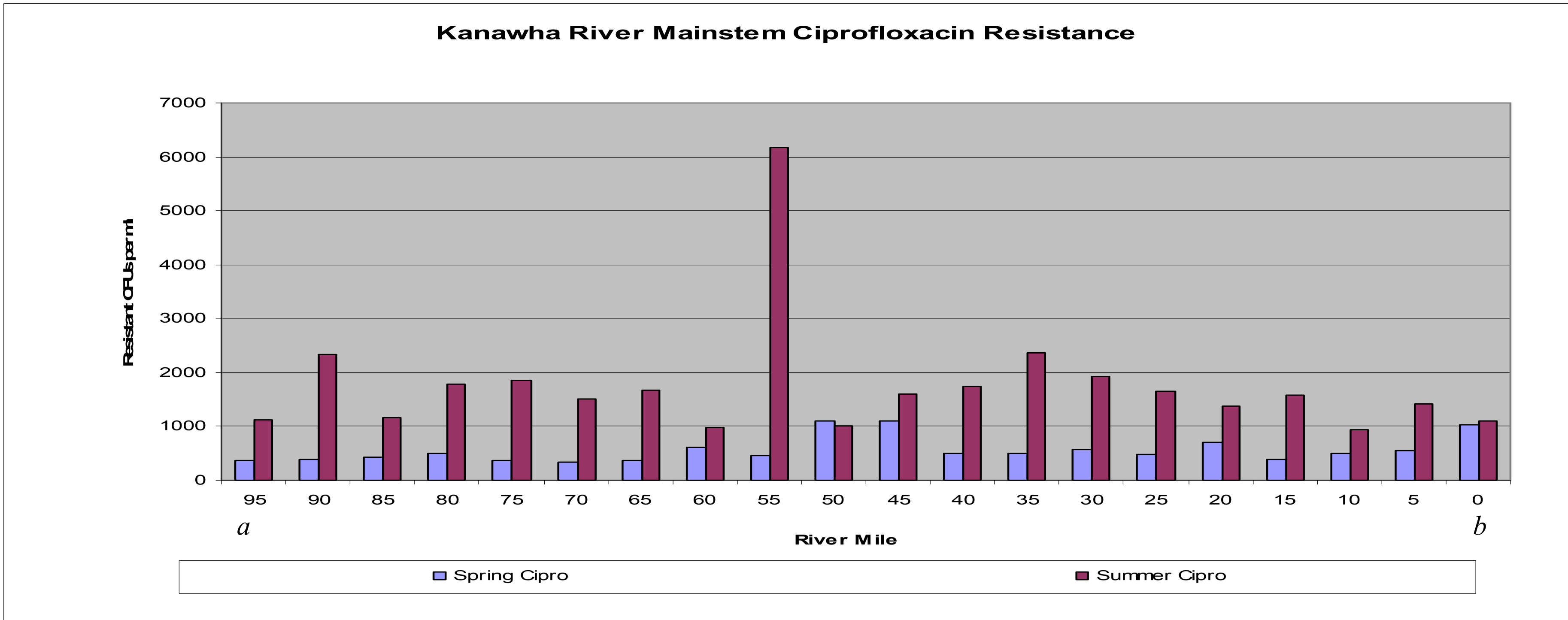


Figure 2. Ciprofloxacin (4 μg/ml) resistance comparison between the means of all main stem sample site counts in spring vs. summer.

Ciprofloxacin resistant counts were higher during the summer sampling than during the spring ($P < 0.01$).

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

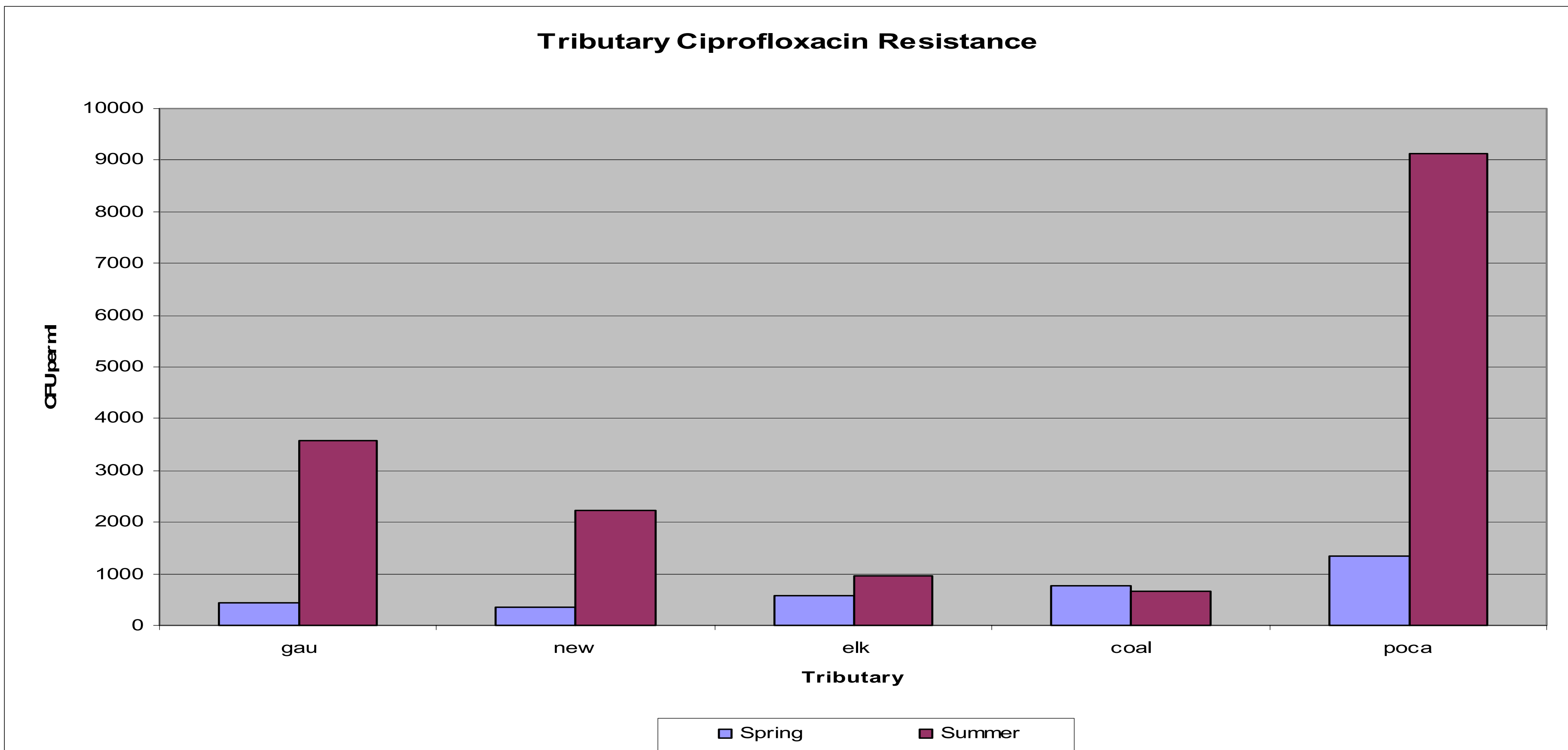


Figure 3. Comparison between the mean Ciprofloxacin (4 µg/ml) resistance counts at tributary sample sites in spring vs. summer samples.

Mean ciprofloxacin resistance counts were higher during the summer in four of the five tributaries samples.

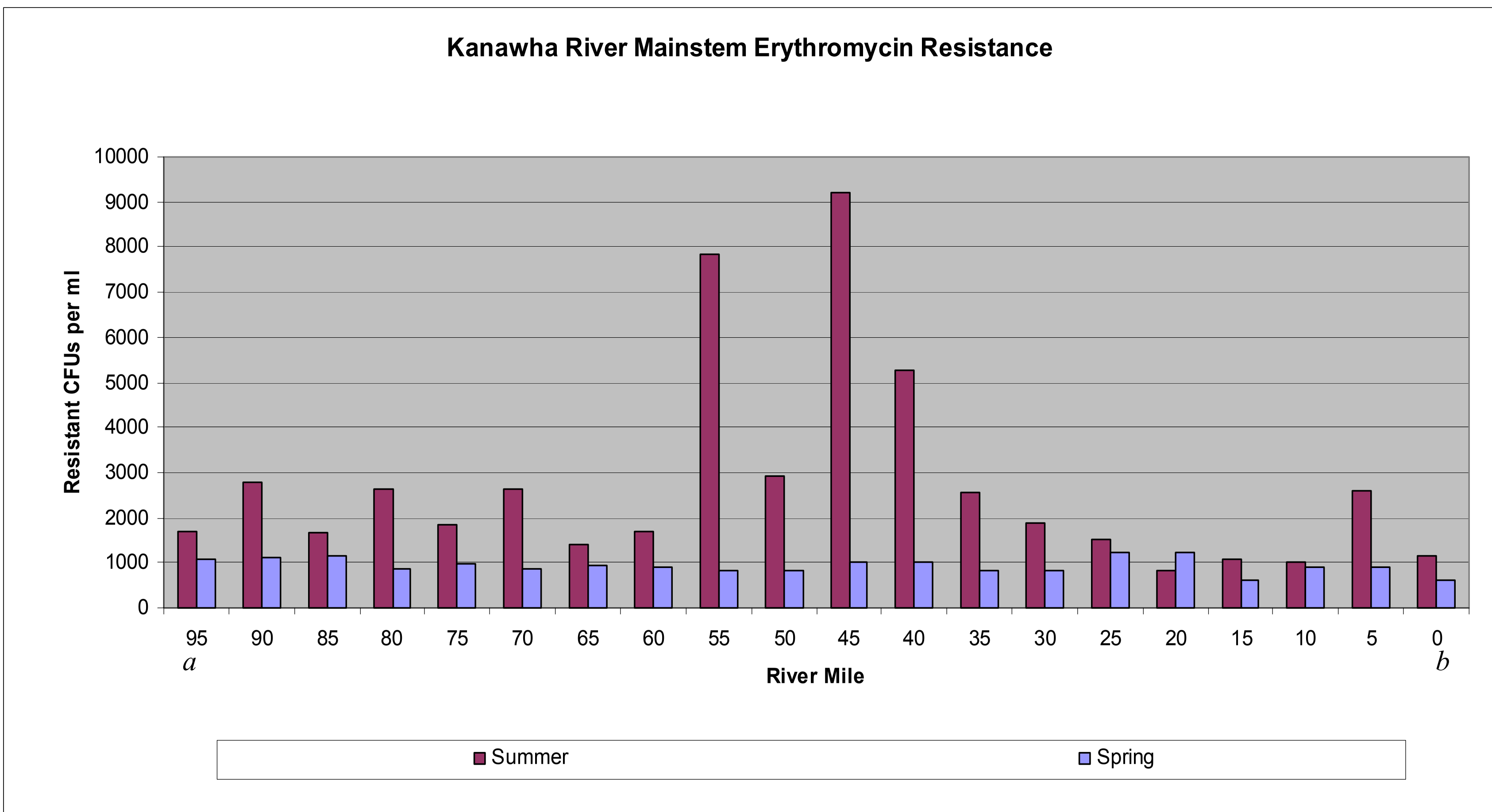


Figure 4. Comparison of erythromycin (8µg/ml) resistance counts for all mainstem sample sites during spring vs. summer.

Erythromycin resistant counts were higher during the summer sampling than during the spring ($P < 0.01$).

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

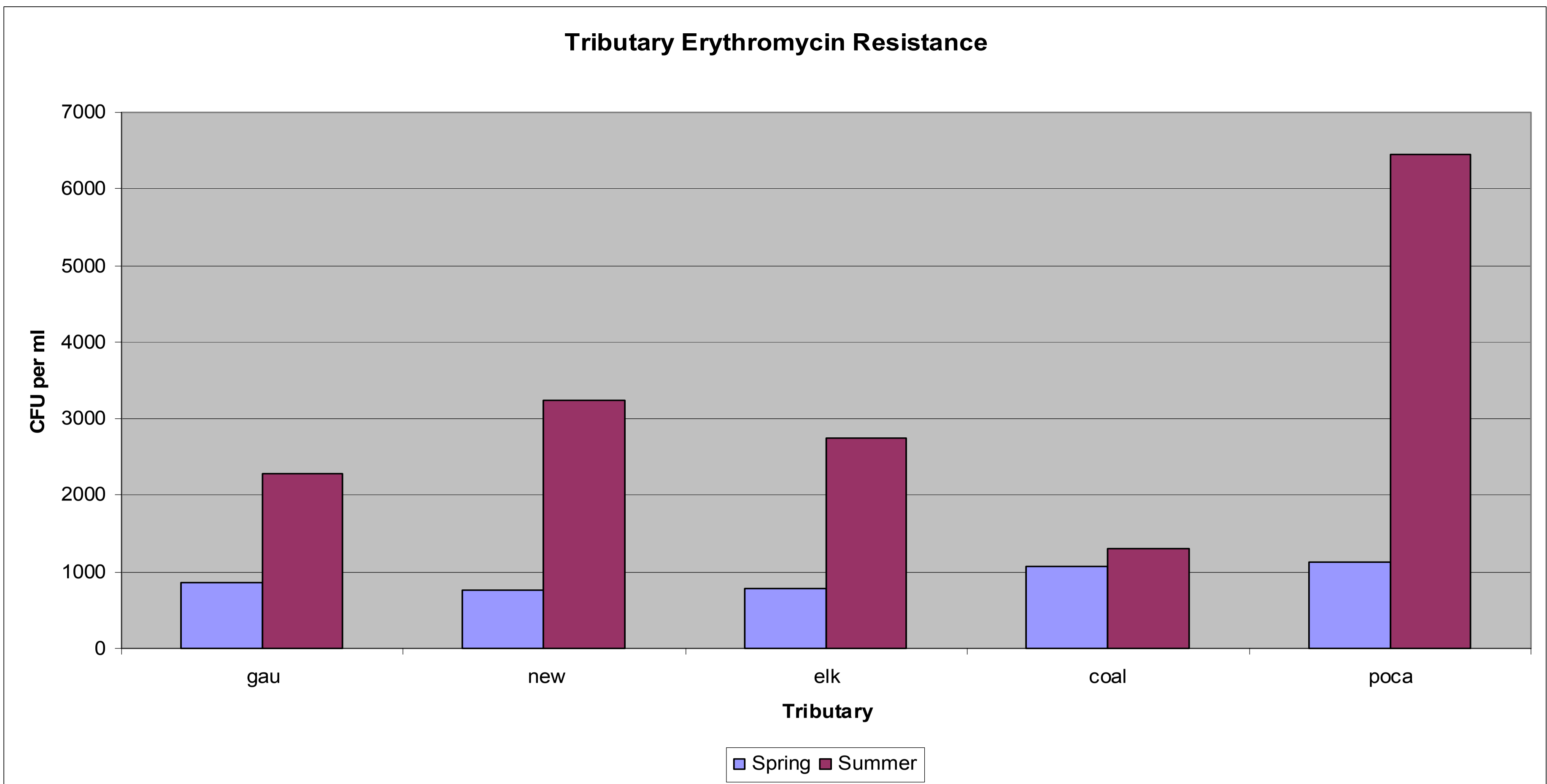


Figure 5. Comparison between the mean erythromycin (8 µg/ml) resistance counts at tributary sample sites in spring vs. summer. Mean erythromycin resistant counts were higher during the summer in all five tributaries sampled.

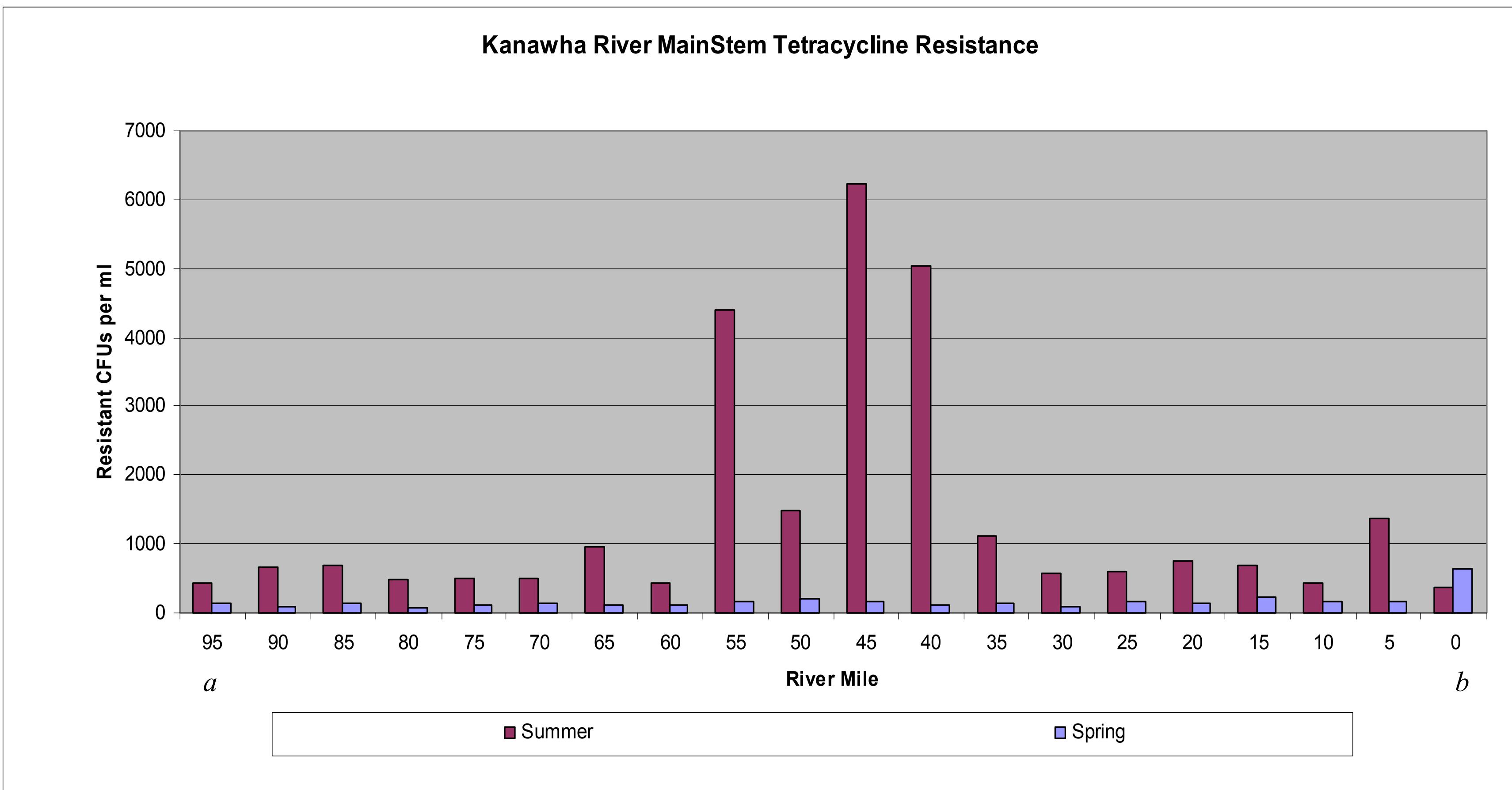


Figure 6. Tetracycline (12.5 µg/ml) resistance comparison between the means of all main stem sample site counts in spring vs. summer.

tetracycline resistant counts were higher during the summer sampling than during the spring ($P < 0.01$).

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

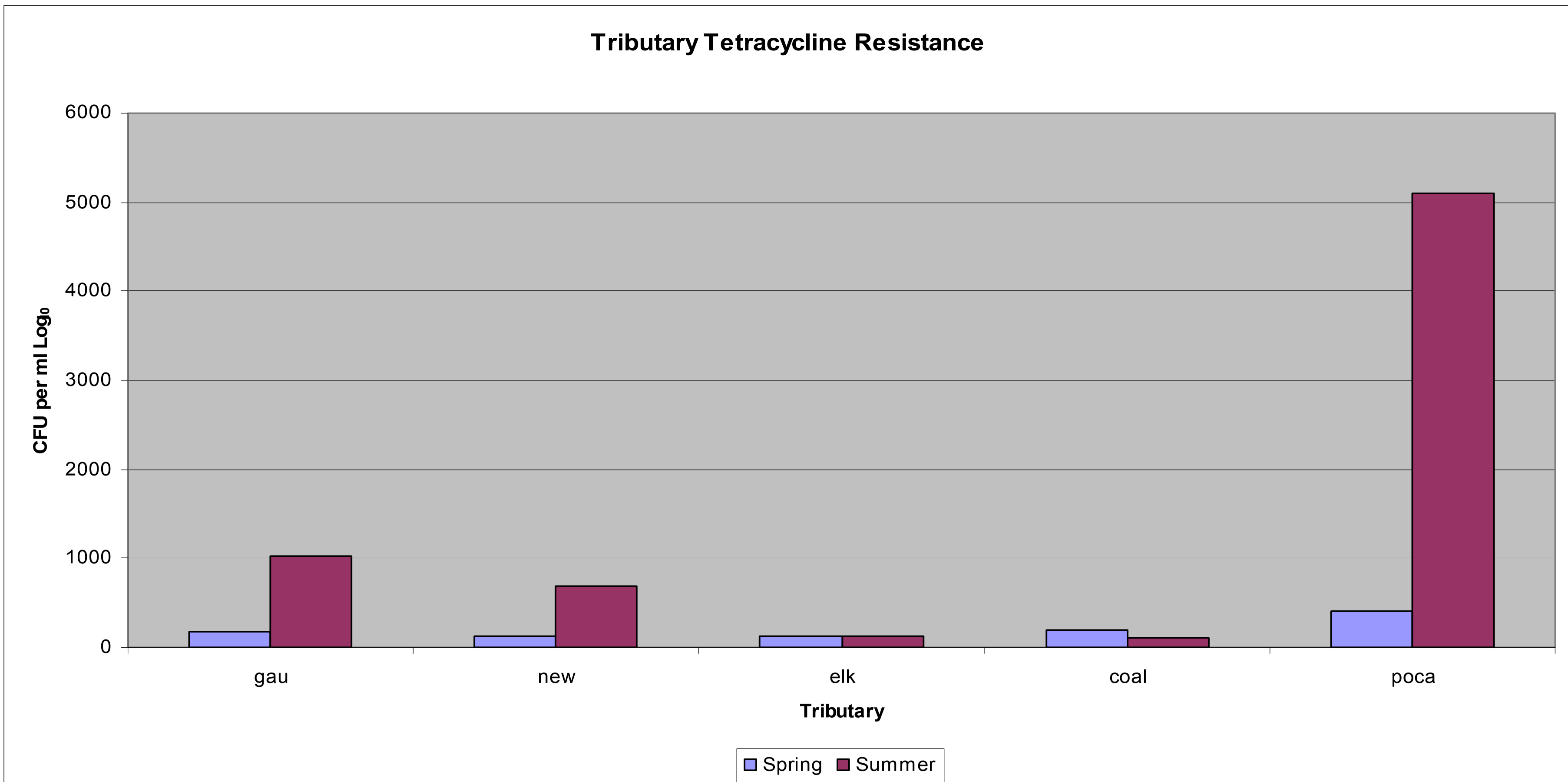


Figure 7. Comparison between the mean tetracycline (12.5 μ/ml) resistance counts at tributary sample sites in spring vs. summer. Mean tetracycline resistance counts were higher during the summer in three of the five tributaries samples.

