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Antibiotic Resistant and Coliform Bacteria in the Ohio River; 2002 to 2004

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Antibiotic Resistant and Coliform Bacteria in the Ohio River; 2002 to 2004

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Abstract

ANTIBIOTIC RESISTANT AND COLIFORM BACTERIA IN THE OHIO RIVER; 2002 TO 2004

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During 2002 and 2003 samples, collected every five miles along the Ohio River, were analyzed for total cultivable bacteria, ciprofloxacin resistant bacteria, erythromycin resistant bacteria, tetracycline resistant bacteria, and fecal coliforms. During 2004 samples were analyzed for total cultivable bacteria, ciprofloxacin resistant bacteria, sulfamethizole resistant bacteria, tetracycline resistant bacteria, Virginiamycin resistant bacteria, total coliforms, and *Escherichia coli.* The objectives of this study were to systematically collect data on fecal coliforms, *E. coli*, and antibiotic resistant bacteria in the Ohio River and its major tributaries; to determine if antibiotic resistance populations are correlated to each other or to coliforms; and to investigate antibiotic resistance patterns, spatially, over time, along the Ohio River. Data from 2002 and 2003 suggest that ciprofloxacin resistant, erythromycin resistant, tetracycline resistant, and coliform bacteria represented significantly different ($P< 0.05$) populations. Data from 2004 showed ciprofloxacin resistant, sulfamethizole resistant, tetracycline resistant, Virginiamycin resistant, total coliform bacteria and *E. coli* represented significantly different ($P< 0.05$) bacterial populations. This lack of correlation between resistant bacterial populations and coliform bacteria suggests antibiotic resistance in the Ohio River stems from sources other than fecal contamination. It can also be concluded from this study that antibiotic resistant populations and coliform bacteria are present in higher numbers when river flows are above harmonic mean flow.

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Chapter 1

Literature Review

"Antibiotic therapy, if indiscriminately used, may
turn out to be a medicinal flood that temporarily
cleans and heals, but ultimately destroys life itself."

Felix Marti-Ibanez, 1955

"La Belle Rivière", "The Beautiful", The Ohio River

The Ohio River basin (Fig 1.) is home to more than 25 million people while the river supplies drinking water to nearly 3 million of those inhabitants. Forty-nine power generating facilities along the banks of the Ohio supply more than six percent of the United States electricity. Around 150 species of fish live in the waters of the Ohio River and more than 230 tons of cargo are transported on the river each year (34). Research contributing to a better understanding of this great river system is paramount to its economic stability, the survival of its people and maintaining its beauty.

Why Test For Coliform Bacteria?

It was realized, as early as the 1880's, that certain bacteria were characteristic of human feces. Measures were first developed in the United States in 1914 to control the bacillus coli group of bacteria from human feces in drinking water to prevent the spread of communicable diseases (44). Today, fecal coliforms and *Escherichia coli* are used as bacterial measures of water quality. These groups are normal inhabitants of the human

gut, and therefore, are often used as indicators of human or warm-blooded animal fecal pollution. A presence of *E. coli* in water demonstrates the possibility that pathogens are present. (42)

Bacteria pollution control standards, as of April 2006, on the Ohio River require fecal coliform levels to meet the requirements for a public water supply from November through April (2,000 CFU/ 100 ml) and recreational water from May through October (240 CFU /100 ml) (34). Current bacteriological samples on the Ohio River are taken five times per month during recreational months at four sites near Pittsburgh and three sites near Wheeling, Huntington, Cincinnati, Louisville, and Evansville. Samples are examined in triplicate for fecal coliforms and *Escherichia coli* using standard membrane filtration methods (38). Though fecal coliform bacteria show human and/or warm-blooded animal impact in surface waters, coliforms may not demonstrate industrial or agricultural pollution and do not establish the threat of infection from antibiotic resistant bacteria. Therefore, fecal coliform and *E. coli* measurements may not be the only bacterial parameters that could be collected to determine the presence of bacteria harmful to human health in the Ohio River.

Why Test For Antibiotic Resistant Bacteria?

Some non-medical uses of antibiotics were questioned as early as 1970 and it was stated that antibiotics could be a significant environmental contaminant and a threat to public health (21). Since that time, multiple studies have noted the prevalence of antibiotic resistance and mentioned concern for the health of the global population due to antibiotic resistant bacteria (8, 15, 19, 30, 39, 45). Hospitals have traditionally been

thought of as the reservoirs of resistant bacteria due to over use of antibiotics in clinical settings. These reservoirs are now known to extend from agricultural farm land to fish farms and day care centers (39). According to Weber (43), "Some strains of pathogenic bacteria are now resistant to essentially all available antimicrobial drugs, and some remain susceptible to only one."

Some research has focused on antibiotic resistance levels, patterns and multiple antibiotic resistance (MAR) in isolated groups of bacteria (12, 16, 24). Other research has focused on antibiotic resistant bacteria present in chlorinated water (1, 2, 15, 27). Though this research does add significantly to the subject of antibiotic resistance and the mechanisms by which certain bacteria may become resistant, it does not demonstrate the trends of antibiotic resistance that would be encountered in the microbial fauna of an industrialized and navigable river system such as the Ohio River.

Other research has examined the prevalence and patterns of antibiotic resistance in *Escherichia coli* and some *Enterobacteriaceae* genera (17, 18, 22, 25, 32). The idea of using *Enterobacteriaceae* genera to determine resistance levels is based on the preconceived notion that antibiotic resistance arises from intestinal flora of warm-blooded animals ingesting antibiotics. However, antibiotic resistance has also been correlated to water quality problems other than sewage, such as heavy metals (7, 29, 40).

The Need For Multiple Microbiological Assays in Determining Water Quality

Kolpin (26) stated, "The continued exponential growth in human population has created a corresponding increase in the demand for the Earth's limited supply of freshwater. Thus, protecting the integrity of our water resources is one of the most

essential environmental issues of the 21st century". El-Zanfaly (15), urged that antibiotic-resistant bacteria in drinking water is a prime concern to public health and that data on multiple antibiotic resistant bacteria should be included in future water quality standards. Grabow (19), presented evidence that coliform bacteria should no longer be considered as harmless but should be re-evaluated for their role in water quality standards because they may carry transferable drug-resistance and contribute to resistance among bacteria involved in disease. Standard Methods 20th ed. (10) recommends focusing studies on the known or suspected pathogenic organisms and studies have shown this should include antibiotic resistant organisms. The influence of the Ohio River on the human population demands the highest water quality and future research should focus on any factor that may jeopardize human health including antibiotic resistant bacteria.

What Influences Antibiotic Resistance?

McArthur *et* al. (29) and Dhakephalker *et* al. (12), have both demonstrated an association between antibiotic resistance and heavy metals in bacteria from different environmental sources. Alvero (1), found high amounts of resistance in bacteria from lake waters that were not directly polluted with sewage effluent. These observations raise the question, is antibiotic resistance influenced from contaminants other than waste water effluent and sewage outfalls?

Kelch *et* al. (24), surveyed the antibiotic resistance of nearly 2,500 isolates from surface runoff in pastures, tributaries, and the waters of Tillamook Bay, Oregon. They demonstrated spatial correlations among antibiotic resistant fecal coliform bacteria. This data suggests that antibiotic resistant coliforms from run-off in pastures contributed to

tributary antibiotic resistant coliforms which contributed to antibiotic resistant coliforms in the bay. The authors state, "These results strongly suggest that the antibiotic resistance patterns in these bacterial groups are very similar, perhaps indicating similar mechanisms for the development of this resistance…" They describe later that the similarities in antibiotic resistance are probably due to some interaction that occurs between the different bacteria, not because they all evolved the same way.

Jones (23), determined incidences of antibiotic resistance in over 2,000 bacteria isolated from an aquatic environment. His results showed higher antibiotic resistance in *Pseudomonas,* a clinically important bacterium, than in *E. coli* and other coliforms. This lends more evidence that water quality standards should incorporate antibiotic resistant bacteria into current testing methods.

Antibiotic Classes and Mechanisms

Ciprofloxacin, (Fig. 2) is a member of the flouroquinolone class of antibiotics used only in human health. Quinolones are potent antibacterial agents that specifically target bacterial DNA gyrase and topoisomerase IV (41). Previous studies have shown genetic mutations cause quinolone resistance but recent studies have also demonstrated plasmid mediated resistance (37). The mechanism by which the plasmid borne gene protects DNA gyrase remains unknown.

Erythromycin, (Fig. 3) a macrolide, is produced by a strain of *Streptomyces erythraeus* and has been in use since 1952. Erythromycin is usually prescribed for respiratory tract infections (RTI's) and sexually transmitted diseases. It binds to the 50 S ribosomal subunit and inhibits translation of peptides. Intrinsic resistance to

erythromycin can be found in Gram-negative *bacilli* due to the low permeability of the outer membrane. Transferred resistance usually involves some modification of the ribosomal target (37).

Sulfamethizole, (Fig. 4) is in the class of antibiotics known as sulfonamides which interfere with the production of dihydrofolic acid (14, 31). Sulfamethizole is a competitive inhibitor of bacterial para-aminobenzoic acid (PABA), a substrate of the enzyme dihydropteroate synthetase. The inhibited reaction is necessary in these organisms for the synthesis of folic acid.

The tetracycline (Fig. 5) class of antibiotics was introduced in 1948 and welcomed in clinical settings because it is a broad-spectrum antibiotic inhibiting both Gram-negative and positive bacteria. Tetracycline specifically targets ribosomal protection proteins and inhibits the elongation of proteins (11). Tetracycline is used in veterinary medicine as a growth promoter and in human health for respiratory tract infections, acne, and other illnesses.

Virginiamycin, (Fig. 6) is in the streptogramin class of antibiotics and produced is by various S*treptomyces* species. It has two synergistic components, Virginiamycin M and Virginiamycin S that alone are bacteriostatic and when combined are bactericidal. Both components of Virginiamycin bind to the 50 S subunit and cause a block in protein synthesis (9). The FDA approved Virginiamycin in 1974 for use in veterinary medicine and/or as a growth promoter for chickens, turkeys, swine and cattle. Intrinsic and acquired resistance to Virginiamycin are comparable to that of erythromycin.

Intrinsic Antibiotic Resistance

Intrinsic resistance, natural resistance to an antibiotic due to production of the antibiotic as a secondary metabolite or a function of genetic inheritance, has been blamed to a small degree for resistance to antibiotics in environmental isolates (4). Though production of antibiotics from some bacteria is natural, high levels of antibiotics have been found in human impacted areas. Hamscher *et* al. (20), found tetracycline concentrations higher than 150 μg/kg in sandy soil that had been fertilized with liquid manure. Ash (4), also found bacteria collected from aquatic environments to be commonly resistant to chemically modified antibiotics and synthesized compounds that are not produced in the natural environment. These studies support the idea that intrinsic resistance can not fully explain high levels of problematic resistance organisms in medical settings and that anthropogenic influences affect the microbial flora of soil and aquatic habitats.

Antibiotic Resistance by Genetic Mutation

Antibiotic resistance initially arose by spontaneous mutation or recombination before the medical use of these substances, but during this period there was no great selective advantage for pathogenic microorganisms to possess the characteristic of antibiotic resistance (5). A recent example of a bacterium becoming resistant to an antibiotic due to mutation was found in a strain of *Enterococcus.* It was resistant to linezolid, a completely synthesized structure (13). Clinical resistance was reported less than one year after the drug was accepted by the U.S. Food and Drug Administration. Bacteria resistant to naturally occurring, chemically modified and synthesized antibiotics are now widespread in aquatic environments (4). These antibiotics select for mutant or

resistant cells allowing them to become dominant in microhabitats. Due to the uninhibited use of antibiotics, bacteria possessing antibiotic resistance now have a complete advantage over susceptible organisms.

Plasmid Mediated Resistance

The extensive overuse of antibiotics since World War II has selected for bacteria with plasmids carrying antibiotic resistant genes. This selective pressure has allowed for the growth of bacterial populations that may carry R-factors. R-factors are double stranded circular DNA that can carry single or multiple genes that encode resistance to heavy metals and/or antibiotics. R-factors can be transmitted from a resistant bacterial cell to a susceptible cell through conjugation or transferred to a daughter cell during cell replication. Plasmid mediated resistance has been noted in all of the antibiotics used in this study (8, 9, 37, 41).

Study Objectives

McArthur and Tuckfield (29) noted in 2000 that few studies have focused on antibiotic resistance in streams and none have included samples collected systematically to determine spatial patterns in aquatic systems.

> "The river continuum concept provides a theoretical basis for the distribution of organisms and biogeochemical transformation along river systems. Interestingly, microbes are mentioned in this concept but no meaningful predictions of their distributions are presented. Since bacteria are important components of all river systems, it is important to know if they further validate the continuum concept." (29)

There were three objectives of this study. One was to systematically collect data on fecal coliforms, *E. coli*, and antibiotic resistant bacteria that may provide descriptive information about antibiotic resistance among environmental bacterial isolates in the Ohio River and its major tributaries. Second, we wanted to determine if antibiotic resistance populations can be statistically correlated to each other or to coliforms. Lastly, we wanted to investigate spatial and temporal antibiotic resistance patterns along the Ohio River.

Chapter 2

Materials and Methods

Site Description

The headwaters of the Ohio River are formed at the confluence of the Monongahela and Allegheny Rivers in Pittsburgh, Pennsylvania where it is an 8th order river (6). It extends 981 miles southwest from Pennsylvania and borders the states of Ohio, West Virginia, Kentucky, Indiana, and Illinois. The Ohio River is the second largest tributary of the Mississippi River at its confluence in Cairo, Illinois where it ends as a 9th order stream (6). The drainage basin of the Ohio River (Fig. 1) is 204,000 sq. mi. and extends through eleven states as far north as southern New York and as far south as North Carolina. Four eco-regions are dissected by the Ohio River: the Western Allegheny Plateau, the Interior Plateau, the Interior River Lowland and the Mississippi Alluvial Plain (33). Along the river there are over 600 permitted discharges, 20 dams, and 49 electrical plants powered by gas and oil, coal, hydro-electricity, or nuclear energy. The average depth of the river is 24 feet and its widest point is at Smithland Dam (RM 918.5) where it stretches one mile across (34).

During 2002, the first 505 miles of the Ohio River were sampled from Pittsburgh, PA to Rising Sun, IN. During 2003, all 981 miles were sampled downstream from Pittsburgh, PA to Cairo, IL. During 2004, all 981 miles were sampled upstream from Cairo, IL to Pittsburgh, PA. Sampling sites were located by dead reckoning, using Ohio River Navigation Charts (United States Army Corps of Engineers) and a Garmin GPS Map 188 Sounder.

Bacteriological Sampling

During 2002 and 2003 samples were analyzed for total cultivable bacteria, ciprofloxacin (4 μg/ml) resistant bacteria erythromycin (8 μg/ml) resistant bacteria, and tetracycline (12.5 μg/ml) resistant bacteria. During 2004 samples were analyzed for total cultivable bacteria, ciprofloxacin (4 μg/ml) resistant bacteria, sulfamethizole (128 μg/ml) resistant bacteria, tetracycline (12.5 μg/ml) resistant bacteria, and Virginiamycin (16 μg/ml) resistant bacteria. Sources for all antibiotics used from 2002 to 2004 are included in appendix A.

Mid-channel sub-surface water samples (~ 400 ml) were collected in sterile glass jars every five miles on the mainstem of the Ohio River and on all major tributaries above the debris line. All samples were packed on ice, transported each evening to a field laboratory where they were processed within 8 hours of collection.

We inoculated 0.1 ml of dilute (10^{-2}) river water in triplicate on R2A (34) (Becton Dickinson and Co., Sparks, MD) agar plus fungizone (BioWhittaker, Walkersville, MD) (250 ng/ml; R2Af) for the enumeration of total cultivable bacteria. During 2004 fungizone concentration was increased to 1.5X the recommended concentration (375 ng/ml) due to fungal contamination during the previous year. Aliquots (0.1 ml) of each sample were plated in triplicate on R2Af supplemented with the published Gram-negative minimum inhibitory concentration (MIC) of one antibiotic. After inoculation, five sterilized glass plating beads (5mm) were added to each plate. Lids were replaced, triplicate sets of plates were stacked together and gently shaken rolling the beads in a random motion for about 30 seconds or until plates appeared dry (35). Beads were discarded and each set of triplicate plates were wrapped in parafilm, inverted and incubated for one week at room temperature.

The antibiotics used in this study were noted as emerging contaminants by Kolpin *et* al. (26) during a nationwide reconnaissance of the occurrence of pharmaceuticals, hormones, and other organic wastewater contaminants in water resources. To prevent overlap of data and conserve resources, one antibiotic, was chosen from each chemical class targeted by Kolpin (26).

Fecal coliform counts were analyzed in 2002 and 2003 by filtering suitable aliquots of water through sterile 0.45 µm cellulose filters (Nalgene, Rochester, NY). Aliquots of water were determined based on the turbidity at each sampling site. Less water was filtered with increased turbidity. Membranes were incubated on m-FC medium (Gelman Laboratory, Ann Arbor, MI) at 44.5°C in a mobile incubator for 24 hours. Blue colonies were counted and recorded as fecal colifom CFU's / 100 ml.

Total coliform and *E. coli* counts were analyzed in 2004 using the EPA approved Idexx Quanti-Tray/ 2000© method. Reagent powder packs were added to aliquots (100 ml) of whole water , transferred to the 97 well Quanti-Tray®, sealed, and incubated for 24 hours at 37° C. After incubation, clear wells were negative for coliform bacteria and positive wells turned yellow due to the break down of 2-Nitrophenyl-ß-D-galactopyranoside (ONPG). Wells that fluoresced under UV light were positive for *E. coli* since these bacteria produce a diagnostic enzyme capable of hydrolyzing 4-Methylumbelliferyl-ß-D-glucuronide (4-MUG).

Data Analysis

Colony forming units (CFU's) were counted and recorded for each plate (fungal contaminated plates were excluded) and CFU's /ml were determined by averaging the counts for each triplicate set of plates and multiplying by the dilution factor.

Fecal coliform to antibiotic resistant bacteria ratios were calculated at each site. Statistica 7.0 was used to compare tributary ratios to mainstem ratios using the Mann-Whitney Rank Sum test for non-normal distributions of data. Ratios from tributary data that were statistically different from mainstem data would suggest the populations of fecal coliforms and antibiotic resistant bacteria are different, e.g. they are surviving at different rates. Linear regression analyses and 2D box plots, and Spearman correlations (Statistica 7.0) were used to determine any relationships between antibiotic resistant cells and fecal coliforms at the same site within the mainstem for 2002, 2003, and 2004. The null hypothesis of a Spearman Correlation test is that the populations in question are not different from what might have occurred as due to chance. The significance level set for the Spearman correlations was <0.05. Linear regression analysis was also used to determine any spatial trends among bacterial populations along the river on a yearly basis.

Daily flow data for each sampling period was obtained from ORSANCO along with harmonic mean flow (HMF) for each pool on the Ohio River. Daily flows, in cubic feet per second (cfs) were converted into percent of HMF to compare flow data across years.

Chapter 3

Results

2002

Linear regression analyses showed fecal coliform counts were not significantly correlated to the three antibiotic resistance bacterial counts along the mainstem of the Ohio River (Fig. 7-9). While R-values above 0.95 were not achieved, Figure 8 does show a positive trend (R= 0.6116) between fecal coliforms and erythromycin antibiotic resistant bacteria. Positive linear trends were also observed when antibiotic resistance counts from the mainstem sampling sites were compared to each other (Fig. 10-12). Figures 13, 15, and 16 show a decrease downstream in fecal coliform bacteria, ciprofloxacin resistant bacteria and tetracycline resistant bacteria. Erythromycin resistant bacteria (Fig. 16) demonstrated ultimately no trend downstream (R = 0.00637). The difference in fecal coliform counts when compared to all three antibiotic resistant bacterial counts is demonstrated in Figure 17.

The Mann-Whitney Rank Sum test (Table 1) was used to compare independent ratios of fecal coliforms/ antibiotic resistant bacteria from tributaries to matching ratios from every site sampled in the mainstem of the Ohio River. The ratios of fecal coliforms to ciprofloxacin resistant bacteria and fecal coliforms to erythromycin resistant bacteria were found to be significantly different between tributaries and the mainstem ($P< 0.05$).

2003

Results from 2003 demonstrate positive linear trends between fecal coliforms and antibiotic resistant bacterial counts in the mainstem but R- values were less than 0.95 (Fig 18-20). Figures 21-23 show a positive linear trend between the different antibiotic resistant bacterial populations measured but again the R-values show these trends are not strong. Scatter plots comparing river mile to the different bacterial populations showed a decrease in each population in the lower part of the river (Fig. 24-27). The box and whisker plot in Figures 28 illustrates the difference between antibiotic resistant population levels and fecal coliform levels.

Ratios of fecal coliforms to antibiotic resistant counts from tributaries were compared to all the sites on the mainstem with a Mann-Whitney Rank Sum test (Table 3). Results show a significant difference between the fecal coliform to erythromycin resistant bacteria ratios in the mainstem and those of the tributaries.

A Spearman correlation test compared average CFU/ml for each bacterial population to all other bacterial measurements taken. The resulting coefficients between each comparison were significantly different at $P < 0.05$.

2004

Linear regression analysis showed populations of total coliform and *E. coli* increased along with the four antibiotic resistance bacterial counts in the mainstem of the Ohio River in 2004. R-values for all comparisons were less than 0.95 (Fig 29-38). This positive linear trend is not as apparent in Fig. 36 comparing total coliforms and *E. coli*. When *E. coli*/ml were compared to antibiotic resistant CFU/ml for all four antibiotics

there was a strong positive linear trend for all comparisons along with increased R values (Fig 34-37) .

During August 2004, linear regression analyses showed an increase in population size with coliform bacteria and three of the antibiotic resistant bacteria (Fig 44 and 46-49). Figure 45, shows a small negative downstream trend with sulfamethizole resistant bacteria. Also, seen in Figures 44-47 and 49, is an increase in all four antibiotic resistant bacterial populations and *E. coli* just after Ohio River mile 600 near Louisville, KY.

The Mann-Whitney Rank Sum test was used to compare independent ratios of coliform bacteria to antibiotic resistant bacteria from tributaries to matching ratios calculated from all sites on the Ohio River. *P*-values on Table 7 shows there were no tributary ratios significantly different from the mainstem ratios during 2004.

Comparison of Data from All Three Years

Box and whisker plots comparing total cultivable, coliform, and antibiotic resistant bacterial counts from 2002, 2003, and 2004 show an increase in all bacterial population counts during the 2003 sampling season (Fig 55-61). This trend is also seen when the flows from each sampling season are compared as in Fig. 62. The flows during the 2002 sampling season were well below the 2003 and 2004 flows while the 2003 flows were the highest among the three sampling periods.

Chapter 4

Discussion

2002

During 2002 linear regression analyses showed positive linear trends when comparing each of the bacterial populations measured to one another. It is important to note here that correlation does not imply causation. To better understand if these populations act dependently or independently of one another, a Spearman correlation test was run on the mainstem data alone. Table 2 shows each bacterial population to be significantly different ($P < 0.05$) from all the another bacterial populations enumerated. Figure 17 demonstrates the difference between the population counts through box and whisker plots. Boxes encompass 50% of the population data while the whiskers extend to include 95% of the data. Non-overlapping boxes generally represent significantly different outcomes or in this case, different sized populations.

Figures 13, 15, and 16 show a negative linear trend of coliform and resistant bacteria, downstream. The first 100 miles of the Ohio River showed increased counts of fecal coliforms and antibiotic resistant bacteria. This area of the Ohio River also has the most industrialized flood plane. The towns of Pittsburgh and Wheeling, which have been large steel producing cities, are located at river miles 0.0 and 87.0, respectively. Our results are supported by McArthur (29), who showed increased antibiotic resistance in aquatic bacterial isolates when heavy metal concentrations were also increased. The lower 300 miles of flood plane along the Ohio River, where we see decreased fecal and antibiotic resistant bacteria counts, are heavily farmed and used for crops and agriculture instead of heavy industry.

Downstream of river mile 100, all four of the populations measured increased between river mile 270 and 300. This is just 4.5 miles downstream of the mouth of the Kanawha River, one of the largest tributaries on the upper Ohio River which is also very industrialized. Downstream of river mile 300 erythromycin resistant, tetracycline resistant and coliform bacteria increased near river mile 350 downstream of the Big Sandy River, Ashland, Portsmouth and the Scioto River; and near river mile 465 at Cincinnati, OH.

After observing these trends, a Mann-Whitney Rank Sum test was run to better understand how the bacterial populations in the tributaries along the Ohio River were influencing the bacterial populations within the mainstem. Table 1 shows the *P*-values when comparing ratios of fecal coliform to antibiotic resistant bacteria in tributaries to matching ratios of mainstem sampling sites. These results show there was a significant difference between the tributary and mainstem fecal coliform to erythromycin resistant ratios and the fecal coliform to ciprofloxacin resistant ratios. A significant difference demonstrates the measured populations are dying off or surviving differently therefore, they may represent different populations. This data also suggests the populations of fecal coliforms and tetracycline resistant bacteria in tributaries are influencing the Ohio River in similar ways; they may be surviving or dying off at the same rate.

A Spearman correlation test was run on the bacterial measurements of the data, only from the mainstem, to see how correlated different populations would be to one another within a single site. The null hypothesis of this correlation test is that any observed difference is due to chance alone. If correlation coefficients are significant the

null hypothesis is rejected and the alternative is accepted. Table 2 shows each correlation coefficient for be significant at $P< 0.05$.

2003

During 2003 linear regression analyses showed positive trends among each of the bacterial populations measured (Fig. 18-23) which follows the trend observed in 2002. Although the bacterial counts showed similar trends, the Spearman correlation in Table 4 shows that any one of the bacterial measurements is not representative of any of the other bacterial populations measured.

Figures 24-27 show a negative trend of coliform and resistant bacteria downstream. During 2003 ciprofloxacin resistant counts peaked at river mile 435.0 with more than 2400 CFU/ml. USACOE Ohio River navigation charts do not indicate any industrial influence or tributary that could cause this drastic increase in ciprofloxacin resistant bacteria is probably an outlier by chance. Erythromycin resistant, tetracycline resistant, and fecal coliform bacteria all peaked within the first 100 miles of the Ohio River as was seen in 2002.

Between river mile 350 and 400, noticeable increases were observed in three of the bacterial populations measured. This stretch of river is downstream of Ashland, KY, Portsmouth, OH and the Scioto River which may be contributing to the coliforms and antibiotic resistance. Increases in all four population counts were noticeable near river mile 570 which is less than 10 miles downstream of Madison, IN and a power plant fueled by coal.

A Mann-Whitney Rank Sum test was run to better understand how the bacterial populations in the tributaries along the Ohio River were influencing the bacterial populations in the mainstem. Table 3 shows the *P*-values achieved when comparing ratios of fecal coliform to antibiotic resistant bacteria in tributaries compared to matching ratios from the nearest downstream site in the mainstem. These results, due to significantly different ratios ($P < 0.05$), suggest fecal coliforms populations and erythromycin resistant populations are surviving differently between tributaries and the mainstem of the Ohio River. This was also concluded in 2002. Significant differences were not observed between tributaries and the mainstem populations of fecal coliforms, ciprofloxacin, and tetracycline resistant bacteria.

2004

Analysis of the 2004 data showed trends comparable to 2002, and 2003. Linear regression analyses showed positive linear trends between all six bacterial populations surveyed. These trends are questionable due to the distribution among the populations measured in 2004. The strongest correlation was between ciprofloxacin resistant and Virginiamycin resistant bacteria with an R-value of 0.8749 (Fig 40). 2004 was the only year Virginiamycin resistant populations were measured so data is not available to show if this is a consistent trend.

Interestingly, during 2004 the highest population counts for all bacteria populations measured were not within the first 100 miles of the river as in 2002 and 2003. All populations, with the exception of total coliforms, spiked near river mile 630.0, just 20 miles downstream of Louisville KY. Due to the resolution of the most probable

number chart associated with the total coliform test, several sites along the entire river reached the maximum probable number associated with the IDEXX system. Data is not available to show if total coliforms would have also spiked downstream of Louisville.

A Mann-Whitney Rank Sum test (Table 5) showed no significant difference between tributary population ratios and mainstem population ratios. However, the Spearman correlation results (Table 6) show the bacterial populations numbers meausered in the mainstem were significantly different ($P< 0.05$) from one another. These same results were seen in 2002 and 2003.

