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# Effects of Land Use in the Ohio River Basin on the Distribution of Coliform and Antibiotic Resistant Bacteria in the Ohio River

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**EFFECTS OF LAND USE IN THE OHIO RIVER BASIN ON THE  
DISTRIBUTION OF COLIFORM AND ANTIBIOTIC RESISTANT  
BACTERIA IN THE OHIO RIVER**

Thesis submitted in partial fulfillment of the requirements for  
the Master of Science (M.S.) degree in Biological Sciences

by

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# Table of Contents

<b>Table of Contents</b> .....	ii
<b>LIST OF FIGURES</b> .....	iii
<b>LIST OF TABLES</b> .....	iii
<b>Abstract</b> .....	iv
<b>Introduction</b> .....	5
<b>Site Description</b> .....	6
<b>Material and Methods</b> .....	6
<b>ArcGIS Methods</b> .....	11
<b>Data Analysis</b> .....	13
<b>Results</b> .....	15
Canoco Correspondence Analysis Results.....	20
Linear Regression Results.....	24
Effect of Precipitation on Bacterial Growth.....	29
<b>Discussion</b> .....	31
<b>Conclusions</b> .....	37
<b>Further directions</b> .....	37
<b>References</b> .....	39
<b>Appendix A – Significant Land Use/Microbe Relationships</b> .....	43

## LIST OF FIGURES

Figure 1.....	13
Figure 2.....	16
Figure 3.....	17
Figure 4.....	18
Figure 5.....	19
Figure 6.....	20
Figure 7.....	22
Figure 8.....	23
Figure 9.....	24
Figure 10.....	30
Figure 11.....	31

## LIST OF TABLES

Table 1.....	9
Table 2.....	25
Table 3.....	26
Table 4.....	27
Table 5.....	28
Table 6.....	29

## Abstract

Recent studies indicate that antibiotic resistant bacteria can be useful as indicators of water quality (1, 2, 5, 7, 8, 9, 10). Studies in our laboratory have shown that fecal pollution did not fully explain the distribution or the frequency of antibiotic resistant bacteria in the Ohio River (27, 28). Therefore, it is important to understand the factors that affect the distribution of antibiotic resistant bacteria in aquatic habitat. The purpose of this study was to examine the correlations between land use, water quality, and concentration of antibiotic resistant bacteria in the Ohio River.

Mid-channel water samples were collected at five mile intervals in the Ohio River and all major tributaries. Total cultivable bacteria and selected antibiotic resistant bacteria were cultivated on R2A agar. Antibiotic resistant total coliforms and *Escherichia coli* were enumerated using Colilert™ reagent (IDEXX Laboratories, Inc., Westbrook, ME) and Quanti-Tray/2000™. Land use features were obtained from the national land cover data (NLCD) gathered from the USGS website. The data were then put into ArcGIS® (ESRI, Redlands, CA) and were used with microbiological data to analyze the association between land use and microbial communities. CANOCO 4.5 was used to determine the spatial differences between each site. Linear regression models were used to determine trends between land use and individual microbial communities. The data suggested residential, commercial and, in some cases, wetland land use types have a significant and proportional relationship and that farming and forested areas have a significant but inverse relationship between land use and bacterial abundance.

## Introduction

The Ohio River plays an important role in the eastern United States. It provides drinking water for more than three million people, provides cooling water for 44 electric generating facilities, and serves as a major transportation route for cargo in the eastern United States. More than 25 million people, approximately 10 percent of the U.S. population, live in the Ohio River basin (Orsanco.org). The scale of the resource makes comprehensive monitoring of water quality time consuming and expensive. In order to determine the role that human impact plays on the river, a simple, accurate, rapid, and inexpensive method to determine water quality is needed.

Fecal indicator bacteria (e.g. *E. coli*, fecal strep, fecal coliforms) have long been used to indicate recent fecal contamination of water. Studies have shown that antibiotic resistant bacteria (ARB) may also be useful in determining water quality (1, 3,5,10, 28). Previous work in our laboratory has shown that fecal contamination does not adequately explain the frequency or the distribution of antibiotic resistant bacteria in the Ohio River (27, 28). So this important group of microorganisms is not adequately modeled by current method. These studies have shown that cultivable ARB outnumber cultivable fecal indicator bacteria and that the distributions of the two population patterns are distinct. These data suggest that fecal contamination alone cannot explain the distribution of ARB in the environment. Recent studies suggest that the number and distribution of ARB in the environment may be influenced by human impacts on the environment such as agricultural and storm water management practices (1, 3,5,10, 28). But a comprehensive analysis of how land use impacts the distribution of ARB has not previously been done.

In this study we quantified total cultivable, fecal indicator bacteria and ARB and compared their distribution patterns to land use. Microbiological data, along with land use data and ArcGIS<sup>®</sup>, were used to examine the association between the frequency and distribution of specific microbial populations. The data were also used to determine if there were correlations between certain types of land use, ARB and fecal coliform bacteria.

### Site Description

The Ohio River is the largest tributary by volume of the Mississippi River. The Ohio forms at the confluence of the Allegheny and Monongahela rivers in Pittsburgh, PA. From Pittsburgh, it flows southwest until it joins the Mississippi near the city of Cairo, IL. It is approximately 981 miles (1,579 km) long, and flows through or along the border of six states, and its drainage basin includes parts of 14 states, including many of the states of the southeastern U.S. The Ohio's drainage basin covers 189,422 square miles (490,603 km<sup>2</sup>), including the eastern-most regions of the Mississippi Basin. Because the Ohio River basin covers such a large area in the eastern part of the United States, the variety of land use along the river ranges from agricultural to highly industrialized urban areas.