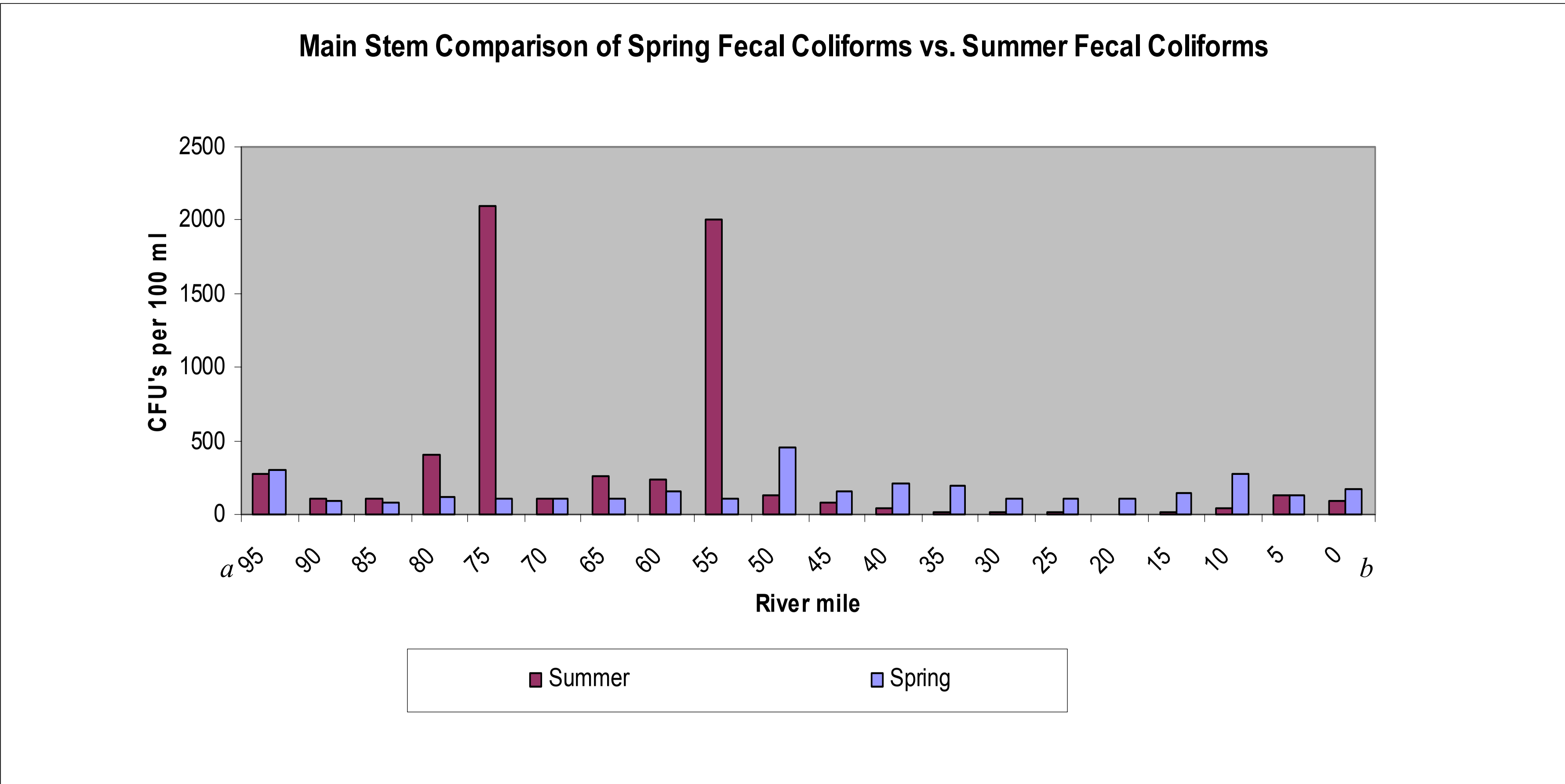


Figure 8. Fecal coliform comparison between the means of all mainstem sample site counts in spring vs. all mainstem sample site counts in summer.

$P < 0.01$ indicates average fecal coliform counts were higher at KR50-KR00 during the spring compared to summer.
$P = 0.01$ Indicates average fecal coliform counts were lower at KR95-KR55 during the summer compared to spring.

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

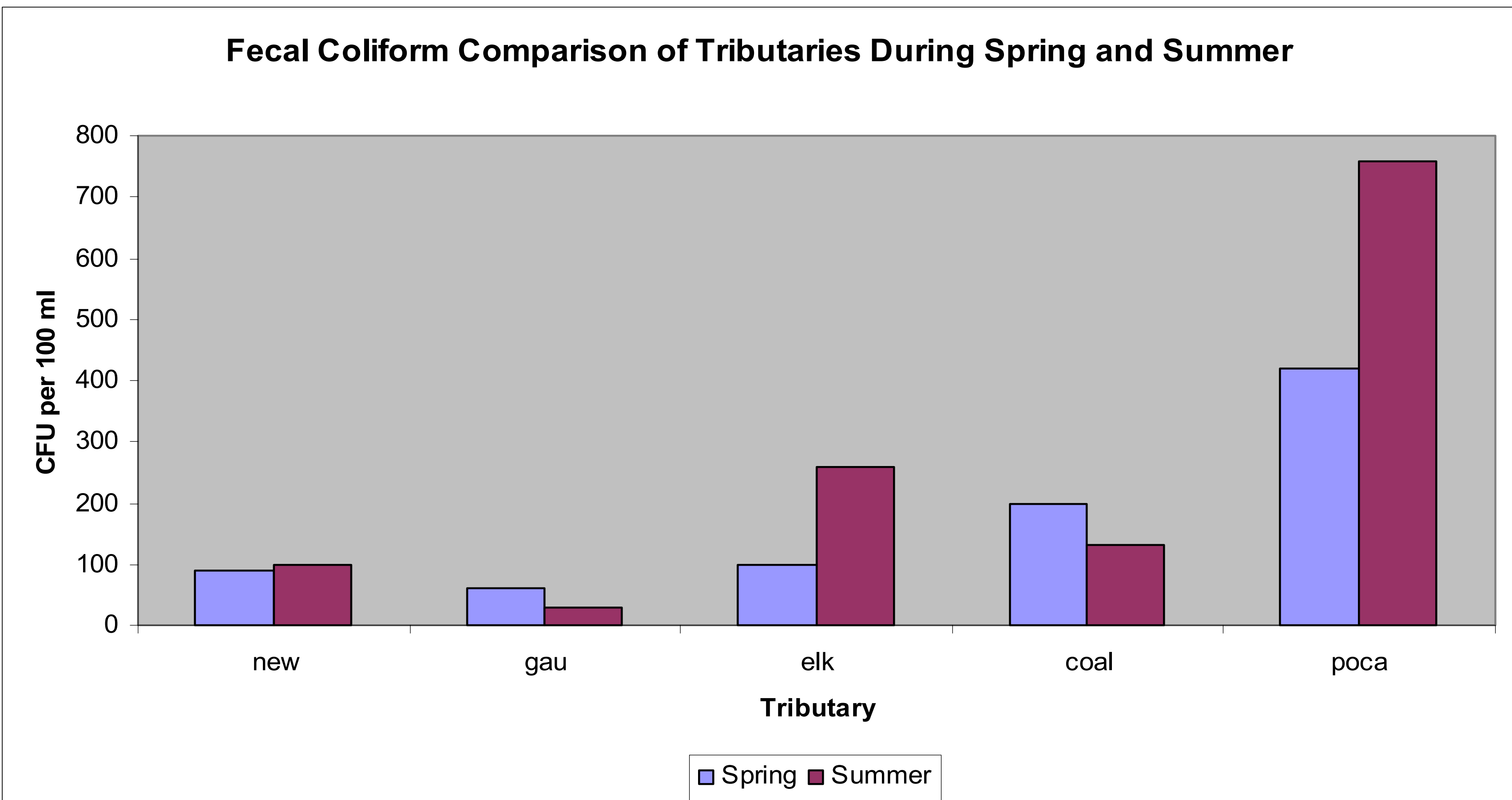


Figure 9. Comparison between fecal coliform means of the tributary sample site counts in the spring vs. the tributary sample site counts in summer.

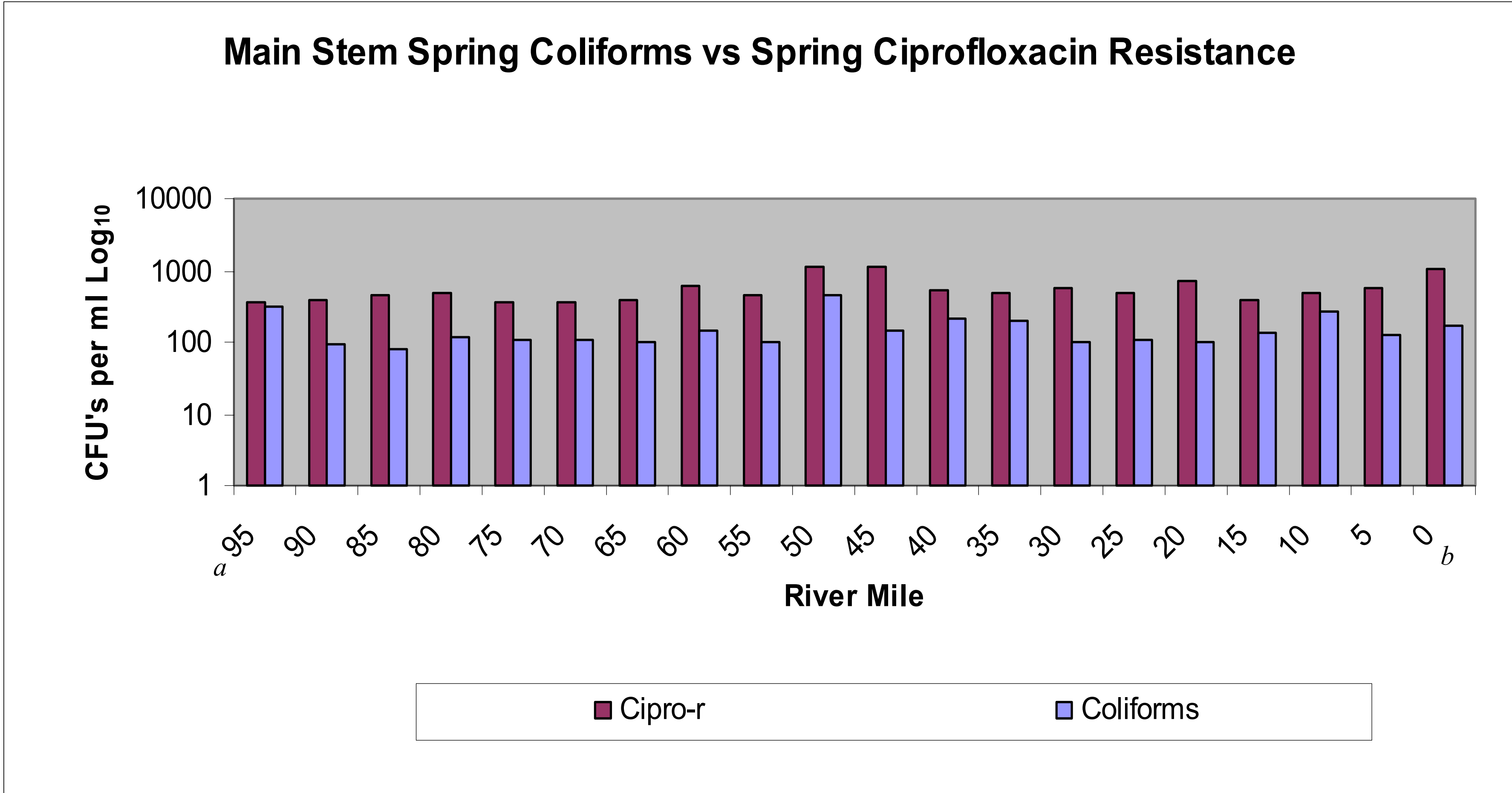


Figure 10. Comparison of all main stem mean fecal coliform counts to all main stem mean ciprofloxacin (4 µg/ml) resistance counts during the spring.

$P < 0.01$ indicates mean fecal coliform counts are significantly lower than mean ciprofloxacin resistance counts during the spring.

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

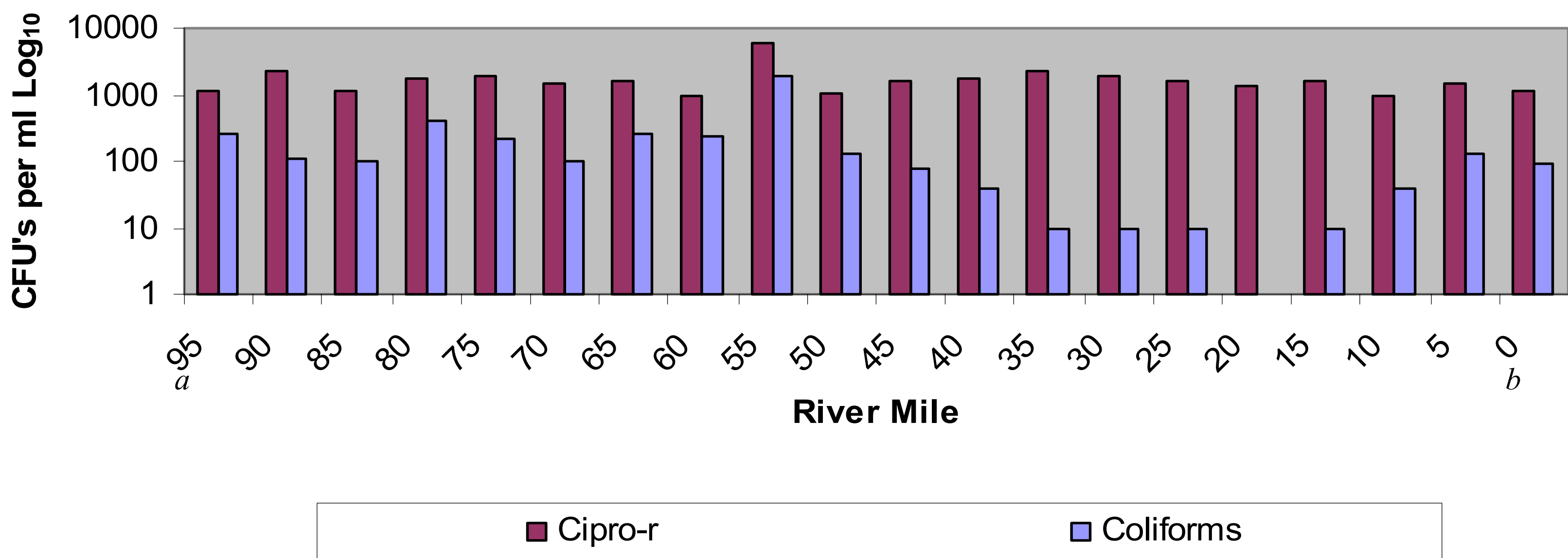


Figure 11. Comparison of all main stem mean fecal coliform counts to all main stem mean ciprofloxacin (4 µg/ml) resistance counts during the summer.

$P < 0.01$ in the Lower Kanawha (KR50-KR00) indicates mean fecal coliform counts are significantly lower than mean ciprofloxacin counts during the summer.

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

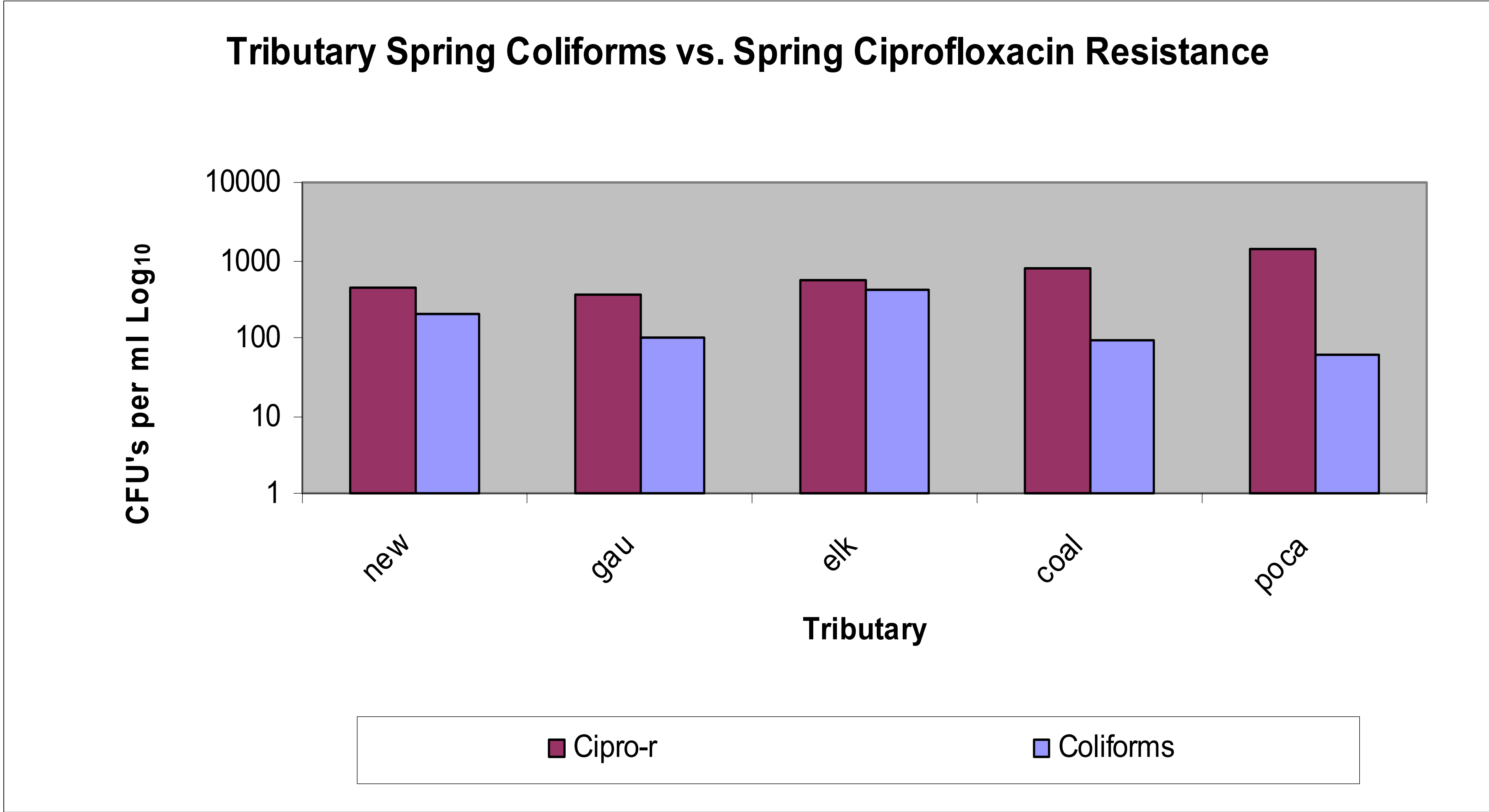


Figure 12. Comparison between the spring mean fecal coliform counts of the tributaries vs. spring mean ciprofloxacin (4 µg/ml) resistance counts.

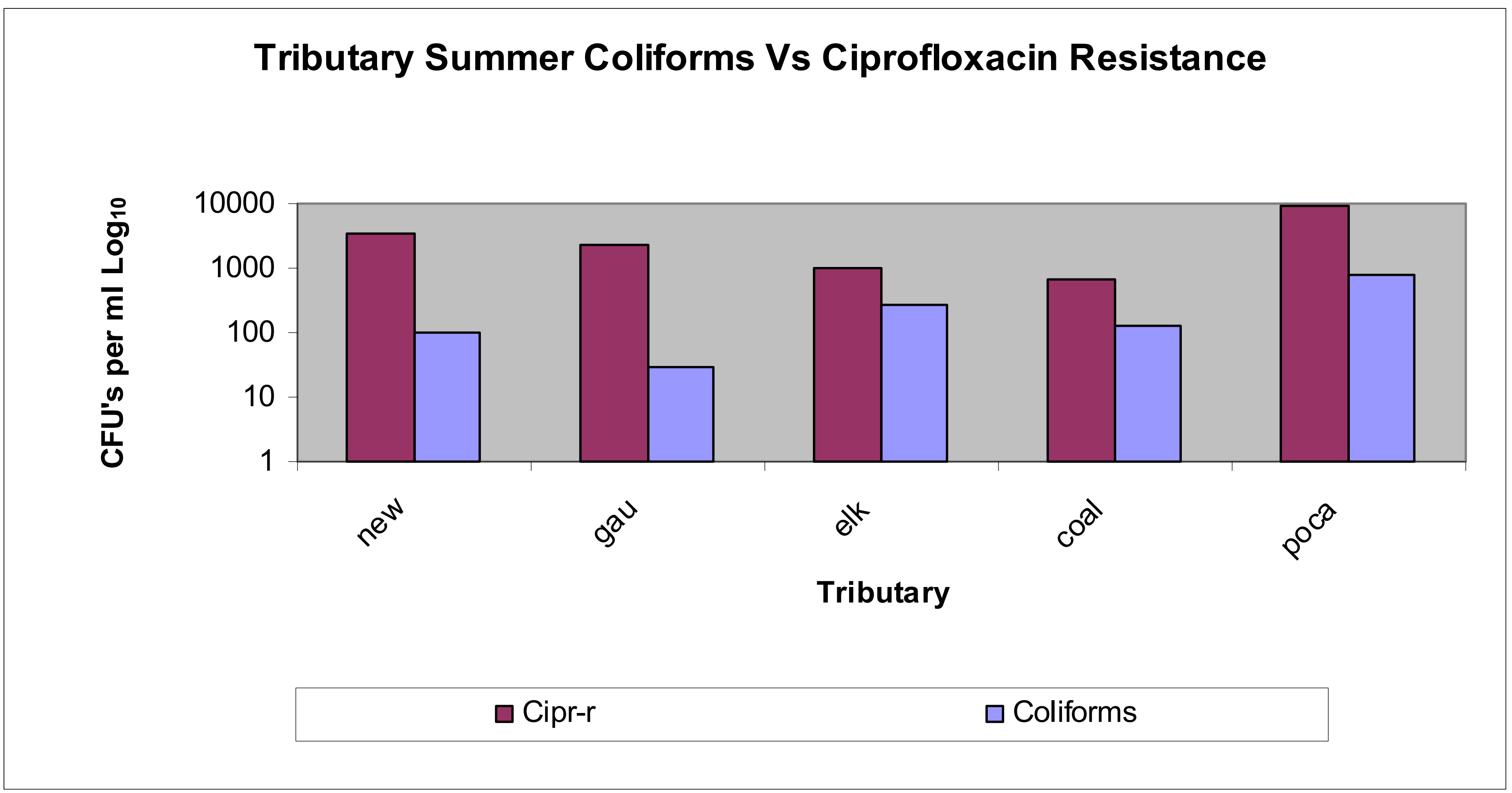


Figure 13. Comparison between the summer mean fecal coliform counts of the tributaries vs. summer mean ciprofloxacin (4 µg/ml) resistance counts.