Comparison of Data from All Three Years

All three sampling years had similar results showing some relationship between the populations sampled. Spearman correlations, each year, also showed the coliform and antibiotic resistant bacteria represent different sub-populations within a sample. Linear regressions from 2002 and 2003 showed a negative downstream trend between river mile and the bacterial populations measured. This decrease in coliforms and resistant populations follows trends of industrialization along the river which are believed to influence antibiotic resistance. (29) This decrease in bacterial enumeration downstream was not seen in 2004 shown by the increase in population counts near Louisville (Rmi 630.0).

Figures 52-56 show the levels of antibiotic resistant bacteria were higher during the 2003 sampling event when compared to 2002 and 2004. Flow data provided by ORSANCO showed flow during the 2003 season was higher than the flows sampled during the other two years. Increased flows are likely to affect the bacterial populations

due to runoff and higher turbidity. This was also the case when bacterial counts spiked at Louisville during 2004. The highest flows experienced during that sampling period were in Louisville where flows were above 260 percent of the mean harmonic flow for that area. During 2002 samples were collected during flow events well under mean harmonic flow resulting in bacterial counts that were lower than the years to follow. Lower flow also resulted in bacterial numbers that gave higher resolution between populations during 2002.

In 2002 and 2003, Mann-Whitney tests showed erythromycin resistant bacterial populations to be significantly different from coliform populations when compared between tributaries and the mainstem of the Ohio River. This data is not available for a third year because erythromycin was not used during 2004. It can be suggested by this data that erythromycin resistance comes from a source other than fecal material, that erythromycin resistance is easily transferable among different species of bacteria or that erythromycin resistance may increase the survival of a bacterium.

Using Impact Scores to Show Spatial Distribution

Due to the variance of levels of bacterial populations along the river, there is a need to rank the data to better understand the spatial distribution of antibiotic resistant bacteria. This was done using the impact score method as described in Loughman (28). Figures 57-59 show areas in the river that have increased levels of antibiotic resistant and coliform bacteria.

Chapter 5

Conclusions

The three objectives of this study were (i) to systematically collect data on fecal coliforms, *E. coli*, and antibiotic resistant bacteria that may provide descriptive information about antibiotic resistance among environmental bacterial isolates in the Ohio River and its major tributaries; (ii) to determine if antibiotic resistance can be correlated to fecal coliforms; (iii) to investigate any spatial and temporal patterns of antibiotic resistance in the Ohio River.

Data collected from these studies showed antibiotic resistant bacteria and coliforms in recreational waters are surviving at noteworthy levels. These data provide a snapshot of bacterial levels along the Ohio River with increased antibiotic resistant bacteria in the first 100 miles of river; at the confluence of the Kanawha River; near the industrial region of Ashland, KY and just downstream of Louisville, KY.

Data from 2002 and 2003 suggest that ciprofloxacin resistant, erythromycin resistant, tetracycline resistant, and coliform bacteria represent different populations of bacteria in the Ohio River. Data from 2004 showed ciprofloxacin resistant, sulfamethizole resistant, tetracycline resistant, Virginiamycin resistant, total coliform bacterial and *E. coli* also represent different bacterial populations in the mainstem of the Ohio River.

This study demonstrated the influence that daily flow can have on levels of bacterial populations such as coliforms and antibiotic resistant bacteria. Of the three sampling seasons 2003 had the highest bacterial counts across the populations measured along with the highest average flows. During 2004, the sampling sites between river mile

620 and 660 had the highest bacterial counts across the populations measured along with the highest average flow.

With this information, it can be concluded that antibacterial resistant populations and coliform bacteria are present in higher numbers when flows increase. Also, it can be concluded that low numbers of coliforms in the Ohio River do not infer low numbers of antibiotic resistant bacteria.

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Tables and Figures

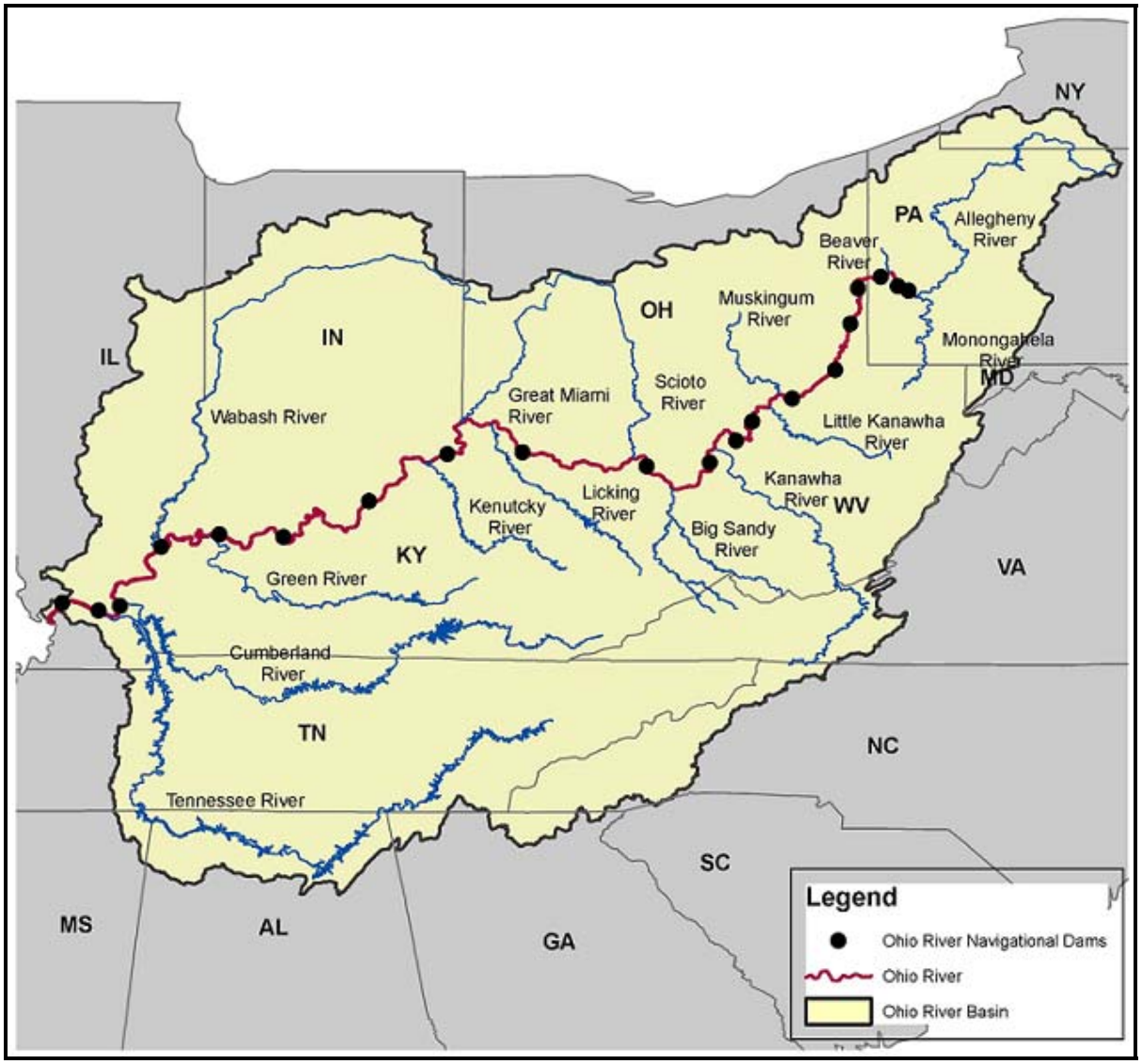

Fig. 1: Ohio River basin with states, tributaries, and navigational dams.

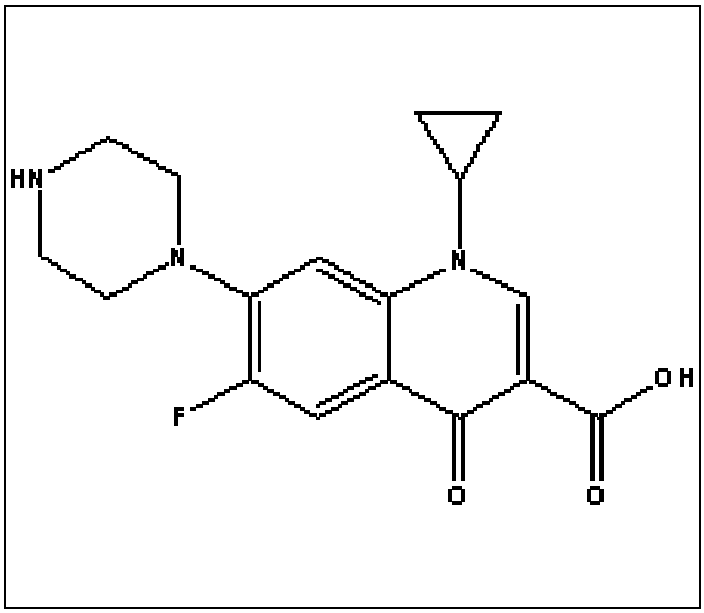

Figure 2: Chemical structure of ciprofloxacin

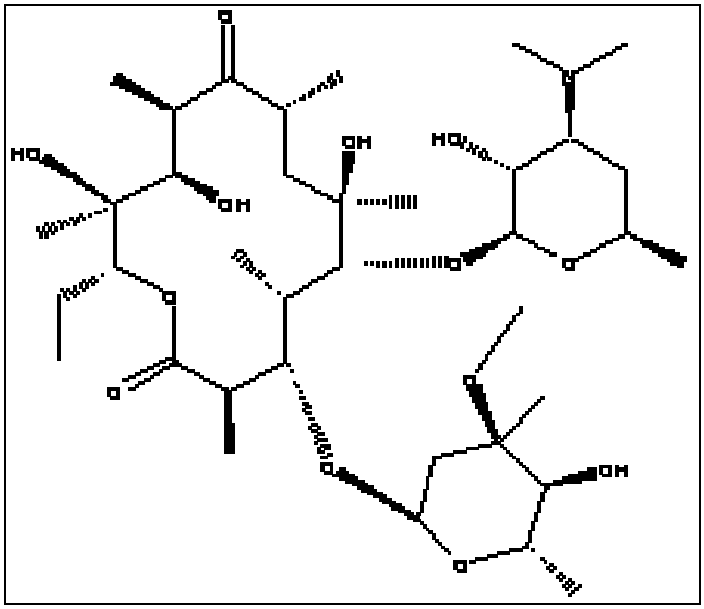

Figure 3: Chemical structure of erythromycin

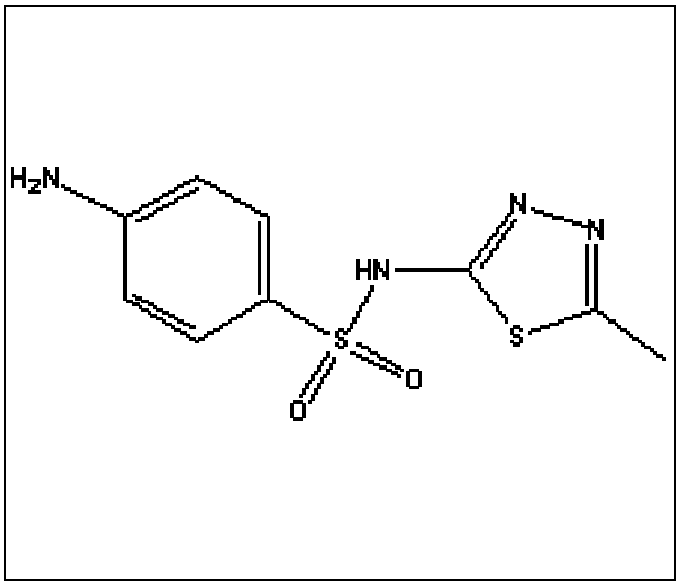

Figure 4: Chemical structure of sulfamethizole

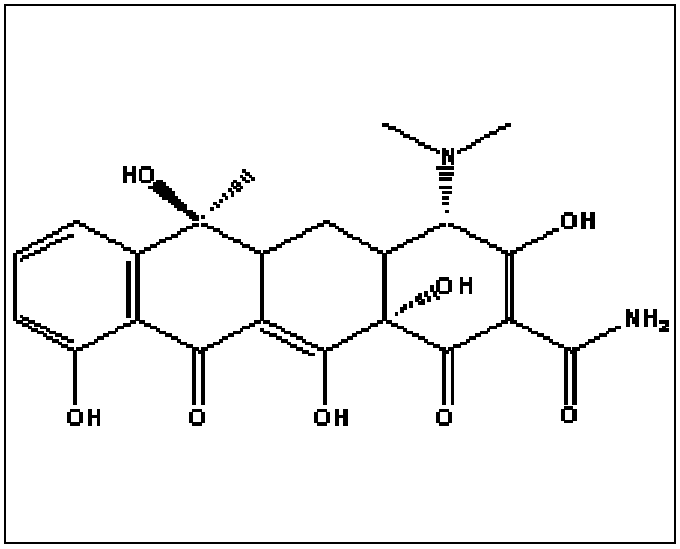

Figure 5: Chemical structure of tetracycline

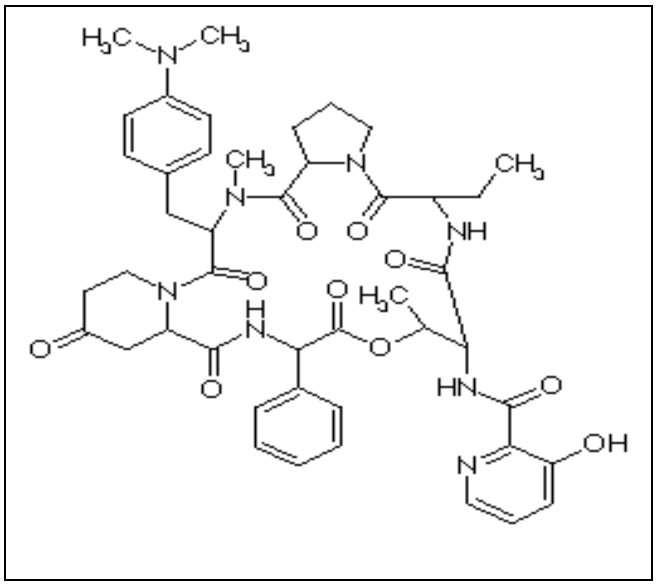

Figure 6: Chemical structure of Virginiamycin S1.

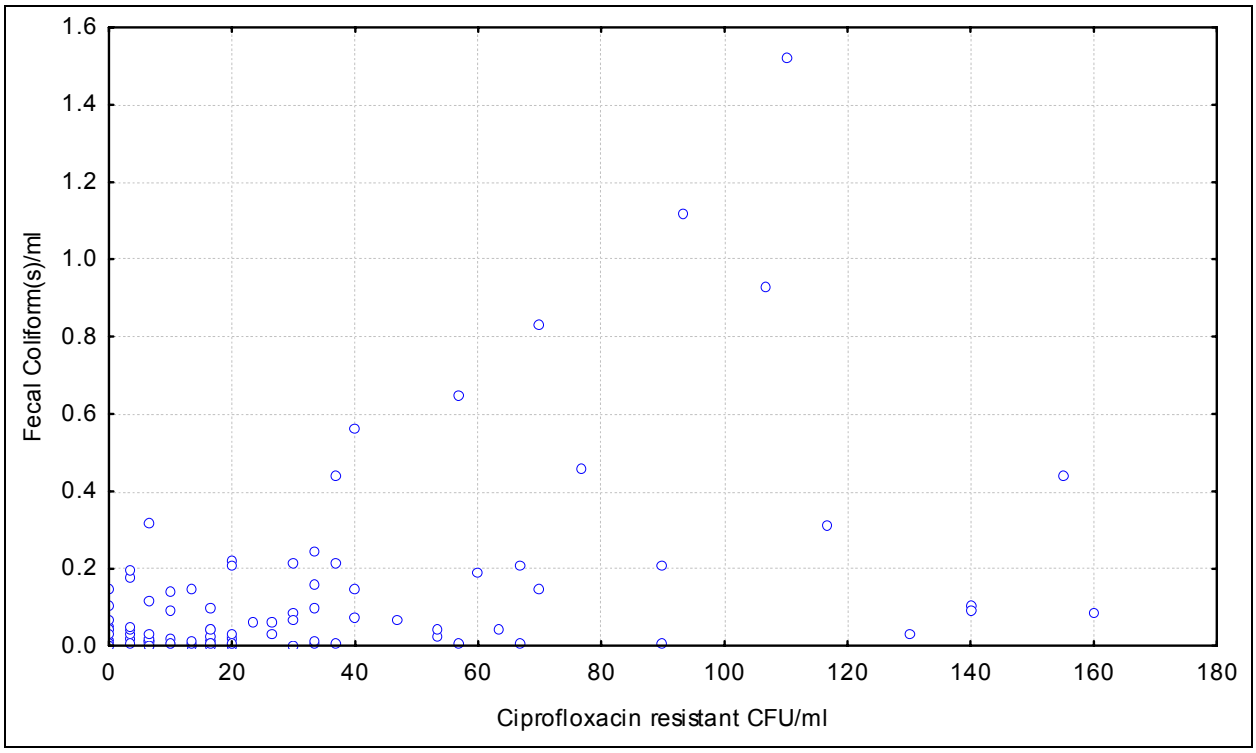

Fig 7: Linear regression analysis comparing ciprofloxacin resistant CFU/ml and fecal coliform(s)/ml in the mainstem of the Ohio River during Aug 2002. (R = 0.4683)

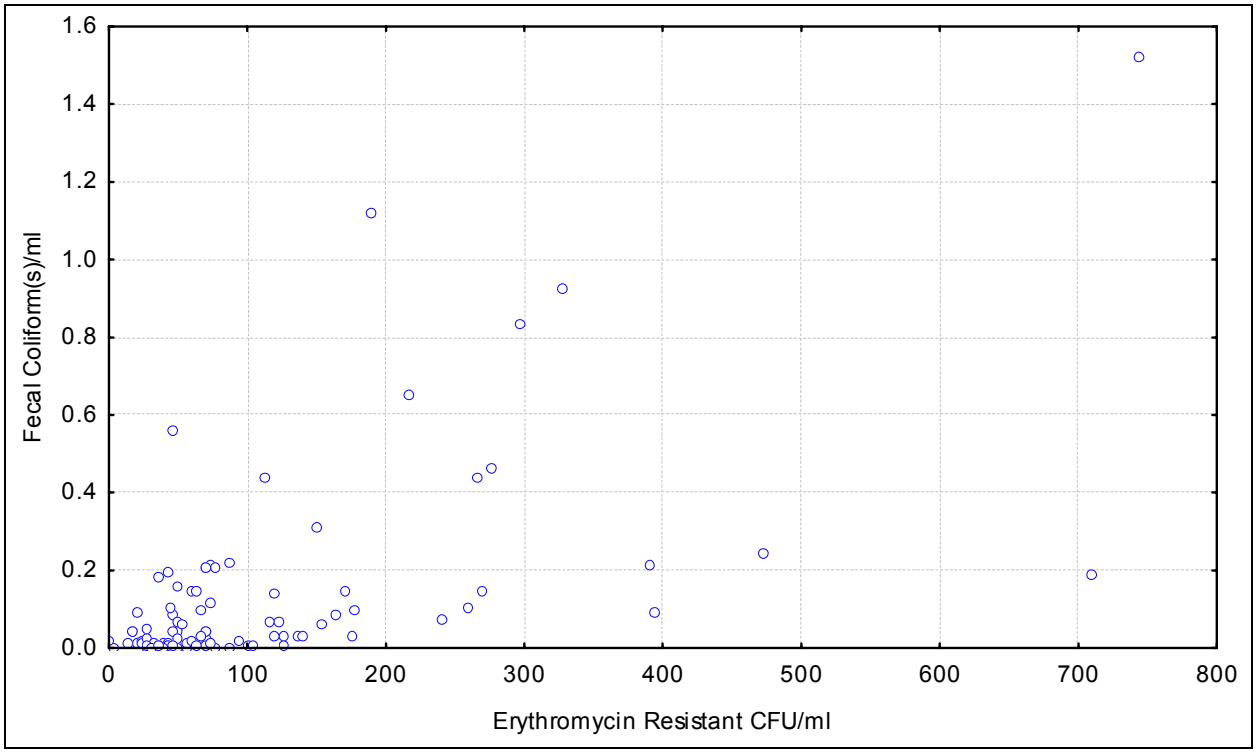

Fig. 8: Linear regression analysis comparing erythromycin resistant CFU/ml and fecal coliform(s)/ml in the mainstem of the Ohio River during Aug 2002. (R = 0.6116)

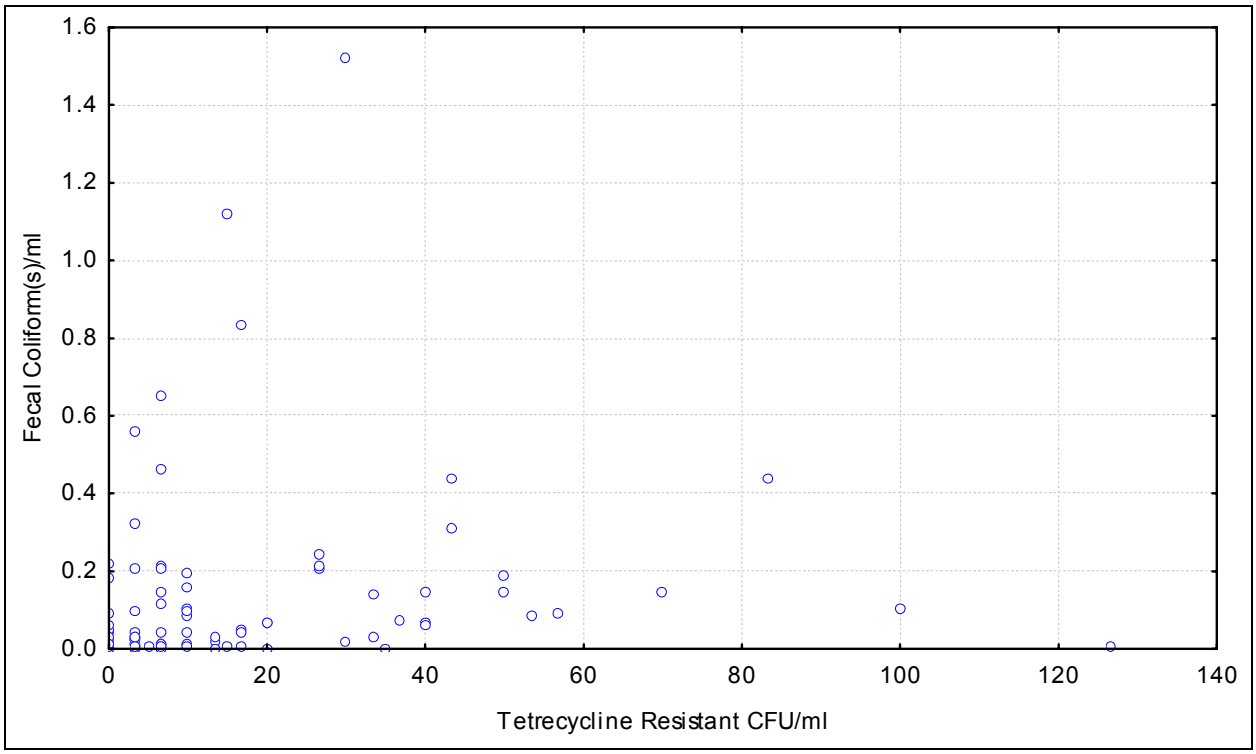

Fig. 9: Linear regression analysis comparing tetracycline resistant CFU/ml and fecal coliform(s)/ml in the mainstem of the Ohio River during Aug 2002. (R = 0.1644)

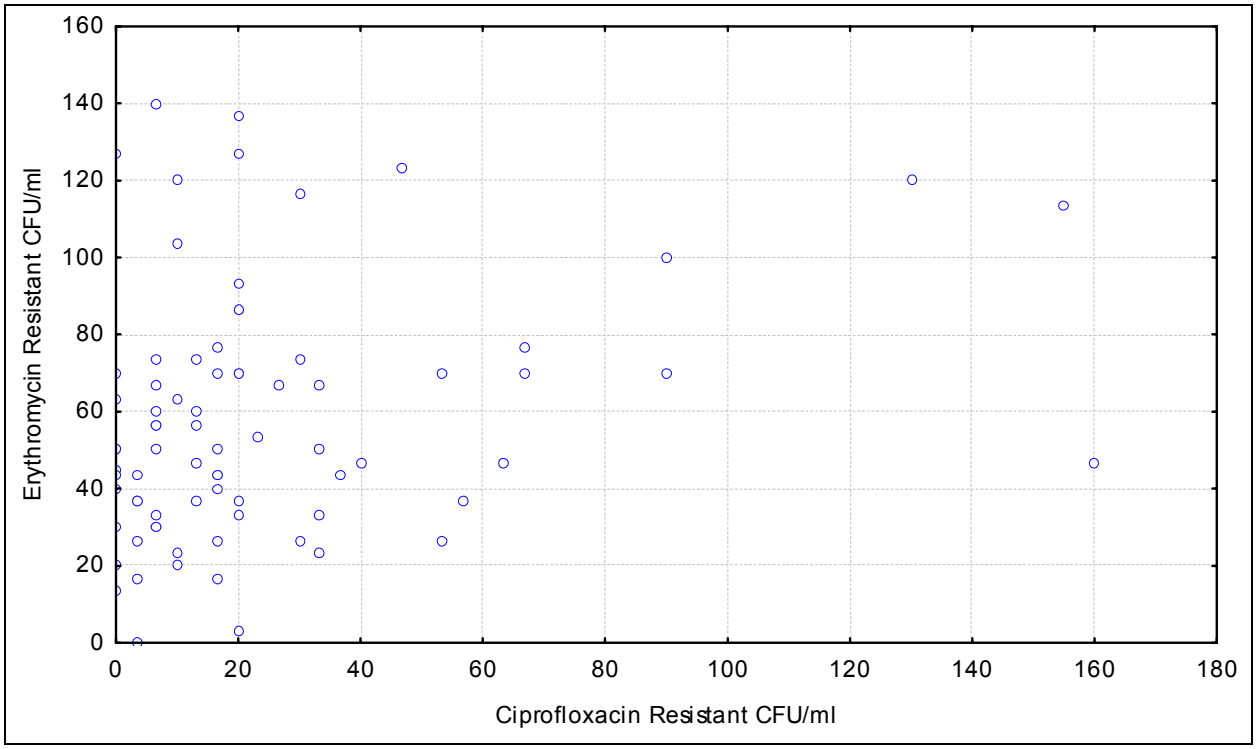

Fig. 10: Linear regression analysis comparing ciprofloxacin resistant CFU/ml and erythromycin resistant CFU/ml) in the mainstem of the Ohio River during Aug 2002. (R = 0.2728)

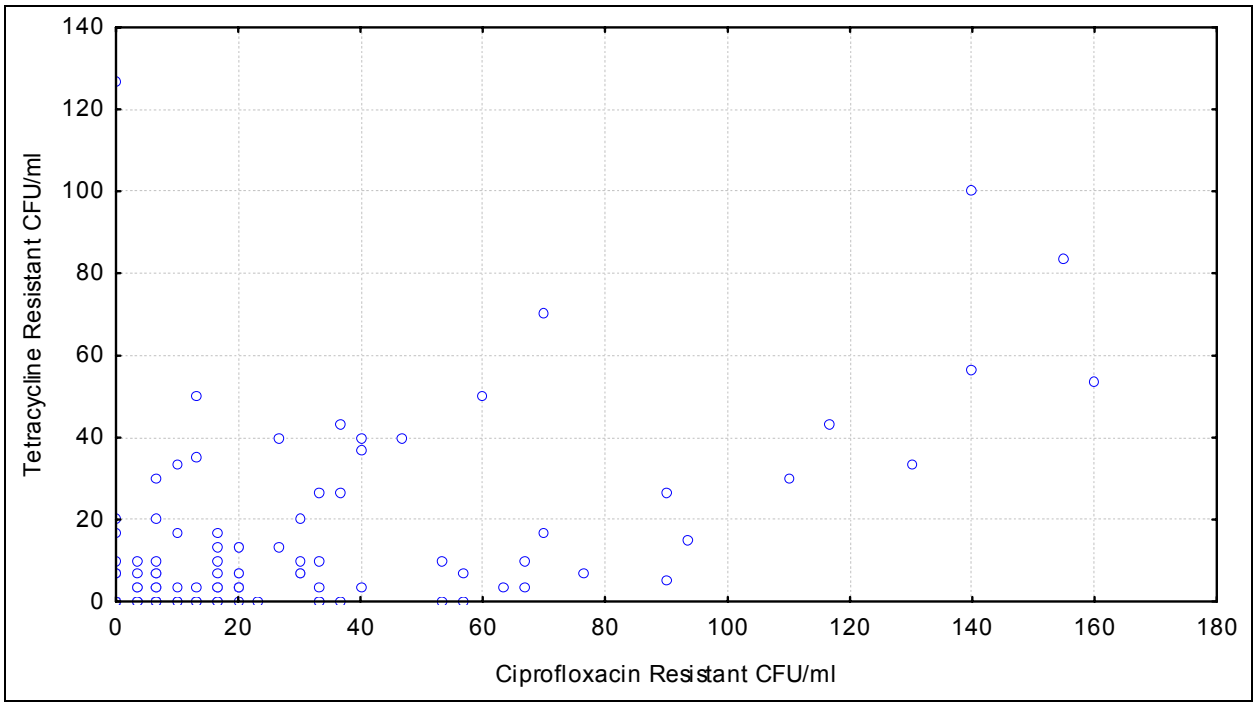

Fig. 11: Linear regression analysis comparing ciprofloxacin resistant CFU/ml and tetracycline resistant CFU/ml in the mainstem of the Ohio River during Aug 2002. (R = 0.5076)