### Methods

Mid-channel, sub-surface water samples were collected at 5-mile intervals along the mainstem of the river. In 2001 and 2002 samples were taken from Pittsburgh, PA to Rising Sun, IN (505 miles, 101 sites). From 2003 to 2006 samples were taken from Pittsburgh, PA to Cairo, IL (981 miles, 198 sites). Samples were placed on ice and

transported to a field laboratory for analyses. R2A agar and fungizone (see Table 1 for working concentrations) were used to cultivate total cultivable heterotrophic bacteria. R2A agar, fungizone, and selected antibiotics (see Table 1 for working concentration) were used to cultivate antibiotic resistant bacteria.

From 2001 to 2004 fecal coliform bacteria were collected by vacuum filtration using a 0.2 $\mu$  membrane filter and cultivated on m-FC medium, according to the manufacture's instructions. For sampling seasons 2005 and 2006, Colilert™ reagent (IDEXX Laboratories, Inc., Westbrook, ME) and Quanti-Tray/2000™ were used for the cultivation and enumeration of total coliforms and *E. coli*. Antibiotic resistant coliforms and *E. coli* were cultivated using the same method and reagents plus selected antibiotics (see Table 1 for working concentration). These antibiotics were chosen because they were listed under the USGS toxic substances hydrology program, which listed target compounds for national reconnaissance of emerging contaminants in US streams.

The antibiotics, solvents, stock concentrations and working concentrations used in all sampling years are shown in Table 1.

**Table 1.** Antibiotics used and recommended concentrations<sup>a</sup> supplied in solution<sup>b</sup> 50:50 by volume

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

Stock solutions were made by weighing out sufficient antibiotic to make a 10 ml of river water solution and the antibiotic powder added to a sterile, 15 ml of river water test tube. The appropriate solvent (Table 1) was added to the tube to transform the total volume of 10ml of river water. Suspensions were vortexed and placed in a bath sonicator until the antibiotic was fully dissolved. Aqueous and ETOH: H<sub>2</sub>O solutions were filtered through a sterile 0.2µm syringe filter and all stock solutions were stored at -20°C until used. Stock solutions were made fresh each month and all agar media were only used up to one week after preparation.

R2A agar (9.1 g) was suspended in 500 ml of river water or purified water. The mixture was stirred and then autoclaved at 121°C and 15 psi for 20 minutes. After autoclaving, the medium was moved to a 48°C water bath until tempered. After the medium was cooled, it was gently mixed to make sure the agar was evenly distributed. After mixing, 750 µl of fungizone stock was added aseptically. The agar was stirred again for about 15 to 30 seconds. After mixing, the medium was then poured into a pre-sterilized petri plates, and the plates were allowed to cool and age for 24 hours. For the preparation of antibiotic resistant agar plates, the same steps were used for preparation of R2A agar; however, after the addition of fungizone, 500 µl of antibiotic stock was added and mixed again for 15 to 30 seconds prior to pouring.

Upon bringing samples into the lab, 0.1 ml of river water was aseptically transferred into a sterile 9.9 ml of river water dilution blank and was mixed with a vortex. An aliquot (0.1 ml of river water) of diluted sample was then transferred to plates with R2A and fungizone only, in triplicate. The diluted water sample was then spread using sterile glass beads. The beads were removed, and the plates were wrapped with parafilm and inverted and incubated at room temperature in the dark for one week. The number of colonies on each plate was then counted and an average count of all three plates was determined. Colony forming units (CFU) per ml of river water of original sample were calculated by multiplying the average count by the total dilution factor ( $10^3$ ). These counts represent the total cultivable heterotrophic bacteria in the H<sub>2</sub>O sample. For antibiotic resistant bacteria, aliquots of 0.1 to 0.2 ml of river water of undiluted river water were transferred to R2A/fungizone/selected antibiotic plates, in triplicate. The water sample was then evenly spread over the medium using sterile glass beads and

incubated as described above. After one week, the number of colonies on each plate was counted, and an average colony count was calculated. The CFU per ml of river water of antibiotic resistant cells were calculated by multiplying the average count by the dilution factor (10 for a 0.1ml of river water inoculum and 5 for a 0.2 ml of river water inoculum).

For the enumeration of fecal coliforms (2001-2004) three different volumes were plated for each site sampled. A sample of water (1 to 25 ml of river water) was aseptically transferred into a sterile filtration funnel containing a 0.2  $\mu\text{m}$  membrane filter and 20 to 30 ml of river water or sterile water. The samples were then filtered by vacuum. After the water was completely filtered, the membrane was removed and placed into a dish containing a sterile pad saturated with m-FC broth. Each dish was incubated for 24 hours at 35°C.

For the sampling season of 2005 and 2006, Colilert™ reagent (IDEXX Laboratories, Inc., Westbrook, ME) and Quanti-Tray/2000™ incubation trays were used for the enumeration of total coliforms and *Escherichia coli*. The reagent that was added to the 100 ml of river water sample contained two nutrient indicators; one of these indicators was o-Nitrophenyl- $\beta$ -galactopyranoside (ONPG) which is a lactose analog. When split by  $\beta$ -galactosidase, an enzyme present in lactose fermenting bacteria, the nitrophenyl group turns yellow. The reagent also contained Methylumbelliferyl- $\beta$ , D-glucuronide (MUG), a glucuronic acid analog. When split by  $\beta$ -glucuronidase, an enzyme characteristic of *E. coli*, the methylumbelliferyl group produces a distinct blue fluorescence. One pre-measured reagent pack was combined with 100 ml of river water of water sample, mixed well until dissolved and transferred to a Quanti-Tray/2000™. Trays were heat sealed, and incubated for 24 hours at 35°C. Most Probable Number