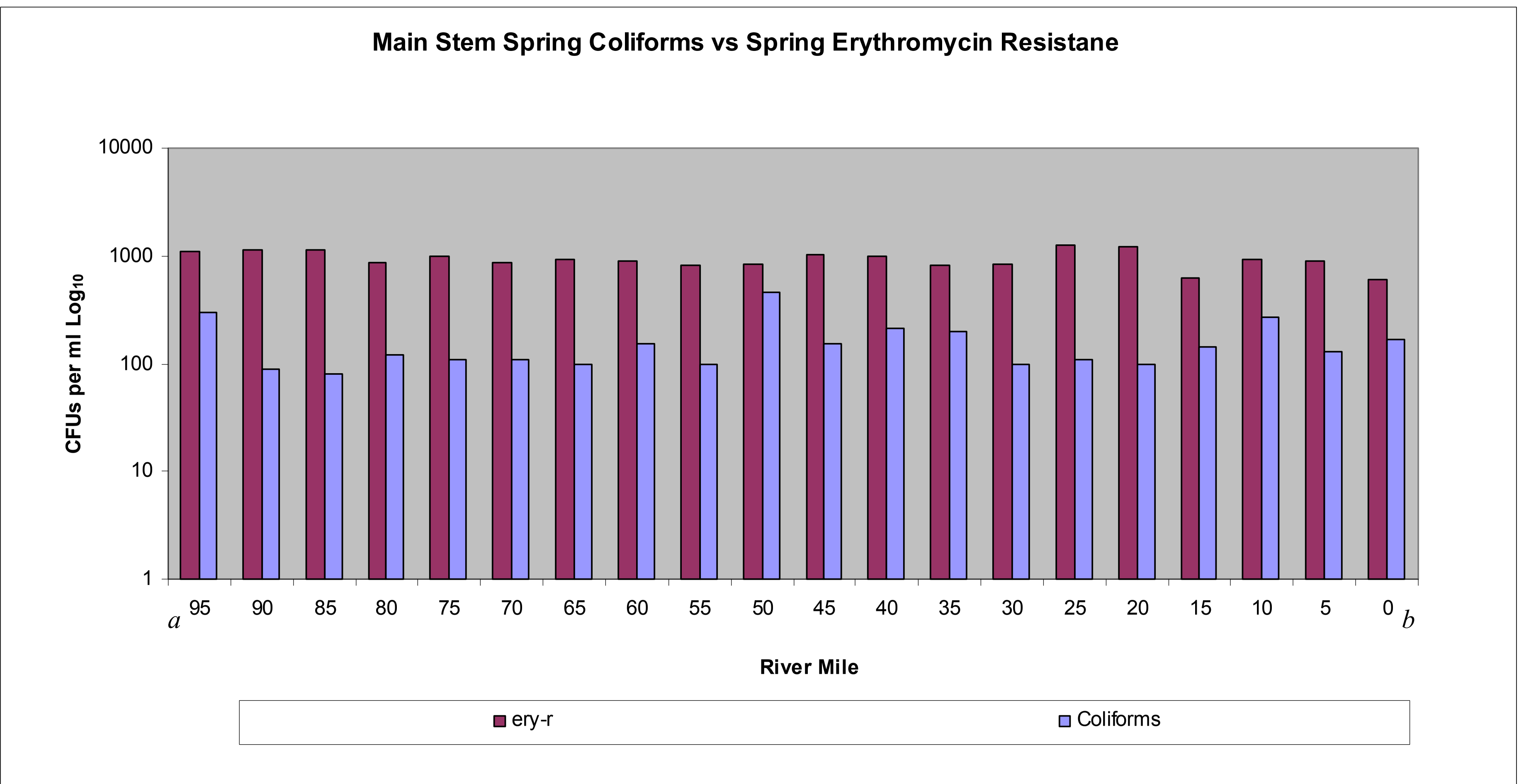


Figure 14. Comparison of all main stem mean fecal coliform counts to all main stem mean erythromycin (8µg/ml) resistance counts during the spring.

$P < 0.01$ indicates mean fecal coliform counts are significantly lower than mean erythromycin resistance counts during the spring.

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

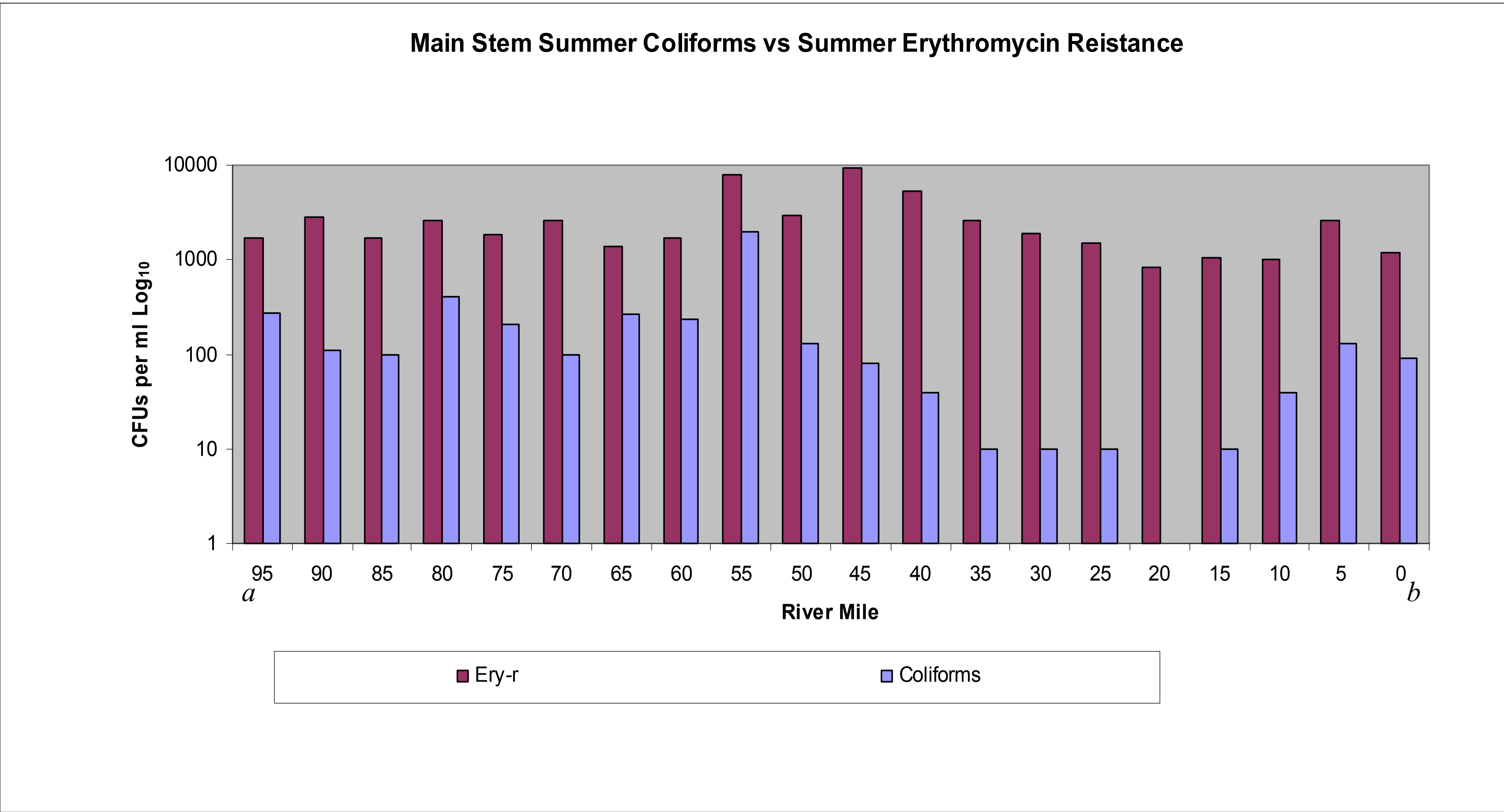


Figure 15. Comparison of all main stem mean fecal coliform counts to all main stem mean erythromycin (8µg/ml) resistance counts during the summer.

$P < 0.01$ indicates mean fecal coliform counts were significantly lower than mean erythromycin counts.

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

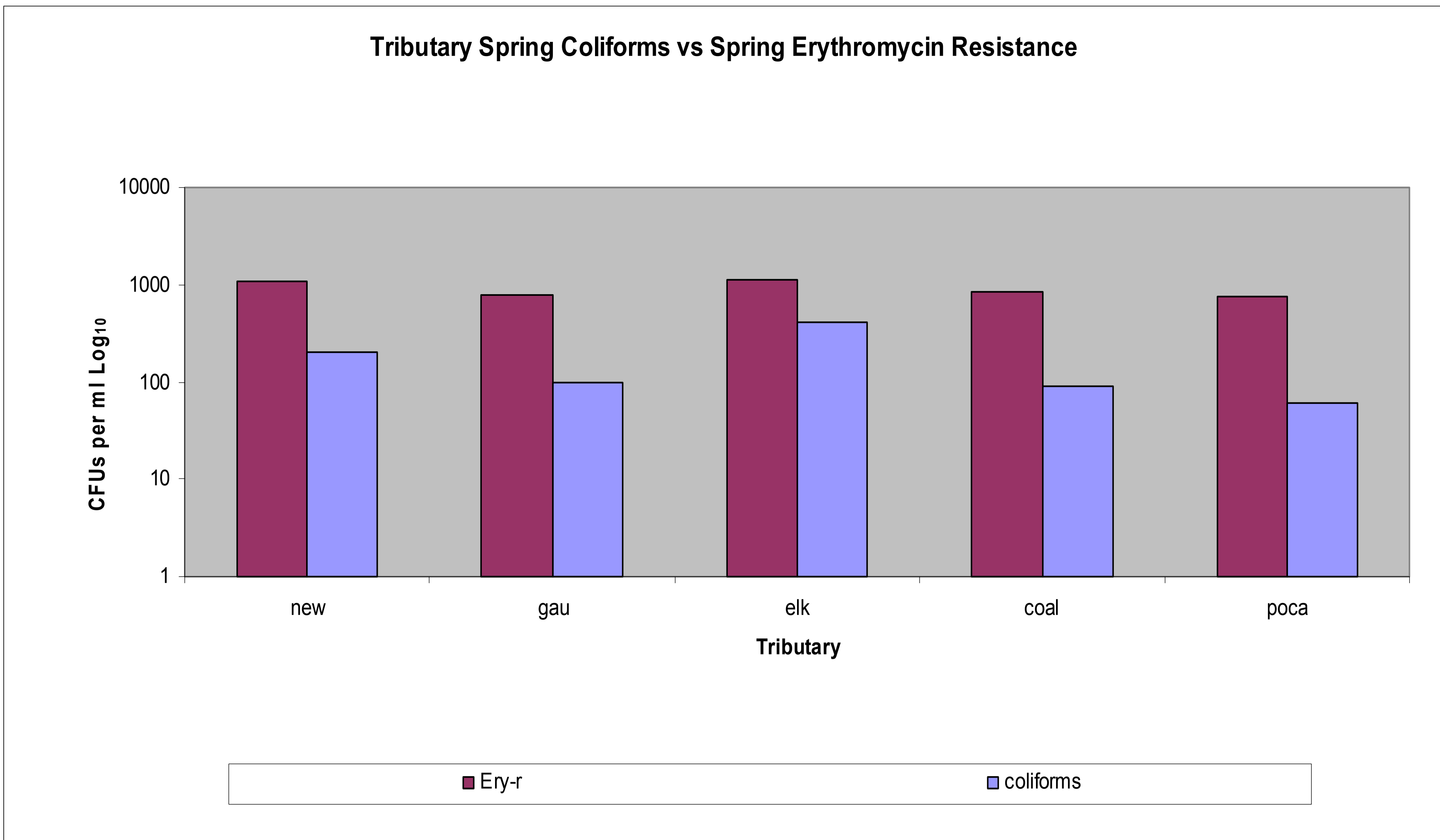


Figure 16. Comparison between the spring mean fecal coliform counts of the tributaries vs. summer mean erythromycin (8 µg/ml) resistance counts during the summer.

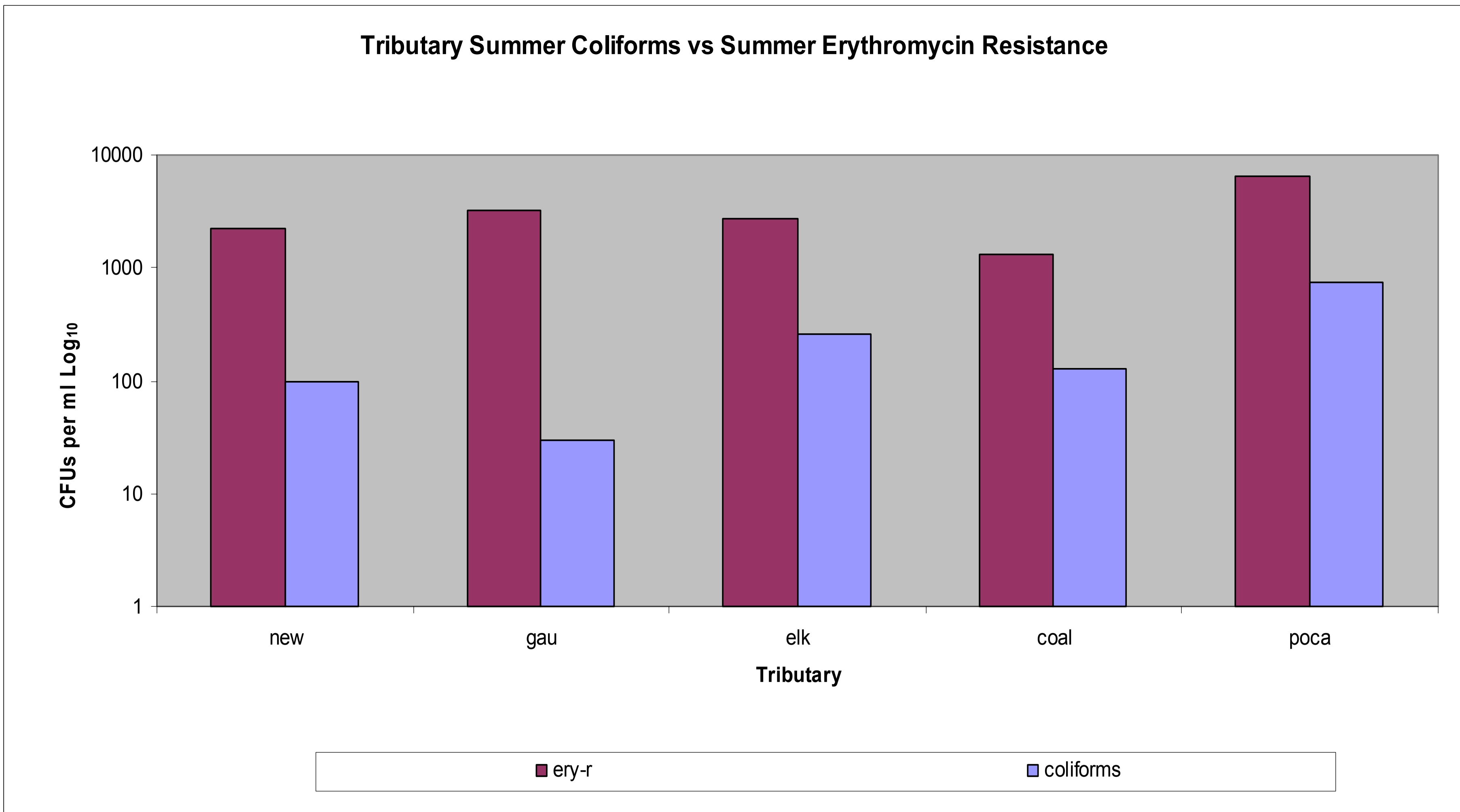


Figure 17. Comparison between the summer mean fecal coliform counts of the tributaries vs. summer mean erythromycin (8 µg/ml) resistance counts.

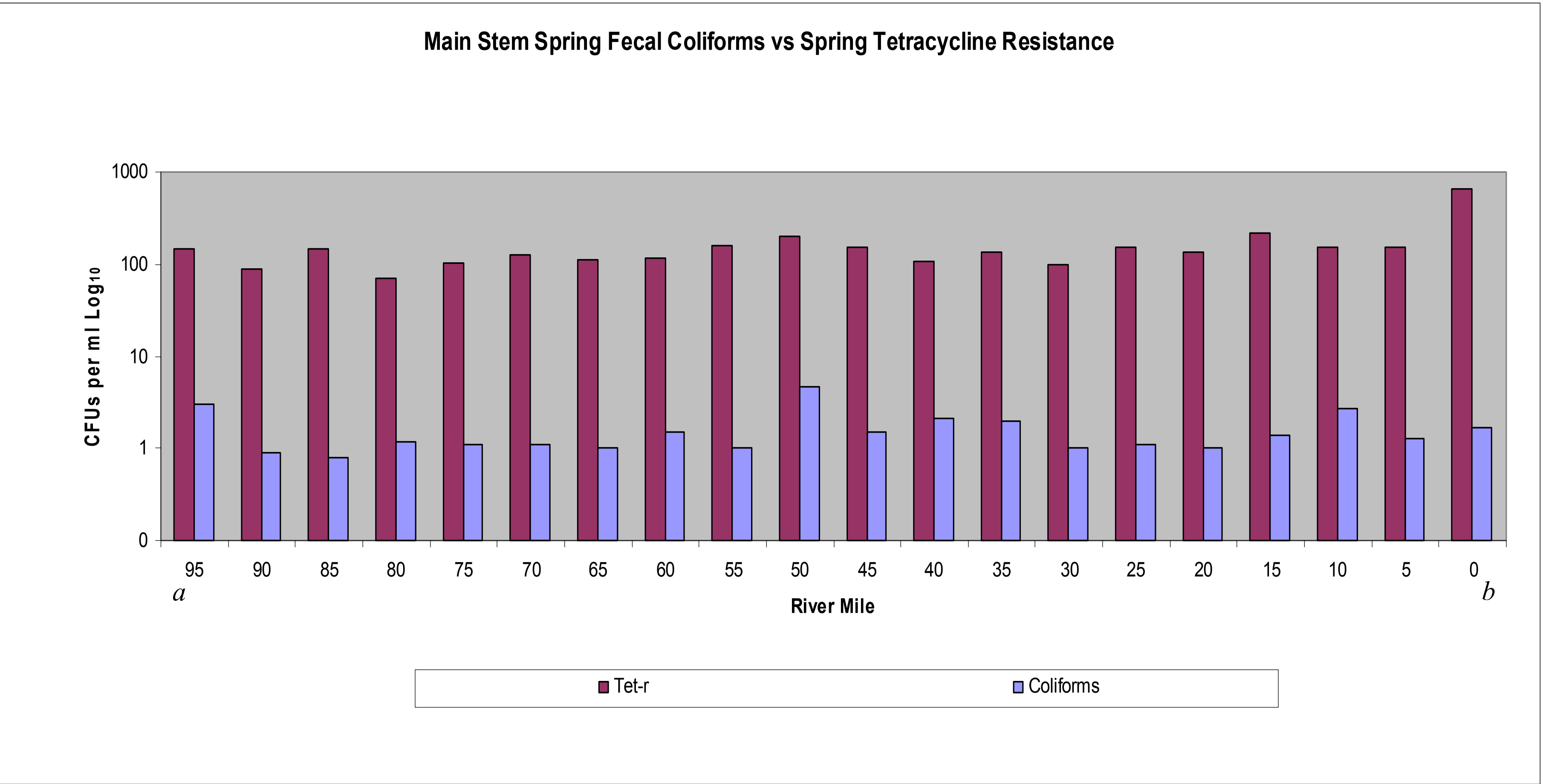


Figure 18. Comparison of all main stem mean fecal coliform counts to all main stem mean tetracycline (12.5 µg/ml) resistance counts during the spring.

P = 0.49 indicates mean fecal coliform counts were on average significantly lower than mean tetracycline counts.

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

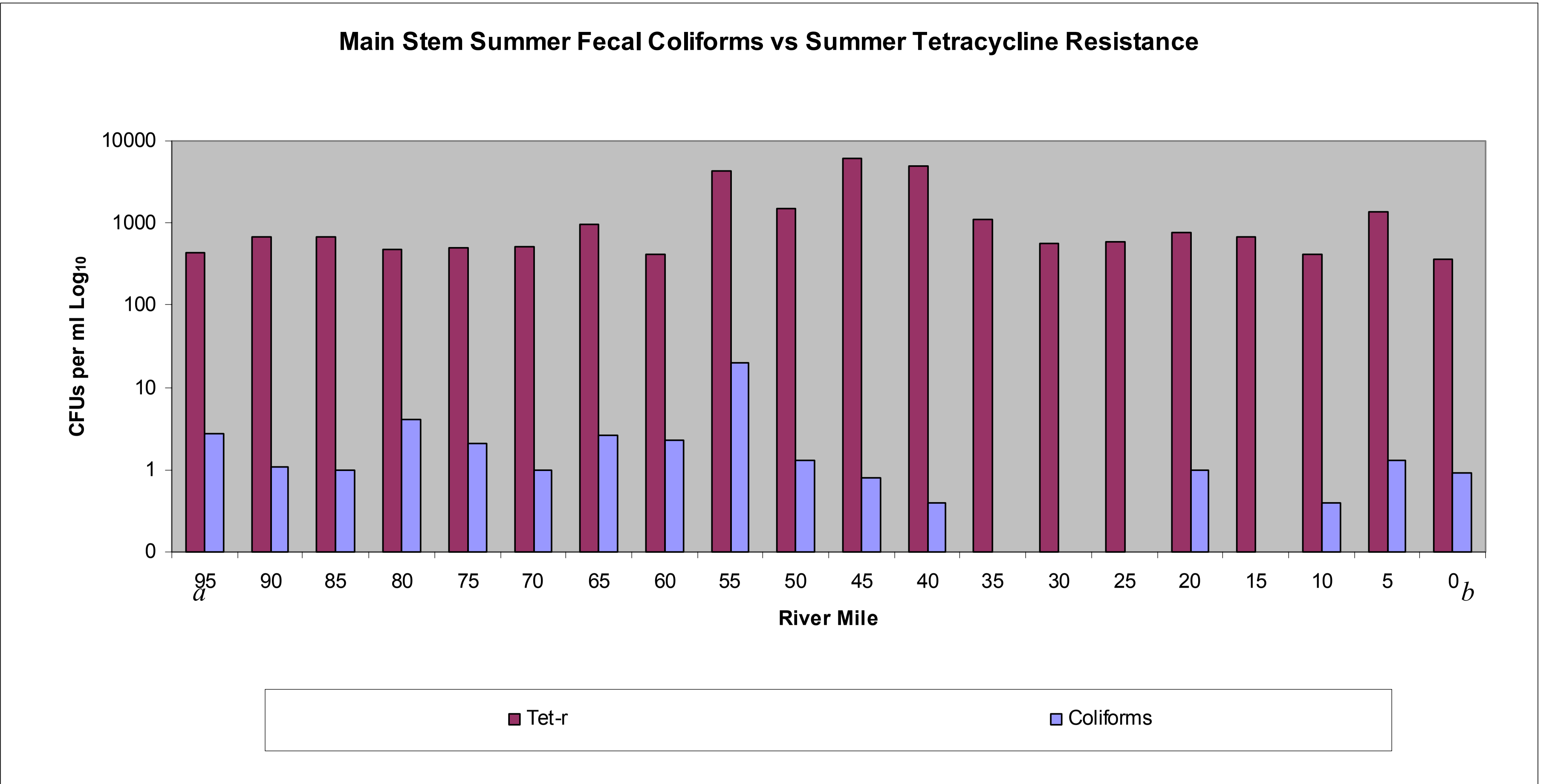


Figure 19. Comparison of all main stem mean fecal coliform counts to all main stem mean tetracycline (12.5 µg/ml) resistance counts during the summer.