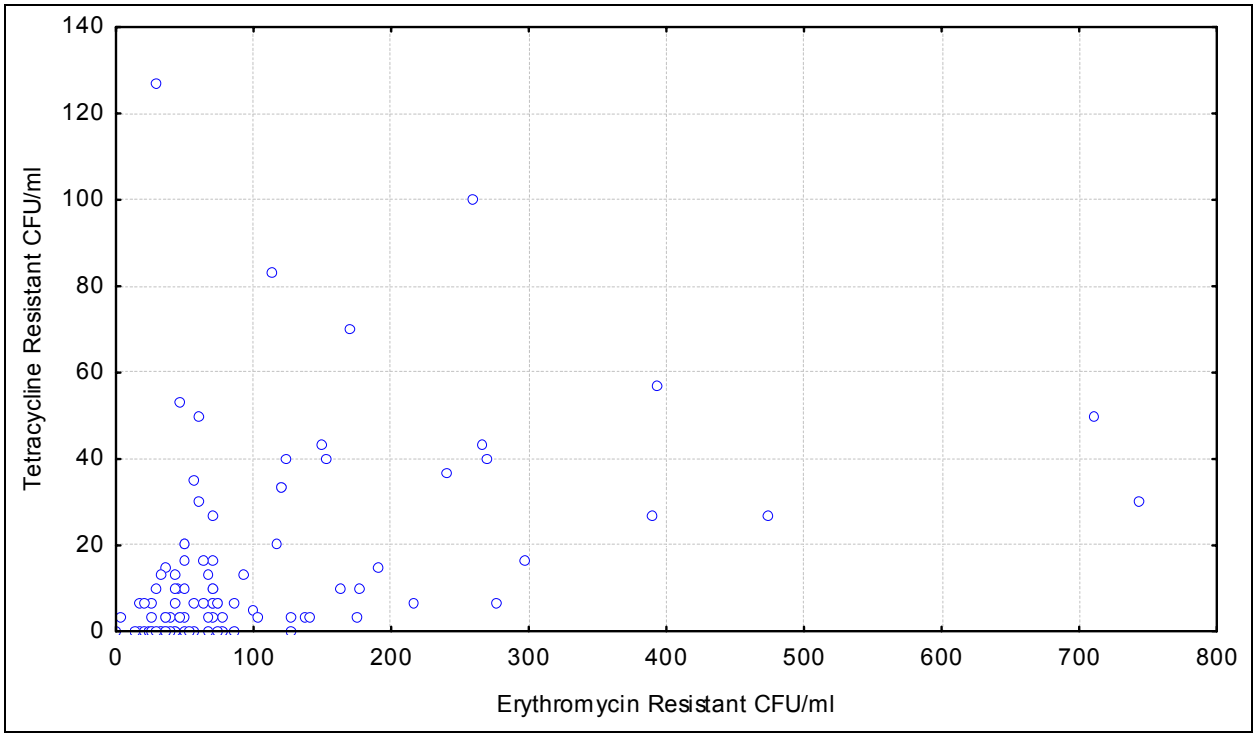

Fig. 12: Linear regression analysis comparing erythromycin resistant CFU/ml and tetracycline resistant CFU/ml in the mainstem of the Ohio River during Aug 2002. (R = 0.3733)

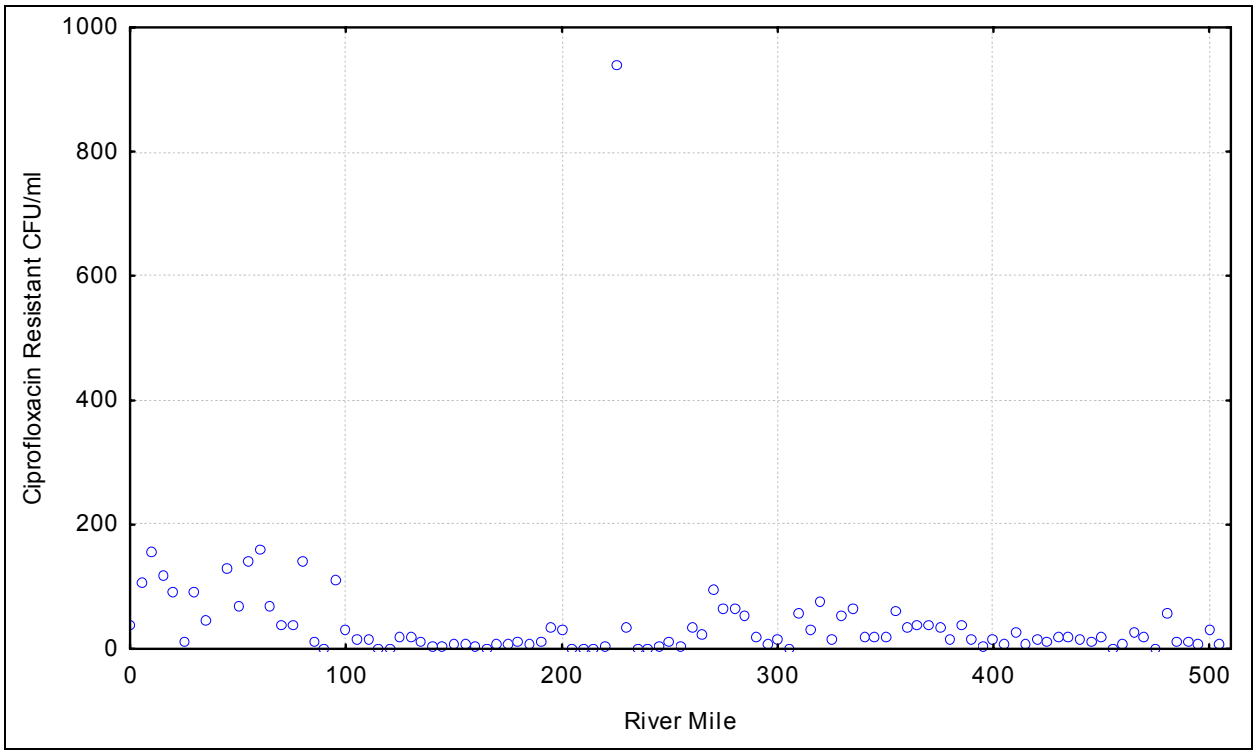

Fig. 13: Linear regression analysis comparing Ohio River miles and ciprofloxacin resistant CFU/ml in the mainstem of the Ohio River during Aug 2002. (R = -0.1714)

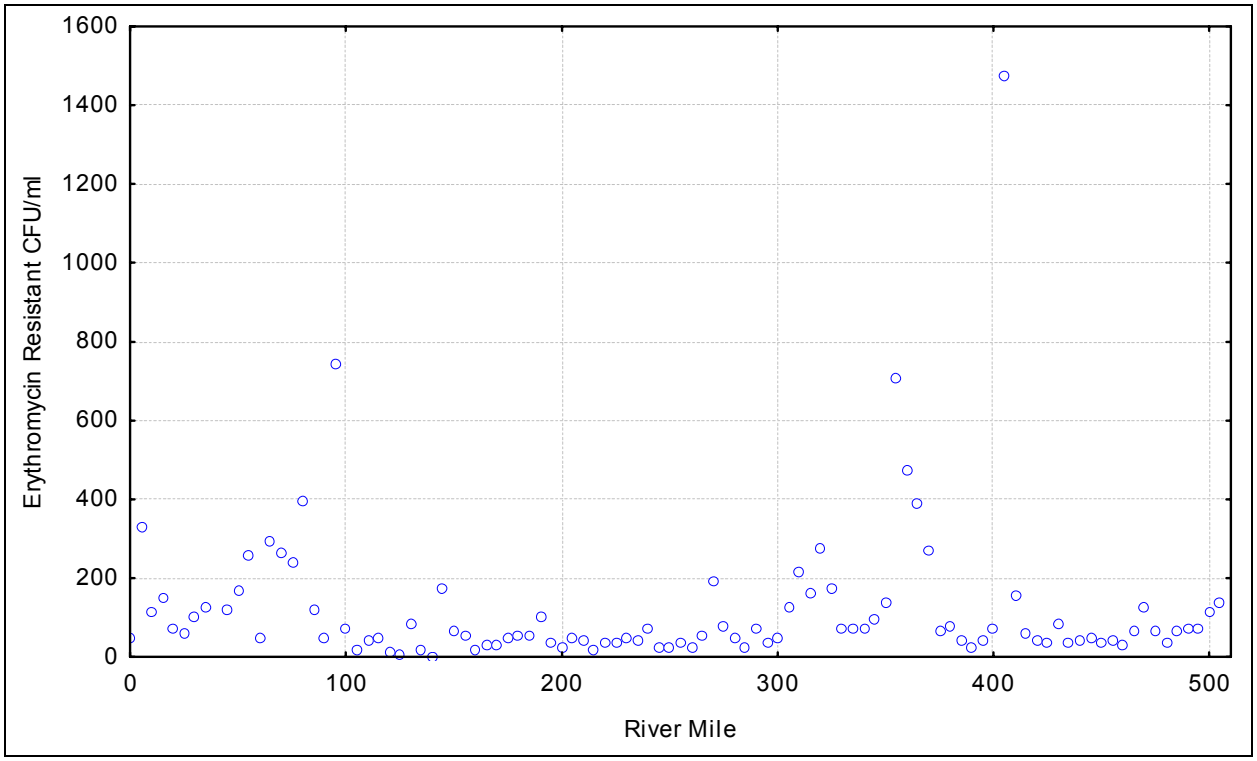

Fig. 14: Linear regression analysis comparing Ohio River miles and erythromycin resistant CFU/ml in the mainstem of the Ohio River during Aug 2002. (R = 0.00637)

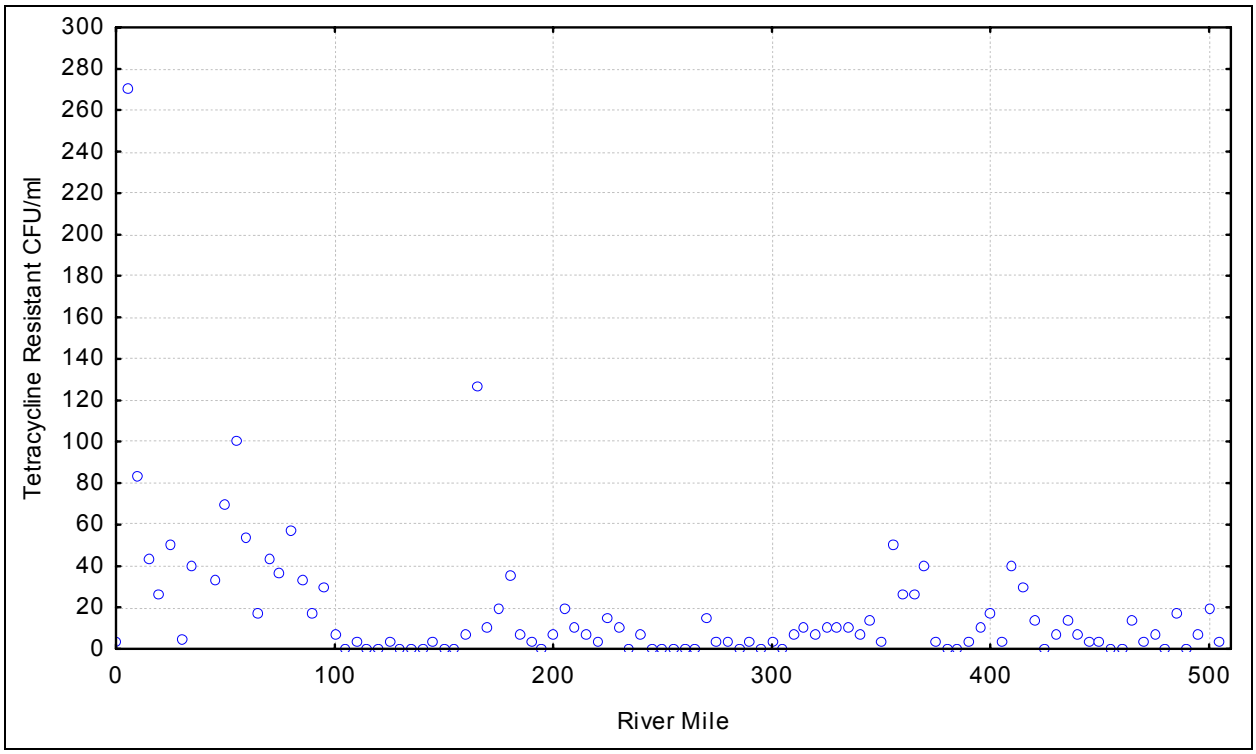

Fig. 15: Linear regression analysis comparing Ohio River miles and tetracycline resistant CFU/ml in the mainstem of the Ohio River during Aug 2002. (R = -0.3638)

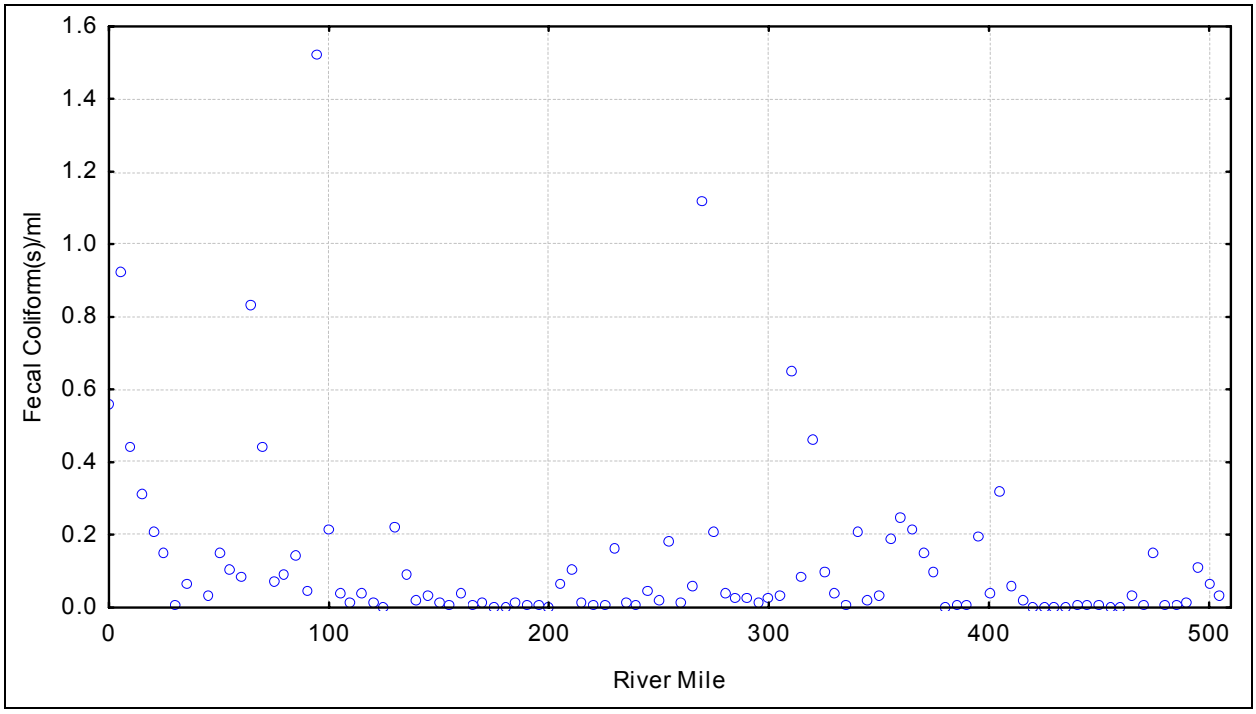

Fig. 16: Linear regression analysis comparing Ohio River miles and fecal coliform(s)/ml in the mainstem of the Ohio River during Aug 2002. (R = -0.2723)

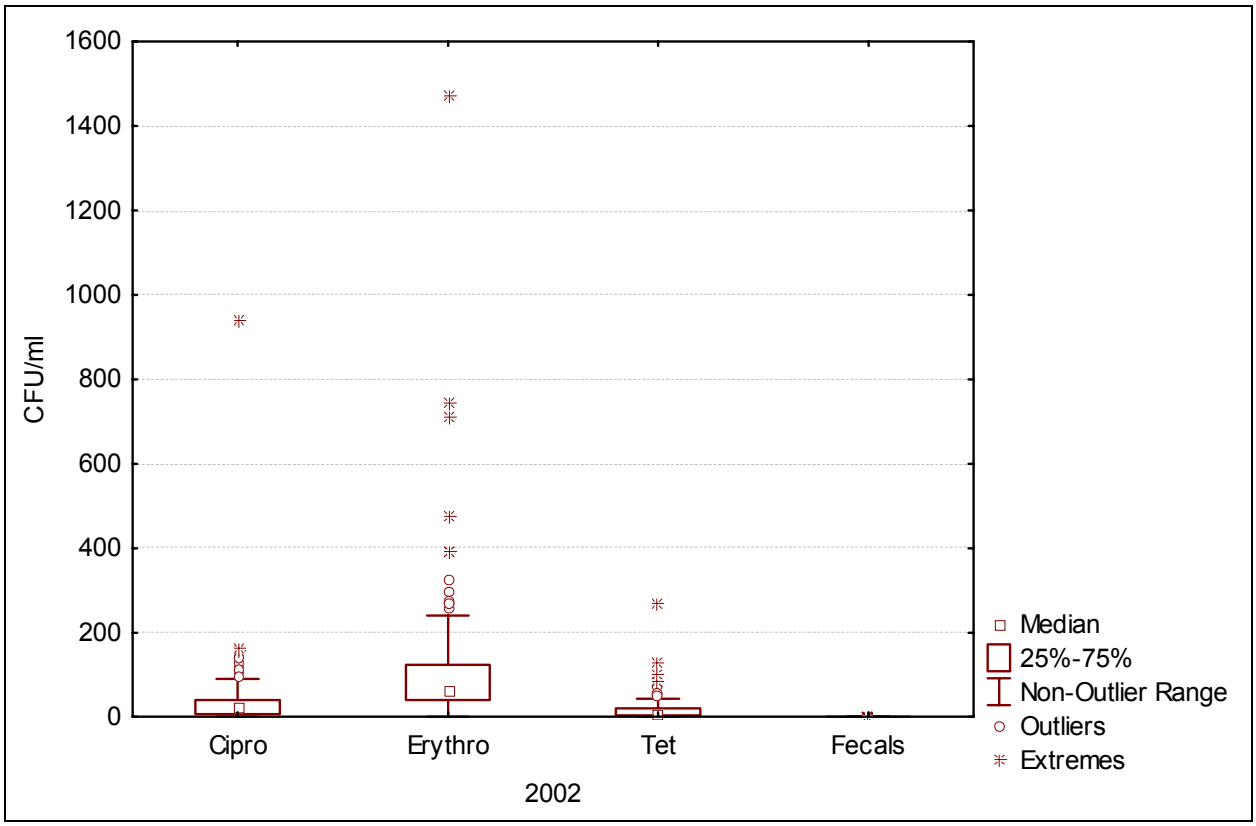

Fig 17: Box and whisker plot comparing total cultivable, antibiotic resistant and coliform bacteria counts measured during August 2002. Non-overlapping boxes suggest populations are probably significantly different.

Table 1: Mann-Whitney Rank Sum Test comparing ratios of fecal coliform bacteria / antibiotic resistant bacteria in tributaries to matching ratios from all of the mainstem sampling sites during August 2002. Italicized *P*-values are significant at <0.05.

	FC/Cipro		
FC/Cipro	*0.0357*	**FC/Erythro**	
FC/Erythro		*0.0199*	**FC/Tet**
FC/Tet			0.0738

Table 2: Spearman Correlation Test comparing each average CFU / ml to all other bacterial measurements at the same site in the mainstem samples of the Ohio River during August 2002. Correlation coefficients that are italicized show that the two populations are different at a significance level of $P<0.05$

	Totals	Cipro	Erythro	Tet	FC
Totals	1.0				
Cipro	*0.3464*	1.0			
Erythro	*0.4196*	*0.4486*	1.0		
Tet	*0.3569*	*0.41*	*0.5135*	1.0	
FC	*0.226*	*0.3593*	*0.5605*	*0.3858*	1.0

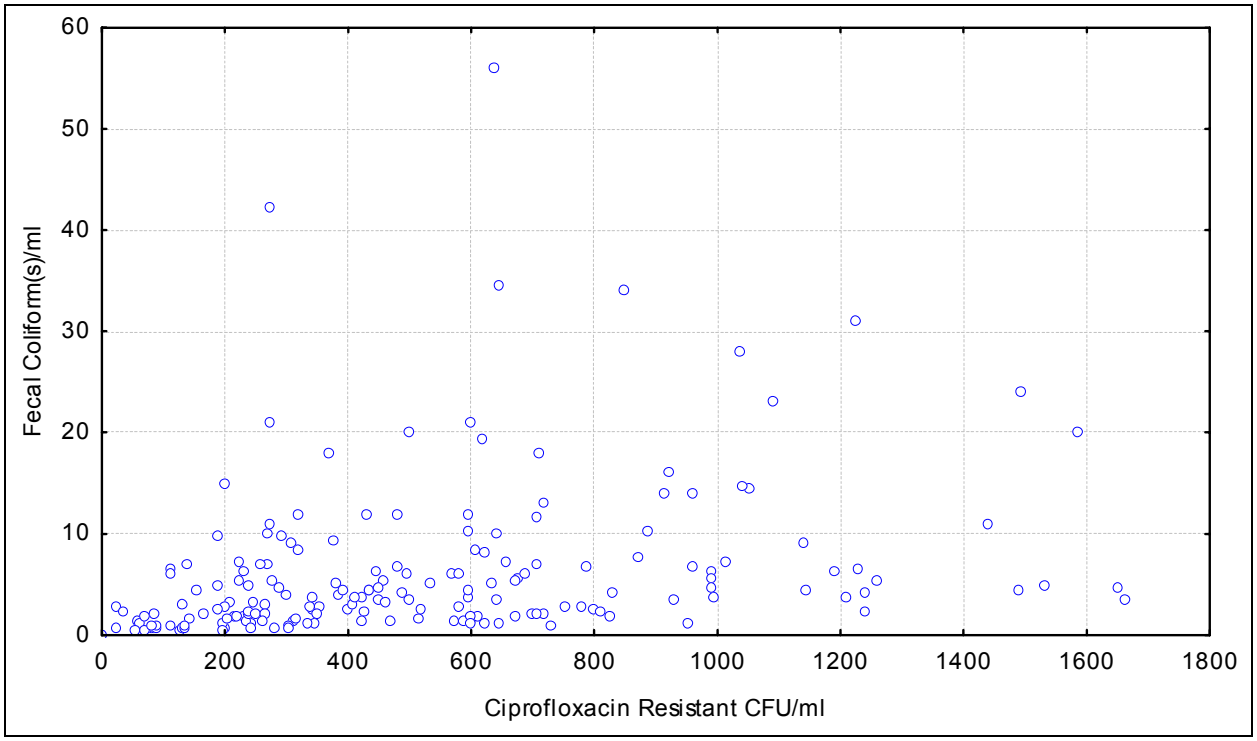

Fig. 18: Linear regression analysis comparing ciprofloxacin resistant CFU/ml and fecal coliform(s)/ml in the mainstem of the Ohio River during Aug 2003. (R = 0.2978)

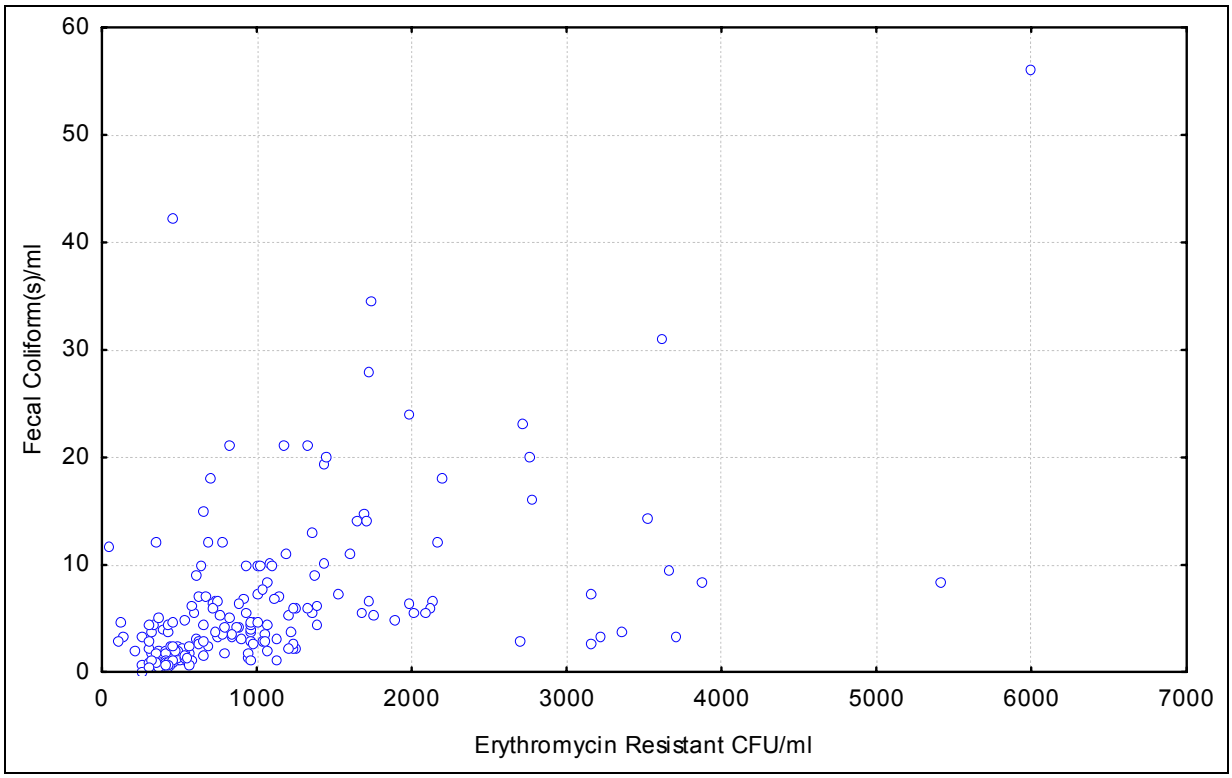

Fig. 19: Linear regression analysis comparing erythromycin resistant CFU/ml and fecal coliform(s)/ml in the mainstem of the Ohio River during Aug 2003. (R = 0.5092)