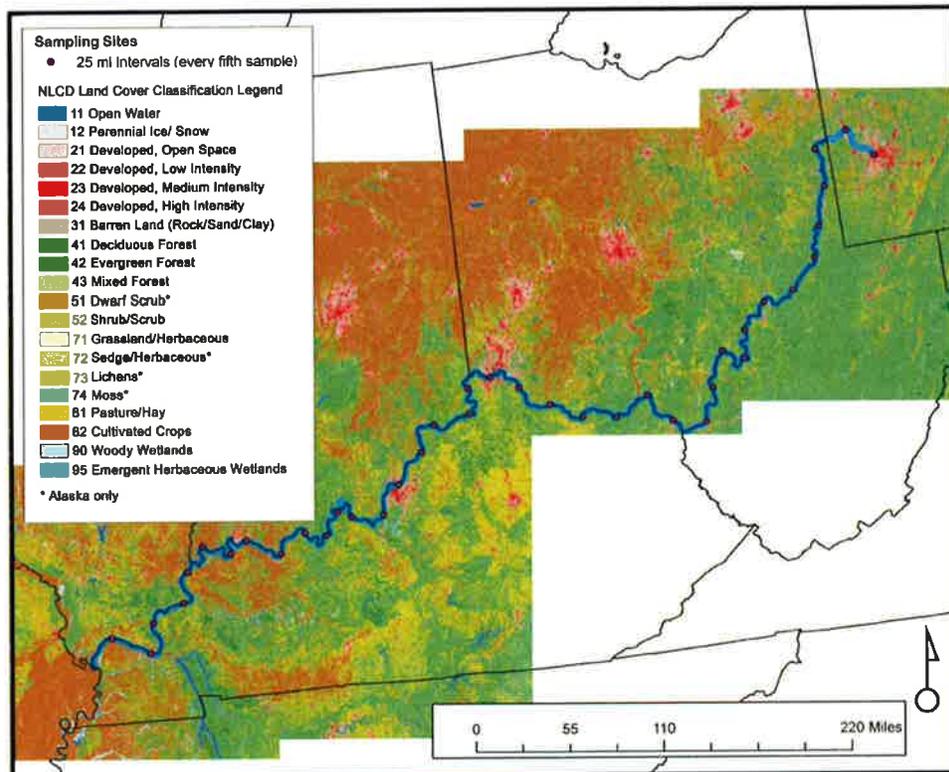
(MPN) estimation for total coliforms and *E. coli* were determined, after 24 hours, by counting the number of large and small wells that had turned yellow and entering those numbers in an MPN chart provided by IDEXX Laboratories, Inc. MPN for *E. coli* were determined by counting the number of large and small fluorescent wells under UV luminations. Antibiotic resistant coliforms and *E. coli* were also determined using the coliert reagents with the addition of individual antibiotics.

Impact Scores were used as an indicator of the overall water quality of the sampling site. All microbiological counts were entered into an Excel spreadsheet. For each population (e.g. the antibiotic treatments) a percentile score was determined by using the PERCENTRANK function in Excel. Boundaries were then chosen at the 10<sup>th</sup> and 90<sup>th</sup> percentiles. A population score of 1 was assigned to all data points above the 90<sup>th</sup> percentile, and a score of -1 score was assigned to a population below the 10<sup>th</sup> percentile. A score of zero 0 was assigned to all points that fell between the boundaries. This step was repeated for each population. The individual population scores were then added together to obtain the impact score for each site. Higher impact scores are intended to indicate sites with relatively high levels of disturbance.

#### ArcGIS® Methods

Global positioning satellites (GPS) coordinates were taken at each of the sampling sites. Land use raster data were gathered from the national land cover data set (NLCD) from the USGS website. These data and the GPS coordinates were then put into ArcGIS® (ESRI, Redlands, CA), which was used to analyze the effects of different types of land use on the measured microbial populations. Using the GPS coordinates collected

at each of the sample site and the collected GIS data, a comprehensive map of the Ohio River basin was created in ArcGIS®. In ArcGIS® the land use features for each state surrounding the Ohio River basin were merged together using Spatial Analyst. First, the area of the raster for each raster with no data was replaced with 0 by using a conditional statement in map algebra, `con(isnull(filename),0,filename)`. After the no data areas were replaced by 0, each of the areas were combined in map algebra using an over statement, `(filename) over (filename)`. The area in purple shows an area with no data that were replaced by 0. After all land use rasters were merged together, land use information was extracted for the 5-mile buffer around sampling site using the clip tool. However, to get an accurate representation of the amount of land use that would affect each sampling site, only the land use features that were upstream from the sampling site were considered. To do so, the five mile buffer from the previous site was used, which touched the sampling site of interest and also included five miles of land use features upstream of the site.