$P < 0.01$ indicates mean tetracycline counts were higher than mean fecal coliform counts.

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

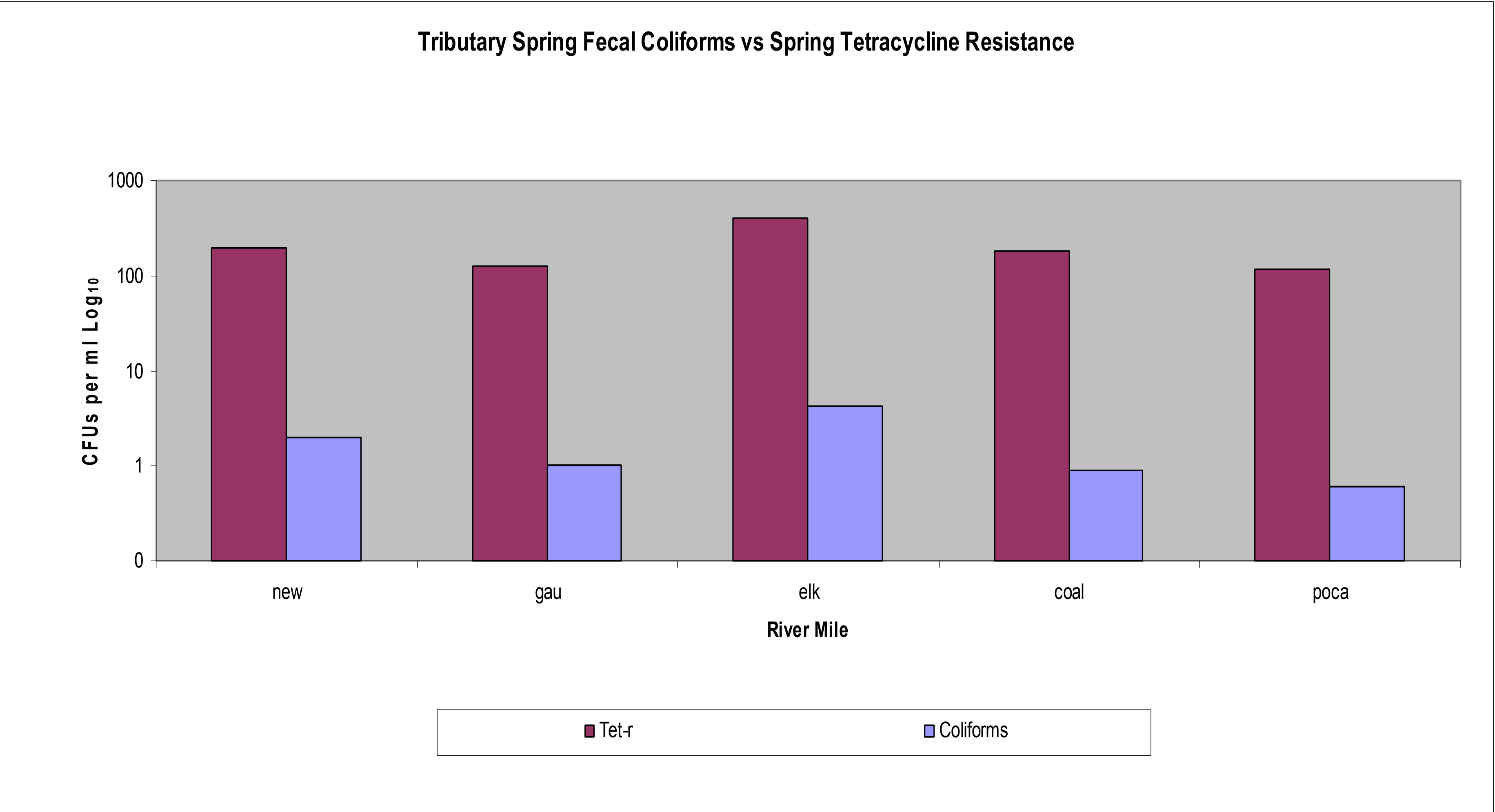


Figure 20. Comparison between the spring mean fecal coliform counts of the tributaries vs. summer mean tetracycline (12.5 µg/ml) resistance counts.

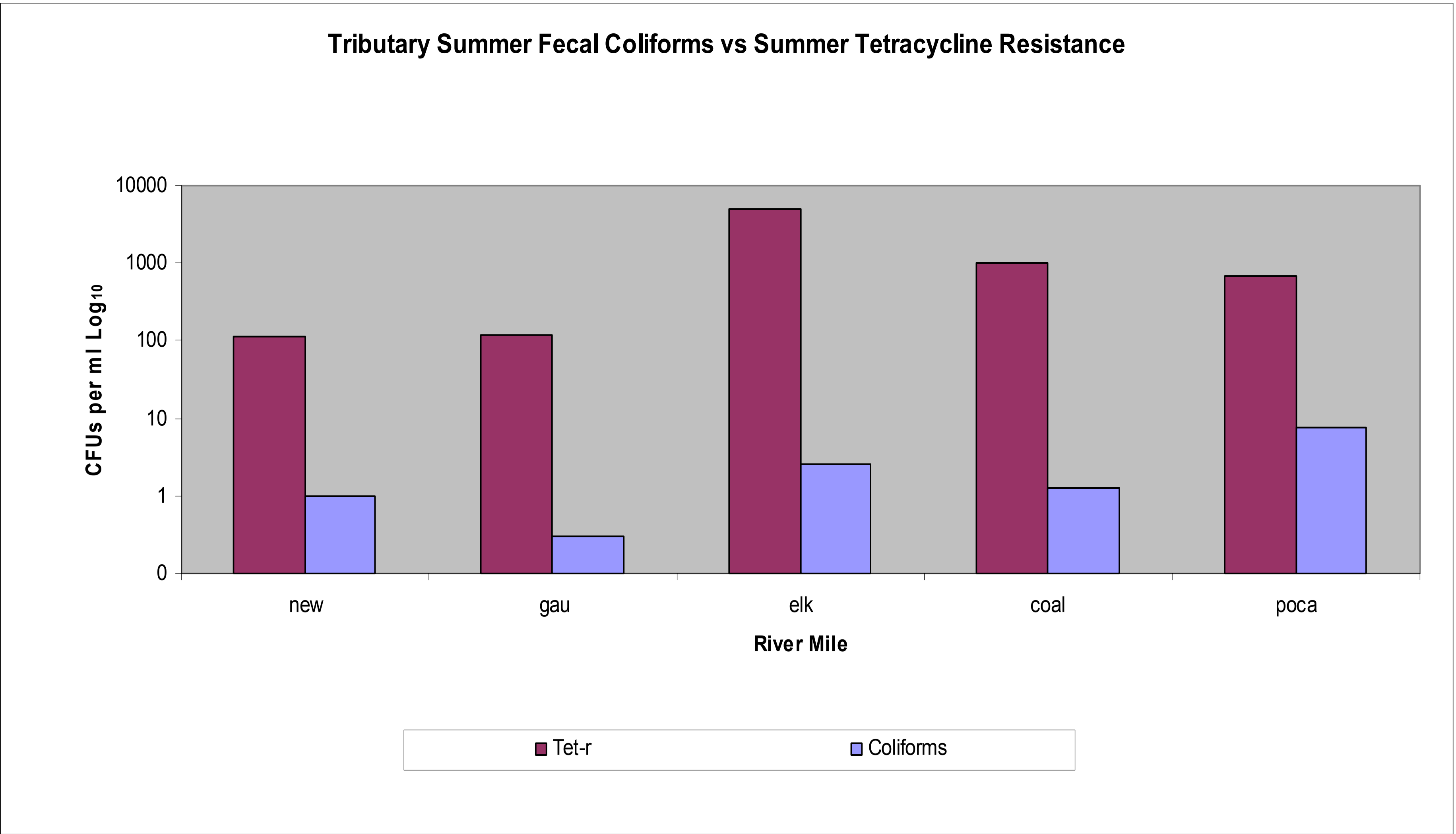


Figure 21. Comparison between the summer mean fecal coliform counts of the tributaries vs. summer mean tetracycline (12.5 µg/ml) resistance counts.

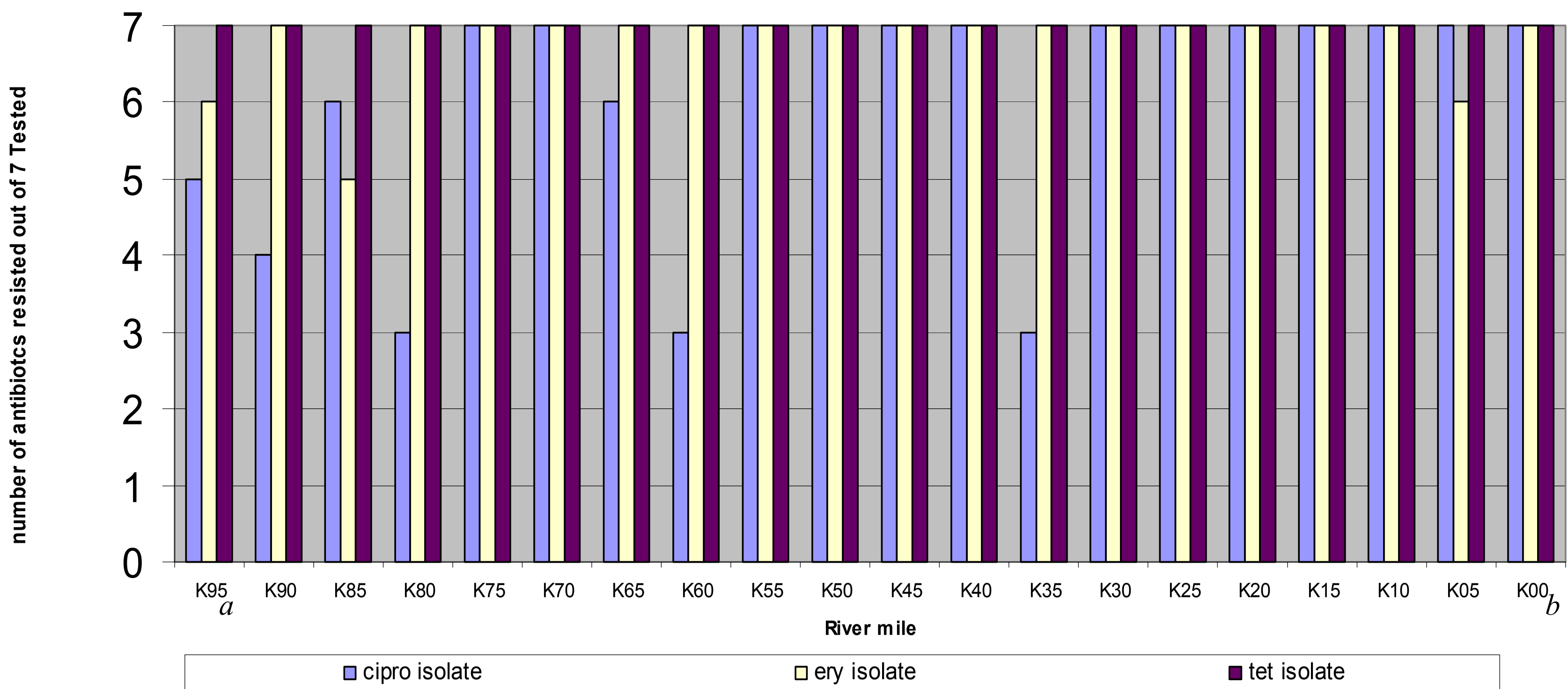


Figure 22. Distribution of multiple antibiotic resistance (MAR) from the main stem sample sites during the summer sample season using seven antibiotics.

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

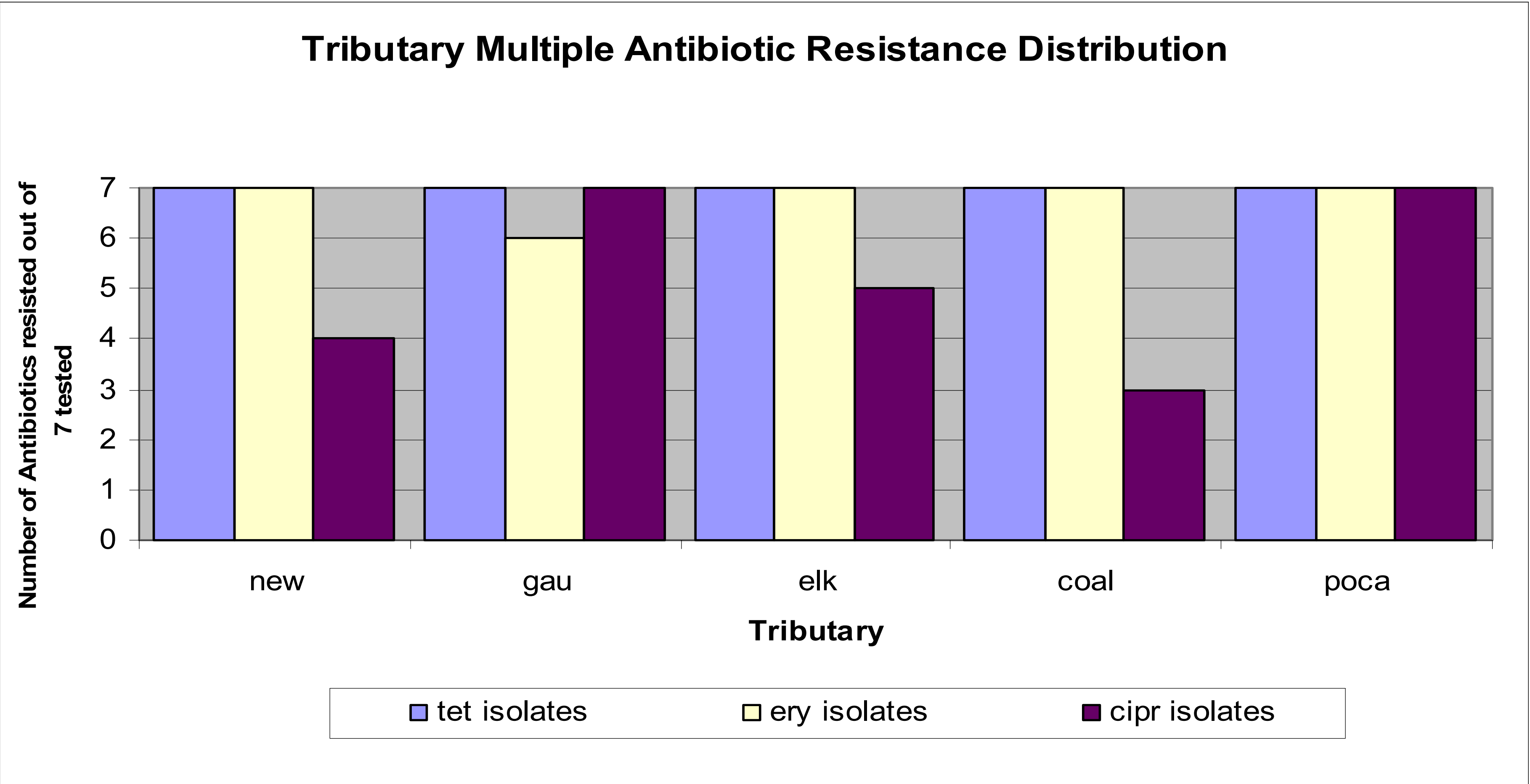


Figure 23 Distribution of multiple antibiotic resistance (MAR) from the tributary sample sites during the summer sample season using seven antibiotics.

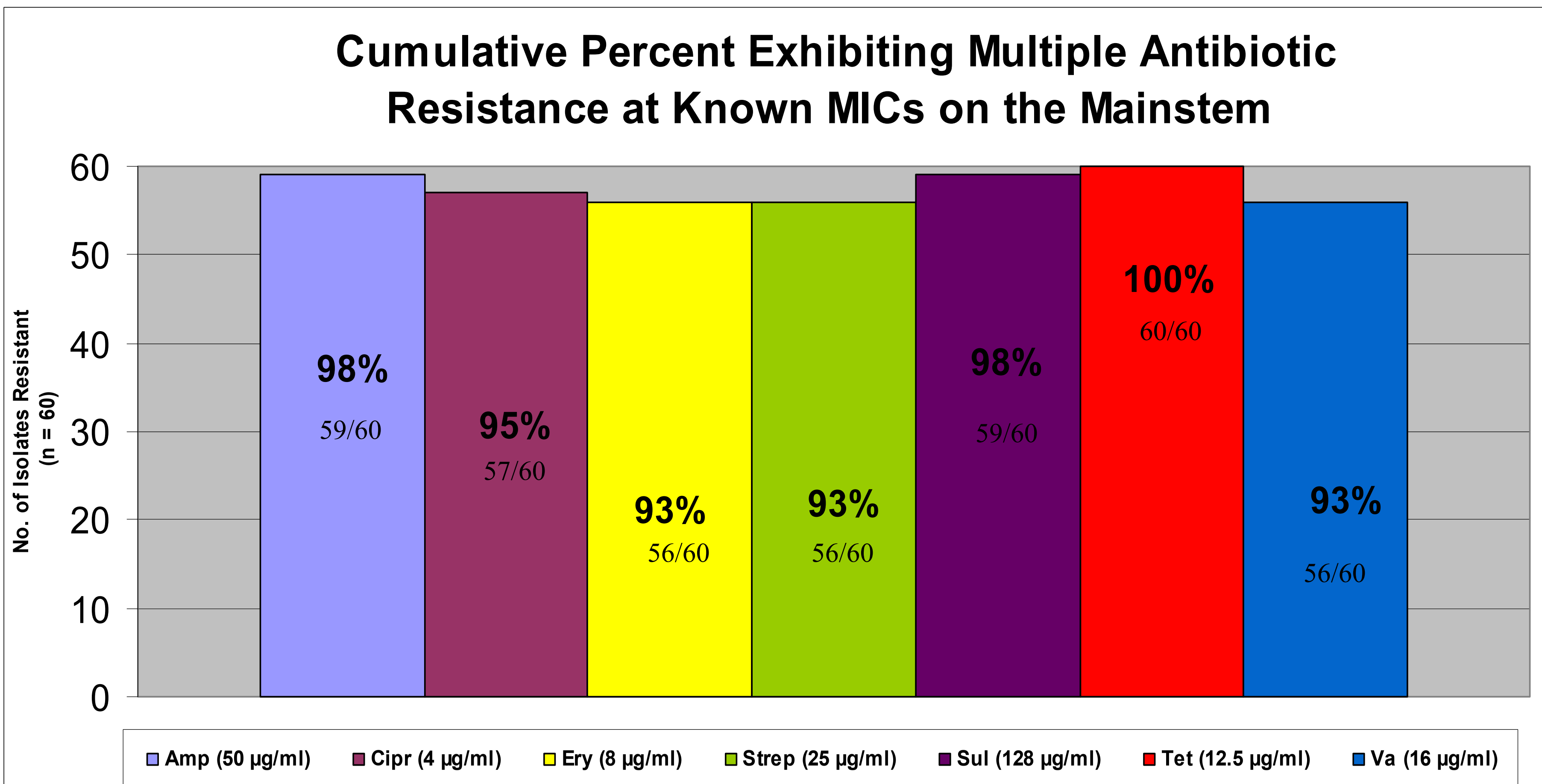


Figure 24. Cumulative percentage of isolates (n = 60) from the main stem of the Kanawha River exhibiting multiple antibiotic resistance.

100 % of isolates (n = 60) were resistant to tetracycline, 98 % of the isolates were resistant to ampicillin and sulfamethizole, 93 % were resistant to erythromycin, streptomycin and, virginiamycin and 95% were resistant to ciprofloxacin.

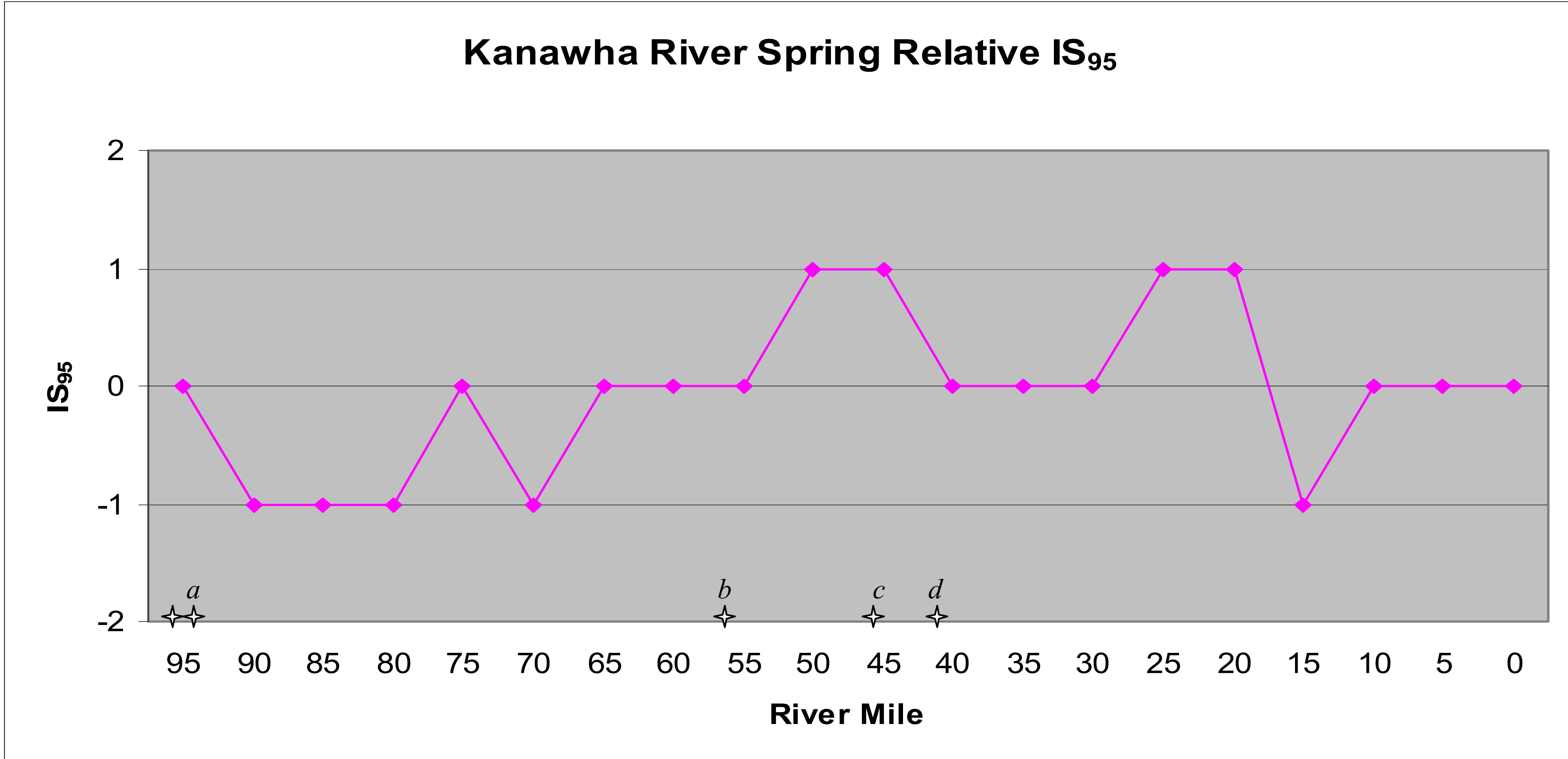


Figure 25. Kanawha River spring relative impact score for the mainstem using the 95th percentile boundary.