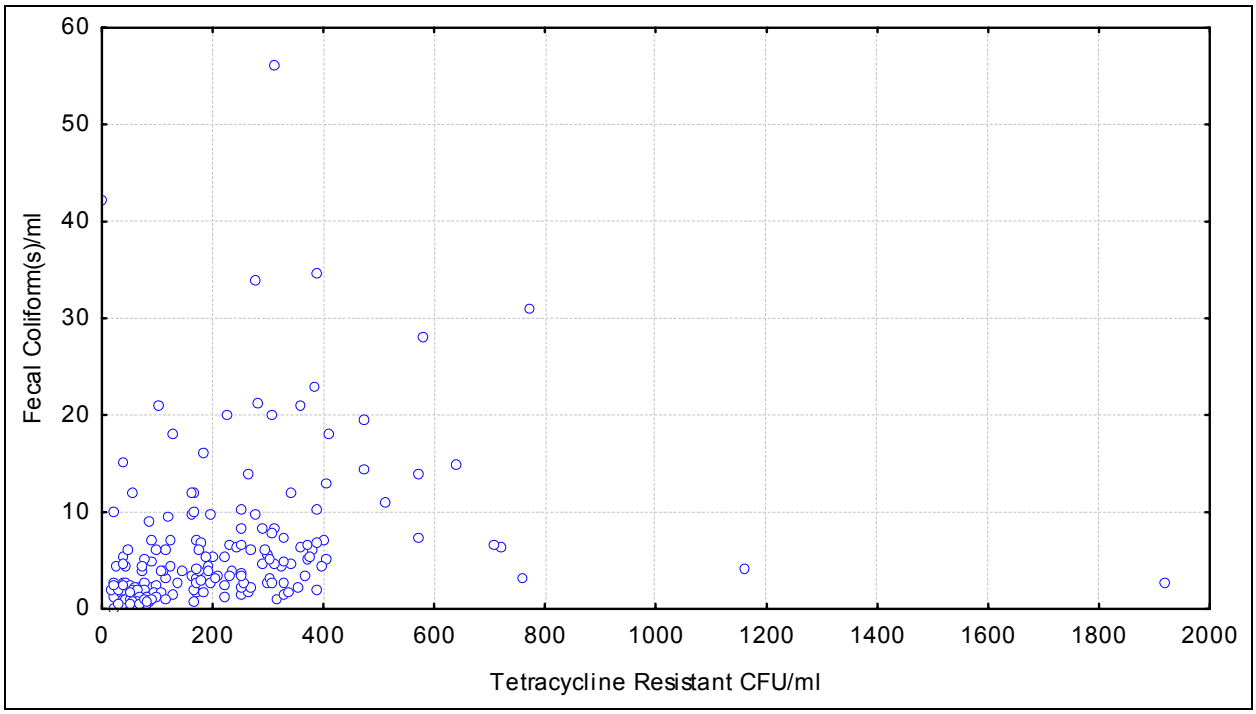

Fig. 20: Linear regression analysis comparing tetracycline resistant CFU/ml and fecal coliform(s)/ml in the mainstem of the Ohio River during Aug 2003. (R = 0.2472)

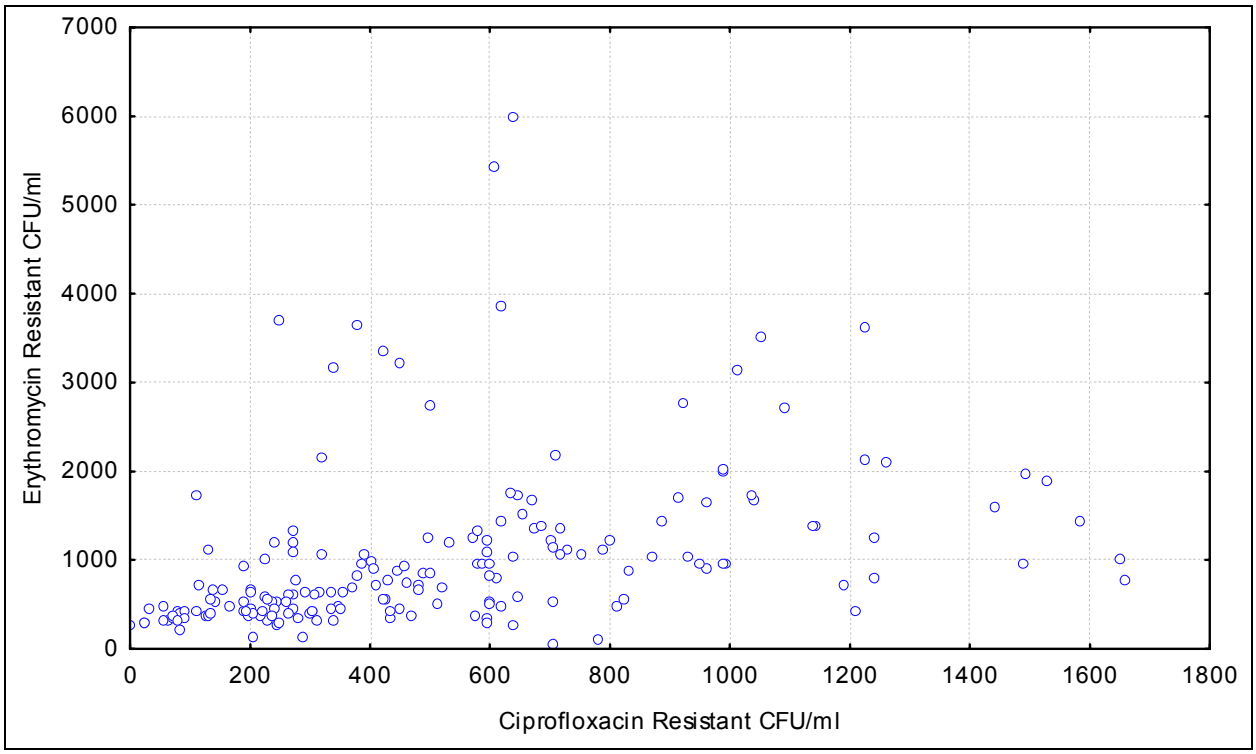

Fig. 21: Linear regression analysis comparing ciprofloxacin resistant CFU/ml and erythromycin resistant CFU/ml in the mainstem of the Ohio River during Aug 2003. (R = 0.3723)

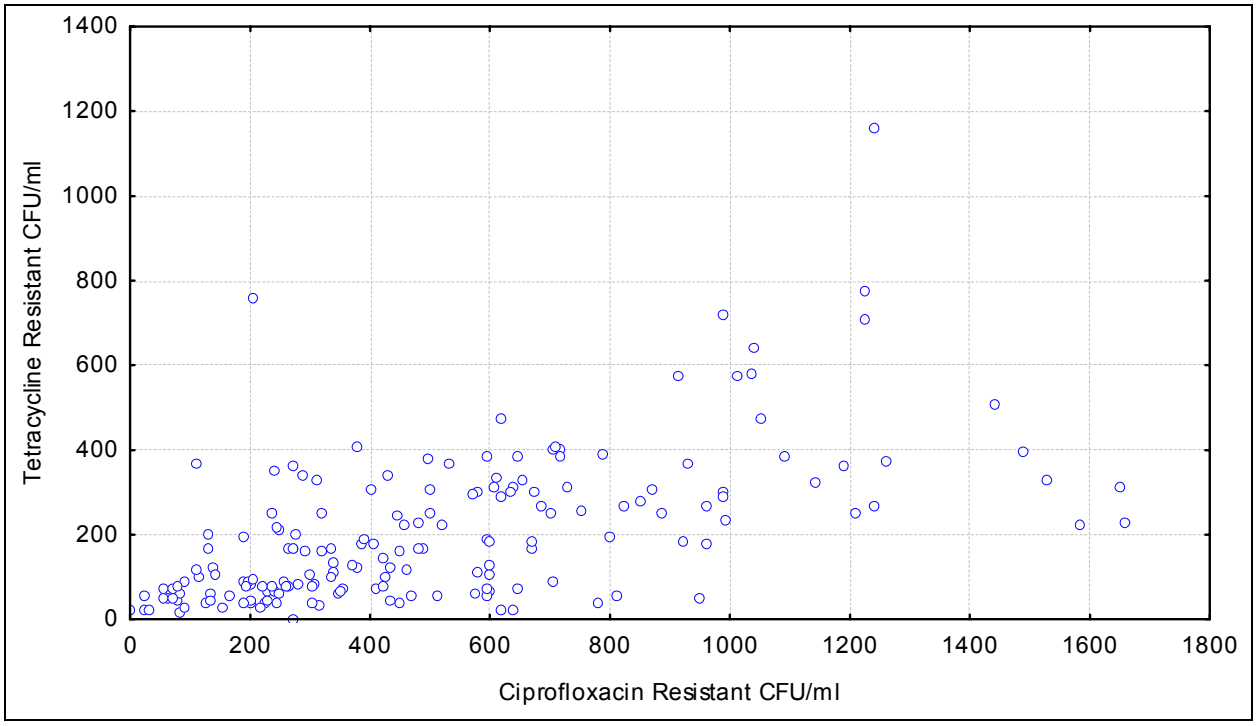

Fig. 22: Linear regression analysis comparing ciprofloxacin resistant CFU/ml and tetracycline resistant CFU/ml in the mainstem of the Ohio River during Aug 2003. (R = 0.5847)

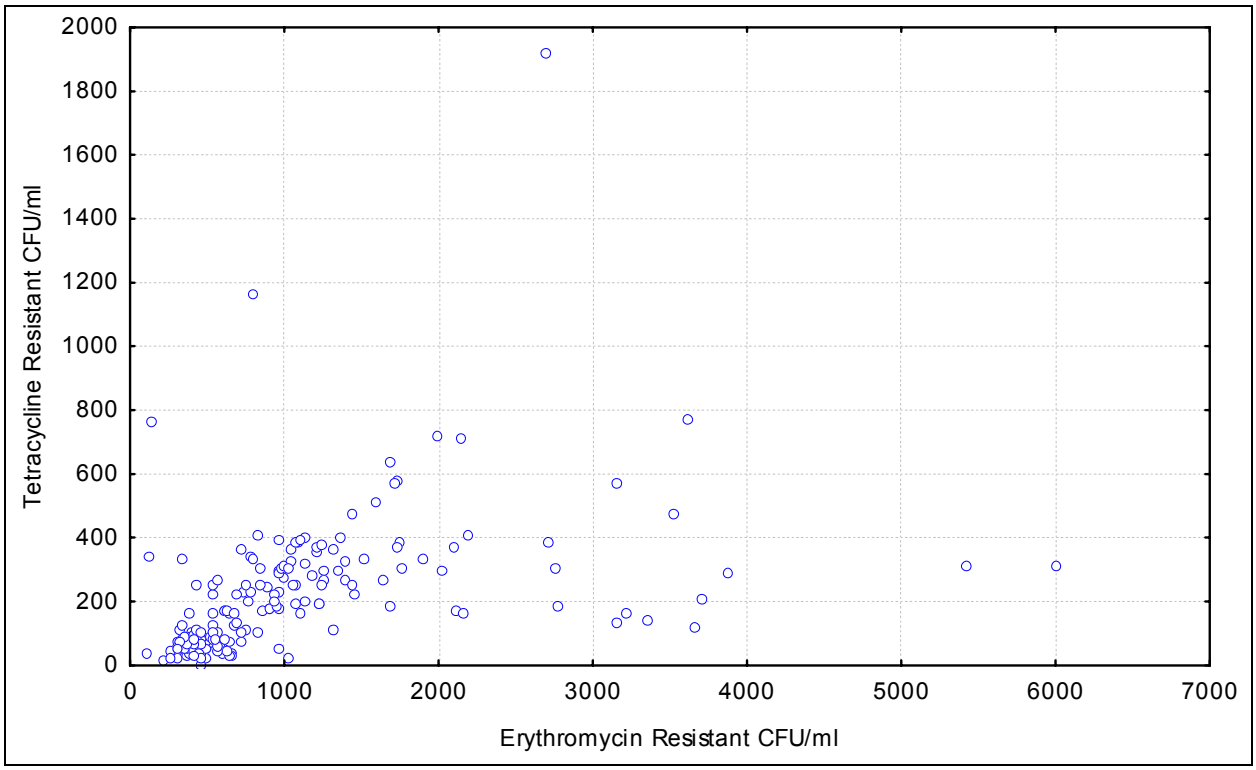

Fig. 23: Linear regression analysis comparing erythromycin resistant CFU/ml and tetracycline resistant CFU/ml in the mainstem of the Ohio River during Aug 2003. (R = 0.4240)

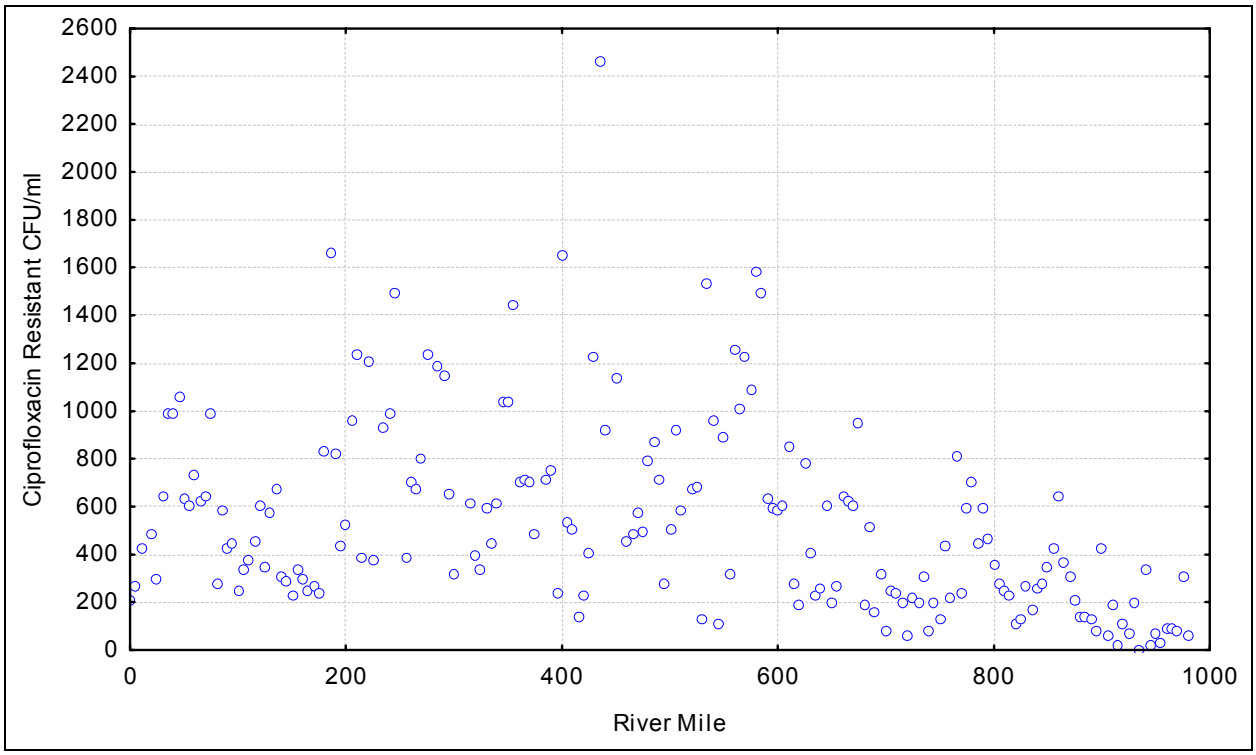

Fig. 24: Linear regression analysis comparing Ohio River miles and ciprofloxacin resistant CFU/ml in the mainstem of the Ohio River during Aug 2003. (R = -0.3712)

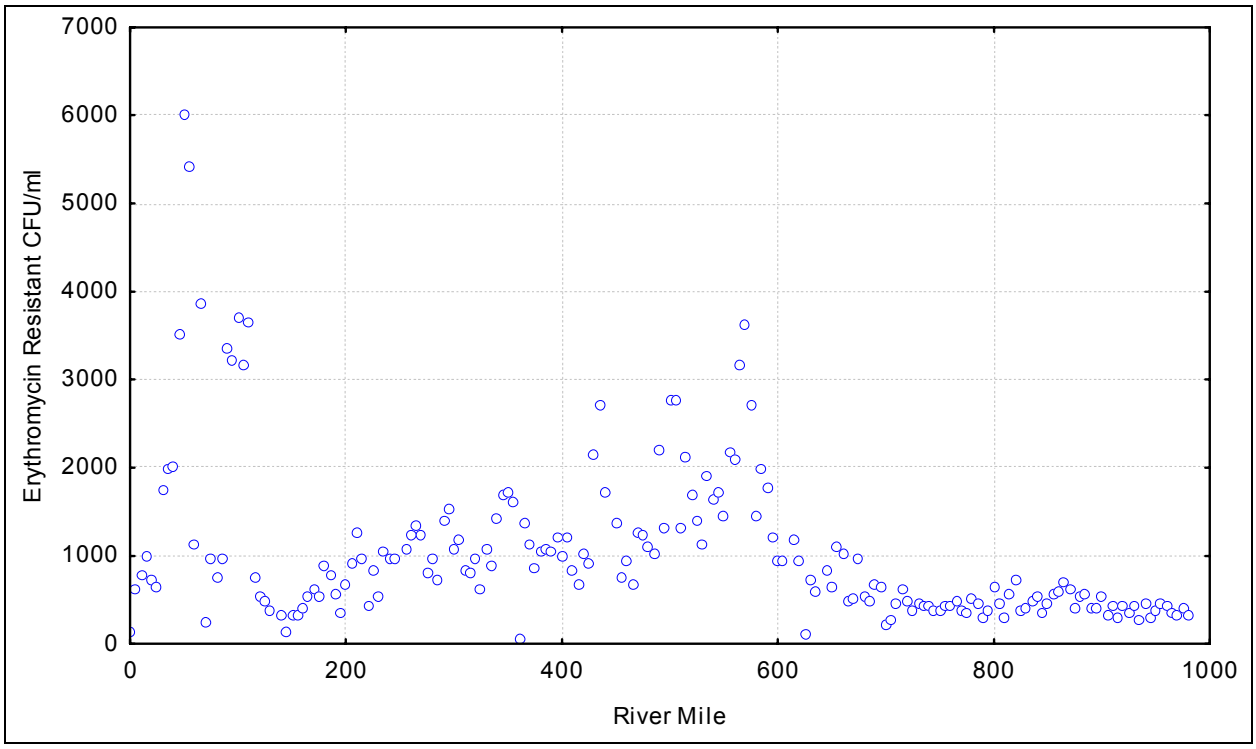

Fig. 25: Linear regression analysis comparing Ohio River miles and erythromycin resistant CFU/ml in the mainstem of the Ohio River during Aug 2003. (R = -0.3914)

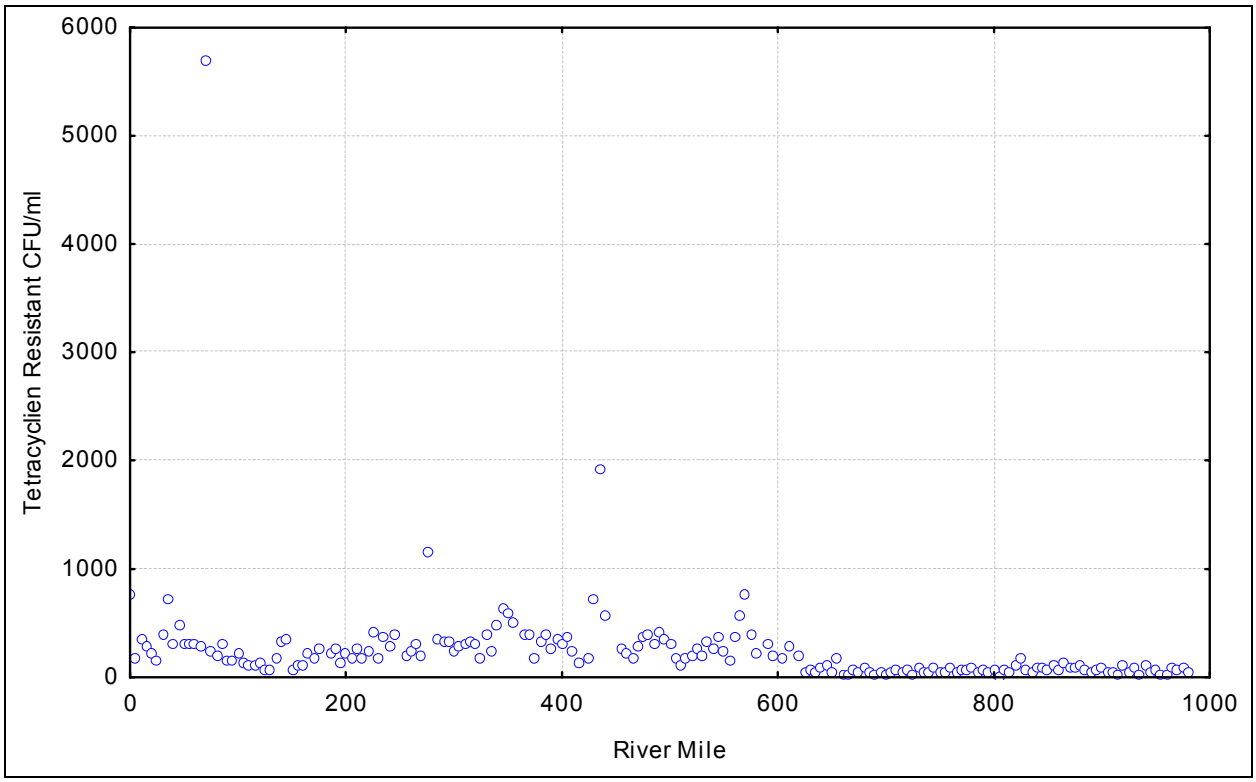

Fig. 26: Linear regression analysis comparing Ohio River miles and tetracycline resistant CFU/ml in the mainstem of the Ohio River during Aug 2003. (R = -0.2928)

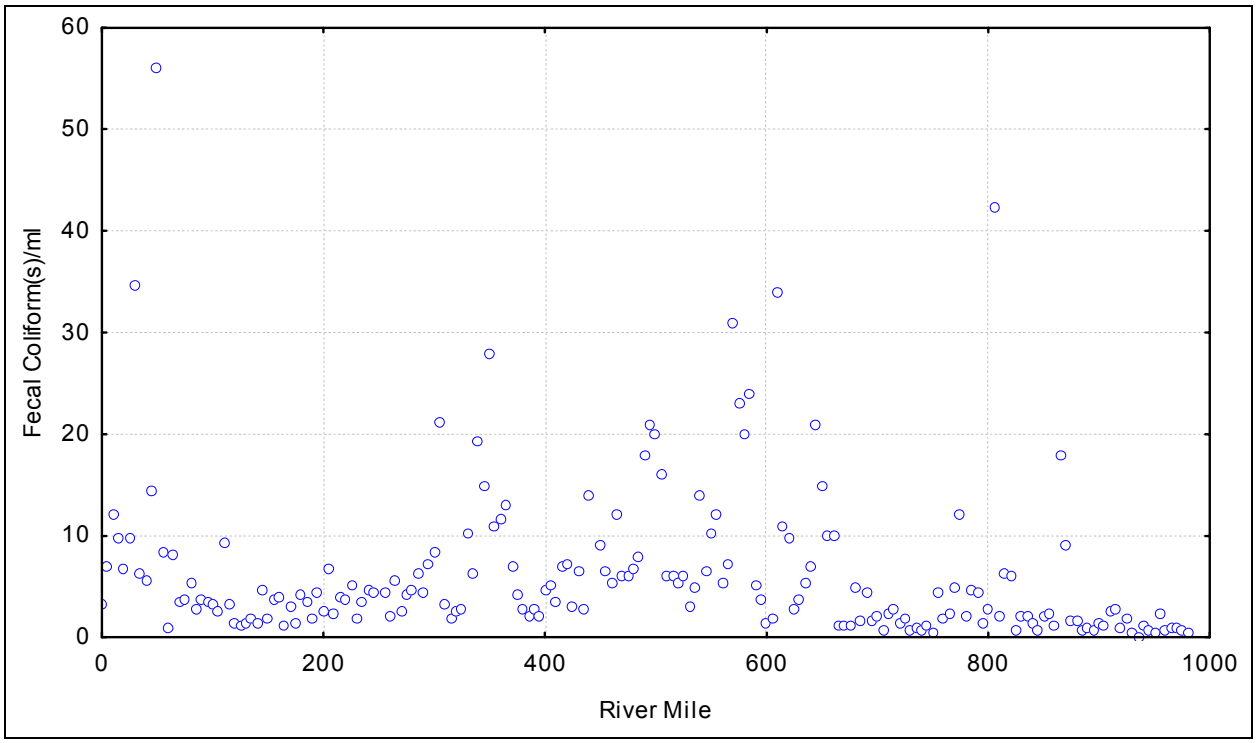

Fig. 27: Linear regression analysis comparing Ohio River miles and fecal coliform(s)/ml in the mainstem of the Ohio River during Aug 2003. (R = -0.1645)

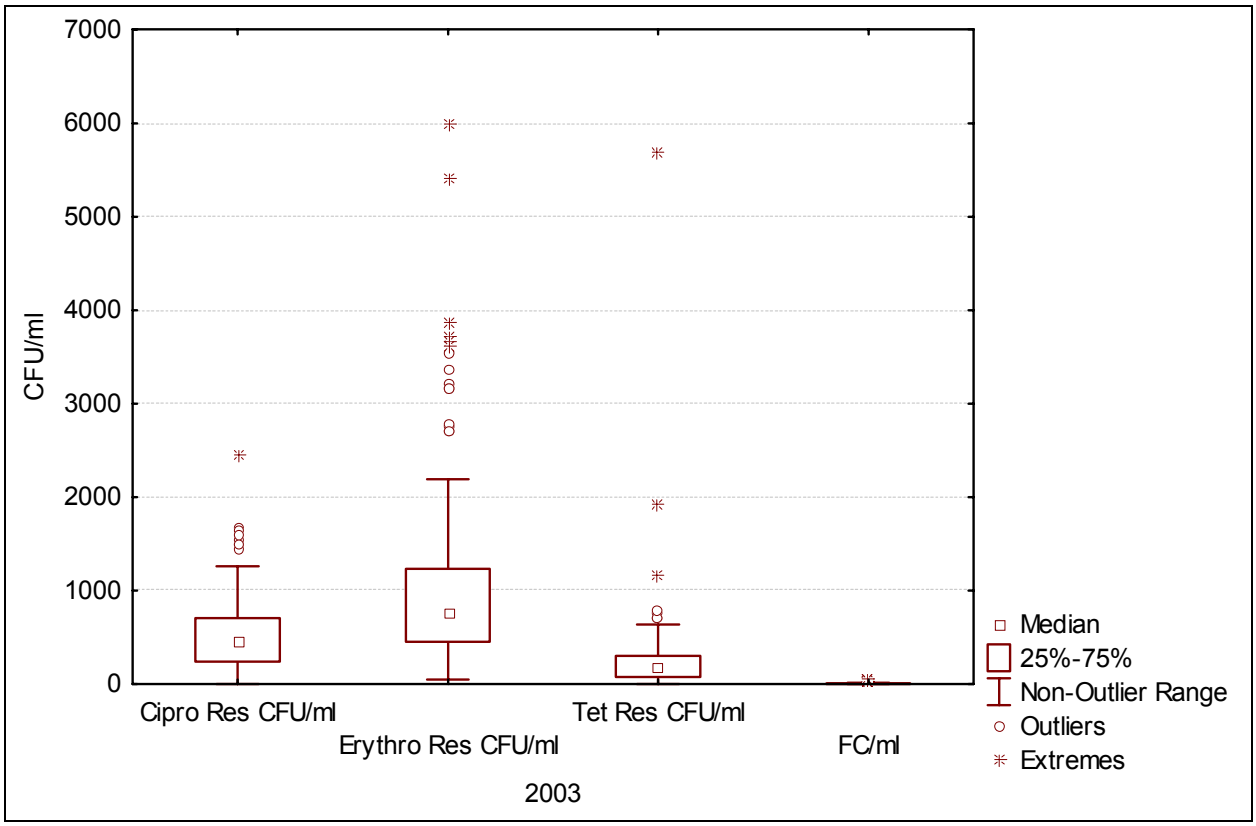

Fig 28: Box and whisker plot comparing bacterial population measurements collected on the Ohio River during August 2003. Non-overlapping boxes suggest populations are probably significantly different.

Table 3: Mann Whitney Rank Sum Test comparing ratios of fecal coliform bacteria / antibiotic resistant bacteria in tributaries to matching ratios from all of the mainstem sampling sites during 2003.