**Figure 1.** Map showing the study area after all files were merged using spatial analysis.

## Data Analysis

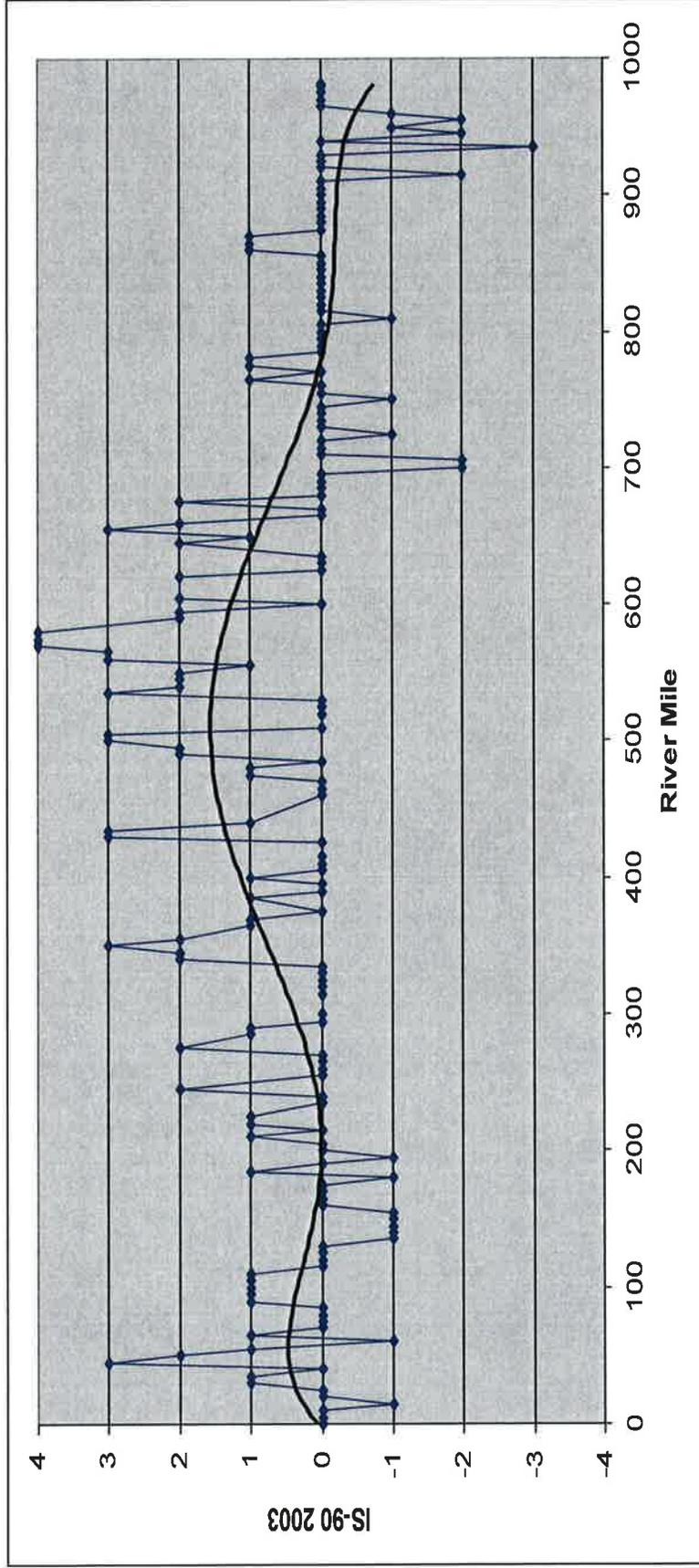
Microbial data were compared to land use features to determine association between land use and specific microbial populations. Impact scores were graphed versus river mile, and a polynomial trend line was used to determine the trend of the data. Using the software CANOCO 4.5, canonical correspondence analysis (CCA) was used to analyze spatial differences between the sample sites. This tool allowed for the analysis of the impact of all land use features on all microbial data and their interaction. CCA is a tool used in ecology to see how multiple species would respond to external factors such as environmental variables, pollutants, and management regimes. Data were collected on

microbial population composition and the external variables at a number of points in space and time.

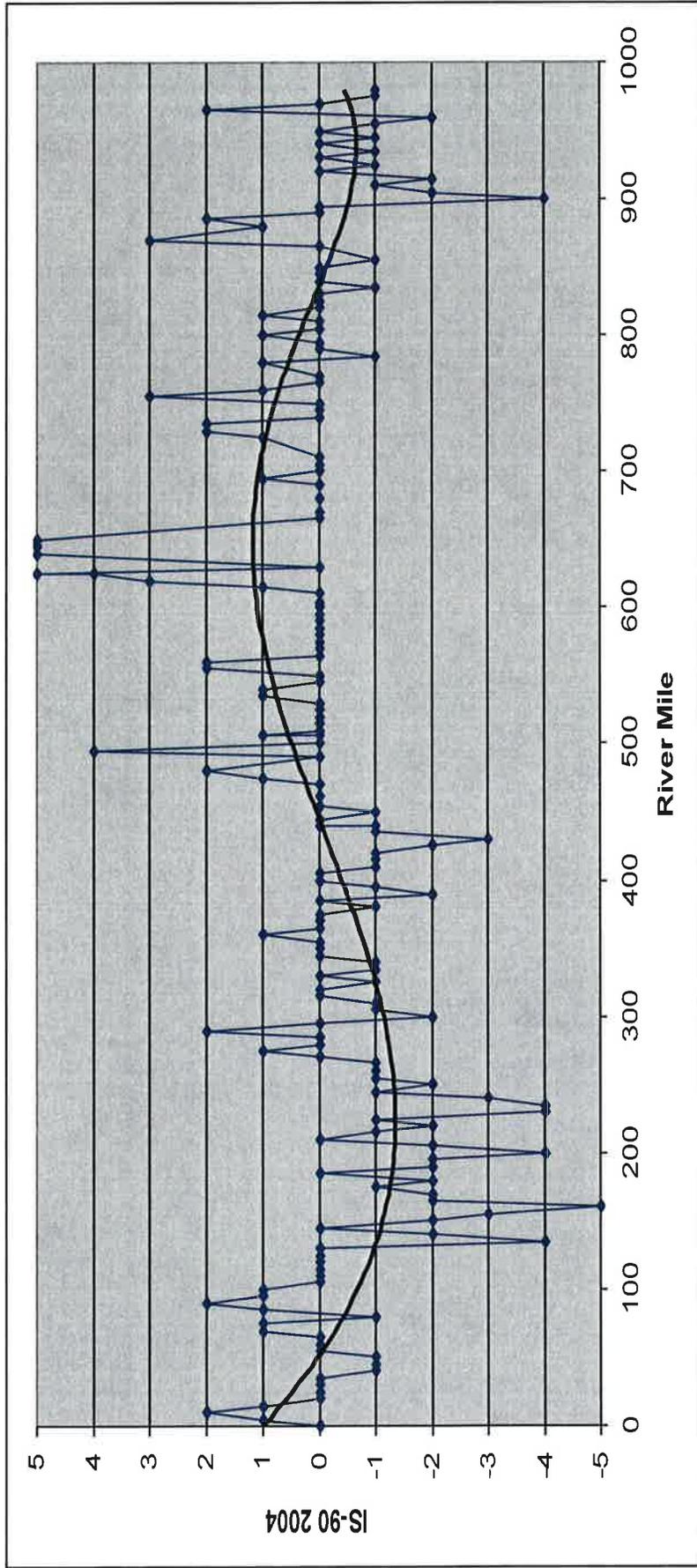
Adaptations were made to use this tool, usually used in ecology, for this research. ARB data and other microbiological data were treated as individual species. For example, all ciprofloxacin resistant bacteria were considered one species, all tetracycline resistant bacteria were considered another species, and so on. Environmental data were obtained by ArcGIS<sup>®</sup> and each land use type was classified as a percentage of overall land use for the five miles above a sampling site. Biplots, plots of environmental variables versus species data, were made for each data to determine the correlation between population changes and environmental variables.