✧ 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

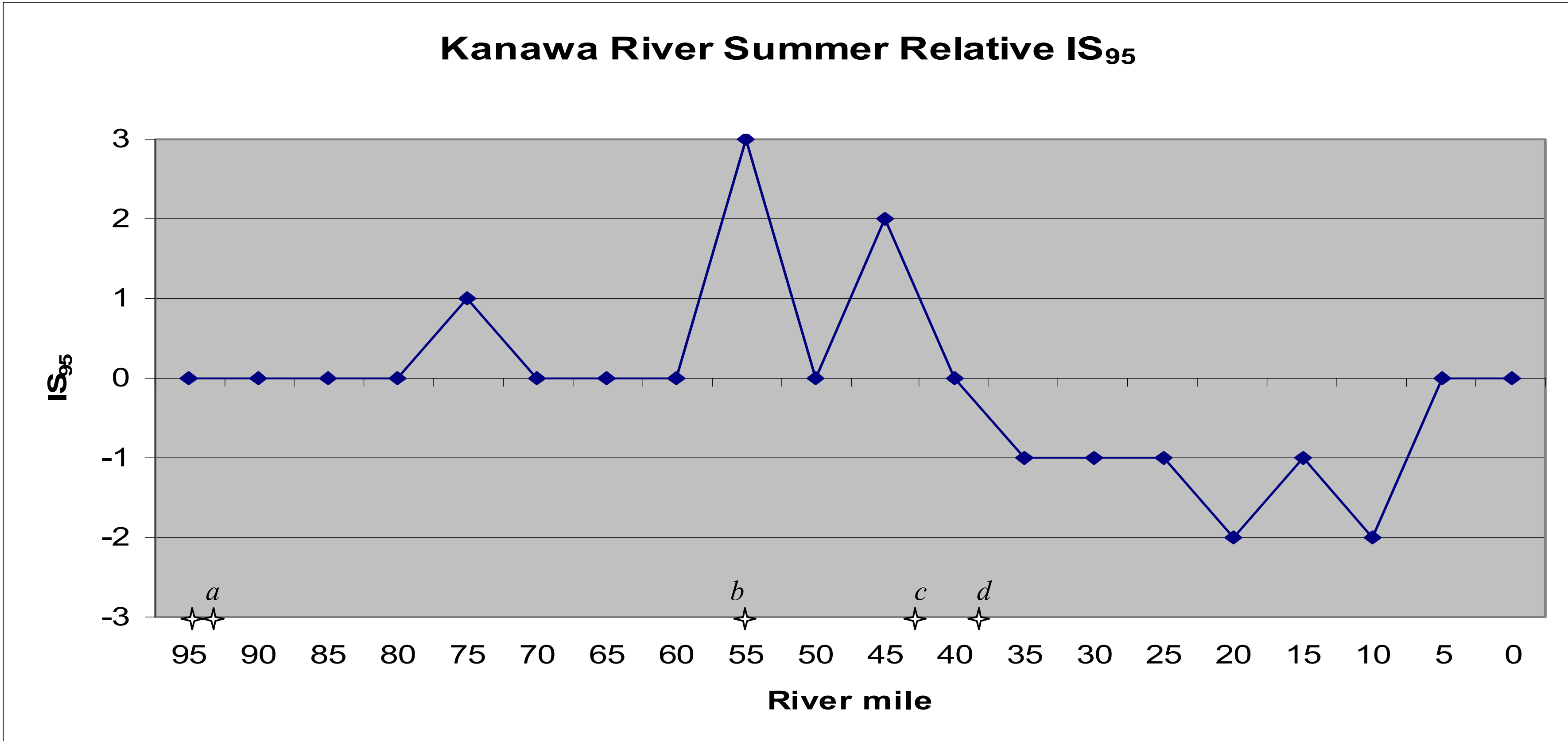


Figure 26. Kanawha River summer relative impact score for the mainstem using the 95th percentile boundary.

✧ 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

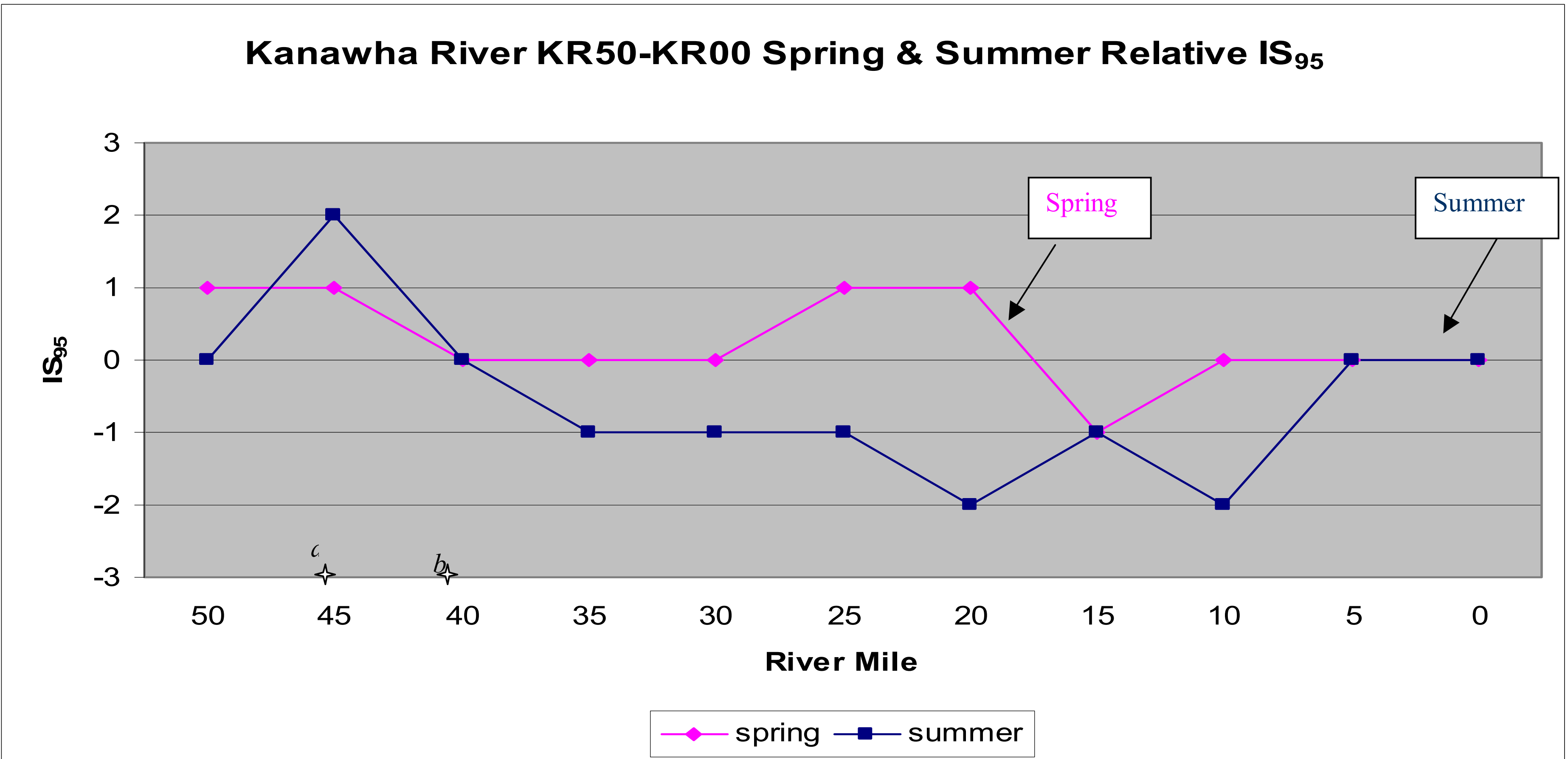


Figure 27. Mainstem comparison of relative impact scores for the 95th percentile boundary at KR50-KR00 for spring and summer.

✧ Indicates entry point of a tributary
a Coal River enters the main stem at KR45
b Pocatalico River enters the main stem at KR41

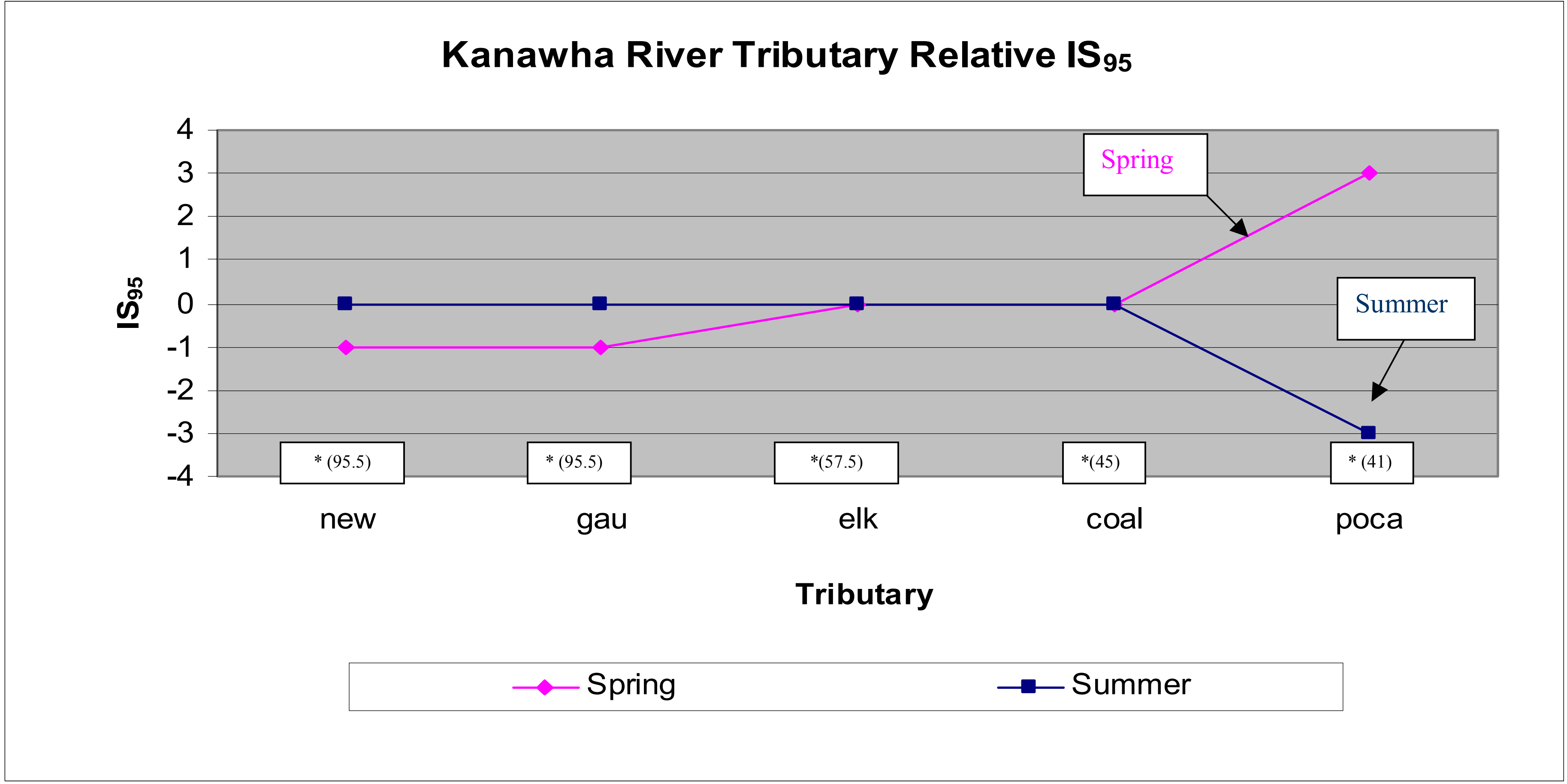


Figure 28. Tributary comparisons of relative impact scores for the 95th percentile boundary during spring and summer.

* Indicates the River mile at which the tributary enters the mainstem.

Table 1. Sample site locations along the mainstem from KR55-KR00

Site	Latitude	Longitude	Site Description
KR00	38.50'14"N	82.8'21"W	Located on the Kanawha River Upstream of Rt. 2 bridge at the mouth of the Kanawha emptying into the Ohio River.
KR05	38.48'18"N	82.3'31"W	Located on Kanawha River. Near ambrosia near Rockcastle Creek, 5.0 miles from the mouth.
KR10	38.46'15"N	81.59'7"W	Located on the Kanawha River. Near confluence with Ten Mile Creek 10.0 miles from the mouth.
KR15	38.42'30"N	81.57'6"W	Located on the Kanawha River. Near Arbuckle 15.0 miles from the mouth.
KR20	38.38'17"N	81.58'7"W	Located on the Kanawha River. Site is 1.5 miles downstream of the Buffalo boat launch 20.0 miles from the mouth
KR25	38.34'57"N	81.59'58"W	Located on the Kanawha River. Near Frazier's Bottom 25.0 miles from the mouth.
KR30	38.31'42"N	81.55'52"W	Located on the Kanawha River. Site is 1.2 miles downstream from the Winnfield Locks 30.0 miles from the mouth
KR35	38.31'52"N	81.511'20"W	Located on the Kanawha River. Site is 4.0 miles below the Pocatalico River and 3.6 miles upstream of the Winfield Locks, 35.0 miles from the mouth
KR40	38.27'37"N	81.49'13"W	Located on the Kanawha River. Site is 1 mile upstream from the Pocatalico River, 40.0 miles from the mouth.
KR45	38.23'53"N	81.50'34"W	Located on the Kanawha River. Site is 400 meters downstream from the Coal River, 45.0 miles from the mouth.
KR50	38.21'59"N	81.45'41"W	Located on the Kanawha River. Site is near Dunbar, 50.0 miles from the mouth
KR55	38.22'20"N	81.41'33"W	Located on the Kanawha River. Site is on the downstream side of Union Carbide island, 3 miles downstream of the Elk River, 55.0 miles from the mouth

Table 1 (Continued). Sample site locations along the mainstem from KR95-KR60

Site	Latitude	Longitude	Site Description
KR60	38.20'3"N	81.36'411"W	Located on the Kanawha River. Site is in Charleston near the capital, 60.0 miles from the mouth.
KR65	38.17'13"N	81.34'3"W	Located on the Kanawha River. Site is 2.5 miles downstream of the Marmet Locks near Rand, 65.0 miles from the mouth.
KR70	38.13'38"N	81.32'19"W	Located on the Kanawha River. Site is 2.5 miles upstream of the Marmet Locks, 70.0 miles from the mouth.
KR75	38.12'20"N	81.27'56.22"W	Located on the Kanawha River. Site is 1.6 miles upstream of the Chelyan Bridge at Goat Island.
KR80	38.12'25"N	81.23'57"W	Located on the Kanawha River. Site is in the town of Riverside, 80.0 miles from the mouth.
KR85	38.12'56"N	81.19'53"W	Located on the Kanawha River. Site is <1 mile downstream of the Montgomery bridge, 85.0 miles from the mouth.
KR90	38.8'0"N	81.16'43"W	Located on the Kanawha River. Site is behind Alloy plant in Alloy, 90.5 miles from the mouth.
KR95	38.8'48"N	81.12'39.7"W	Located on the Kanawha River. Site is on the right descending bank below island near Kanawha Falls.

Table 2. Sample site locations of the Kanawha Rivers 5 main tributaries

Elk River	38.21'21.5"N	81.38'35.4"W	Tributary. On Elk River, one tenth of a mile from the mouth, on the left descending bank
Gauley River	38.9'12.3"N	81.50'24.7"W	Tributary. Two miles upstream of Kanawha Falls on the right bank.
New River	38.9'12.3"N	81.10’53.1”W	Tributary. Site is approximately 1/4 mile up the channel out of the mixing zone.
Pocatalico	38.28'40.9"N	81.48'48.1"W	Tributary. Left bank of Pocatalico River, just above WV-62 bridge.
Coal River	38.23'4.38"N	81.50'24.7"W	Tributary. Site is approximately 1/4 mile up the channel out of the mixing zone.

Ampicillin (µg/ml)	**990**	**495**	**247.5**	**123.75**	**61.88**	**30.94**	**15.469**	**7.734**	**3.867**	**1.934**	**0.9667**
Ciprofloxacin (µg/ml)	**70**	**35**	**17.5**	**8.75**	**4.375**	**2.188**	**1.094**	**0.5469**	**0.2734**	**0.1367**	**0.0684**
Erythromycin (µg/ml)	**150**	**75**	**37.5**	**18.75**	**9.375**	**4.688**	**2.344**	**1.172**	**0.5859**	**0.2930**	**0.1465**
Tetracycline (µg/ml)	**240**	**120**	**60**	**30**	**15**	**7.5**	**3.75**	**1.875**	**0.9375**	**0.4688**	**0.2344**
Streptomycin (µg/ml)	**490**	**245**	**122.5**	**61.25**	**30.625**	**15.313**	**7.656**	**3.828**	**1.914**	**0.9570**	**0.4785**
Sulfamethizole (µg/ml)	**2550**	**1275**	**637.5**	**318.75**	**159.375**	**79.688**	**39.844**	**19.922**	**9.961**	**4.981**	**2.490**
Virginiamycin (µg/ml)	**310**	**155**	**77.5**	**38.75**	**19.375**	**9.688**	**4.844**	**2.422**	**1.2109**	**0.6055**	**0.3027**

Table 3. Concentrations of antibiotics tested in microtiter format.

Fields are highlighted in varying colors representing the concentrations associated with the antibiotic tested. Antibiotic ranges are from highest concentration to lowest concentration (i.e. 1000-0.9766 µg/ml ampicillin, 80-0.078 µg/ml ciprofloxacin, 160-0.156 µg/ml erythromycin, 500-0.4883 µg/ml streptomycin, 2560-2.50 µg/ml sulfamethizole, 250-0.244µg/ml tetracycline and 320-0.313µg/ml for virginiamycin).

Antibiotic	Season	Tributary				
		New River	Gauley River	Elk River	Coal River	Pocatalico River
Ciprofloxacin (4 µg/ml)	Spring (CFU/ml)	347	440	563	**763**	1353
	Summer (CFU/ml)	**2213**	**3560**	**963**	670	**9120**
Erythromycin (8 µg/ml)	Spring (CFU/ml)	760	850	780	1067	1133
	Summer (CFU/ml)	**1300**	**2750**	**6460**	**2273**	**3237**
Tetracycline (12.5 µg/ml)	Spring (CFU/ml)	117	**183**	**123**	**197**	407
	Summer (CFU/ml)	**690**	**1015**	117	113	**5097**

Table 4. Mean antibiotic resistance counts from each of the five tributaries sampled.

Fields highlighted yellow indicate higher average resistance counts relative to the seasonal comparison within the tributaries.

Table 5. Growth of Ciprofloxacin (4 µg/ml) resistant isolates from the Kanawha River and its tributaries exposed to varying concentrations of ampicillin.

Ampicillin conc. (µg/ml)	No. of Isolates growing on Mueller-Hinton Broth	
	Tributaries (n=5)	Main Stem (n=20)
990	4 (*80)	19 (*95)
495	4 (80)	19 (95)
247.5	4 (80)	20 (100)
123.75	4 (80)	20 (100)
61.88	4 (80)	20 (100)
30.94	4 (80)	20 (100)
15.469	4 (80)	20 (100)
7.734	4 (80)	20 (100)
3.867	5 (100)	20 (100)
1.934	5 (100)	20 (100)
0.9667	5 (100)	20 (100)

* indicates the percentage of isolates resistant at the given concentration

Table 6. Growth of Erythromycin (8 µg/ml) resistant isolates from the Kanawha River and its tributaries exposed to varying concentrations of ampicillin.

Ampicillin conc. (µg/ml)	No. of Isolates growing on Mueller-Hinton Broth	
	Tributaries (n=5)	Main Stem (n=20)
990	5 (*100)	19 (*95)
495	5 (100)	19 (95)
247.5	5 (100)	19 (95)
123.75	5 (100)	19 (95)
61.88	5 (100)	19 (95)
30.94	5 (100)	20 (100)
15.469	5 (100)	20 (100)
7.734	5 (100)	20 (100)
3.867	5 (100)	20 (100)
1.934	5 (100)	20 (100)
0.9667	5 (100)	20 (100)

* indicates the percentage of isolates resistant at the given concentration

Table 7. Growth of Tetracycline (12.5 µg/ml) resistant isolates from the Kanawha River and its tributaries exposed to varying concentrations of ampicillin.

Ampicillin conc. (µg/ml)	No. of Isolates growing on Mueller-Hinton Broth	
	Tributaries (n=5)	Main Stem (n=20)
990	5 (*100)	20 (*100)
495	5 (100)	20 (100)
247.5	5 (100)	20 (100)
123.75	5 (100)	20 (100)
61.88	5 (100)	20 (100)
30.94	5 (100)	20 (100)
15.469	5 (100)	20 (100)
7.734	5 (100)	20 (100)
3.867	5 (100)	20 (100)
1.934	5 (100)	20 (100)
0.9667	5 (100)	20 (100)

* indicates the percentage of isolates resistant at the given concentration

Table 8. Growth of Ciprofloxacin (4 µg/ml) resistant isolates from the Kanawha River and its tributaries exposed to varying concentrations of ciprofloxacin.

Ciprofloxacin conc. (µg/ml)	No. of Isolates growing on Mueller-Hinton Broth	
	Tributaries (n=5)	Main Stem (n=20)
70	5 (*100)	19 (*95)
35	5 (100)	19 (95)
17.5	5 (100)	19 (95)
8.75	5 (100)	19 (95)
4.375	5 (100)	20 (100)
2.188	5 (100)	20 (100)
1.094	5 (100)	20 (100)
0.5469	5 (100)	20 (100)
0.2734	5 (100)	20 (100)
0.1367	5 (100)	20 (100)
0.0684	5 (100)	20 (100)

* indicates the percentage of isolates resistant at the given concentration

Table 9. Growth of Erythromycin (8 µg/ml) resistant isolates from the Kanawha River and its tributaries exposed to varying concentrations of ciprofloxacin.