	FC/Cipro		
FC/Cipro	0.3993	**FC/Erythro**	
FC/Erythro		*0.0248*	**FC/Tet**
FC/Tet			0.1419

Table 4: Spearman Correlation test comparing each average CFU / ml to all other bacterial measurements at the same site in the mainstem samples of the Ohio River during 2003. Correlation coefficients that are italicized show that the two populations are different at a significance level of $P<0.05$

	Totals				
Totals	1.0	**Cipro**			
Cipro	*0.6106*	1.0	**Erythro**		
Erythro	*0.7322*	*0.5942*	1.0	**Tet**	
Tet	*0.7412*	*0.6257*	*0.67*	1.0	**FC**
FC	*0.6320*	*0.457*	*0.6229*	*0.5132*	1.0

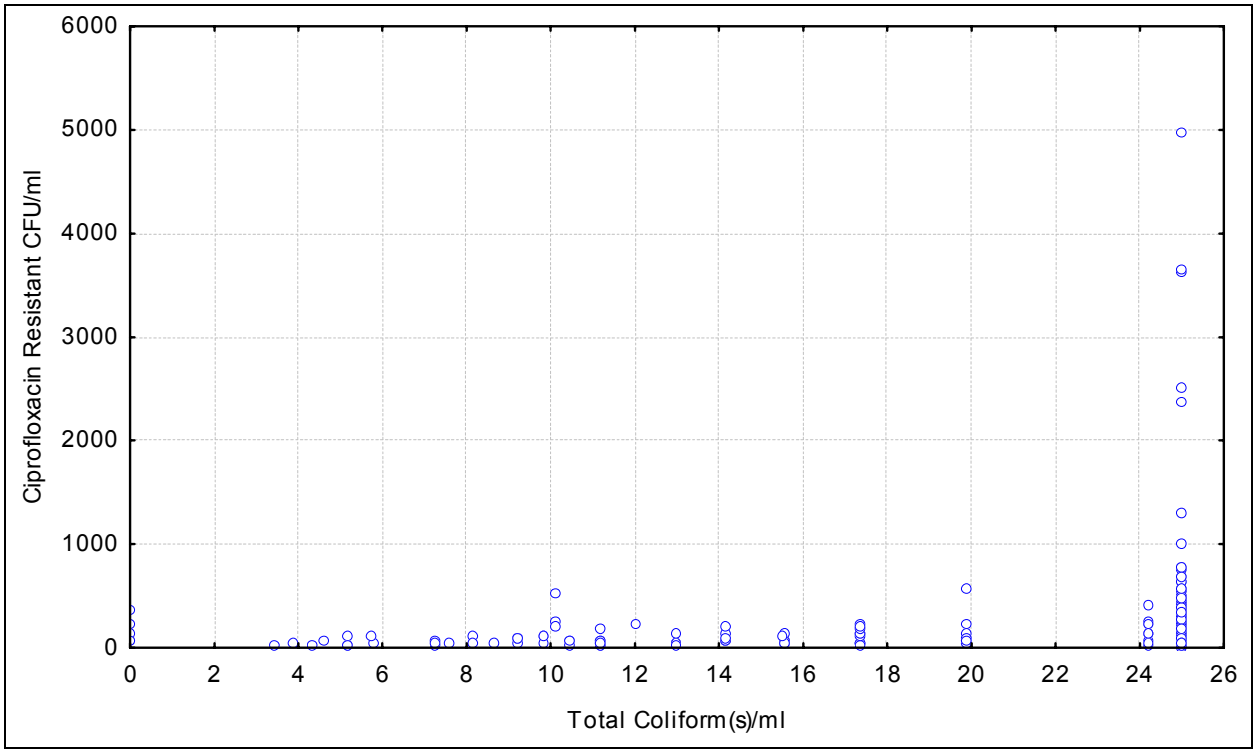

Fig. 29: Linear regression analysis comparing ciprofloxacin resistant CFU/ml and total coliform(s)/ml in the mainstem of the Ohio River during Aug 2004. (R = 0.2359)

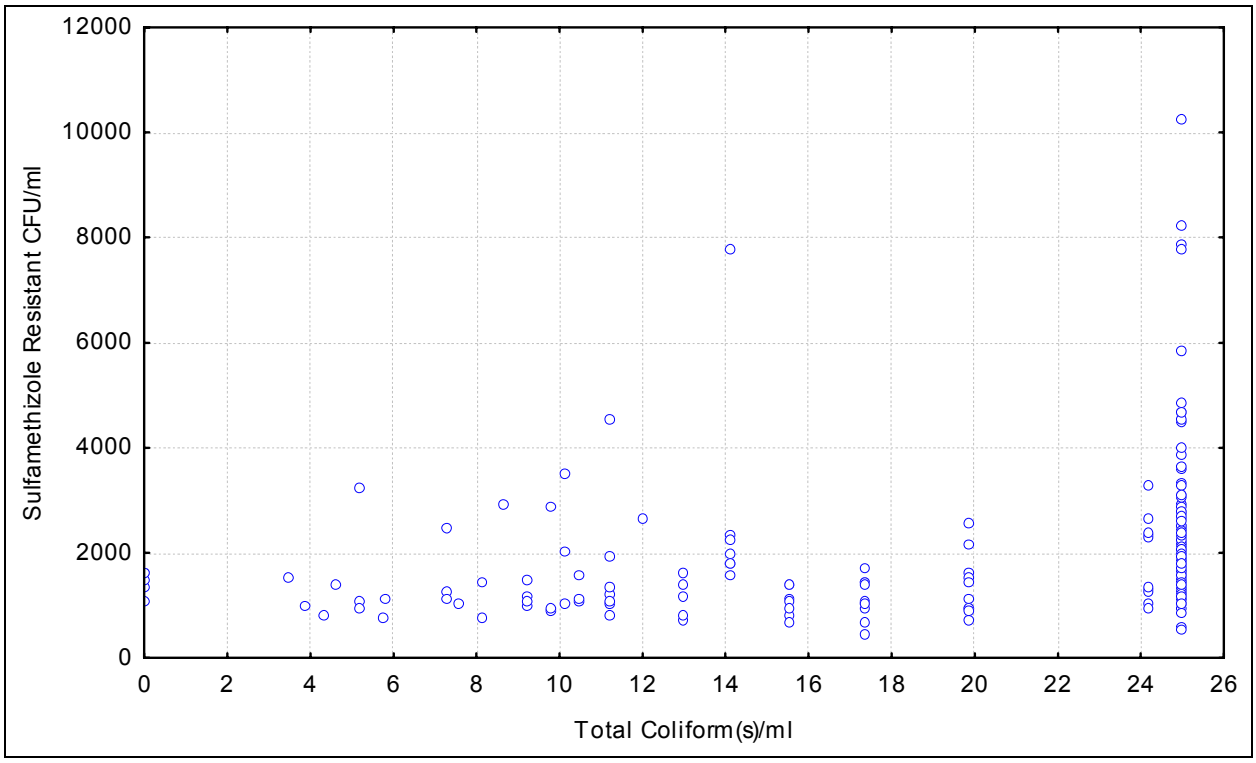

Fig. 30: Linear regression analysis comparing sulfamethizole resistant CFU/ml and total coliform(s)/ml in the mainstem of the Ohio River during Aug 2004. (R = 0.2467)

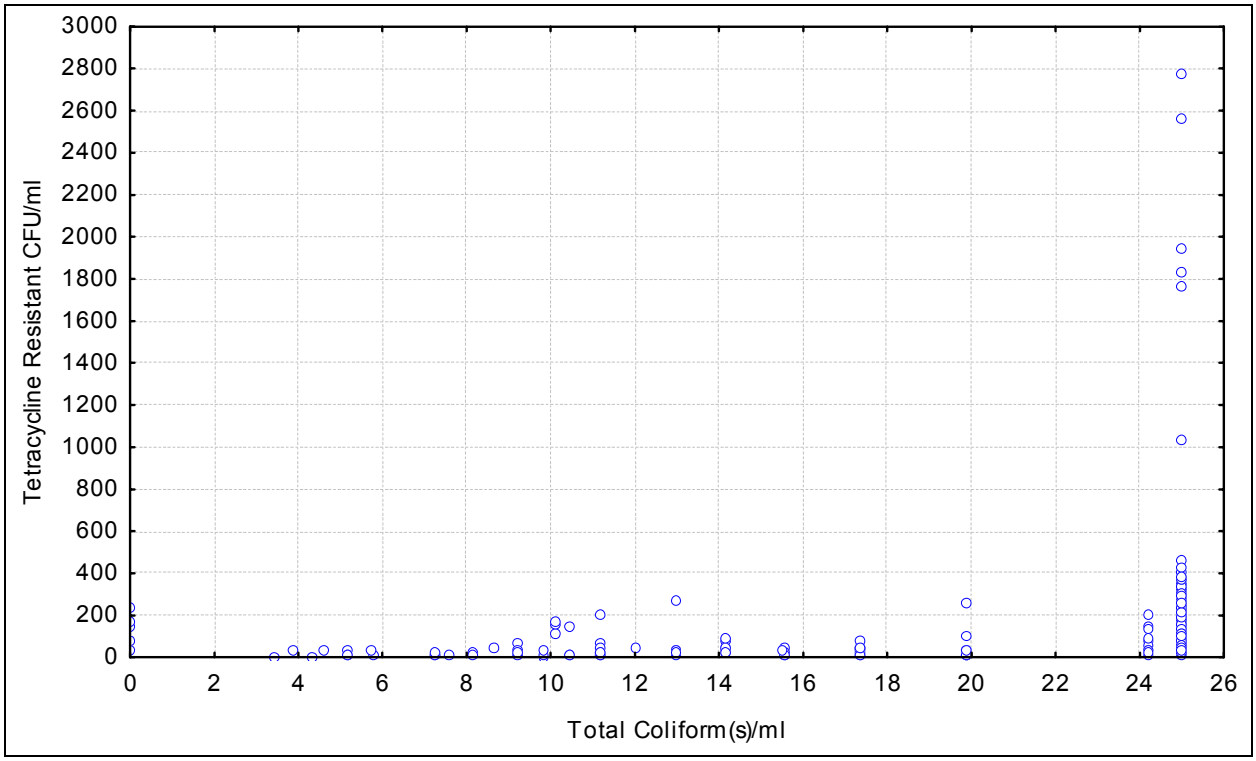

Fig. 31: Linear regression analysis comparing tetracycline resistant CFU/ml and total coliform(s)/ml in the mainstem of the Ohio River during Aug 2004. (R = 0.2354)

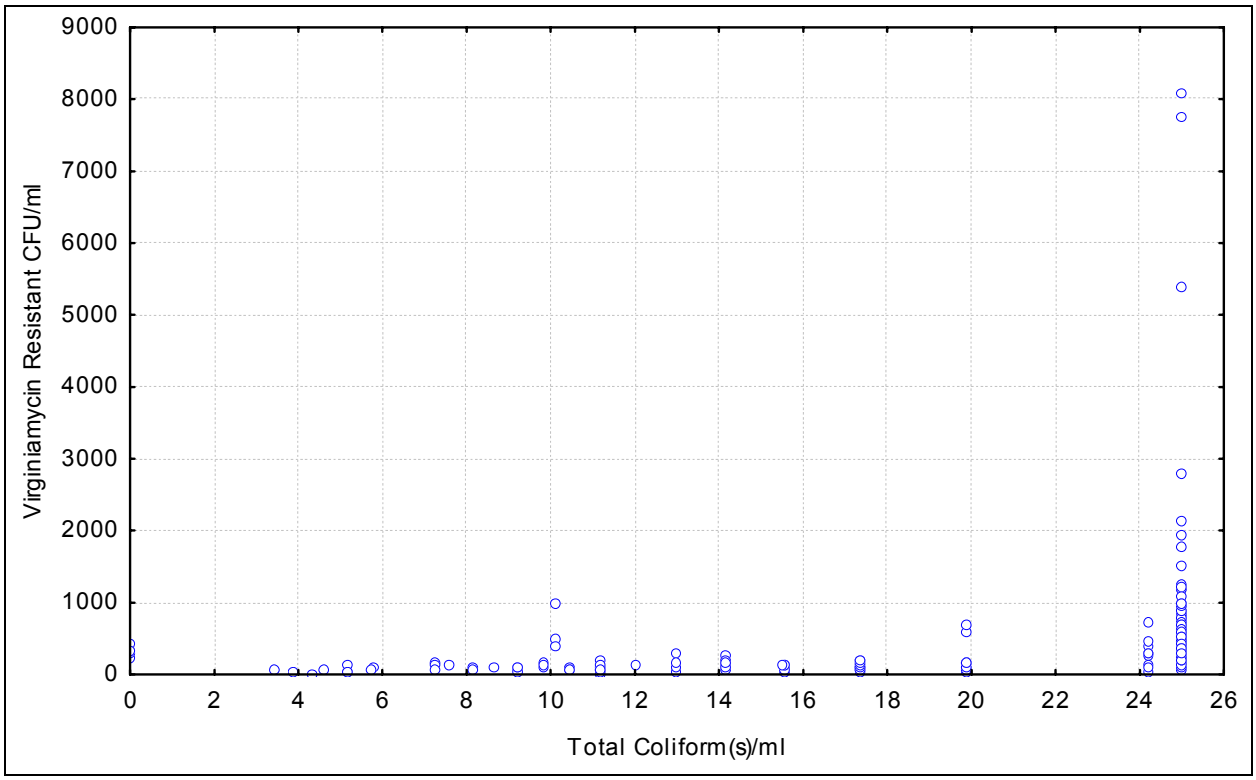

Fig. 32: Linear regression analysis comparing Virginiamycin resistant CFU/ml and total coliform(s)/ml in the mainstem of the Ohio River during Aug 2004. (R = 0.2757)

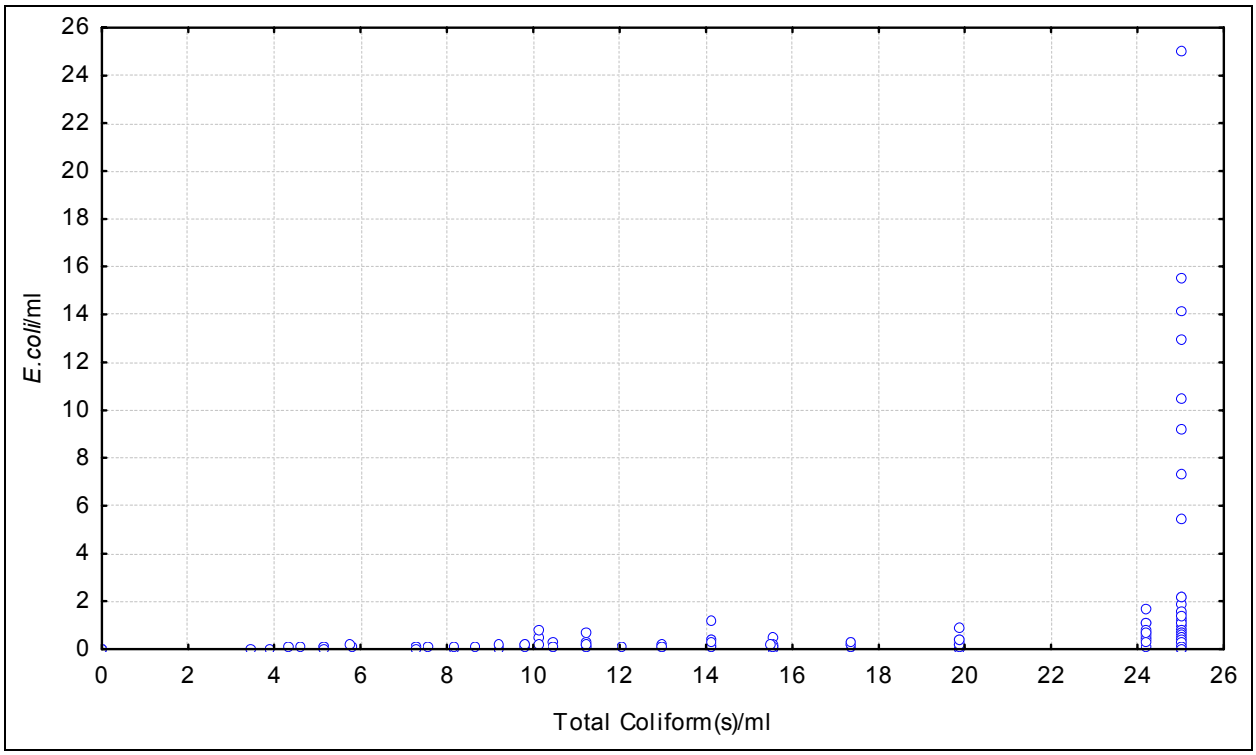

Fig. 33: Linear regression analysis comparing *E. coli*/ml and total coliform(s)/ml in the mainstem of the Ohio River during Aug 2004. (R = 0.1908)

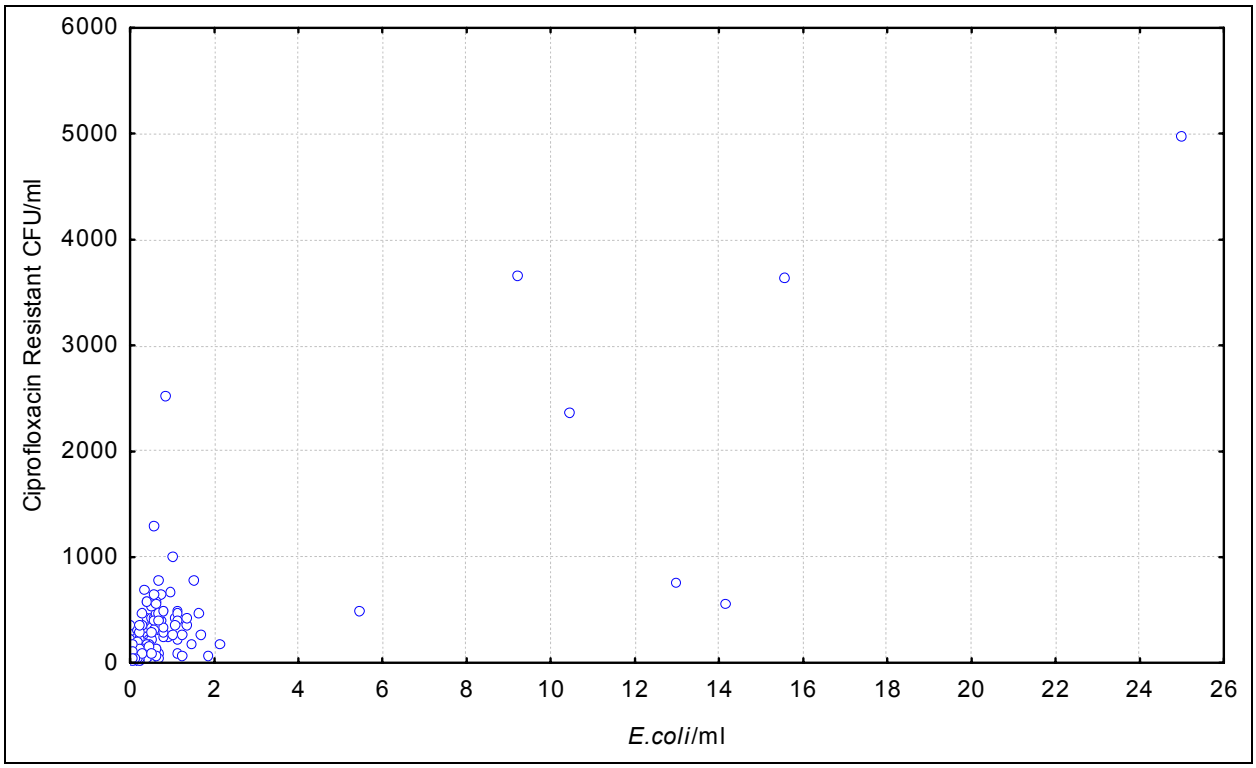

Fig. 34: Linear regression analysis comparing *E. coli*/ml and ciprofloxacin resistant CFU/ml in the mainstem of the Ohio River during Aug 2004. (R = 0.8000)

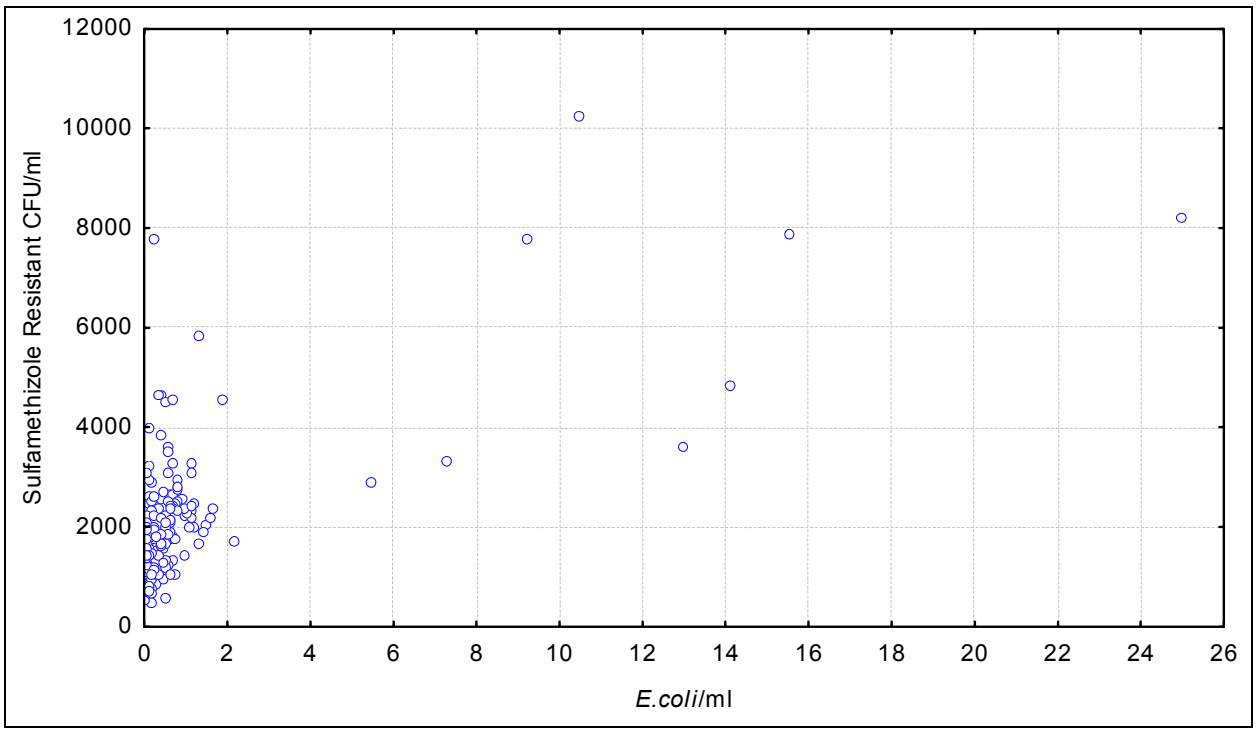

Fig. 35: Linear regression analysis comparing *E. coli*/ml and sulfamethizole resistant CFU/ml in the mainstem of the Ohio River during Aug 2004. (R = 0.6535)

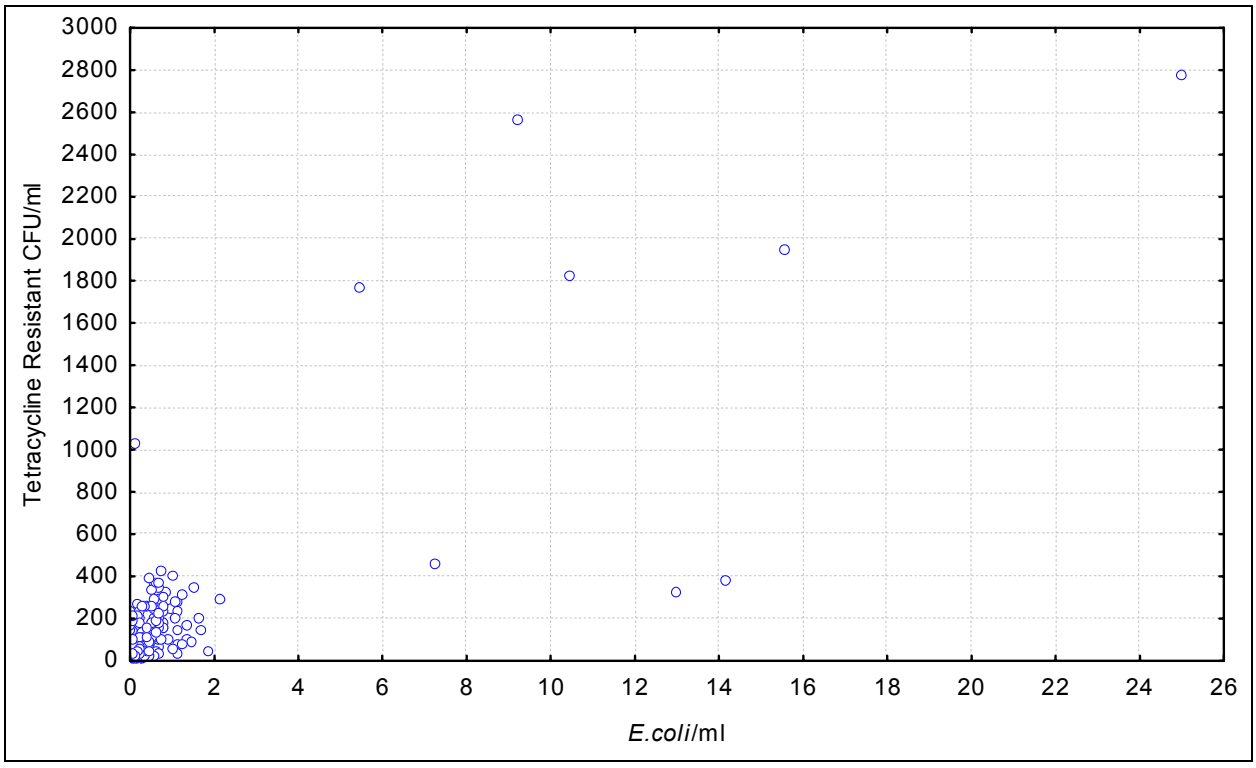

Fig. 36: Linear regression analysis comparing *E. coli*/ml and tetracycline resistant CFU/ml in the mainstem of the Ohio River during Aug 2004. (R = 0.7897)

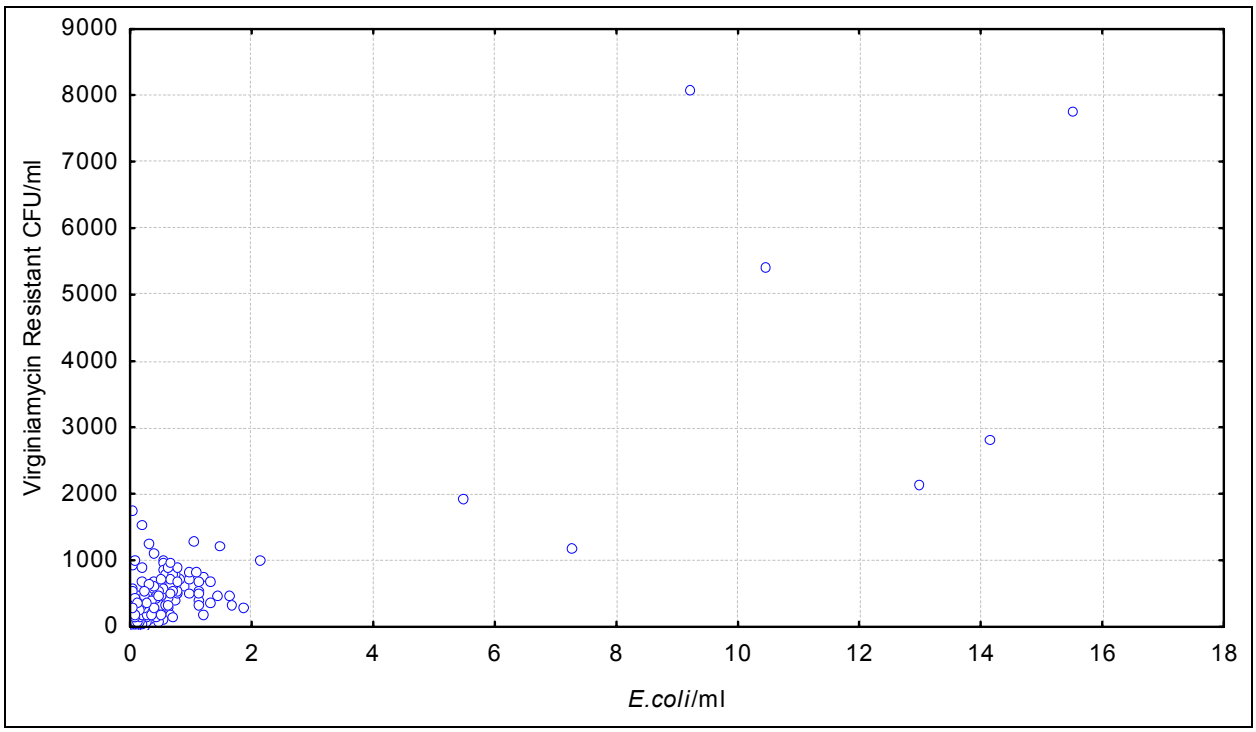

Fig. 37: Linear regression analysis comparing *E. coli*/ml and Virginiamycin resistant CFU/ml in the mainstem of the Ohio River during Aug 2004. (R = 0.8062)