Linear regression models were also used to determine the degree of association between land use features and bacterial populations. A regression graph was made for each environmental variable versus each microbial species. R-values were determined to indicate the strength of the relationship between the bacterial species and land use features. P-values were determined to indicate confidence in these relationships. Tables were created for each of the sampling years and include both R-values and P-values.

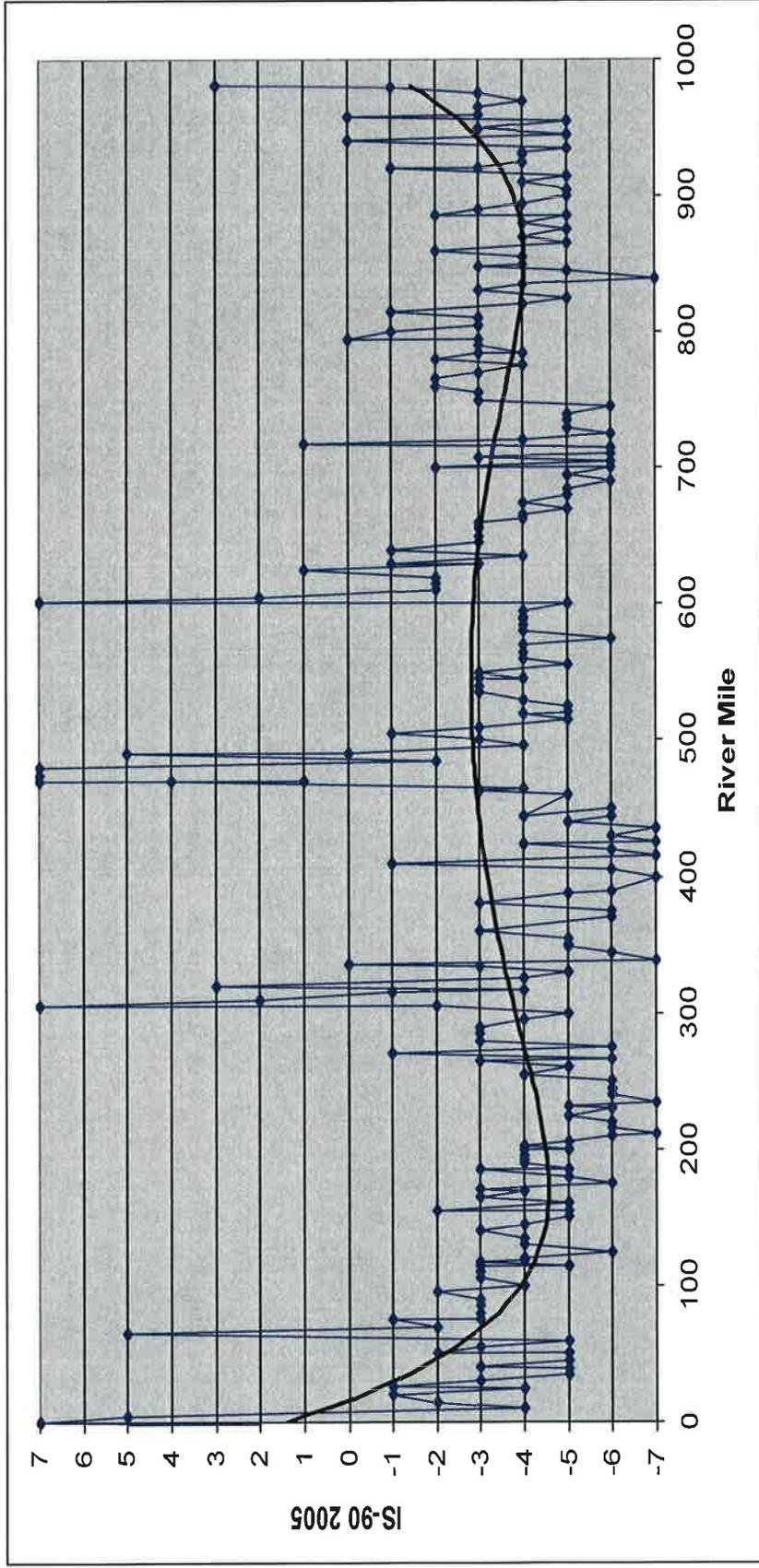
Results



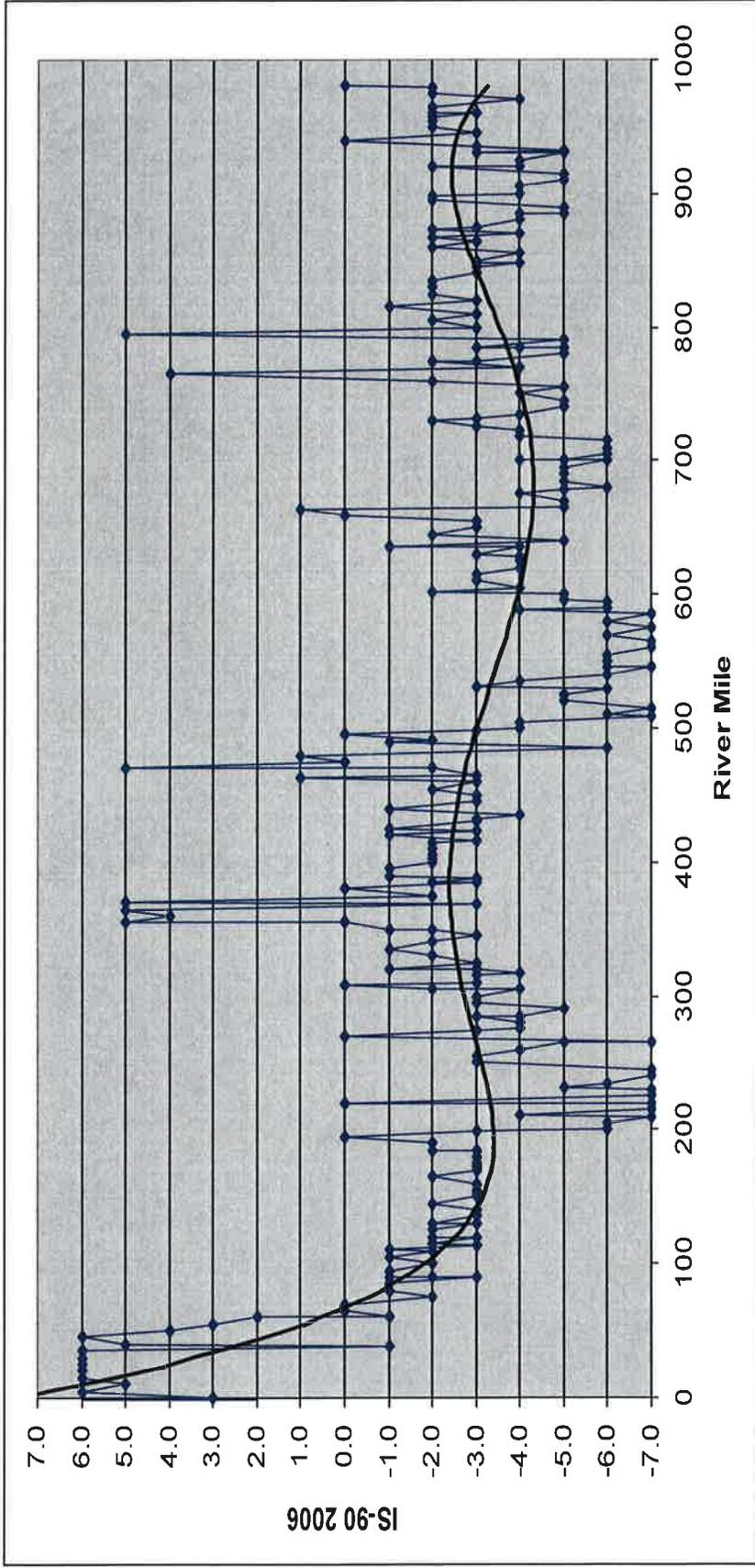
**Figure 2.** Impact score versus river mile for the 2003 sampling set. This figure also includes a polynomial trend line to show broad trends in impact score along the river. Some major tributaries and population centers are listed for reference: Pittsburgh, PA, river mile 0, Parkersburg, WV, river mile 183, Kanawha River, river mile 266, Huntington, WV river mile 307, Portsmouth, OH, river mile 356, Cincinnati, OH, river mile 464, Louisville, KY, river mile 557.



**Figure 3.