Ciprofloxacin conc. (µg/ml)	No. of Isolates growing on Mueller-Hinton Broth	
	Tributaries (n=5)	Main Stem (n=20)
70	5 (*100)	20 (*100)
35	5 (100)	20 (100)
17.5	5 (100)	20 (100)
8.75	5 (100)	20 (100)
4.375	5 (100)	20 (100)
2.188	5 (100)	20 (100)
1.094	5 (100)	20 (100)
0.5469	5 (100)	20 (100)
0.2734	5 (100)	20 (100)
0.1367	5 (100)	20 (100)
0.0684	5 (100)	20 (100)

* indicates the percentage of isolates resistant at the given concentration

Table 10. Growth of Tetracycline (12.5 µg/ml) resistant isolates from the Kanawha River and its tributaries exposed to varying concentrations of ciprofloxacin.

Ciprofloxacin conc. (µg/ml)	No. of Isolates growing on Mueller-Hinton Broth	
	Tributaries (n=5)	Main Stem (n=20)
70	5 (*100)	20 (*100)
35	5 (100)	20 (100)
17.5	5 (100)	20 (100)
8.75	5 (100)	20 (100)
4.375	5 (100)	20 (100)
2.188	5 (100)	20 (100)
1.094	5 (100)	20 (100)
0.5469	5 (100)	20 (100)
0.2734	5 (100)	20 (100)
0.1367	5 (100)	20 (100)
0.0684	5 (100)	20 (100)

* indicates the percentage of isolates resistant at the given concentration

Table 11. Growth of Ciprofloxacin (4 µg/ml) resistant isolates from the Kanawha River and its tributaries exposed to varying concentrations of erythromycin.

Erythromycin conc. (µg/ml)	No. of Isolates growing on Mueller-Hinton Broth	
	Tributaries (n=5)	Main Stem (n=20)
150	3 (*60)	15 (*75)
75	3 (60)	15 (75)
37.5	3 (60)	15 (75)
18.75	3 (60)	16 (*80)
9.375	3 (60)	16 (80)
4.688	3 (60)	16 (80)
2.344	3 (60)	17 (*85)
1.172	3 (60)	18 (*90)
0.5859	3 (60)	19 (*95)
0.2930	4 (*80)	19 (95)
0.1465	4 (80)	19 (95)

* indicates the percentage of isolates resistant at the given concentration

Table 12. Growth of Erythromycin (8 µg/ml) resistant isolates from the Kanawha River and its tributaries exposed to varying concentrations of erythromycin.

Erythromycin conc. (µg/ml)	No. of Isolates growing on Mueller-Hinton Broth	
	Tributaries (n=5)	Main Stem (n=20)
150	4 (*80)	19 (95)
75	4 (80)	19 (95)
37.5	4 (80)	19 (95)
18.75	4 (80)	19 (95)
9.375	4 (80)	19 (95)
4.688	5 (*100)	20 (*100)
2.344	5 (100)	20 (100)
1.172	5 (100)	20 (100)
0.5859	5 (100)	20 (100)
0.2930	5 (100)	20 (100)
0.1465	5 (100)	20 (100)

* indicates the percentage of isolates resistant at the given concentration

Table 13. Growth of Tetracycline (12.5 µg/ml) resistant isolates from the Kanawha River and its tributaries exposed to varying concentrations of erythromycin.

Erythromycin conc. (µg/ml)	No. of Isolates growing on Mueller-Hinton Broth	
	Tributaries (n=5)	Main Stem (n=20)
150	5 (*100)	20 (*100)
75	5 (100)	20 (100)
37.5	5 (100)	20 (100)
18.75	5 (100)	20 (100)
9.375	5 (100)	20 (100)
4.688	5 (100)	20 (100)
2.344	5 (100)	20 (100)
1.172	5 (100)	20 (100)
0.5859	5 (100)	20 (100)
0.2930	5 (100)	20 (100)
0.1465	5 (100)	20 (100)

* indicates the percentage of isolates resistant at the given concentration

Table 14. Growth of Ciprofloxacin (4 µg/ml) resistant isolates from the Kanawha River and its tributaries exposed to varying concentrations of streptomycin.

Streptomycin conc. (µg/ml)	No. of Isolates growing on Mueller-Hinton Broth	
	Tributaries (n=5)	Main Stem (n=20)
490	2 (*40)	16 (*80)
245	3 (*60)	16 (80)
122.5	3 (60)	16 (80)
61.25	3 (60)	18 (*90)
30.625	3 (60)	20 (*100)
15.313	3 (60)	20 (100)
7.656	3 (60)	20 (100)
3.828	5 (*100)	20 (100)
1.914	5 (100)	20 (100)
0.9570	5 (100)	20 (100)
0.4785	5 (100)	20 (100)

* indicates the percentage of isolates resistant at the given concentration

Table 15. Growth of Erythromycin (8 µg/ml) resistant isolates from the Kanawha River and its tributaries exposed to varying concentrations of streptomycin.

Streptomycin conc. (µg/ml)	No. of Isolates growing on Mueller-Hinton Broth	
	Tributaries (n=5)	Main Stem (n=20)
490	4 (*80)	17 (*85)
245	4 (80)	17 (85)
122.5	4 (80)	18 (*90)
61.25	4 (80)	18 (90)
30.625	5 (*100)	18 (90)
15.313	5 (100)	20 (*100)
7.656	5 (100)	20 (100)
3.828	5 (100)	20 (100)
1.914	5 (100)	20 (100)
0.9570	5 (100)	20 (100)
0.4785	5 (100)	20 (100)

* indicates the percentage of isolates resistant at the given concentration

Table 16. Growth of Tetracycline (12.5 µg/ml) resistant isolates from the Kanawha River and its tributaries exposed to varying concentrations of streptomycin.

Streptomycin conc. (µg/ml)	No. of Isolates growing on Mueller-Hinton Broth	
	Tributaries (n=5)	Main Stem (n=20)
490	5 (*100)	20 (*100)
245	5 (100)	20 (100)
122.5	5 (100)	20 (100)
61.25	5 (100)	20 (100)
30.625	5 (100)	20 (100)
15.313	5 (100)	20 (100)
7.656	5 (100)	20 (100)
3.828	5 (100)	20 (100)
1.914	5 (100)	20 (100)
0.9570	5 (100)	20 (100)
0.4785	5 (100)	20 (100)

* indicates the percentage of isolates resistant at the given concentration

Table 17. Growth of Ciprofloxacin (4 µg/ml) resistant isolates from the Kanawha River and its tributaries exposed to varying concentrations of sulfamethizole.

Sulfamethizole conc. (µg/ml)	No. of Isolates growing on Mueller-Hinton Broth	
	Tributaries (n=5)	Main Stem (n=20)
2550	5 (*100)	17 (*85)
1275	5 (100)	18 (*90)
637.5	5 (100)	18 (90)
318.75	5 (100)	18 (90)
159.375	5 (100)	19 (*95)
79.688	5 (100)	19 (95)
39.844	5 (100)	20 (*100)
19.922	5 (100)	20 (100)
9.961	5 (100)	20 (100)
4.981	5 (100)	20 (100)
2.490	5 (100)	20 (100)

* indicates the percentage of isolates resistant at the given concentration

Table 18. Growth of Erythromycin (8 µg/ml) resistant isolates from the Kanawha River and its tributaries exposed to varying concentrations of sulfamethizole.

Sulfamethizole conc. (µg/ml)	No. of Isolates growing on Mueller-Hinton Broth	
	Tributaries (n=5)	Main Stem (n=20)
2550	5 (*100)	20 (*100)
1275	5 (100)	20 (100)
637.5	5 (100)	20 (100)
318.75	5 (100)	20 (100)
159.375	5 (100)	20 (100)
79.688	5 (100)	20 (100)
39.844	5 (100)	20 (100)
19.922	5 (100)	20 (100)
9.961	5 (100)	20 (100)
4.981	5 (100)	20 (100)
2.490	5 (100)	20 (100)

* indicates the percentage of isolates resistant at the given concentration

Table 19. Growth of Tetracycline (12.5 µg/ml) resistant isolates from the Kanawha River and its tributaries exposed to varying concentrations of sulfamethizole.

Sulfamethizole conc. (µg/ml)	No. of Isolates growing on Mueller-Hinton Broth	
	Tributaries (n=5)	Main Stem (n=20)
2550	5 (*100)	20 (*100)
1275	5 (100)	20 (100)
637.5	5 (100)	20 (100)
318.75	5 (100)	20 (100)
159.375	5 (100)	20 (100)
79.688	5 (100)	20 (100)
39.844	5 (100)	20 (100)
19.922	5 (100)	20 (100)
9.961	5 (100)	20 (100)
4.981	5 (100)	20 (100)
2.490	5 (100)	20 (100)

* indicates the percentage of isolates resistant at the given concentration

Table 20. Growth of Ciprofloxacin (4 µg/ml) resistant isolates from the Kanawha River and its tributaries exposed to varying concentrations of tetracycline.

Tetracycline conc. (µg/ml)	No. of Isolates growing on Mueller-Hinton Broth	
	Tributaries (n=5)	Main Stem (n=20)
240	2 (*40)	16 (*80)
120	2 (*40)	16 (80)
60	3 (*60)	16 (80)
30	3 (60)	17 (*85)
15	4 (*80)	17 (85)
7.5	5 (*100)	18 (*90)
3.75	5 (100)	18 (90)
1.875	5 (100)	20 (*100)
0.9375	5 (100)	20 (100)
0.4688	5 (100)	20 (100)
0.2344	5 (100)	20 (100)

* indicates the percentage of isolates resistant at the given concentration

Table 21. Growth of Erythromycin (8 µg/ml) resistant isolates from the Kanawha River and its tributaries exposed to varying concentrations of tetracycline.

Tetracycline conc. (µg/ml)	No. of Isolates growing on Mueller-Hinton Broth	
	Tributaries (n=5)	Main Stem (n=20)
240	4 (*80)	18 (*90)
120	4 (80)	18 (90)
60	5 (*100)	18 (90)
30	5 (100)	20 (*100)
15	5 (100)	20 (100)
7.5	5 (100)	20 (100)
3.75	5 (100)	20 (100)
1.875	5 (100)	20 (100)
0.9375	5 (100)	20 (100)
0.4688	5 (100)	20 (100)
0.2344	5 (100)	20 (100)

* indicates the percentage of isolates resistant at the given concentration

Table 22. Growth of Tetracycline (12.5 µg/ml) resistant isolates from the Kanawha River and its tributaries exposed to varying concentrations of tetracycline.

Tetracycline conc. (µg/ml)	No. of Isolates growing on Mueller-Hinton Broth	
	Tributaries (n=5)	Main Stem (n=20)
240	5 (*100)	20 (*100)
120	5 (100)	20 (100)
60	5 (100)	20 (100)
30	5 (100)	20 (100)
15	5 (100)	20 (100)
7.5	5 (100)	20 (100)
3.75	5 (100)	20 (100)
1.875	5 (100)	20 (100)
0.9375	5 (100)	20 (100)
0.4688	5 (100)	20 (100)
0.2344	5 (100)	20 (100)

* indicates the percentage of isolates resistant at the given concentration

Table 23. Growth of Ciprofloxacin (4 µg/ml) resistant isolates from the Kanawha River and its tributaries exposed to varying concentrations of virginiamycin.

Virginiamycin conc. (µg/ml)	No. of Isolates growing on Mueller-Hinton Broth	
	Tributaries (n=5)	Main Stem (n=20)
310	2 (*40)	13 (*65)
155	2 (40)	13 (65)
77.5	2 (40)	13 (65)
38.75	3 (*60)	13 (65)
19.375	3 (60)	15 (*75)
9.688	3 (60)	16 (*80)
4.844	3 (60)	16 (80)
2.422	5 (*100)	17 (*85)
1.2109	5 (100)	20 (100)
0.6055	5 (100)	20 (100)
0.3027	5 (100)	20 (100)

* indicates the percentage of isolates resistant at the given concentration

Table 24. Growth of Erythromycin (8 µg/ml) resistant isolates from the Kanawha River and its tributaries exposed to varying concentrations of virginiamycin.

Virginiamycin conc. (µg/ml)	No. of Isolates growing on Mueller-Hinton Broth	
	Tributaries (n=5)	Main Stem (n=20)
310	5 (*100)	18 (*90)
155	5 (100)	18 (90)
77.5	5 (100)	18 (90)
38.75	5 (100)	18 (90)
19.375	5 (100)	19 (*95)
9.688	5 (100)	19 (95)
4.844	5 (100)	19 (95)
2.422	5 (100)	20 (*100)
1.2109	5 (100)	20 (100)
0.6055	5 (100)	20 (100)
0.3027	5 (100)	20 (100)

* indicates the percentage of isolates resistant at the given concentration

Table 25. Growth of Tetracycline (12.5 µg/ml) resistant isolates from the Kanawha River and its tributaries exposed to varying concentrations of virginiamycin.

Virginiamycin conc. (µg/ml)	No. of Isolates growing on Mueller-Hinton Broth	
	Tributaries (n=5)	Main Stem (n=20)
310	5 (*100)	20 (*100)
155	5 (100)	20 (100)
77.5	5 (100)	20 (100)
38.75	5 (100)	20 (100)
19.375	5 (100)	20 (100)
9.688	5 (100)	20 (100)
4.844	5 (100)	20 (100)
2.422	5 (100)	20 (100)
1.2109	5 (100)	20 (100)
0.6055	5 (100)	20 (100)
0.3027	5 (100)	20 (100)

* indicates the percentage of isolates resistant at the given concentration

Table 26. Minimum Inhibitory Concentrations for 7 antibiotics using one ciprofloxacin (4 µg/ml) resistant isolate recovered from each of the twenty samples sites from the main stem of the Great Kanawha River.

Antibiotic (µg/ml)	River Mile																			
	K95	K90	K85	K80	K75	K70	K65	K60	K55	K50	K45	K40	K35	K30	K25	K20	K15	K10	K05	K00
Erythromycin	**2.344**	**0.2930**	>150	**4.688**	>150	>150	>150	**37.5**	>150	>150	>150	>150	>150	>150	>150	>150	>150	>150	>150	>150
Tetracycline	**3.75**	**15**	>240	**15**	>240	>240	>240	**60**	>240	>240	>240	>240	>240	>240	>240	>240	>240	>240	>240	>240
Ampicillin	>990	>990	**495**	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990
Streptomycin	>490	**61.25**	**30.625**	**61.25**	>490	>490	>490	>490	>490	>490	>490	>490	**30.625**	>490	>490	>490	>490	>490	>490	>490
Virginiamycin	**38.75**	**2.422**	**310**	**2.422**	>310	>310	**4.844**	**38.75**	>310	>310	>310	>310	**19.375**	>310	>310	>310	>310	>310	>310	>310
Sulfamethizole	>2550	>2550	>2550	>2550	>2550	>2550	>2550	**637.5**	>2550	>2550	>2550	>2550	**79.688**	>2550	>2550	>2550	>2550	>2550	>2550	>2550
Ciprofloxacin	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70

* Fields highlighted in bold print indicate a Minimum Inhibitory Concentration.

** Fields not highlighted indicate the Minimum Inhibitory concentration was greater than the highest antibiotic concentration tested.

Table 27. Minimum Inhibitory Concentrations for 7 antibiotics using one erythromycin (8 µg/ml) resistant isolate recovered from each of the twenty samples sites from the main stem of the Great Kanawha River.

Antibiotic (µg/ml)	River Mile																			
	K95	K90	K85	K80	K75	K70	K65	K60	K55	K50	K45	K40	K35	K30	K25	K20	K15	K10	K05	K00
Erythromycin	>150	>150	>150	**9.375**	>150	>150	>150	>150	>150	>150	>150	>150	>150	>150	>150	>150	>150	>150	>150	>150
Tetracycline	>240	>240	**60**	>240	**60**	>240	>240	>240	>240	>240	>240	>240	>240	>240	>240	>240	>240	>240	>240	>240
Ampicillin	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990	**61.88**	>990
Streptomycin	**30.625**	>490	**30.625**	>490	>490	**245**	>490	>490	>490	>490	>490	>490	>490	>490	>490	>490	>490	>490	>490	>490
Virginiamycin	**38.75**	>310	**4.844**	>310	>310	>310	>310	>310	>310	>310	>310	>310	>310	>310	>310	>310	>310	>310	>310	>310
Sulfamethizole	>2550	>2550	>2550	>2550	>2550	>2550	>2550	>2550	>2550	>2550	>2550	>2550	>2550	>2550	>2550	>2550	>2550	>2550	>2550	>2550
Ciprofloxacin	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70

* Fields highlighted in bold print indicate a Minimum Inhibitory Concentration.

** Fields not highlighted indicate the Minimum Inhibitory concentration was greater than the highest antibiotic concentration tested.

Table 28. Minimum Inhibitory Concentrations for 7 antibiotics using one tetracycline (12.5 µg/ml) resistant isolate recovered from each of the twenty samples sites from the main stem of the Great Kanawha River.

Antibiotic (µg/ml)	River Mile																			
	K95	K90	K85	K80	K75	K70	K65	K60	K55	K50	K45	K40	K35	K30	K25	K20	K15	K10	K05	K00
Erythromycin	>150	>150	>150	>150	>150	>150	>150	>150	>150	>150	>150	>150	>150	>150	>150	>150	>150	>150	>150	>150
Tetracycline	>240	>240	>240	>240	>240	>240	>240	>240	>240	>240	>240	>240	>240	>240	>240	>240	>240	>240	>240	>240
Ampicillin	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990	>990
Streptomycin	>490	>490	>490	>490	>490	>490	>490	>490	>490	>490	>490	>490	>490	>490	>490	>490	>490	>490	>490	>490
Virginiamycin	>310	>310	>310	>310	>310	>310	>310	>310	>310	>310	>310	>310	>310	>310	>310	>310	>310	>310	>310	>310
Sulfamethizole	>2550	>2550	>2550	>2550	>2550	>2550	>2550	>2550	>2550	>2550	>2550	>2550	>2550	>2550	>2550	>2550	>2550	>2550	>2550	>2550
Ciprofloxacin	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70	>70

* Fields highlighted in bold print indicate a Minimum Inhibitory Concentration.

** Fields not highlighted indicate the Minimum Inhibitory concentration was greater than the highest antibiotic concentration tested.

Table 29. Minimum Inhibitory Concentrations for 7 antibiotics using one ciprofloxacin (4 µg/ml) resistant isolate recovered from each of the five primary tributaries of the Great Kanawha River.

Antibiotic (µg/ml)	New	Gauley	Elk	Coal	Pocatalico
Erythromycin	>150	>150	<0.1465	**0.5859**	>150
Tetracycline	**120**	>240	**30**	**7.5**	>240
Ampicillin	**7.5**	>990	>990	>990	>990
Streptomycin	**7.5**	>490	**490**	**7.5**	>490
Virginiamycin	**77.5**	>310	**4.844**	**4.844**	>310
Sulfamethizole	>2550	>2550	>2550	>2550	>2550
Ciprofloxacin	>70	>70	>70	>70	>70

* Fields highlighted in bold print indicate a Minimum Inhibitory Concentration.
** Fields not highlighted indicate the Minimum Inhibitory concentration was greater than the highest antibiotic concentration tested.

Table 30. Minimum Inhibitory Concentrations for 7 antibiotics using one erythromycin (8 µg/ml) resistant isolate recovered from each of the five primary tributaries of the Great Kanawha River.

Antibiotic (µg/ml)	New	Gauley	Elk	Coal	Pocatalico
Erythromycin	>150	**9.375**	>150	>150	>150
Tetracycline	>240	**120**	>240	>240	>240
Ampicillin	>990	>990	>990	>990	>990
Streptomycin	>490	**61.25**	>490	>490	>490
Virginiamycin	>310	>310	>310	>310	>310
Sulfamethizole	>2550	>2550	>2550	>2550	>2550
Ciprofloxacin	>70	>70	>70	>70	>70

* Fields highlighted in bold print indicate a Minimum Inhibitory Concentration.

** Fields not highlighted indicate the Minimum Inhibitory concentration was greater than the highest antibiotic concentration tested.

Table 31. Minimum Inhibitory Concentrations for 7 antibiotics using one tetracycline (12.5 μg/ml) resistant isolate recovered from each of the five primary tributaries of the Great Kanawha River.

Antibiotic (μg/ml)	New	Gauley	Elk	Coal	Pocatalico
Erythromycin	>150	>150	>150	>150	>150
Tetracycline	>240	>240	>240	>240	>240
Ampicillin	>990	>990	>990	>990	>990
Streptomycin	>490	>490	>490	>490	>490
Virginiamycin	>310	>310	>310	>310	>310
Sulfamethizole	>2550	>2550	>2550	>2550	>2550
Ciprofloxacin	>70	>70	>70	>70	>70

* Fields highlighted in bold print indicate a Minimum Inhibitory Concentration.
** Fields not highlighted indicate the Minimum Inhibitory concentration was greater than the highest antibiotic concentration tested.

Table 32. Spring vs. Summer Impact Scores (range -4 to 4) 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	-3
Gauley	T	-1	0
95	U	0	0
90	U	-1	0
85	U	-1	0
80	U	-1	0
75	U	0	1
70	U	-1	0
65	U	0	0
60	U	0	0
Elk	T	0	2
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), L (Lower Kanawha), 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 A

ANTIBIOTIC DESCRIPTIONS

1. **Ampicillin**

   A **penicillin class** of antibiotic with extended spectrum activity against Gram negative species. These are β-lactamase antibiotics that contain penicillin binding proteins that bind to the penicillin binding proteins in the bacterial cell wall to **inhibit peptidoglycan synthesis** which results in cell death. This action makes β-lactamase antibiotics bactericidal.

2. **Ciprofloxacin**

   A **quinilone class** of antibiotic with broad-spectrum activity against Gram negative and Gram positive bacteria. Quinilones are synthetic chemotherapeutic agents that inhibit DNA gyrase or topoisomerases that are required for replication, recombination and repair. As a result **nucleic acid synthesis is inhibited**. Ciprofloxacin is a fluoroqunilone (newer quinilones) derived by alteration of the two ring quinilone nucleus.

3. **Erythromycin**

   A **macrolide class** of antibiotic that is bacteriostatic with a broad-spectrum of activity against Gram-positive and some Gram-negative bacteria (e.g. *Neisseria, Legionella, Mycoplasma, Chlamydia, Chlamydophila, Treponema, and Rickettsia.* Developed from *Streptomyces erythreus).* Macrolides work by reversible binding to the 50s ribosomal subunit, which **blocks polypeptide elongation**.

4. **Streptomycin**

   An **aminoglycoside** class of antibiotic primarily used to treat infections with Gram-negative bacilli. Developed from the *Streptomyces* spp... These antibiotics act by passing through the bacterial outer membrane (in Gram-negative bacteria), cell wall, and cytoplasmic membrane to the cytoplasm where they **inhibit protein synthesis** by irreversibly binding to the 30s ribosomal subunit. Attachment causes misreading of the messenger RNA (mRNA) and interruption of protein synthesis by causing the premature release of the ribosome from mRNA. The action of irreversible binding makes the antibiotic bacteriocidal. Streptomycin has been used for the treatment of tuberculosis, tularemia, and streptococcal or enterococcal infections (in combination with penicillin).