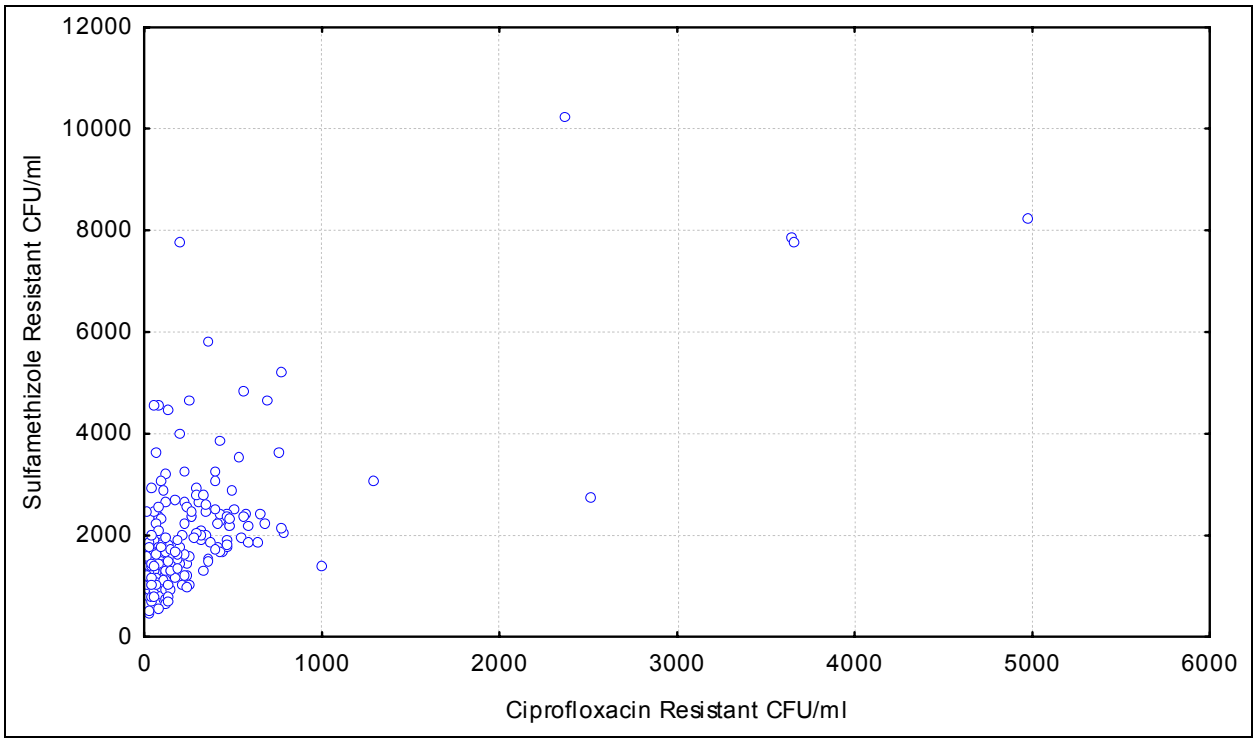

Fig. 38: Linear regression analysis comparing ciprofloxacin resistant CFU/ml and sulfamethizole resistant CFU/ml in the mainstem of the Ohio River during Aug 2004. (R = 0.6801)

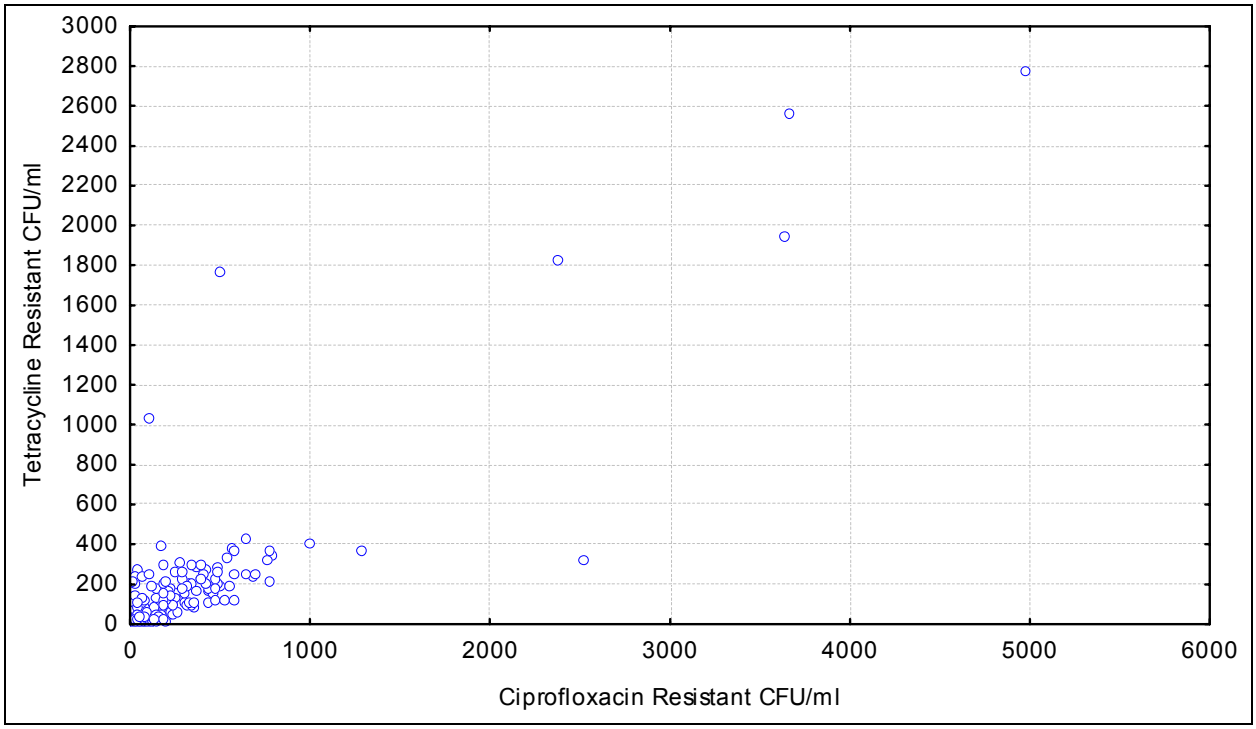

Fig. 39: Linear regression analysis comparing ciprofloxacin resistant CFU/ml and tetracycline resistant CFU/ml in the mainstem of the Ohio River during Aug 2004. (R = 0.8677)

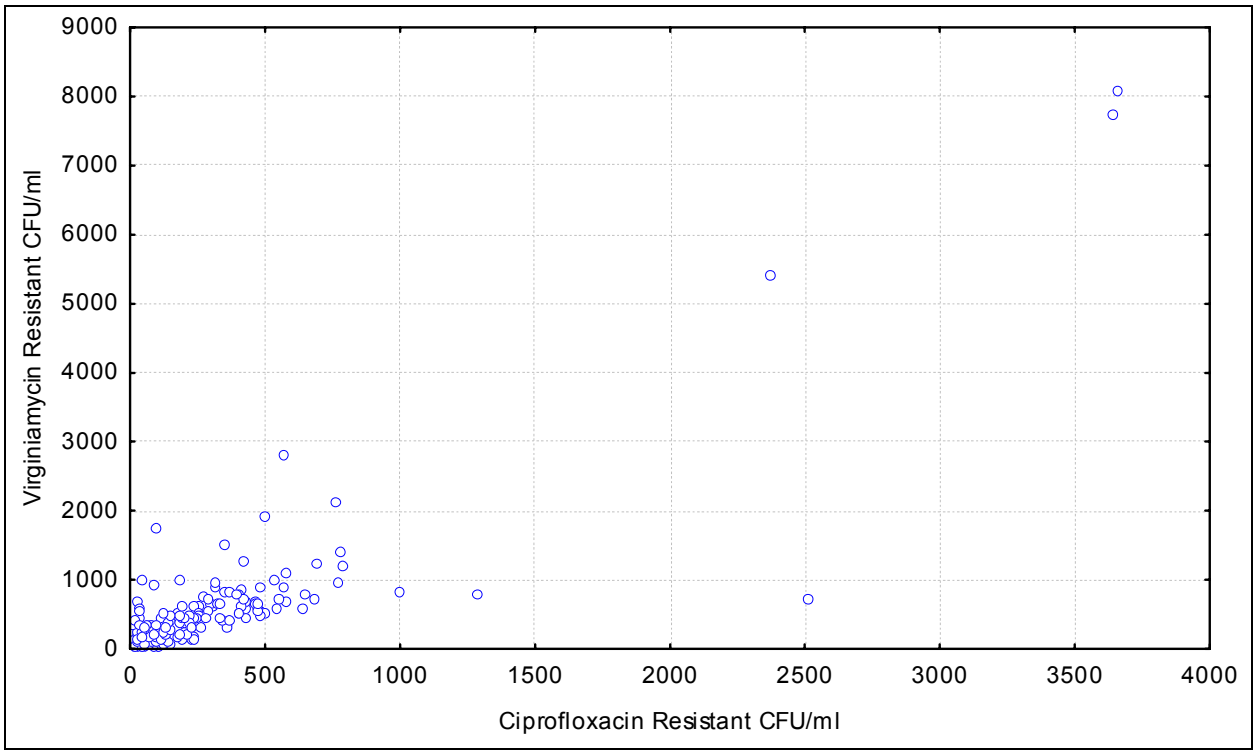

Fig. 40: Linear regression analysis comparing ciprofloxacin resistant CFU/ml and Virginiamycin resistant CFU/ml in the mainstem of the Ohio River during Aug 2004. (R = 0.8749)

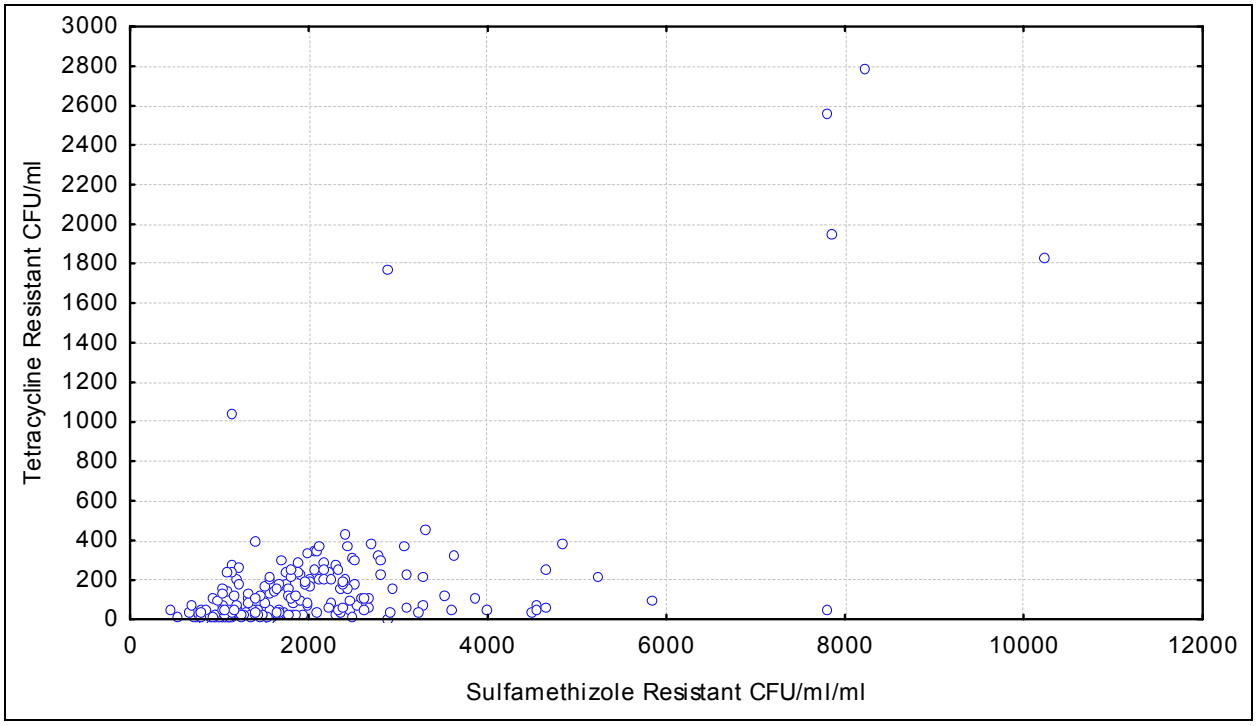

Fig. 41: Linear regression analysis comparing sulfamethizole resistant CFU/ml and tetracycline resistant CFU/ml in the mainstem of the Ohio River during Aug 2004. (R = 0.6431)

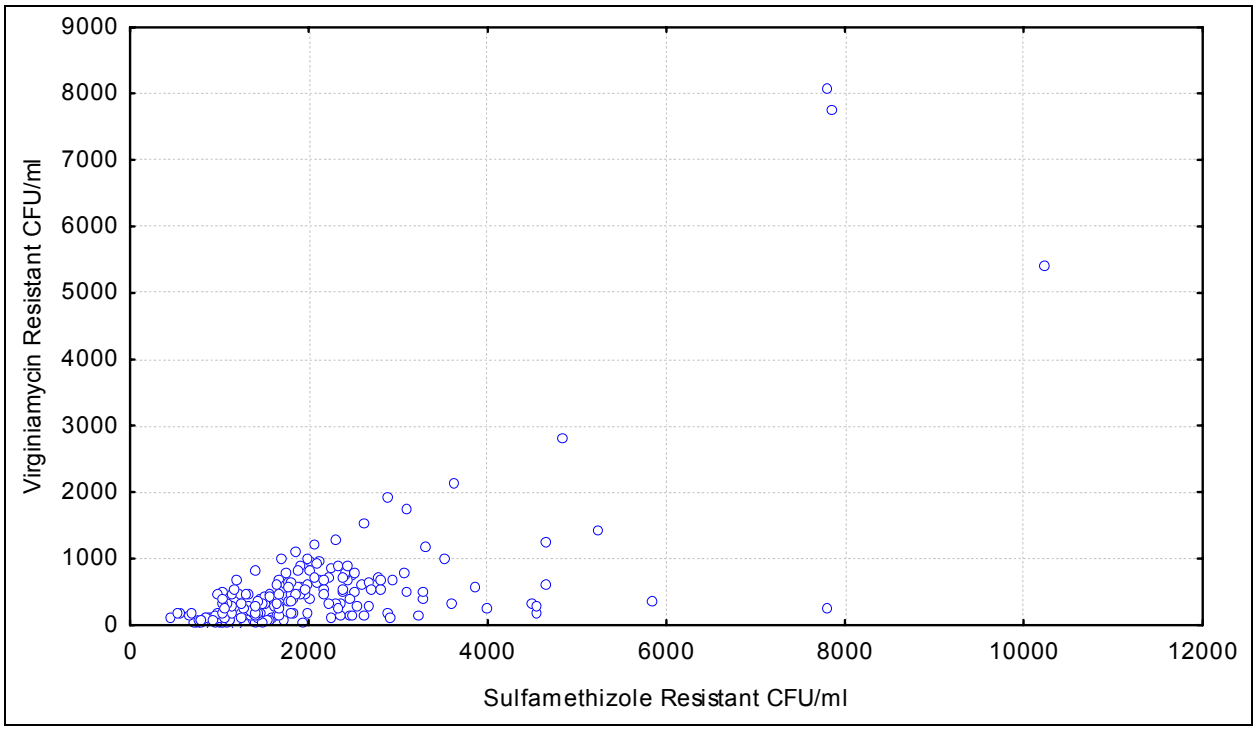

Fig. 42: Linear regression analysis comparing sulfamethizole resistant CFU/ml and Virginiamycin resistant CFU/ml in the mainstem of the Ohio River during Aug 2004. (R = 0.6946)

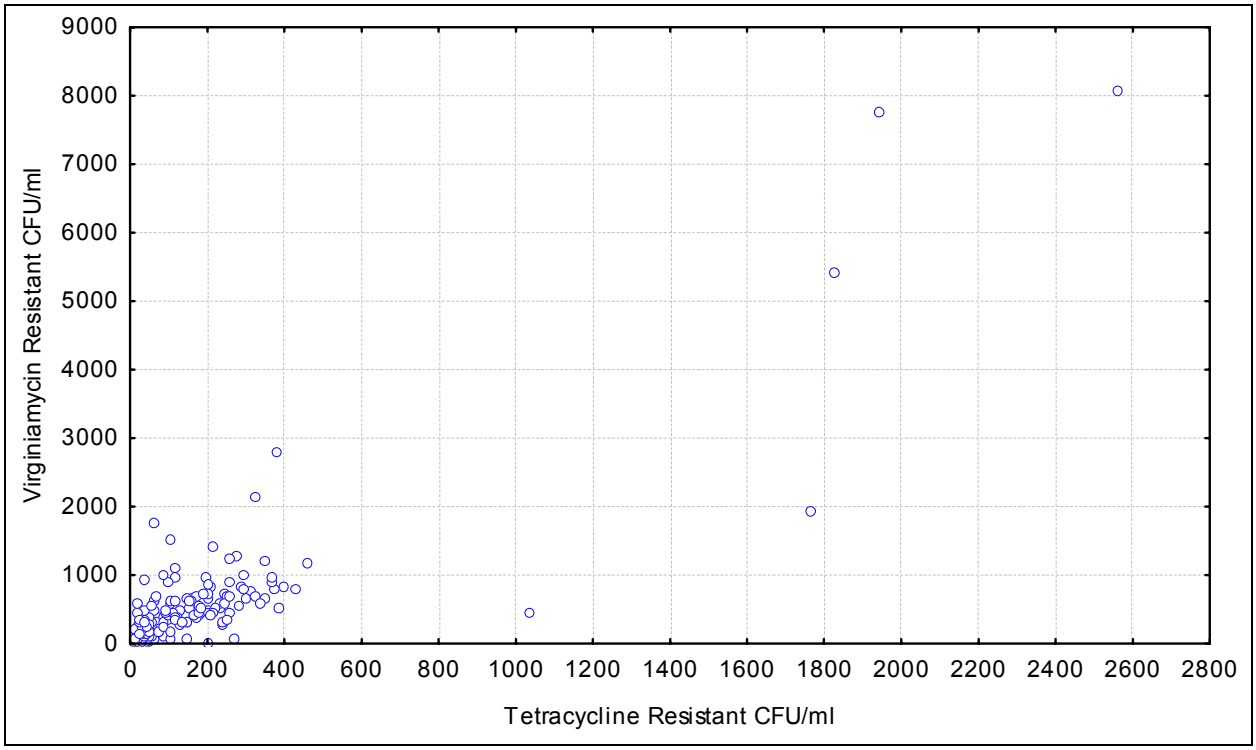

Fig. 43: Linear regression analysis comparing tetracycline resistant CFU/ml and Virginiamycin resistant CFU/ml in the mainstem of the Ohio River during Aug 2004. (R = 0.8770)

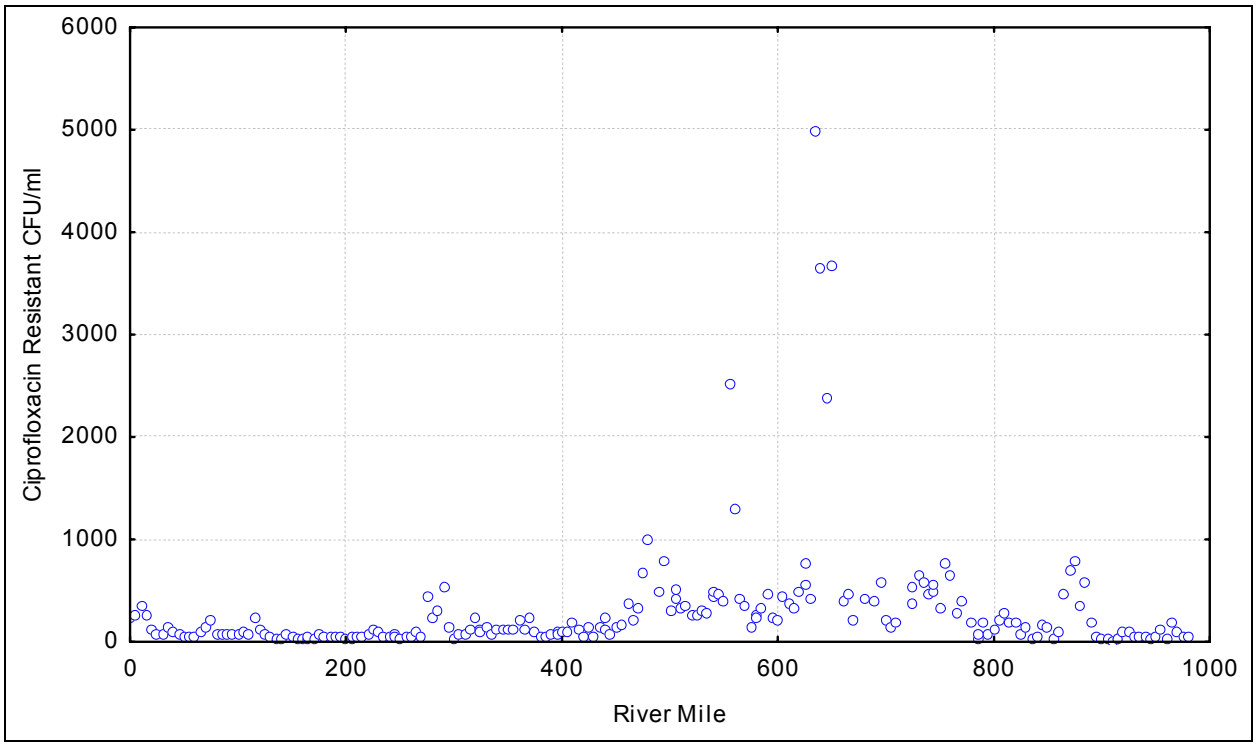

Fig. 44: Linear regression analysis comparing Ohio River miles and ciprofloxacin resistant CFU/ml in the mainstem of the Ohio River during Aug 2004. (R = 0.1687)

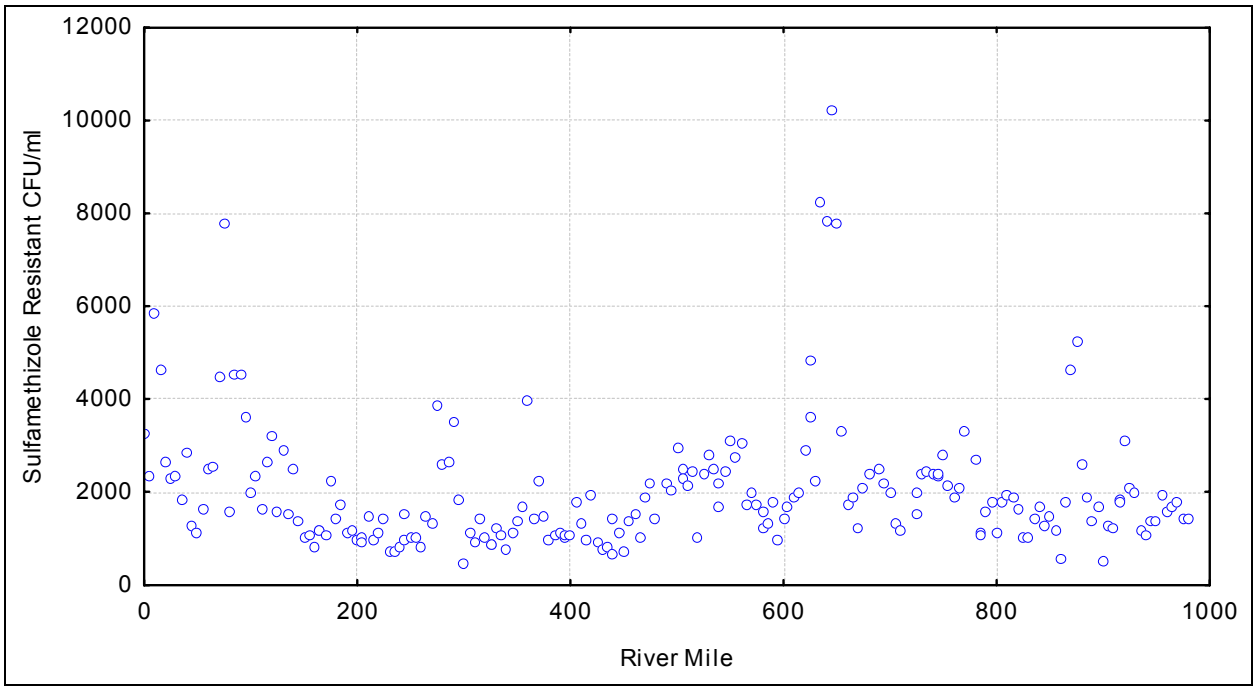

Fig. 45: Linear regression analysis comparing Ohio River miles and sulfamethizole resistant CFU/ml in the mainstem of the Ohio River during Aug 2004. (R = -0.0182)

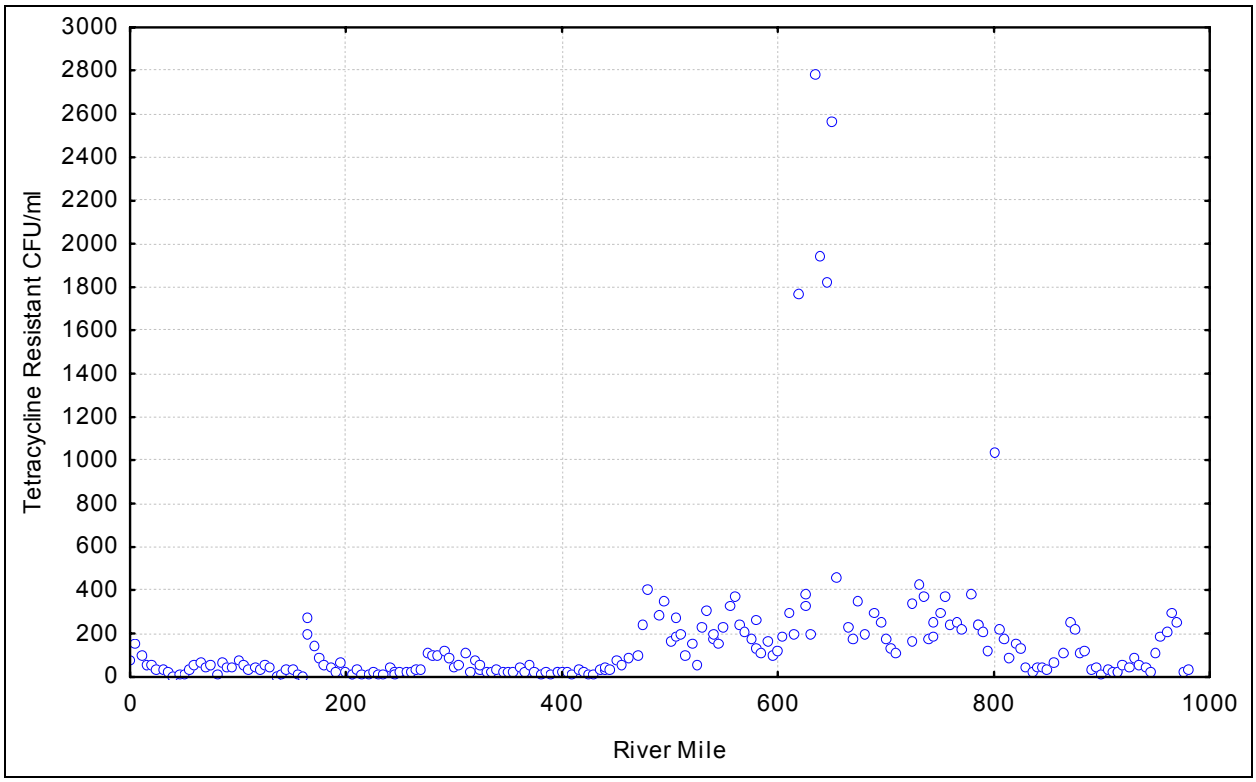

Fig. 46: Linear regression analysis comparing Ohio River miles and tetracycline resistant CFU/ml in the mainstem of the Ohio River during Aug 2004. (R= 0.2206)