** Impact score versus river mile for the 2004 sampling set. This figure also includes a polynomial trend line to show broad trends in impact score along the river. Some major tributaries and population centers are listed for reference. Pittsburgh, PA, river mile 0, Parkersburg, WV, river mile 183, Kanawha River, river mile 266, Huntington, WV river mile 307, Portsmouth, OH, river mile 356, Cincinnati, OH, river mile 464, Louisville, KY, river mile 557.



**Figure 4.** Impact score versus river mile for the 2005 sampling set. This figure also includes a polynomial trend line to show broad trends in impact score along the river. Some major tributaries and population centers are listed for reference. Pittsburgh, PA, river mile 0, Parkersburg, WV, river mile 183, Kanawha River, river mile 266, Huntington, WV river mile 307, Portsmouth, OH, river mile 356, Cincinnati, OH, river mile 464, Louisville, KY, river mile 557.



**Figure 5.** Impact score versus river mile for the 2006 sampling set. This figure also includes a polynomial trend line to show broad trends in impact score along the river. Some major tributaries and population centers are listed for reference. Pittsburgh, PA, river mile 0, Parkersburgh, WV, river mile 183, Kanawha River, river mile 266, Huntington, WV river mile 307, Portsmouth, OH, river mile 356, Cincinnati, OH, river mile 464, Louisville, KY, river mile 557.