5. **Sulfamethizole**

A **sulfonamide** class of antibiotic known as an antimetabolite (a substance which competitively inhibits the utilization, by an organism, of an exogenous substrate or endogenous metabolite (Singleton et al, 2002)). These antimetabolites compete for p-aminobenzoic acid (PABA) preventing folic acid synthesis. Sulfonamides are similar in structure to PABA tricking the bacteria into taking it (sulfonamide) up and **inhibiting folic acid synthesis**. They are effective against a wide range of Gram negative and Gram positive bacteria as well as various protozoa (e.g. *Plasmodium* spp.). In combination with other folic acid antagonists can be used to treat urinary tract infections, Malaria, etc.

6. Tetracycline

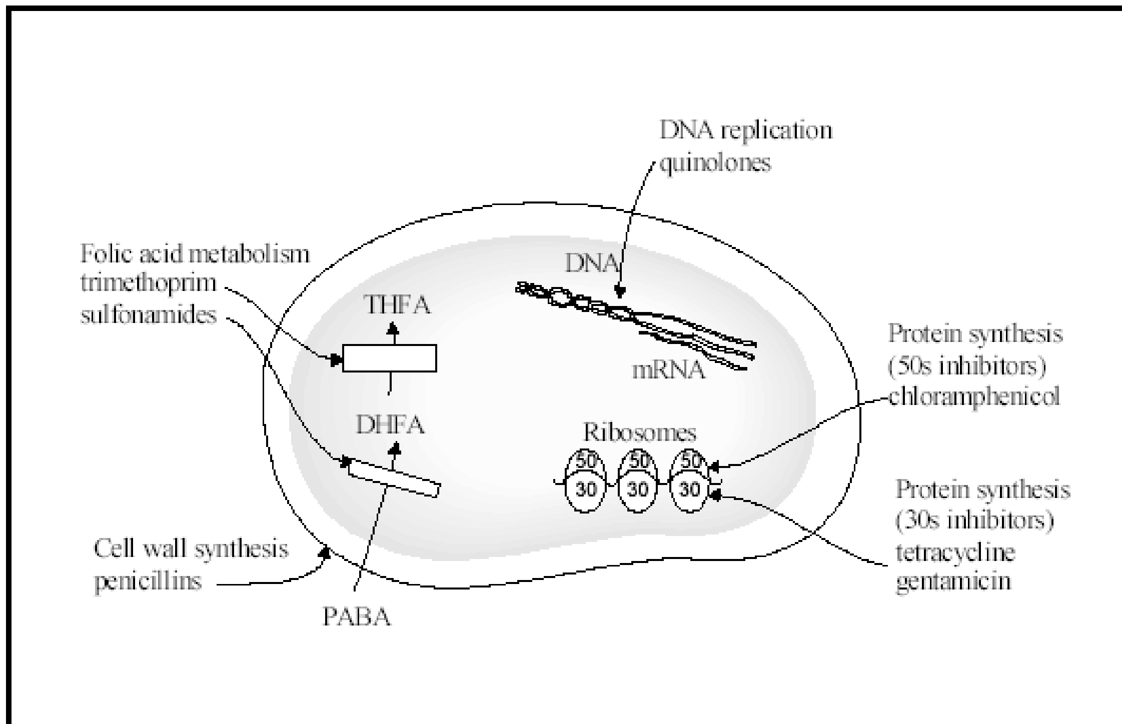
A **tetracycline class** of antibiotic that is bacteriostatic and has broad-spectrum activity which **inhibits protein synthesis** in bacteria by binding reversibly to the 30s ribosomal subunits blocking the binding of aminoacyl transferase. Is effective in the treatment of infections caused by *Chlamydia, Mycoplasma, Rickettsia,* and other selected Gram-positive and Gram-negative bacteria.

7. Virginiamycin

A **streptogramin class** of antibiotic made up of two antibiotic molecules that act synergistically to **prevent protein synthesis**. Primarily a Gram-positive antibacterial. Has been in use for 30 years on poultry, cattle and swine to prevent and control infections and outbreaks of intestinal diseases. It is not absorbed by the systemic circulation of the animals, but remains in the gut.

APPENDIX B

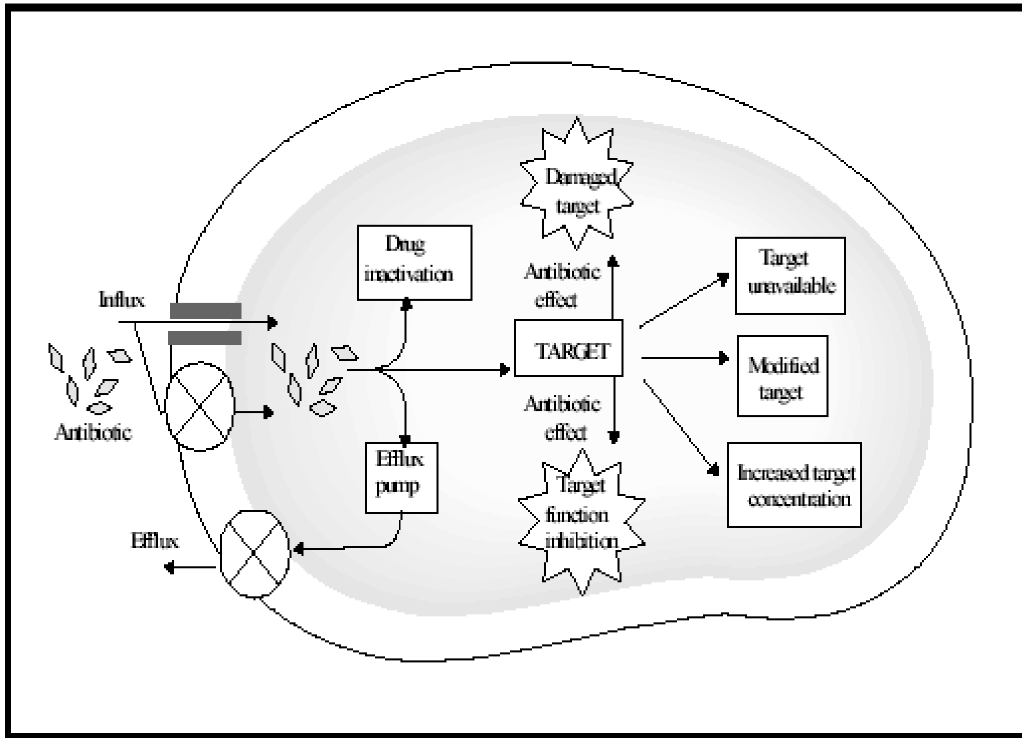
Selective Antibiotic Actions



Guardabassi and Dalsgaard, 2002

APPENDIX C

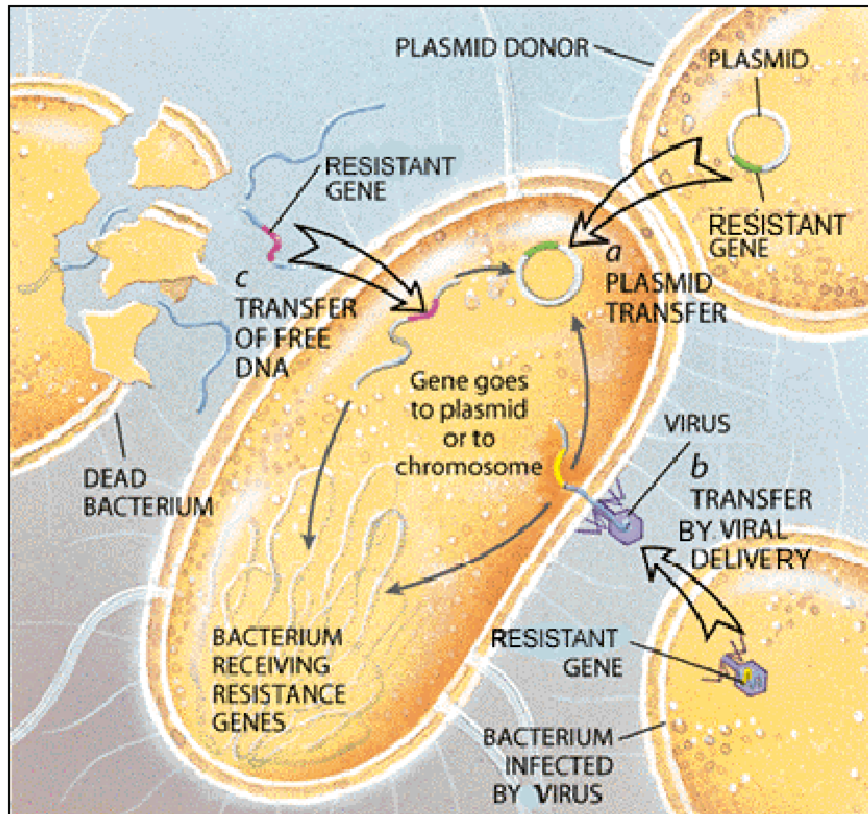
Molecular mechanisms of antibiotic resistance. Modified from Hayes and Wolf, 1996.



Guardabassi and Dalsgaard, 2002

APPENDIX D

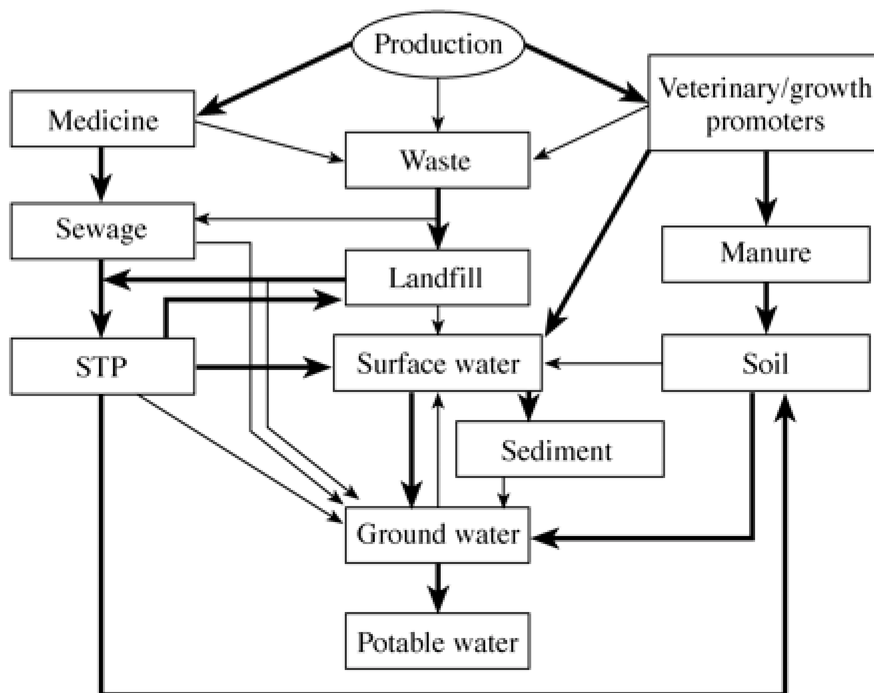
Mechanism of genetic transfer: *a. conjugation; b. transduction; c. transformation*



Levy, 1998

APPENDIX E

Sources and distribution of antibiotics in the environment.



Kümmerer, 2003

STP (Sewage Treatment Plant)

APPENDIX F

Top Antimicrobials Prescribed in 2003		
Brand/Generic Name		**No. prescriptions written**
zithromax	erythromycin	39,535,047
amoxicillin	penicillin	35,768,145
cephalexin	cephalosporin	21,075,715
trimox	penicillin	15,103,044
amox tr/potassium clavulanate	penicillin	14,194,827
levaquin	quinolone	12,642,583
* diflucan		10,733,924
penicillin vk	penicillin	9,724,240
cipro	quinolone	7,983,181
amoxil	penicillin	7,060,402
** cotrim/sulfamethoxazole		6,892,585
biaxin XL	erythromycin	4,848,527
omnicef	cephalosporin	4,699,656
*** macrobid		4,576,805
doxycycline hyclate	tetracycline	4,489,152
cefzil	cephalosporin	4,022,708
bactroban		3,897,112
biaxin	erythromycin	3,584,713
ciprofloxacin HCL	quinolone	3,582,316
avelox		3,042,473
tobradex	aminoglycoside	2,772,278
cefuroxime	cephalosporin	2,568,759
augmentin XR	penicillin	2,463,014
ciloxin	quinolone	2,273,065
banzaclin	erythromycin	2,262,848
tequin	quinolone	2,196,606
tetracycline	tetracycline	21,663,544
Total		**234,060,079**

APPENDIX G

STANDARD OPERATING PROCEDURES (SOPS)

Antibiotic Stock Solutions

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

Table 1. Antibiotics used and recommended concentrations.

Antibiotic	Catalog No.	Solvent[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 µm 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 µl of fungizone stock.

8. Continue stirring for 15 to 30 seconds after the addition of the fungizone to the medium. Tilt the flask to insure that all the fungizone stock solution is transferred to the medium.

9. If you are preparing R2A plus fungizone for the enumeration of total cultivable bacteria, aseptically pour 25 ml per plate into pre-sterilized 100 x 15 mm Petri dishes (Falcon 1029, Becton Dickinson, Sparks, MD or equivalent).

10. If you are preparing R2A plus fungizone and an additional antibiotic for the enumeration of a particular resistant population, ***aseptically*** add 500 µl of the appropriate antibiotic stock to the flask. Stir gently for an additional 15 seconds and tilt the flask to insure that all the antibiotic stock is transferred to the medium.

11. Pour the plates as described in step 9.

12. Clearly mark the plates to indicate media content. E.g. "R2Af " can be used to indicate R2A agar plus fungizone, and "R2Afc" to indicate R2A agar plus fungizone and ciprofloxacin, etc.

13. Allow plates to cure at room temperature for at least 48 hours before use. Plates should be inoculated no later than seven days after pouring.

Sample Collection

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

2. The container should be opened so that the opening is pointing downward, and the inside of the lid does not come into contact with any non-sterile surfaces.

3. Continue holding the opening downward while passing the container through the surface tension layer.

4. When the container is fully submerged, invert it so that it fills with water.

5. Pour off enough water to leave approximately a 10% air headspace.

6. Seal the container and place on ice. Samples should be cultivated within 6 hours of collection.

Enumeration of Total Cultivable Bacteria

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

2. Aseptically transfer 0.1 ml of sample to a sterile 9.9 ml dilution blank in a screw-cap test tube.

3. Tightly cap the tube and mix at full speed on a vortex mixer for at least 5 seconds.

4. Aseptically transfer 0.1 ml of diluted sample to each of three plates of Difco R2A agar plus 375 ng/ml fungizone.

5. Spread the diluted water sample on the surface of the agar plates using a sterile glass spreading rod, a pre-sterilized inoculating loop, or five sterile glass beads (5 mm; see note) until all of the liquid has been absorbed.

   Note – for use of sterile glass beads

   a. Place six glass beads (Fisher Scientific cat no. 11-312C) into a 1000 ml pipette tip (Biolog cat no. 3001; other tips should be tested for suitability). One set of beads is required for each plate inoculated.

   b. Place the tip with beads into the original pipette box, cover all the tips with a sheet of aluminum foil, place the cap on the box, place a piece of autoclave tape on the box, and autoclave at 121°C and 15 psi for 15 minutes.

   c. When plating – open the pipette tip box, roll back the aluminum foil to expose a single row of pipette tips, remove one tip at a time, lift the lid of an inoculated plate, and pour the

sterile beads onto the agar surface. Normally, one bead remains stuck in the bottom of the tip.

d. Repeat step c for all replicate plates.

e. Cover the plates and stack them. Then shake the plates by moving them in a quick back and forth motion while keeping the bottom plate in contact with the bench top - *it is important to avoid allowing the beads to run in a circular motion around the outer edge of the plate*. Shake five times, then rotate the plates by one-quarter turn and shake again five times. Repeat shaking and turning the plates a total of five times.

f. Invert the plates and collect the used beads in a beaker containing 70% ethanol.

6. Plates must be clearly marked with sample number and date of inoculation.

7. Wrap each set of three plates with parafilm and incubate inverted at 25°C for one week (see note)

Note – for incubation of R2A plates

a. R2A agar plates inoculated with river or lake water will continue to develop new microcolonies for 5 to 6 days after inoculation. Therefore, incubation for at least seven days is recommended. Incubation at temperatures above 25°C is not recommended as it may reduce the number of colony forming units.

8. After incubation, count the number of colony forming units (CFU) on each plate and record in a laboratory notebook.

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

10. Determine the CFU per ml of total cultivable bacteria in the original sample by multiplying the average CFU value by a dilution factor of 1,000 (accounts for the initial 10^{-2} dilution and the plating volume of 0.1 ml). Record this value in the laboratory notebook.

Enumeration of Antibiotic Resistant Bacteria

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

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

Note – for selection of plating volume

a. Preliminary tests to determine the volume of sample to be plated are recommended. A plating volume of 0.1 ml is the default volume, but if the number of antibiotic resistant colony forming units is consistently less than 30 per plate, the volume should be increased to 0.2 ml

3. Spread the undiluted water sample on the surface of the agar plates using a sterile glass spreading rod, a pre-sterilized inoculating loop, or five sterile glass beads (5 mm; see note above) until all of the liquid has been absorbed.

4. Plates must be clearly marked with sample number and date of inoculation.

5. Wrap each set of three plates with parafilm and incubate inverted at 25°C for one week (see note above).

6. After incubation, count the number of colony forming units (CFU) on each plate and record in a laboratory notebook.

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

8. Determine the CFU per ml of total cultivable bacteria in the original sample by multiplying the average CFU value by a dilution factor of 10 (for a plating volume of 0.1 ml) or 5 (for a plating volume of 0.2 ml). Record this value in the laboratory notebook.

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 1000 for a filter volume of 0.1 ml of water sample). 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 90th percentile and below the 10th percentile. An IS_{80} score weights sites with population counts above the 80th percentile and below the 20th 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.

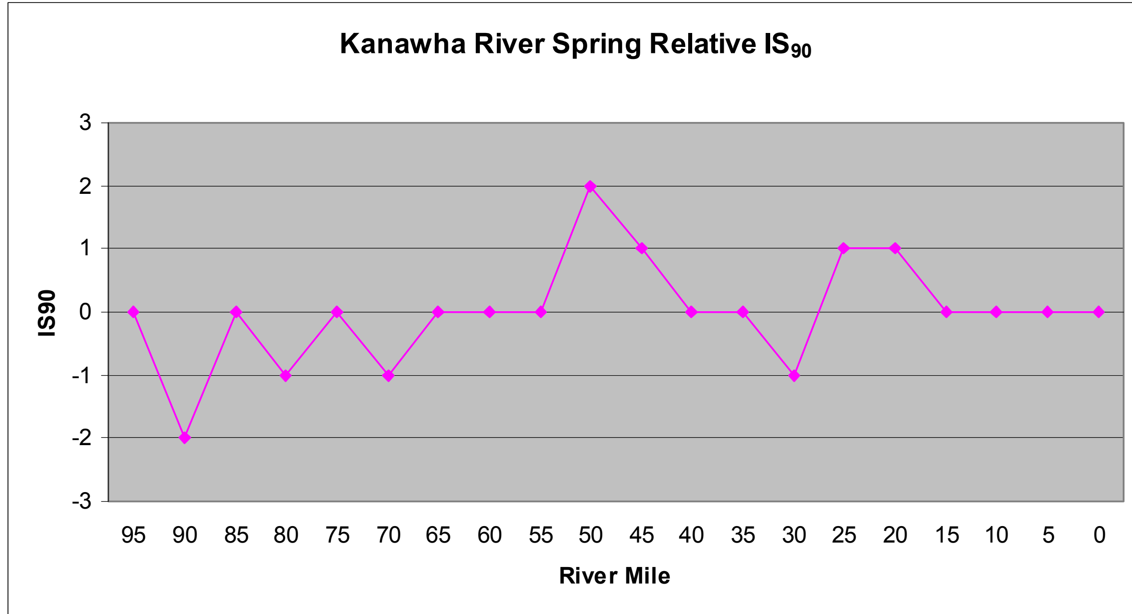
7. Repeat the determination of population scores for all microbial populations enumerated, i.e. for each antibiotic resistant population measured and for the fecal indicator population.

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.

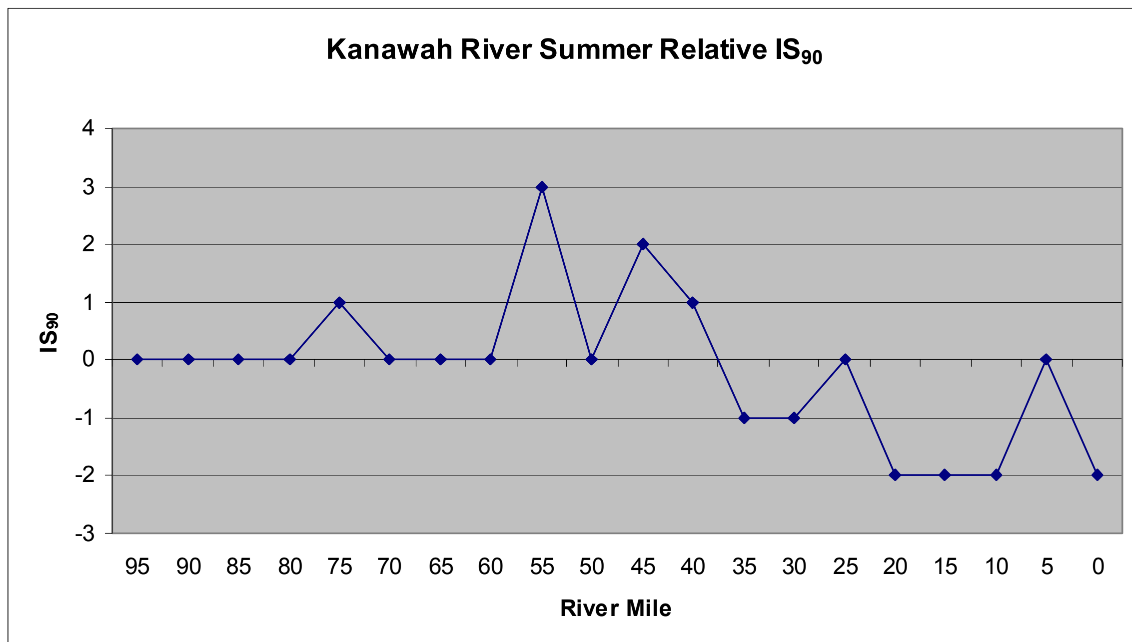
9. Plot IS versus river mile to get a visual representation of water quality variability.