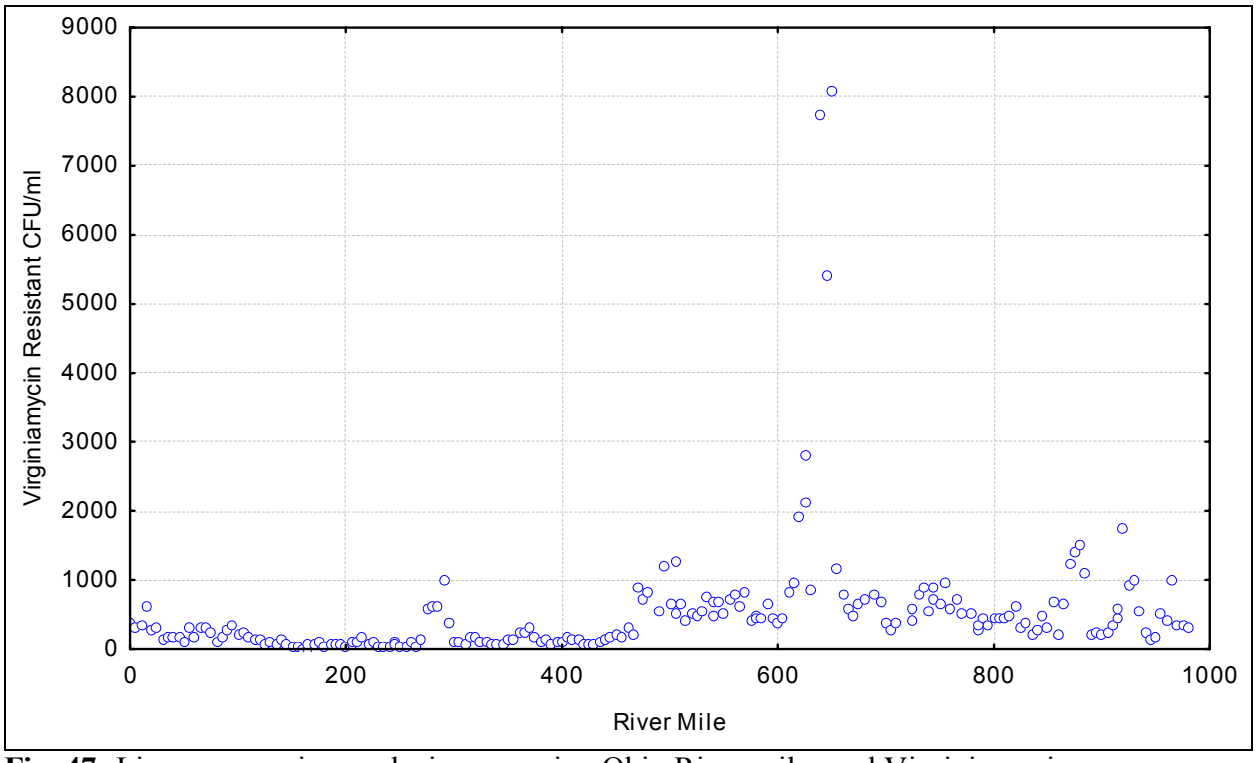

Fig. 47: Linear regression analysis comparing Ohio River miles and Virginiamycin resistant CFU/ml in the mainstem of the Ohio River during Aug 2004. (R = 0.2629)

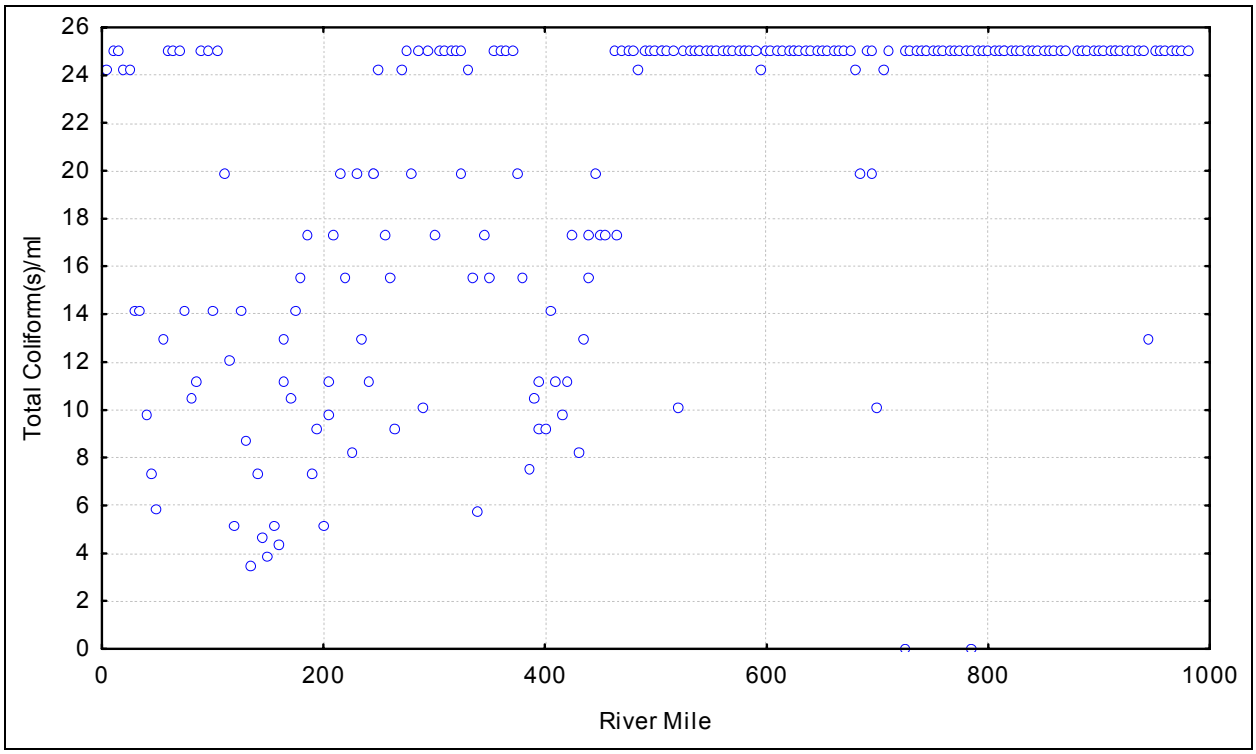

Fig. 48: Linear regression analysis comparing Ohio River miles and total coliform(s)/ml in the mainstem of the Ohio River during Aug 2004. (R = 0.5136)

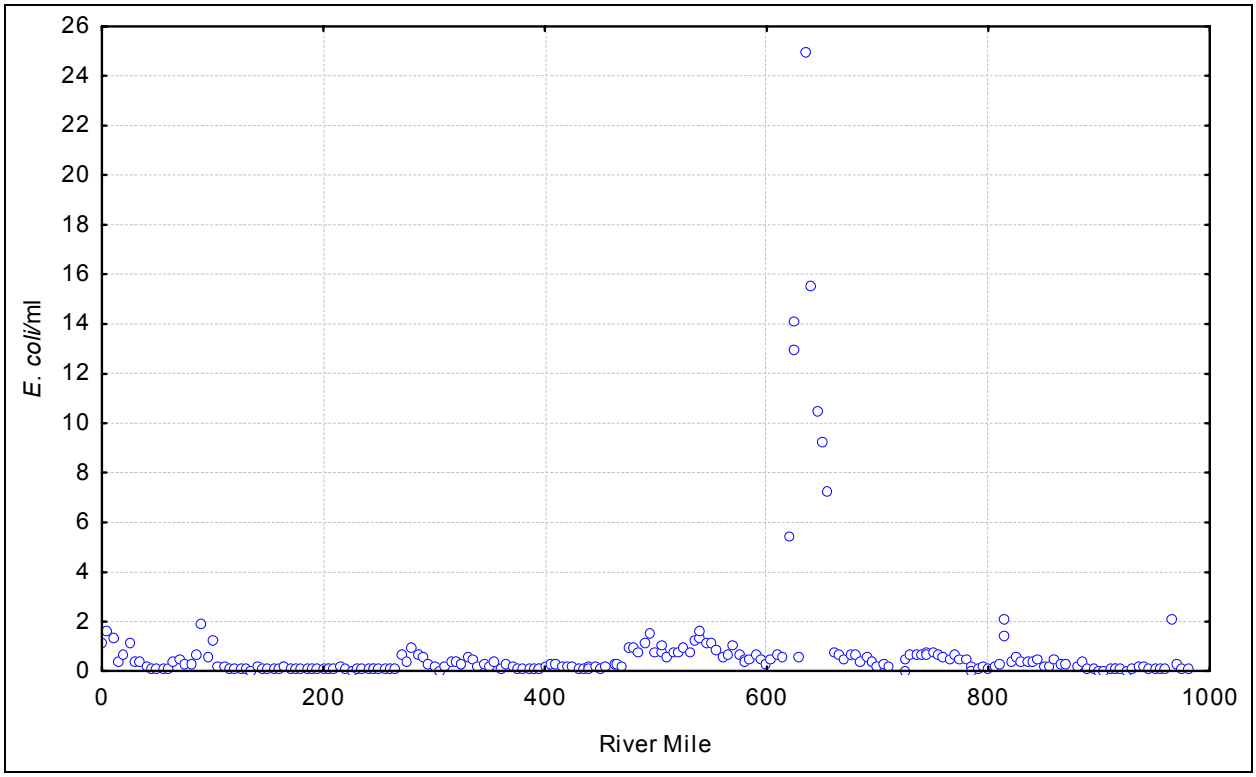

Fig. 49: Linear regression analysis comparing Ohio River miles and *E. coli*/ml in the mainstem of the Ohio River during Aug 2004. (R = 0.1006)

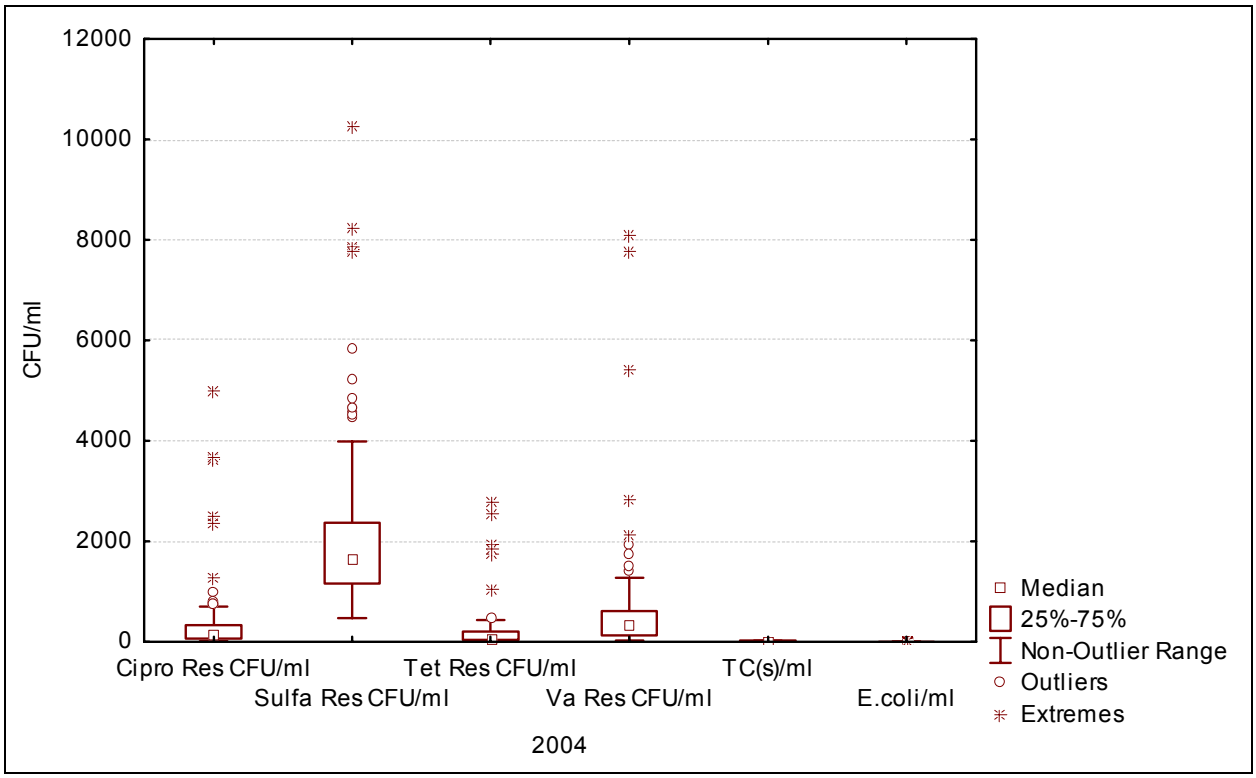

Fig. 50: Box and whisker plot comparing antibiotic resistant and coliform bacterial populations in the mainstem of the Ohio River during August 2004. Non-overlapping boxes suggest populations are probably significantly different.

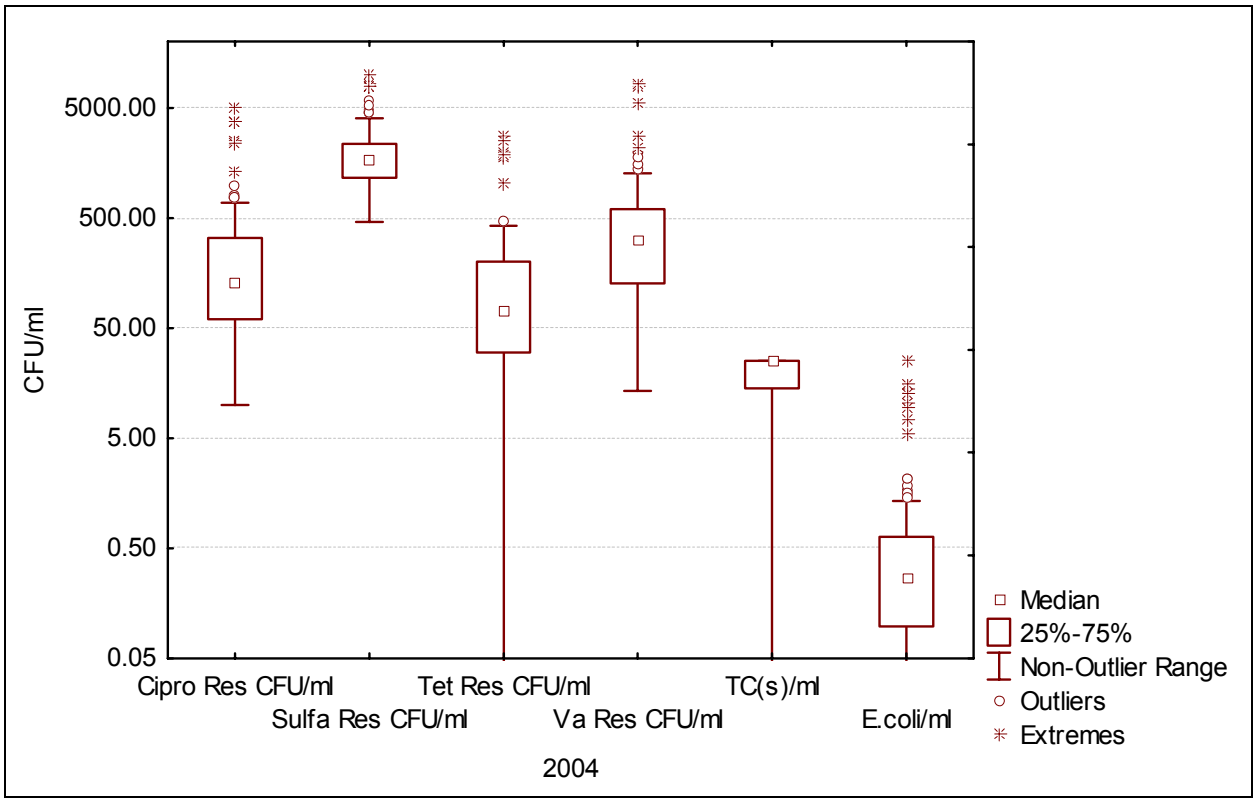

Fig 51: Box and whisker plot comparing antibiotic resistant and coliform bacterial populations in the mainstem of the Ohio River during August 2004 with extremes greater than 6000 CFU/ml removed. Non-overlapping boxes suggest populations are probably significantly different.

Table 5: Mann Whitney Rank Sum Test comparing ratios of fecal coliform bacteria / antibiotic resistant bacteria in tributaries to matching ratios from all of the mainstem sampling sites during 2004. Italicized *P*-values are significant at <0.05

	TC/Cipro								
TC/Cipro	0.4011	**TC/Sulfa**							
TC/Sulfa		0.0367	**TC/Tet**						
TC/Tet			0.2078	**TC/Va**					
TC/Va				0.0526	**TC/*E. coli***				
TC/*E. coli*					0.7998	***E. coli*/Cipro**			
***E. coli*/Cipro**						0.4404	***E. coli*/Sulfa**		
***E. coli*/Sulfa**							0.6239	***E. coli*/Tet**	
***E. coli*/Tet**								0.66382	***E. coli*/Va**
***E. coli*/Va**									0.9219

Table 6: Spearman Correlation Test comparing each average CFU / ml to all other bacterial measurements at the same site in the mainstem samples of the Ohio River during 2004. Correlation coefficients that are italicized show that the two populations are different at a significance level of *P*<0.05

	Totals						
Totals	1.0	**Cipro**					
Cipro	*0.5788*	1.0	**Sulfa**				
Sulfa	*0.5743*	*0.5671*	1.0	**Tet**			
Tet	*0.3842*	*0.7031*	*0.5260*	1.0	**VA**		
VA	*0.4235*	*0.7356*	*0.6416*	*0.7668*	1.0	***TC***	
TC	*0.1595*	*0.4553*	*0.4068*	*0.5671*	*0.6936*	1.0	***E. coli***
E. coli	*0.5234*	*0.7003*	*0.5553*	*0.614*	*0.6169*	*0.5221*	1.0

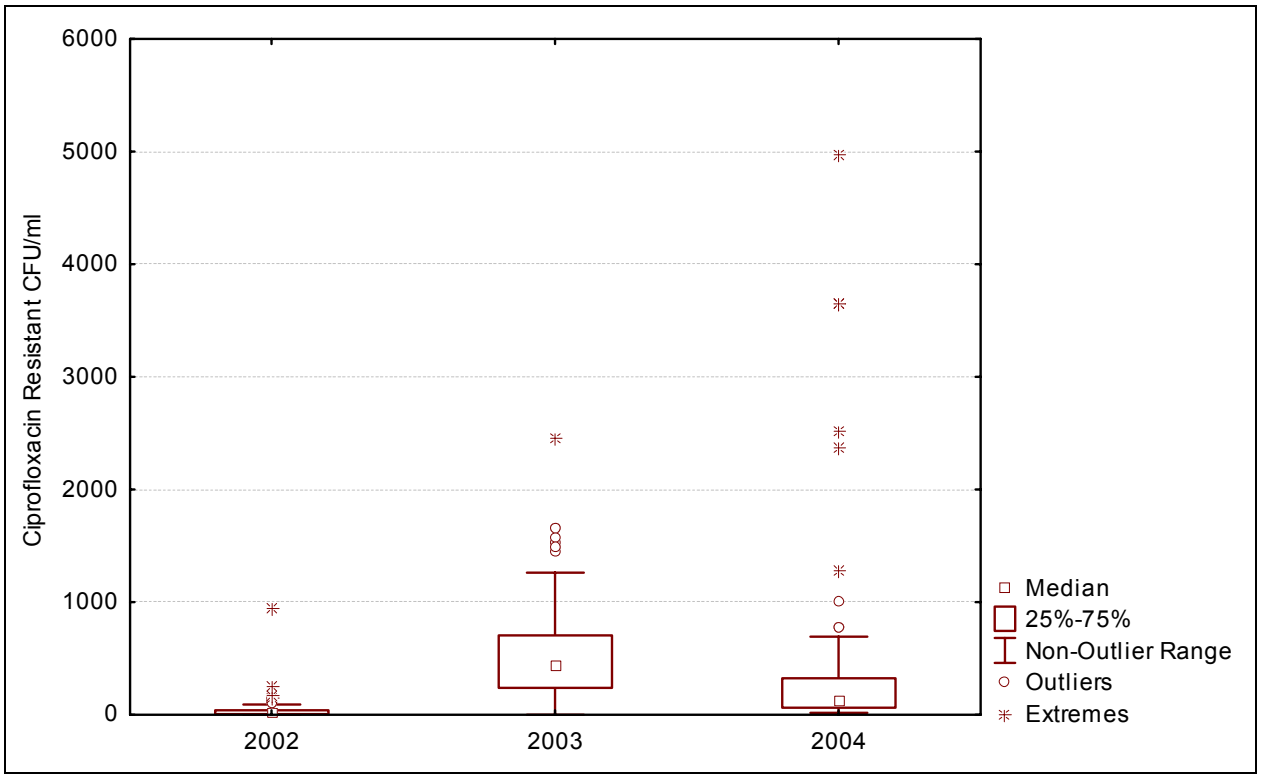

Fig. 52: Box and whisker plot comparing ciprofloxacin resistant CFU/ml from 2002, 2003, and 2004.

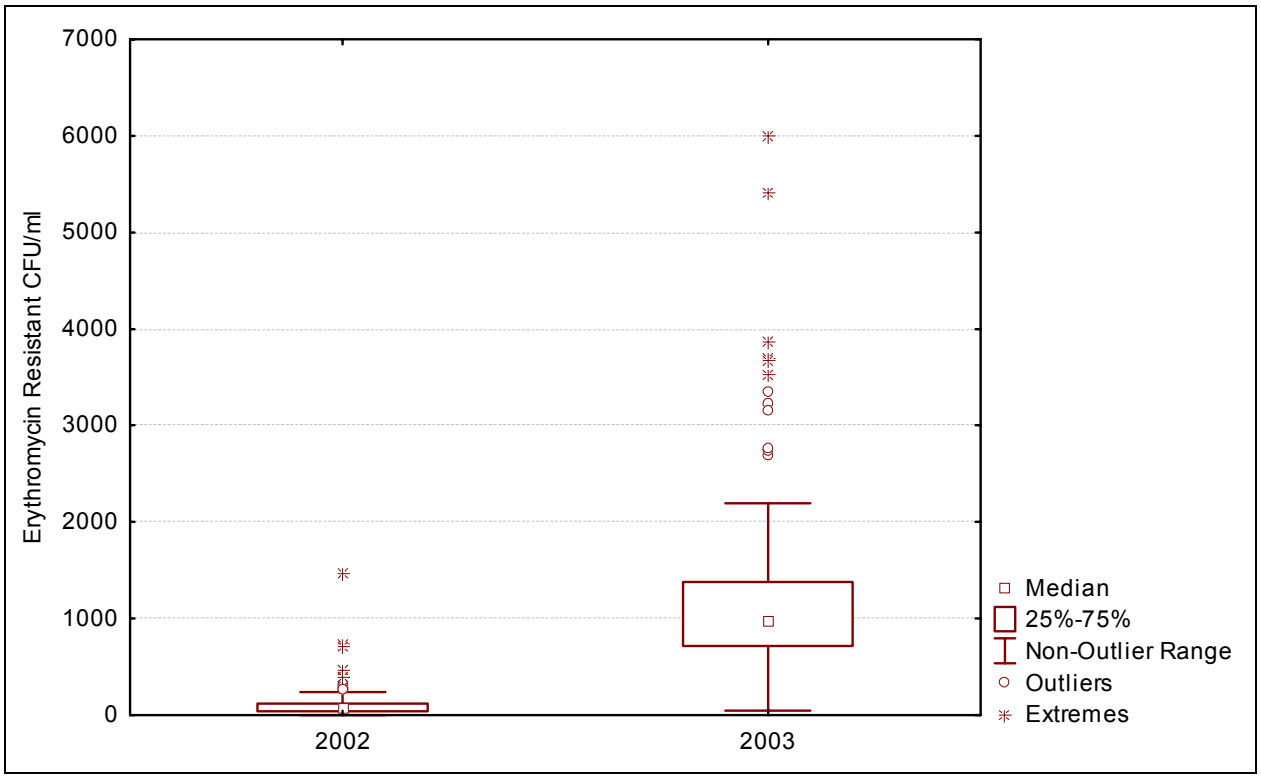

Fig. 53: Box and whisker plot comparing erythromycin resistant CFU/ml from 2002 and 2003.

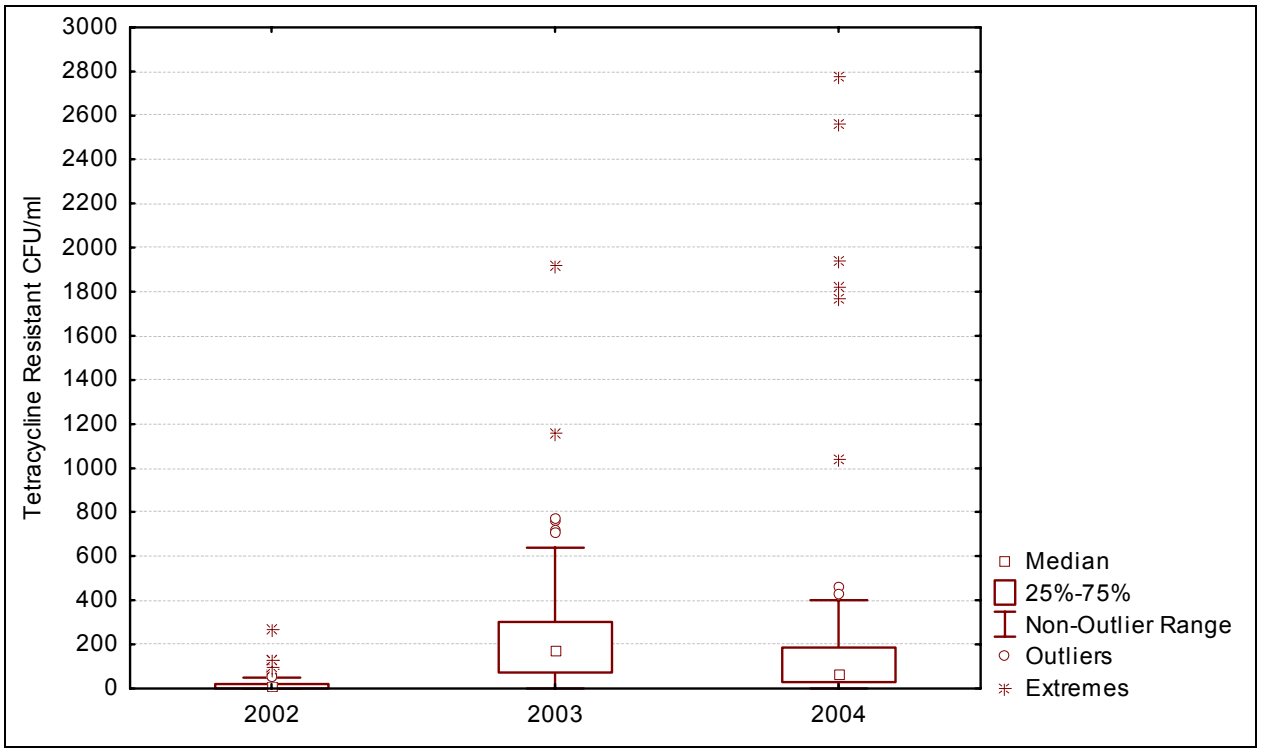

Figure 54: Box and whisker plot comparing tetracycline resistant CFU/ml during 2002, 2003, and 2004.

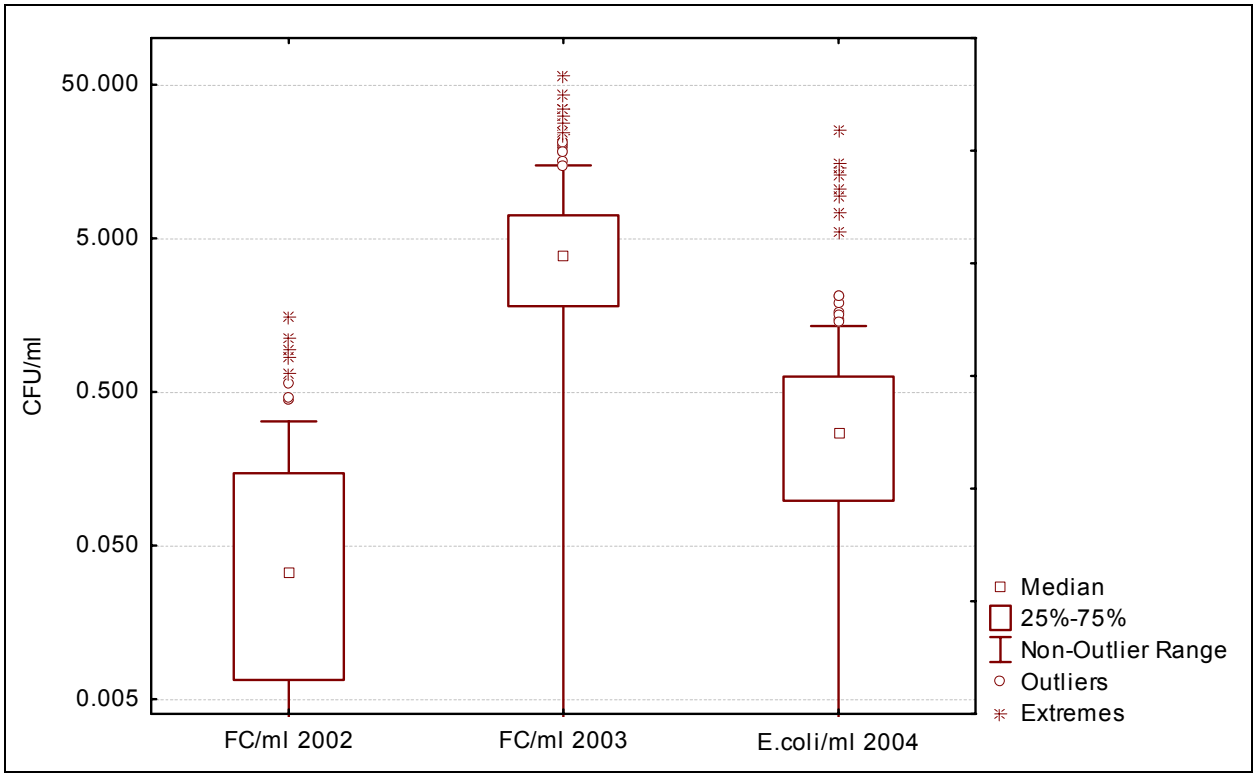

Figure 55: Box and whisker plot (logarithmic scale) comparing coliform counts/ml during 2002, 2003, and 2004.