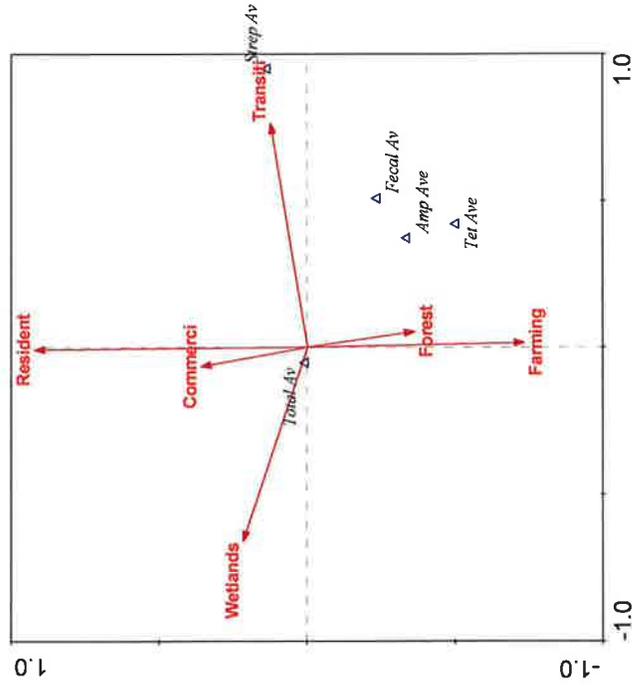
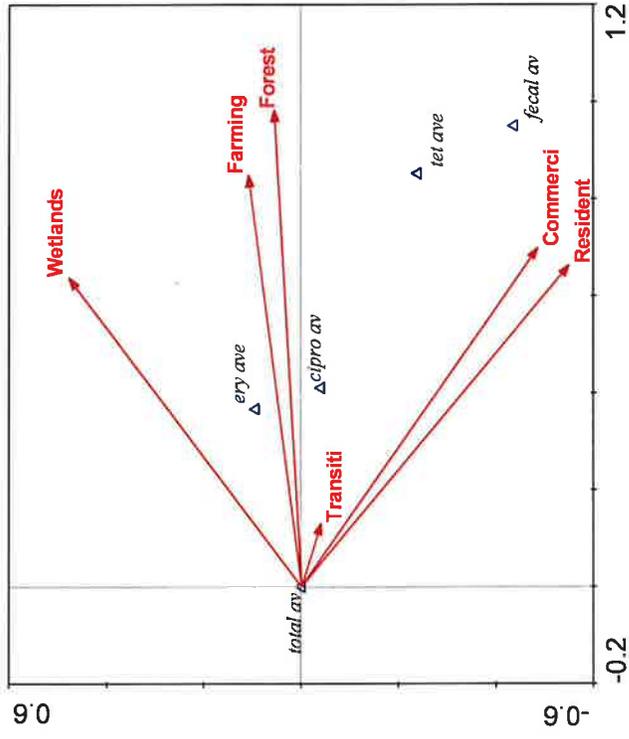
Figures 2 to 5 show the variation in impact scores (IS) trend along the Ohio River for the sampling years of 2003 to 2006. Impact scores are designed to increase with increase perturbation of the habitat and generally show higher values around major population centers (33-34). The figures show that impact scores trend starting high at the origin of the Ohio River and dropping down before river mile 100. Then, impact scores rise again around river mile 200. During sampling seasons 2003 and 2005, high IS value continued until around mile 500. In sampling season 2004, the peak continued until around river mile 650. In sampling season 2006, the peak was seen a little earlier around river mile 400. From there the IS peaks drop until river mile 900 in 2005 and river mile 850 in sampling season 2006 but not in sampling seasons 2003 and 2004.



**Figure 6.** Map of the Ohio River Basin with major cities and their corresponding river mile designation.

## Overview of CCA Output

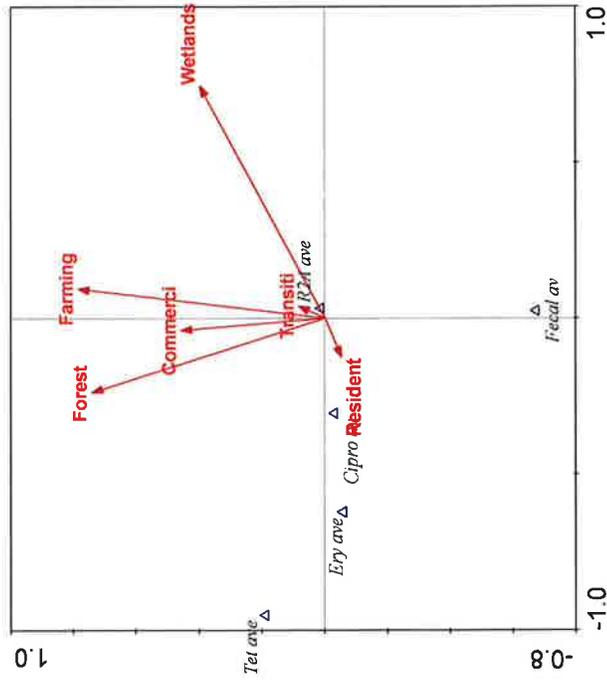
Figures 7- 9 show the relationships between ARB populations and environmental variables for each of the 6 sampling seasons. The figures suggest that residential and commercial land use have a positive impact on ARB abundance, meaning that as the percentage of residential and commercial areas increase so does the abundance of ARB populations. Figure 7- 9 also suggest that farming and forest land use have a negative impact on ARB population density; as the percentage of farming and forested land increases the abundance of ARB decreases. The outputs also show that wetlands at times are associated with low ARB abundance, but at other times are associated with increased ARB abundance. The data also show that total cultivable bacteria and total coliform bacteria did not deviate too much from the origin suggesting that those populations are not affected by changes in land use and are probably not good indicators of environmental perturbation.



2001

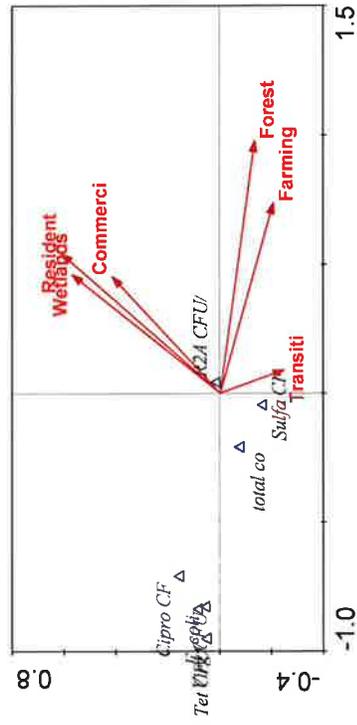
2002

**Figure 7.** CCA output from Canoco 4.5 for sampling seasons 2001 and 2002. This figure shows the relationship between different microbial communities and environmental variables. The vectors are environmental factors, they are true vectors where the length of the vector shows importance of the effect that each variable had on microbial communities. The triangles represent microbial communities. The clustering of the triangles shows that they are similar in their association with environmental conditions. The closeness of the triangles to the environmental vectors shows the strength of the correlation between of the environmental variable and the microbial communities.