APPENDIX H

1. Mainstem relative impact scores for spring using the 90th percentile.

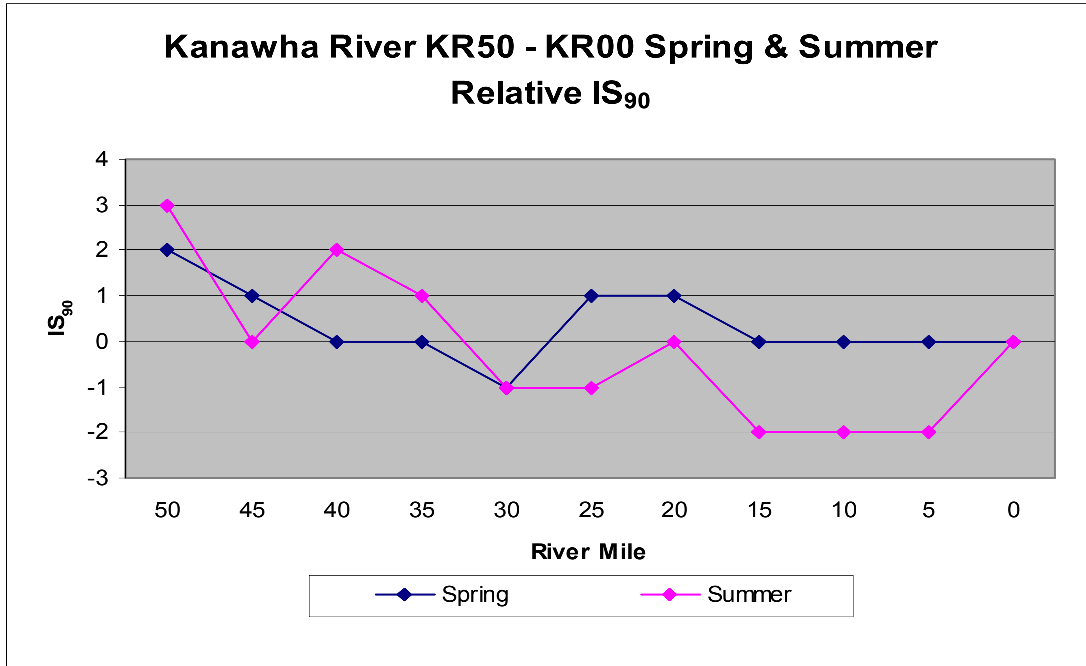


2. Mainstem relative impact scores for summer using the 90th percentile.



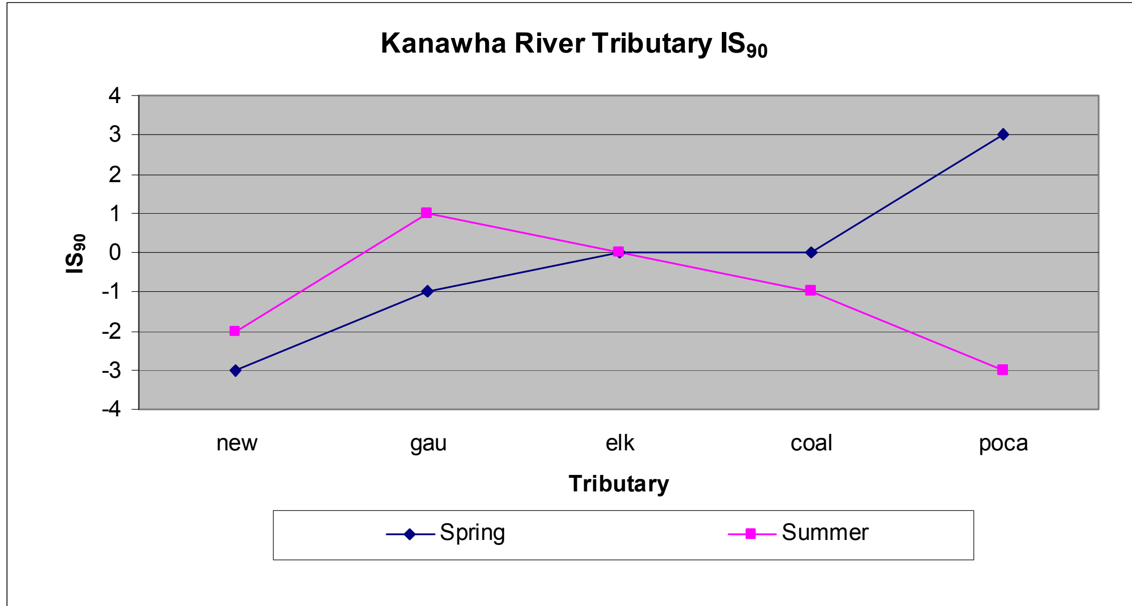
APPENDIX I

Mainstem comparison of relative impact scores for the 90th percentile at KR50-KR00 for spring and summer.



APPENDIX J

Tributary comparisons of relative impact scores for the 90th percentile during spring and summer.



APPENDIX K

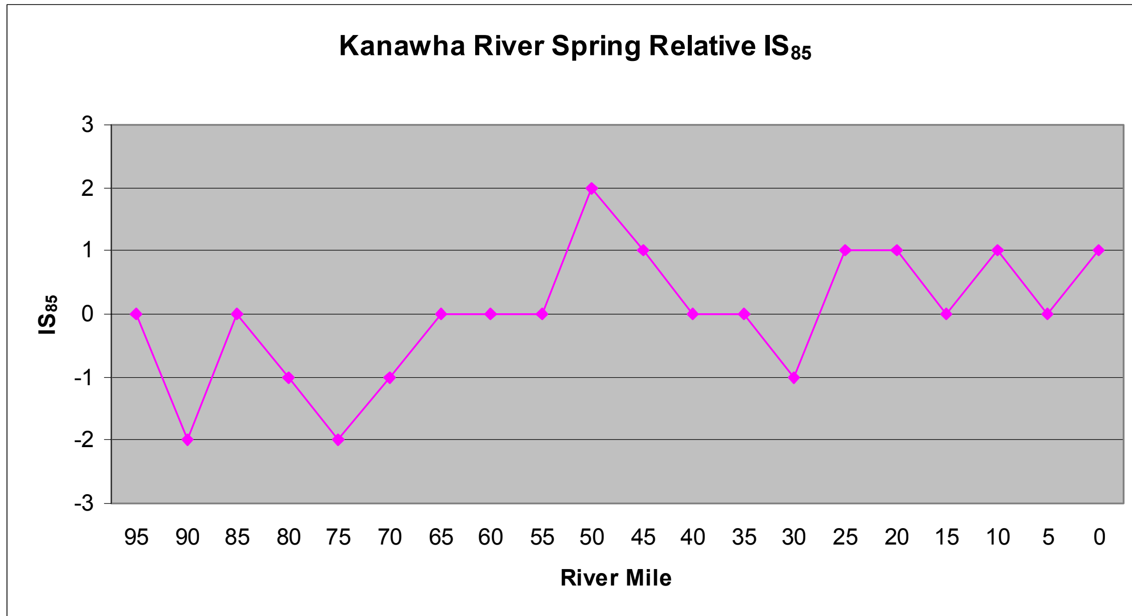
Spring vs. Summer Impact Scores (range -4 to 4) using the 90th Percentile (IS_{90}).

River Mile or Tributary	[a] Site Designation	[b, c] Spring IS_{90}	[b, c] Summer IS_{90}
New	T	-3	-3
Gauley	T	-1	-1
95	U	0	0
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	0
Elk	T	0	4
55	U	0	3
50	L	2	0
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	0
20	L	1	-2
15	L	0	-2
10	L	0	-2
5	L	0	0
0	L	0	-2

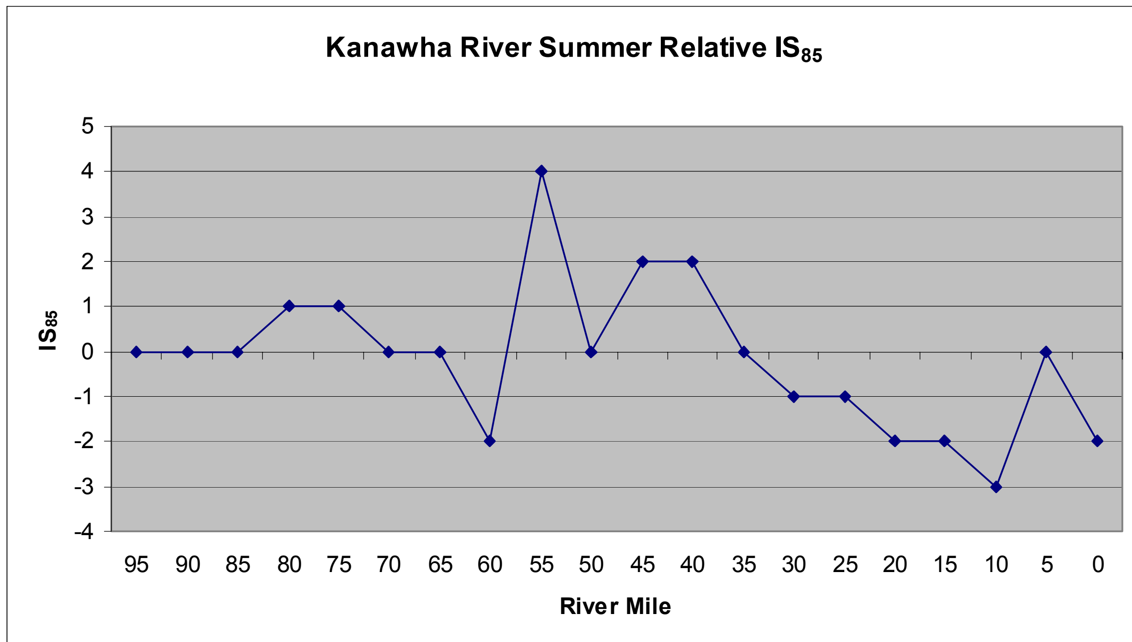
[a] Designation of U (Upper Kanawha), L (Lower Kanawha), or T (Tributary) indicates the region of the River or Tributary entering the river..
[b] Fields highlighted in red indicates an impacted area.
[c] Fields highlighted in blue indicates less impact.

APPENDIX L

1. Mainstem relative impact scores for spring using the 85th percentile.

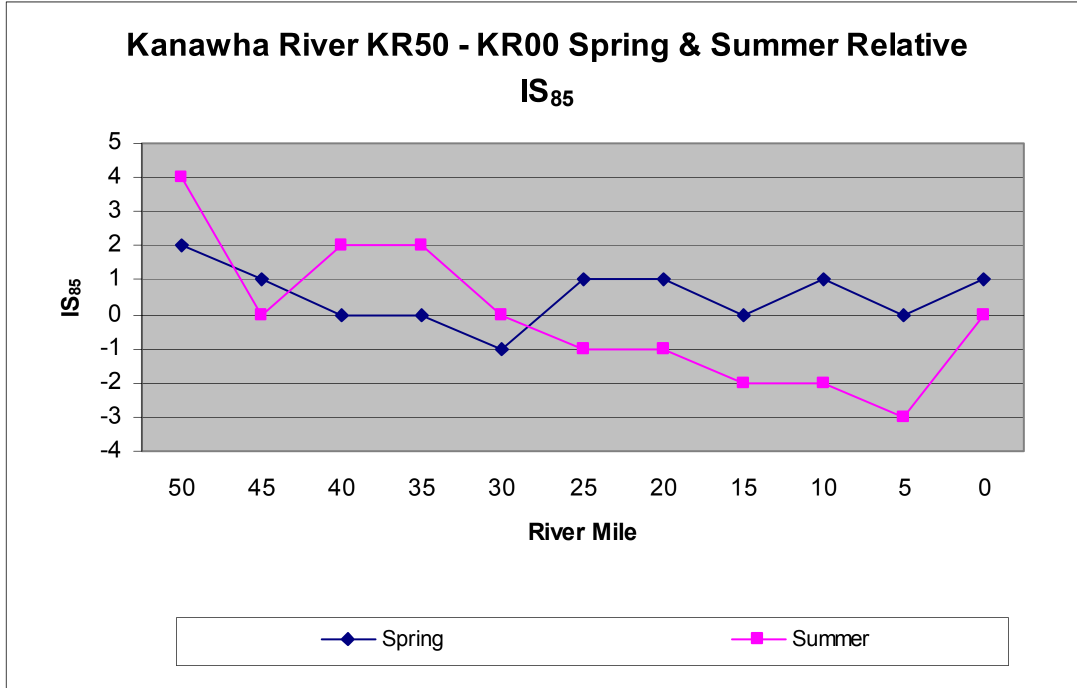


2. Mainstem relative impact scores for summer using the 85th percentile.



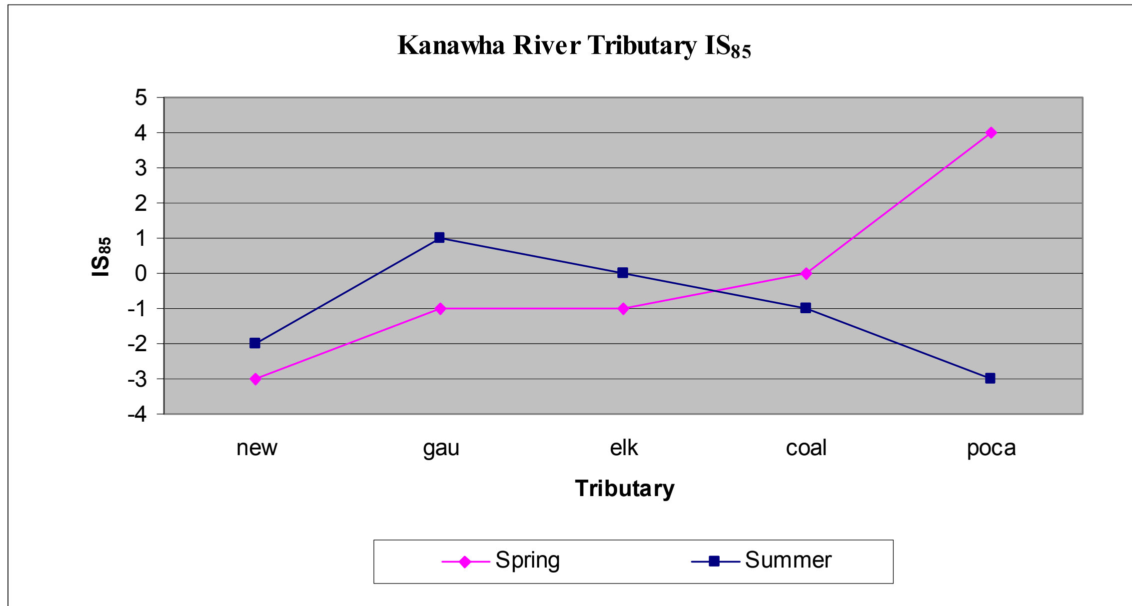
APPENDIX M

Main stem comparison of relative impact scores for the 85th percentile at KR50-KR00 for spring and summer.



APPENDIX N

Tributary comparison of relative impact scores for the 85th percentile during spring and summer.



APPENDIX O

Spring Microbiological Data including average fecal coliforms, ciprofloxacin, erythromycin, and tetracycline counts.

Spring River Mile	Average CFU/ml Total Cultivable	Average CFU/ml ciprofloxacin	Average CFU/ml erythromycin	Average CFU/ml tetracycline	Average CFU/100ml Fecal Coliforms
95	159667	357	1087	147	300
90	208000	387	1127	87	90
85	175000	443	1140	147	80
80	175667	497	853	70	120
75	138000	373	990	103	110
70	131000	353	877	127	110
65	157333	377	923	113	100
60	137333	617	893	117	150
55	141667	455	817	160	100
50	118667	1103	840	197	460
45	109000	1107	1027	150	150
40	112333	510	993	107	210
35	86333	497	823	133	200
30	93667	577	827	100	100
25	81000	483	1237	150	110
20	75500	707	1227	137	100
15	57667	393	620	220	140
10	55000	497	920	153	270
5	11500	553	890	150	130
0	15000	1033	597	640	170
coal	37000	763	1067	197	200
poca	95000	1353	1133	407	420
gau	32333	440	850	183	60
new	162667	347	760	117	90
elk	53667	563	780	123	100

APPENDIX P

Summer Microbiological Data including average fecal coliforms, ciprofloxacin, erythromycin, and tetracycline counts.

Summer River Mile	Average CFU/ml Total Cultivable	Average CFU/ml ciprofloxacin	Average CFU/ml erythromycin	Average CFU/ml tetracycline	Average CFU/100ml Fecal Coliforms
95	20000	1123	1710	443	270
90	10000	2330	2785	667	110
85	14333	1180	1673	690	100
80	12333	1795	2633	485	410
75	11000	1850	1850	503	2100
70	15667	1520	2653	510	100
65	6667	1667	1403	947	260
60	8667	990	1690	423	230
55	38333	6167	7827	4400	2000
50	14667	1010	2923	1480	130
45	40667	1617	9200	6227	80
40	47333	1737	5275	5047	40
35	23333	2353	2577	1123	10
30	50333	1937	1887	563	10
25	30667	1660	1525	600	10
20	168000	1370	813	763	0
15	31667	1590	1070	677	10
10	20000	940	1013	423	40
5	53000	1433	2593	1377	130
0	19000	1107	1170	370	90
coal	77000	3560	2273	1015	100
poca	64000	2213	3237	690	30
gau	28000	963	2750	117	260
new	13333	670	1300	170	130
elk	52333	9120	6460	5097	760

APPENDIX Q

Water chemistry for main stem (KR00- KR50).									
Sample Site	Date, Time		Water Temp (deg C)	Turbidity (NTU)	Sp. Conductance (umho/cm)	Oxygen, Diss (mg/l)	O2 Sat, Diss (%)	pH (units)	Alkalinity, Tot (mg/l)
1KR00005	20040406, 1330		8.9	36	168	12.3	106.44	7.9	
	20040713, 1515		28.5	30	240	8.1	104.02	8.2	92
1KR00505	20040406, 1300		8.6	40	170	12.2	104.79	7.8	
	20040713, 1445		28.2	80	228	8.1	103.5	8.1	80
1KR01005	20040406, 1245		8.5	31	170	12.5	107.1	7.9	
	20040713, 1415		28.8	23	241	8.6	110.99	8.5	92
1KR01505	20040406, 1230		8.4	28	169	12.5	106.84	7.9	
	20040713, 1400		28.4	13	248	9	115.38	8.6	88
1KR02005	20040406, 1215		8.4	26	168	12.5	106.84	7.9	
	20040713, 1345		29.3	6	251	8.5	110.61	8.5	92
1KR02505	20040406, 1200		8.5	26	167	12.5	107.1	7.9	
	20040713, 1330		28.2	7	251	8.5	108.61	8.4	84
1KR03005	20040406, 1200		8.4	25	166	12.7	108.55	8	
	20040713, 1315		28.3	11	253	9.1	116.47	8.2	92
1KR03505	20040406, 1100		8.3	21	169	12.1	103.16	7.9	
	20040713, 1145		28.9	8	258	9.2	118.93	8.7	84
1KR04005	20040406, 1045		8.2	19	178	12	102.05	8	
	20040713, 1100		28.4	6	275	8.3	106.41	8.4	88
1KR04505	20040406, 1030		8.1	18	153	12.2	103.49	8	
	20040713, 1045		29	8	232	8.6	111.36	8.5	80
1KR05005	20040405, 1445		8.1	22	145	12.1	102.64	8.1	
	20040713, 0945		28.7	8	224	8.4	108.23	8.4	72

APPENDIX R

Water chemistry data for main stem (KR55 – KR95).									
Sample Site	Date, Time		Water Temp (deg C)	Turbidity (NTU)	Sp. Conductance (umho/cm)	Oxygen, Diss (mg/l)	O2 Sat, Diss (%)	pH (units)	Alkalinity, Tot (mg/l)
1KR05505	20040405, 1430		8.4	18	164	12	102.56	8	
	20040712, 1430		28.8	8	214	8.1	104.54	8.4	78
	20040805, 1345		26.3	19	192	7.6	94.03	8	72
1KR06005	20040405, 1400		8.4	18	169	12.3	105.13	8.3	
	20040712, 1400		28.6	8	215	7.8	100.33	8.4	80
	20040805, 1315		26.2	23	192	7.6	93.86	8	64
1KR06505	20040405, 1400		8.3	17	167	12.6	107.42	8.4	
	20040712, 1345		28.7	8	217	8	103.08	8.5	80
	20040805, 1245		26.1	28	194	7.6	93.7	8	64
1KR07005	20040405, 1230		8.1	16	159	12.5	106.04	8.4	
	20040712, 1245		29		215	8	103.59	8.6	82
	20040805, 1215		26.5	21	178	7.7	95.59	8	72
1KR07505	20040405, 1215		8	15	157	12.5	105.77	8.3	
	20040712, 1230		28.8	6	208	7.5	96.79	8.4	80
	20040805, 1145		25.6	24	168	7.7	94.11	8	68
1KR08005	20040405, 1200		7.8	15	151	12.8	107.76	8.3	
	20040712, 1200		27.4	6	200	7.6	95.81	8.3	72
	20040805, 1115		25.9	23	167	8	98.29	8.1	60
1KR08505	20040405, 1130		7.5	14	145	12.8	106.94	8.1	
	20040712, 1130		27.8		183	9.3	118.04	8.6	72
	20040805, 1030		25.9	20	170	7.7	94.6	8.1	64
1KR09005	20040405, 1045		7.4	14	156	13	108.33	8.1	
	20040712, 1100		27.2	3	177	8	100.51	8.3	80
	20040805, 1015		25.4	16	188	8.2	99.87	8.1	80
1KR09509	20040405, 1330		6.9	20	132			8.3	
	20040712, 1200		27	5	182	8.8	110.19	8.1	64

Curriculum vitae

April Dawn "Young" Keenan was born on April 25, 1972 to C. Roger Young and Judith Young, in Montgomery, West Virginia. She was educated in public schools and graduated from Dupont High School, Belle, West Virginia in 1990. She entered West Virginia University Institute of Technology (formerly known as West Virginia Institute of Technology) in the spring of 1997 while employed for City National Bank. She graduated earning her Bachelor of Science Degree in May 2001.

After unsuccessfully seeking full-time employment in her field and working part-time as an adjunct laboratory instructor of Anatomy and Physiology for West Virginia University Institute of Technology, Montgomery, West Virginia, Mrs. Keenan began pursuing a Master of Science degree in Biological Sciences at Marshall University, Huntington, West Virginia under the guidance and supervision of Dr. Charles (Chuck) Somerville. During her time at Marshall she worked as a graduate teaching assistant for the Department of Biological Sciences at Marshall University.

In January 2006 Mrs. Keenan found employment with ACCULAB, Inc a privately owned water testing company located at #1 ACCULAB Drive Mt. Gay, West Virginia. Mrs. Keenan was promoted in March 2006 to Manager of the Biological Division and oversees all Biological operations for the company, including an on going survey of impaired streams with the West Virginia DEP.

KEYWORDS:

MIC
Minimum Inhibitory Concentration
Great Kanawha River
Kanawha River
Elk River
Coal River
Pocatalico River
New River
Gauley River
Fecal Coliforms
Membrane filtration
M-FC media
Microdilution
Antibiotic resistance
Multiple Antibiotic Resistances
MAR
Ampicillin
Ciprofloxacin
Erythromycin
Sulfamethizole
Streptomycin
Tetracycline
Virginiamycin