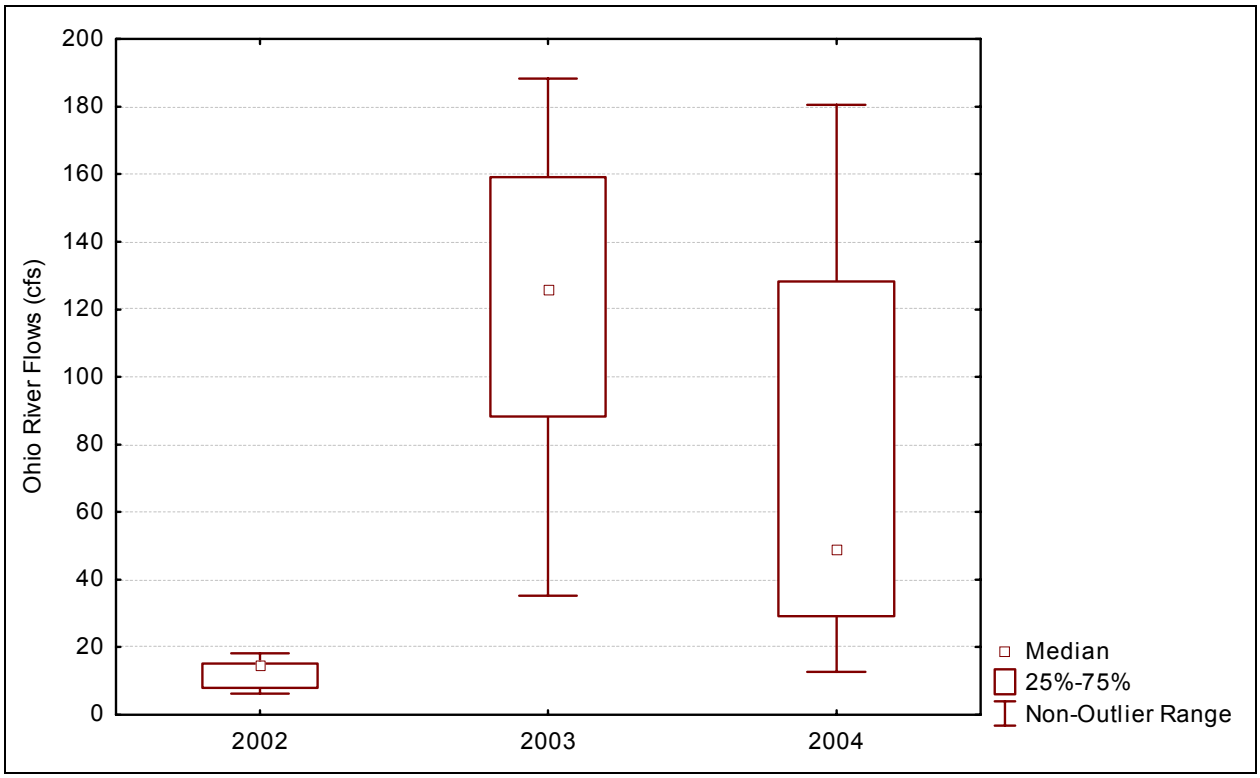

Figure 56: Box and whisker plot comparing the average flow (cfs) along the entire Ohio River during the sampling period for 2002, 2003, and 2004.

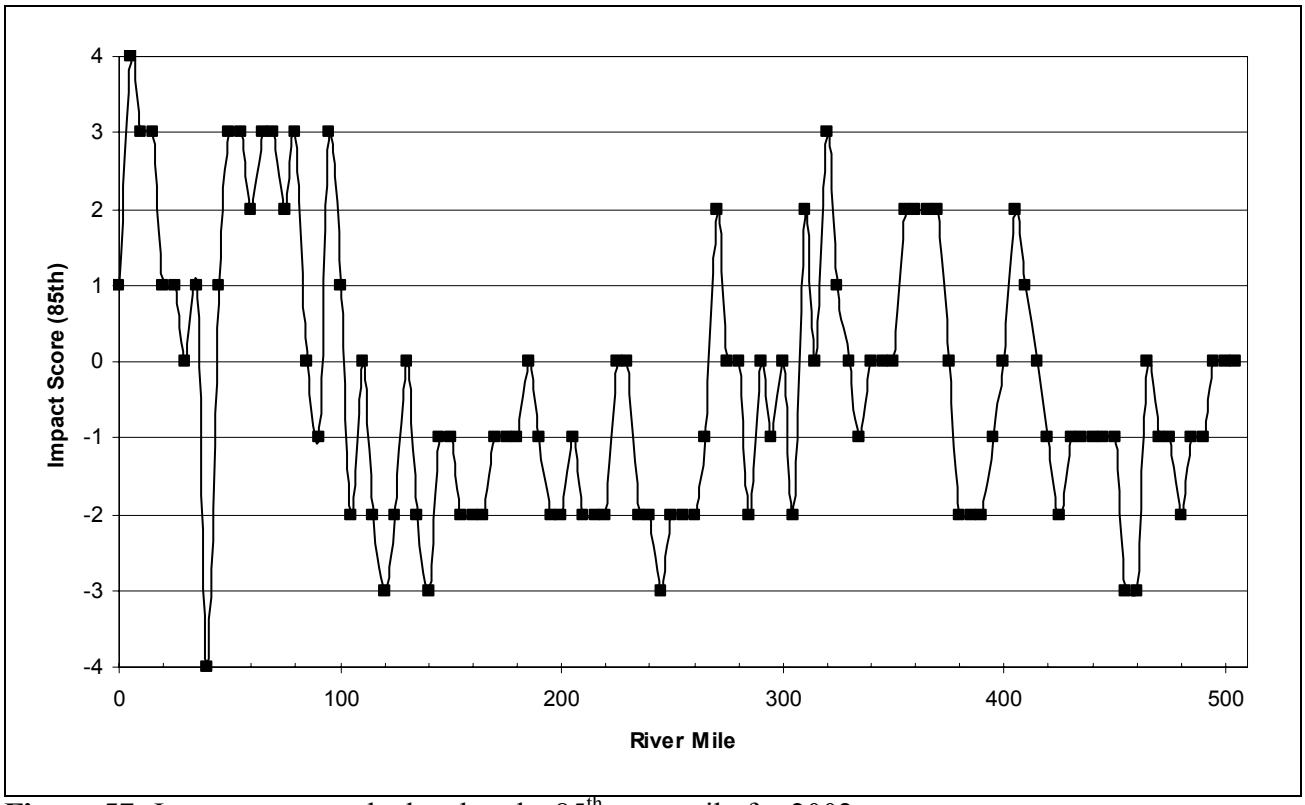

Figure 57: Impact scores calculated at the 85th percentile for 2002

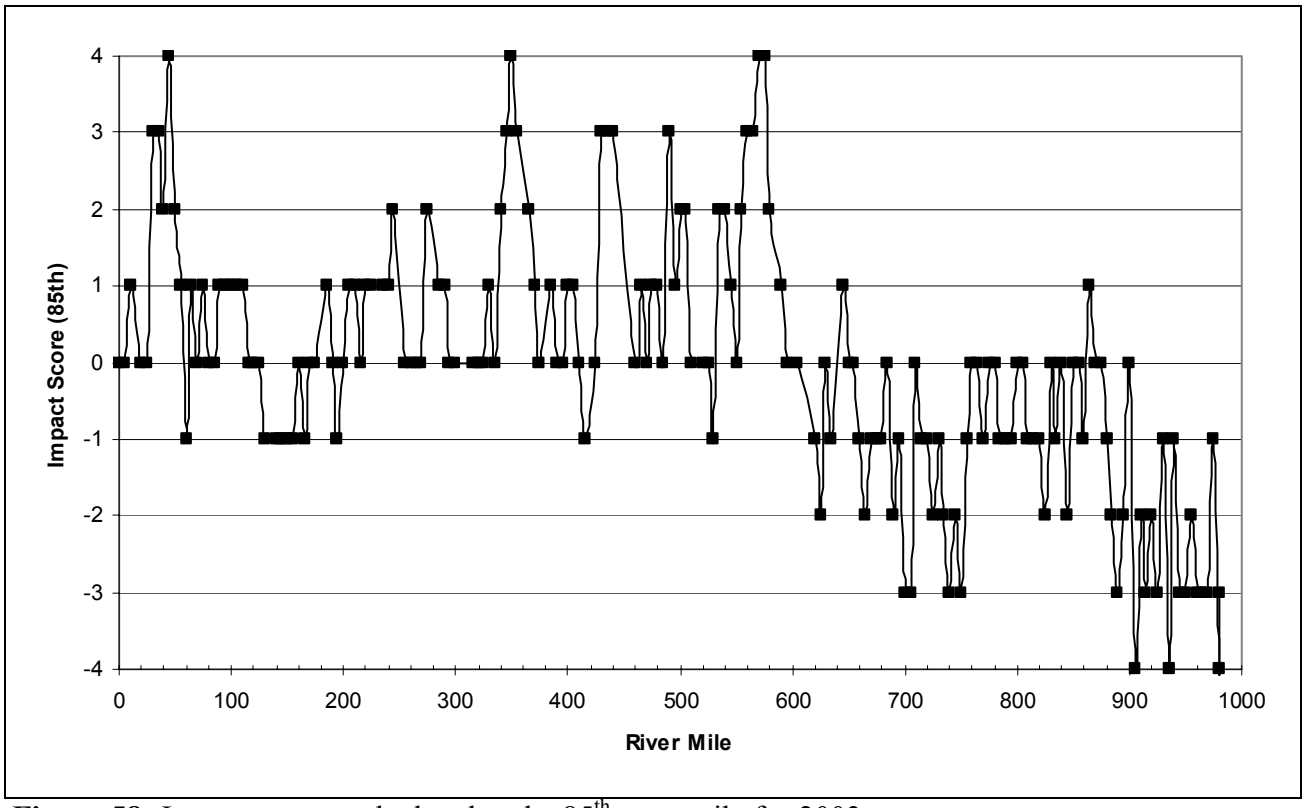

Figure 58: Impact scores calculated at the 85th percentile for 2003

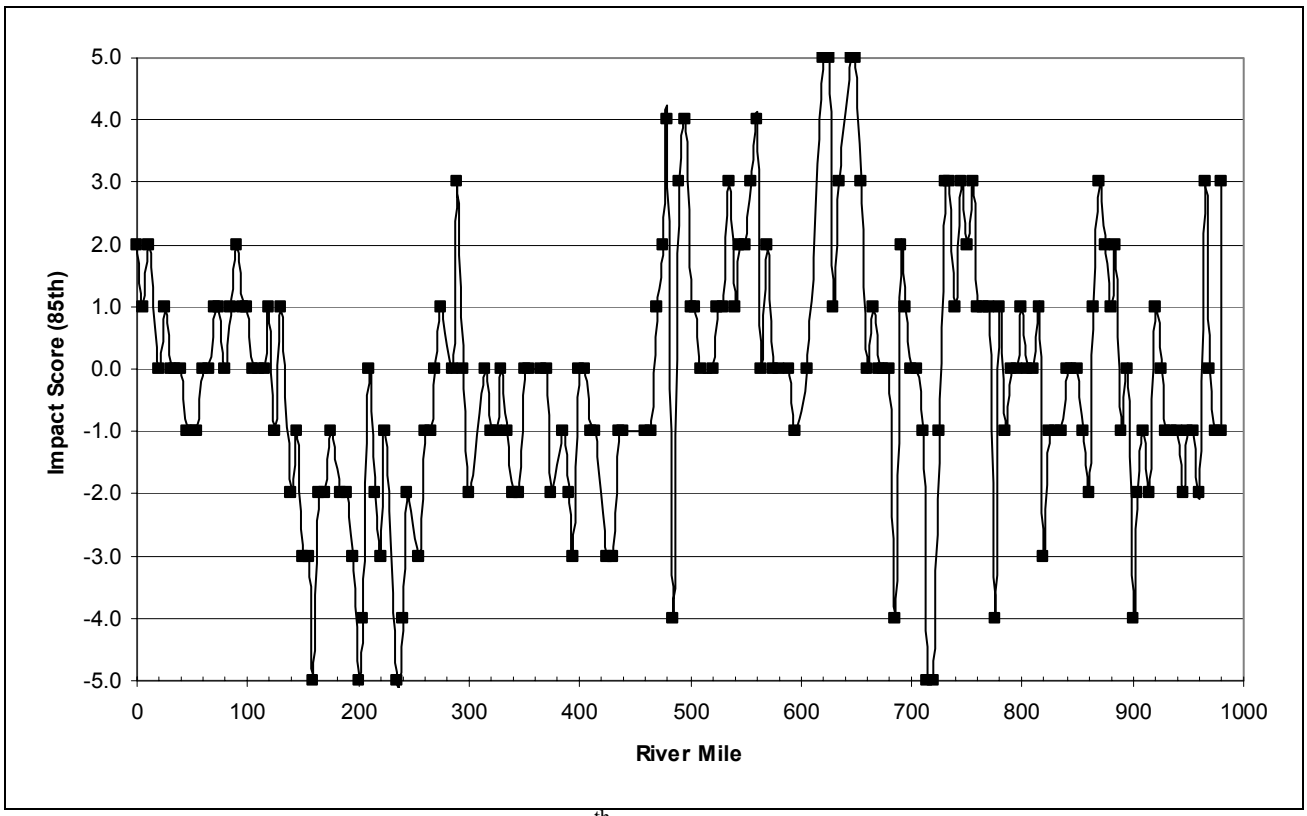

Figure 59: Impact scores calculated at the 85th percentile for 2004

Appendices

Appendix A

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 □g/ml	375 ng/ml
Ampicillin Sodium Salt	Fisher BP1760-25	H_2O	50 mg/ml	50 □g/ml
Ciprofloxacin	Cellgro 61-277-RF	DMSO	4 mg/ml	4 □g/ml
Erythromycin	Fisher BP920-25	EtOH:H_2O	8 mg/ml	8 □g/ml
Streptomycin Sulfate	Fisher BP910-50	Water	25 mg/ml	25 □g/ml
Sulfamethizole	Fisher ICN15671125	DMSO	128 mg/ml	128 □g/ml
Tetracycline Hydrochloride	Fisher BP912-100	EtOH:H_2O	12.5 mg/ml	12.5 □g/ml
Virginiamycin	Fisher 50-213-730	DMSO	16 mg/ml	16 □g/ml

[a] Fungizone is purchased as a stock solution, it is stored frozen and thawed before use. DMSO = dimethylsulfoxide (Certified ACS). EtOH:H_2O = a mixture of equal parts ethanol (100% USP) and reagent grade water (18 MΩ).

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

Note – for determining amount of antibiotic powder to use

a. Be sure to account for the purity of the antibiotic powder by dividing the weight of pure antibiotic required by the purity. For example, ciprofloxacin may be provided as a powder that contains 803 mg ciprofloxacin per gram. To achieve a stock concentration of 4 mg ciprofloxacin per ml, it is necessary to add 4.98 [or 4.0 mg cipro x (1000 mg powder / 803 mg cipro)] mg powder per ml of stock solution.

3. Add 10 ml of the appropriate solvent (see Table 1) to the tube, and vortex to mix.

4. In some cases (e.g. when making stock solutions of ciprofloxacin) the tube can be placed in a bath sonicator to facilitate dissolution of the solute. Take care to be certain that all of the antibiotic has gone into solution.

5. Draw the antibiotic solution into a sterile 10 ml syringe, and sterilize by forcing the solution through a sterile, 0.2 □m syringe filter (Fisher Scientific cat. no. 09-719C or equivalent) into a second sterile plastic centrifuge tube. ***Do not*** *filter sterilize antibiotics dissolved in DMSO.*

6. Store the antibiotic stocks at -20°C until used. Replace antibiotic stocks each month.

Media Preparation

1. Suspend 9.1 grams Difco R2A agar (Becton Dickinson, Sparks, MD; cat no. 218263) in 500 ml of purified water in a 1,000 ml capacity glass Erlenmeyer flask.

2. Add a magnetic stir bar, cover the flask with aluminum foil, place and piece of autoclave tape on the foil, and mark the name of the antibiotic to be added (if appropriate) on the foil.

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

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

5. While the medium is cooling, remove the appropriate antibiotic stock solutions from the freezer and thaw on ice (all antibiotics except ciprofloxacin) or at room temperature (ciprofloxacin).

6. Place the flask on a magnetic stir plate and stir gently until the medium is well mixed. Be careful not to introduce bubbles. Test the temperature of the medium by touching the side of the flask briefly with your bare hand. It should be warm, but not hot. If the flask is hot to the touch, return it to the water bath until it has cooled enough to be handled comfortably. Do not allow the medium to cool below 48°C.

7. Wear disposable latex gloves for the remaining steps of media preparation. When properly tempered, again move the medium to the magnetic stirrer. While stirring gently, ***aseptically*** add 750 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 □l of the appropriate antibiotic stock to the flask. Stir gently for an additional 15 seconds and tilt the flask to insure that all the antibiotic stock is transferred to the medium.

11. Pour the plates as described in step 9.

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

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

Sample Collection

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

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

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

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

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

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

Enumeration of Total Cultivable Bacteria

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

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

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

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

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

   Note – for use of sterile glass beads

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

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

   c. When plating – open the pipette tip box, roll back the aluminum foil to expose a single row of pipette tips, remove one tip at a time, lift the lid of an inoculated plate, and pour the sterile beads onto the agar surface. Normally, one bead remains stuck in the bottom of the tip.

   d. Repeat step c for all replicate plates.

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

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

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

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

Note – for incubation of R2A plates

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

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

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

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

Enumeration of Antibiotic Resistant Bacteria

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

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

Note – for selection of plating volume

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

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

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

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

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

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

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

Appendix B

Latitude and longitude of sites on the mainstem of the Ohio River obtained by GPS mark.

River Mile	Latitude	Longitude	River Mile	Latitude	Longitude	River Mile	Latitude	Longitude
0	40.2673	80.01504	230	38.53983	81.50919	460	39.02507	84.22034
5	40.29733	80.04264	235	38.53182	81.55261	465	39.05869	84.26112
10	40.31384	80.09582	240	38.56628	81.54379	470	39.06139	84.29678
15	40.34099	80.13554	245	38.59576	81.57855	475	39.05175	84.33916
20	40.38086	80.1404	250	39.01407	82.02216	480	39.05493	84.3925
25	40.41356	80.15722	255	38.5762	82.05755	485	39.08274	84.43425
30	40.3983	80.21242	260	38.54399	82.07758	490	39.06702	84.47683
35	40.37543	80.26276	265	38.50995	82.08404	495	39.04216	84.52499
40	40.38182	80.31337	270	38.47905	82.12711	500	39.01161	84.51665
45	40.37457	80.36127	275	38.4422	82.11569	505	38.57455	84.50108
50	40.34915	80.4006	280	38.40138	82.11127	510	38.54024	84.51926
55	40.30865	80.37283	285	38.35914	82.10852	515	38.52732	84.46974
60	40.2711	80.36244	290	38.35602	82.16194	520	38.49401	84.49646
65	40.23462	80.37973	295	38.31967	82.17784	525	38.47329	84.51617
70	40.19822	80.36224	300	38.27381	82.19052	530	38.46422	84.5632
75	40.15713	80.37.299	305	38.26001	82.22947	535	38.4568	85.01231
80	40.12267	80.39928	310	38.2536	82.2791	540	38.43514	85.05891
85	40.08231	80.42375	315	38.24136	82.33585	545	38.41258	85.10481
90	40.0489	80.43763	321	38.28069	82.36578	550	38.43448	85.14559
95	40.00299	80.44341	325	38.31318	82.40853	555	38.4408	85.19715
100	39.566642	80.45726	330	38.33452	82.44041	560	38.43947	85.25164
105	39.5081	80.47355	335	38.34768	82.49825	565	38.4043	85.26708
110	39.51187	80.48638	340	38.37803	82.51464	570	38.36087	85.26003
115	39.4765	80.49817	345	38.41917	82.52683	575	38.32194	85.24971
120	39.44397	80.50944	350	38.45125	82.54502	580	38.29277	85.28697
125	39.41261	80.51.812	355	38.43551	82.59	585	38.27101	85.33201
131	39.37035	80.54328	360	38.41577	83.03273	590	38.24912	85.37154
135	39.915	80.55801	365	38.3894	83.07356	595	38.2027	85.3868
140	39.32627	81.01903	370	38.37008	83.10991	600	38.17197	85.41818
147	39.28211	81.06504	375	38.37033	83.15983	604	38.15587	85.44995
150	39.26553	81.09218	380	38.37736	83.37736	610	38.15532	85.50149
155	39.2356	81.1263	385	38.39798	83.23787	615	38.12343	85.52488
160	39.22296	81.17666	390	38.41408	83.29253	620	38.07738	85.54362
165	39.20461	81.22525	395	38.415	83.34084	625	38.04377	85.54662
170	39.23753	81.24888	400	38.39738	83.38171	630	38.00309	85.56894
175	39.22623	81.29616	405	38.3829	83.42374	635	37.59081	86.02021
180	39.19844	81.33495	410	38.40019	83.46606	640	37.59021	86.04306
185	39.16026	81.34792	415	38.4286	83.49891	645	38.00702	86.08995
190	39.16473	81.39666	421	38.46134	83.54935	650	38.01882	86.13516
195	39.13285	81.41747	425	38.47152	83.58281	655	38.05138	86.16863
200	39.10684	81.45317	430	38.46184	84.03619	660	38.0958	86.17661
205	39.06162	81.44592	435	38.47584	84.08988	665	38.11813	86.21672
210	39.04647	81.48765	440	38.48172	84.14336	670	38.09359	86.19587
215	39.01196	81.45889	445	38.53313	84.14129	675	38.07607	86.22543
220	38.57153	81.46439	450	38.571	84.17471	680	38.0748	86.26194
225	38.56334	81.48241	455	39.01039	84.19049	685	38.04914	86.25786

River Mile	Latitude	Longitude	River Mile	Latitude	Longitude
690	38.02614	86.29455	920	37.08804	88.2538
695	37.54046	86.31489	925	37.05092	88.26856
700	37.54008	86.32935	930	37.04132	88.3304
705	37.54622	86.35808	935	37.05497	88.35471
710	37.50747	86.37386	940	37.07776	88.40243
715	37.5292	86.39214	945	37.09211	88.45472
720	37.54509	86.41449	950	37.11353	88.49557
725	37.55165	86.45481	955	37.13318	88.55277
730	37.58953	86.47734	960	37.13219	88.00319
735	37.58123	86.5247	965	37.10596	89.04141
740	37.55855	86.56309	970	37.06867	89.06877
745	37.54385	87.0179	975	37.03742	89.10586
750	37.50759	87.02762	980	36.59945	89.09453
755	37.47055	87.05005			
760	37.49074	87.08755			
765	37.50495	87.12595			
770	37.52956	87.16709			
775	37.55321	87.21286			
780	37.55771	87.27951			
785	37.54223	87.3096			
790	37.5599	87.35149			
795	37.57235	87.36145			
800	37.53971	87.36217			
805	37.50159	87.36236			
810	37.4994	87.40773			
815	37.54026	87.41099			
820	37.52077	87.45434			
825	37.53131	87.50902			
830	37.55274	87.54346			
835	37.52024	87.56033			
840	37.48153	87.54876			
845	37.46615	87.58013			
850	37.46439	88.02379			
855	37.42932	88.05637			
860	37.40266	88.09222			
865	37.35411	88.07857			
870	37.32724	88.0524			
875	37.29106	88.04041			
880	37.27997	88.08997			
885	37.27336	88.1392			
890	37.2594	88.19397			
895	37.24975	88.23334			
900	37.24041	88.27837			
905	37.19872	88.29391			
910	37.15848	88.30417			
915	37.12981	88.27157			

Appendix C

Tributaries sampled in 2002, 2003, and 2004. Latitude and longitude obtained at site with GPS.

Tributary Name	Ohio River Mile	Bank	Latitude	Longitude	State	2002	2003	2004
Allegheny River	0.0	N/A	40.26617	80.00603	PA			X
Monongahela River	0.0	N/A	40.25994	79.58395	PA			X
Beaver River	25.4	RDB	40.41982	80.17429	PA	X	X	X
Little Beaver Creek	39.5	RDB	40.38715	80.30738	PA	X	X	X
Yellow Creek	50.5	RDB	40.34334	80.40103	OH	X	X	X
Buffalo Creek	74.7	LDB	40.15779	80.36866	WV	X	X	X
Fish Creek	113.9	LDB	39.48539	80.4898	WV	X	X	X
Sunfish Creek	118.0	RDB	39.45813	80.52223	OH	X	X	X
Middle Island Creek	154.0	LDB	39.23614	81.1243	WV	X	X	
Middle Island Creek	154.0	LDB	39.23633	81.12429	WV		X	X
Muskingum River	172.2	RDB	39.24837	81.27474	OH	X	X	X
Little Kanawha River	184.8	LDB	39.15791	81.33969	WV	X	X	X
Little Hocking River	192.0	RDB	39.15814	81.41693	OH	X	X	X
Hocking River	199.4	RDB	39.11154	81.45303	OH	X	X	X
Lee Creek	201.9	LDB	39.09061	81.4437	WV	X	X	X
Shade River	210.8	RDB	39.04085	81.48914	OH	X	X	X
Mill Creek	231.3	LDB	38.53118	81.51543	WV	X	X	X
Leading Creek	254.2	RDB	38.5914	82.04349	OH			X
Great Kanawha River	265.6	LDB	38.50128	82.08104	WV	X	X	X
Raccoon Creek	276.1	RDB	38.4336	82.11501	OH	X	X	X
Guyandotte River	305.2	LDB	38.25766	82.23495	WV	X	X	X
Big Sandy River	317.1	LDB	38.24666	82.35786	WV	X	X	X
Little Sandy River	336.4	LDB	38.34719	82.50478	KY	X	X	X
Little Scioto River	349.0	RDB	38.45365	82.53015	OH	X	X	X
Scioto River	356.6	RDB	38.43878	83.00.767	OH	X	X	X
Kinniconnick Creek	368.2	LDB	38.36897	83.09399	KY	X	X	X
Brush Creek	388.0	RDB	38.40425	83.27069	OH	X	X	X
White Oak Creek	424.0	RDB	38.47308	83.57103	OH	X	X	X
Little Miami River	463.5	RDB	39.04447	84.25841	OH	X	X	X
Licking River	470.3	LDB	39.05164	84.30261	KY	X	X	X
Great Miami River	491.1	RDB	39.06756	84.4927	IN	X	X	X
Paint Lick Creek	521.5	LDB	38.48502	84.4872	KY		X	X
Bryant Creek	527.1	RDB	38.47797	84.52955	IN		X	
Kentucky River	545.9	LDB	38.40762	85.11296	KY		X	
Fourteen Mile Creek	589.4	RDB	38.2545	85.37298	IN		X	
Salt River	629.9	LDB	37.59995	85.56426	KY		X	
Indian Creek	657.1	RDB	38.06846	86.16391	IN		X	X
Blue River	663.0	RDB	38.10978	86.19605	IN		X	X
Little Blue River	678.8	RDB	38.07287	86.24742	IN		X	X
Sinking Creek	701.0	LDB	37.54811	86.31386	KY		X	X
Bull Creek	707.8	LDB	37.52551	86.35436	KY		X	
Millstone Creek	717.3	RDB	37.54339	86.38573	IN		X	
Green River	784.3	LDB	37.54124	87.29705	KY		X	X

Tributary Name	Ohio River Mile	Bank	Latitude	Longitude	State	2002	2003	2004
Wabash River	848.0	RDB	37.485	88.01668	IL		X	X
Tradewater River	873.5	LDB	37.30462	88.03106	KY		X	X
Peters Creek	886.2	RDB	37.27462	88.15557	IL		X	X
Grand Pierre Creek	898.0	RDB	37.25283	88.25864	IL		X	X
Cumberland River	920.3	LDB	37.08889	88.23905	KY		X	X
Tennessee River	932.8	LDB	37.0341	88.32896	KY		X	X
Post Creek Cutoff	957.8	RDB	37.13952	88.57462	IL		X	X