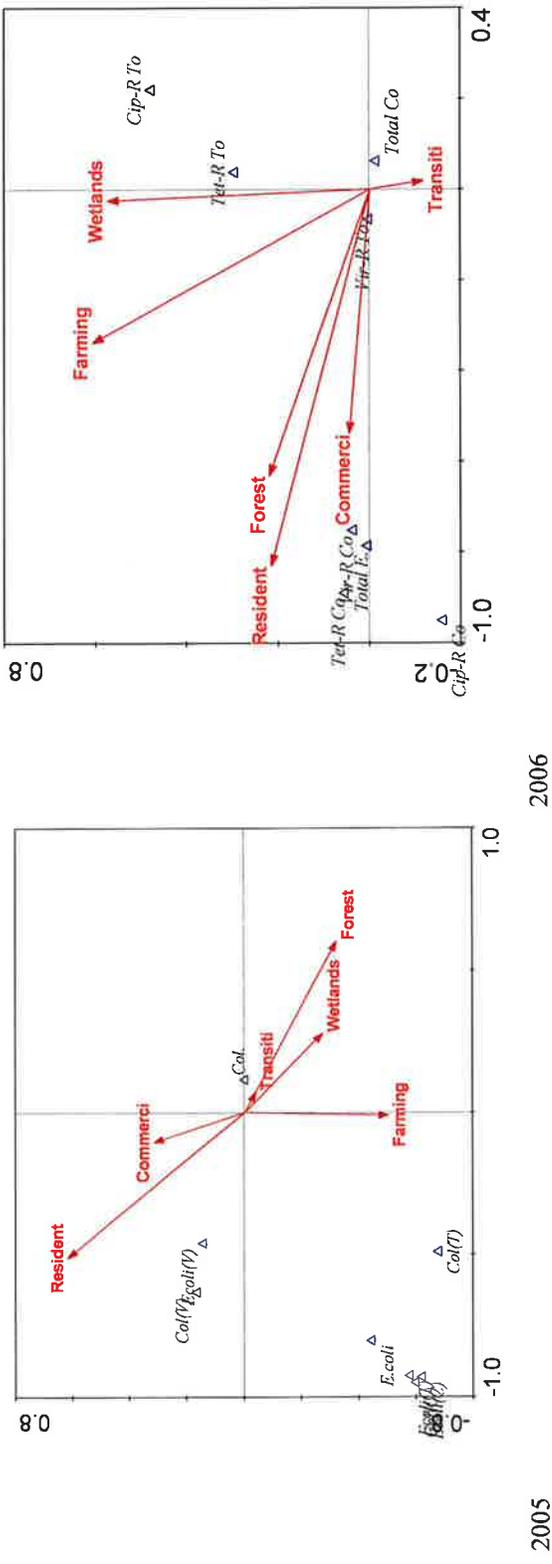


2003

2004



**Figure 8.** CCA output from Canoco 4.5 for sampling seasons 2003 and 2004. This figure shows the relationship between different microbial communities and environmental variables. The vectors are environmental factors, they are true vectors where the length of the vector shows importance of the effect that each variable had on microbial communities. The triangles represent microbial communities. The clustering of the triangles shows that they are similar in their association with environmental conditions. The closeness of the triangles to the environmental vectors shows the strength of the correlation between of the environmental variable and the microbial communities.



**Figure 9.** CCA output from Canoco 4.5 for sampling seasons 2005 and 2006. This figure shows the relationship between different microbial communities and environmental variables. The vectors are environmental factors, they are true vectors where the length of the vector shows importance of the effect that each variable had on microbial communities. The triangles represent microbial communities. The clustering of the triangles shows that they are similar in their association with environmental conditions. The closeness of the triangles to the environmental vectors shows the strength of the correlation between of the environmental variable and the microbial communities

Linear regression models (Table 2) show that there is a positive and significant relationship ( $P < 0.05$ ), between ARB populations and residential land use. There were more significant values in between the years 2004 to 2006. The table suggests a direct relationship between residential land use and increased population growth. The more significant values ( $P < 0.05$ ) was seen in years 2002 and 2004 to 2006. The fact that more significant values were seen during relatively low flow years indicates that this relationship between residential land use and ARB was not driven by runoff but was likely due to resistant point source.

**Table 2.** Summary of  $r^2$  and ( $P$ -value) resulting from linear regression models for residential land use for all six years of study.

Antibiotic Resistant Populations	2001	2002	2003	2004	2005	2006
Amp	-0.177 (0.08)					
Cipro	<sup>a</sup>	0.057 (0.57)	-0.176 (0.09)	<b>0.417 (0.00)</b>		
Ery		0.069 (0.49)	-0.028 (0.78)			
Strp	-0.018 (0.86)					
Sulfa				<b>0.247 (0.01)</b>		
Tet	-0.166 (0.11)	<b>0.350 (0.00)<sup>b</sup></b>	0.076 (0.47)	<b>0.380 (0.00)</b>		
Virg				<b>0.356 (0.00)</b>		
Cipro coliform					<b>0.524 (0.00)</b>	0.067 (0.50)
Tet coliform					<b>0.356 (0.00)</b>	<b>0.391 (0.00)</b>
Virg coliform					<b>0.693 (0.00)</b>	<b>0.276 (0.01)</b>
Cipro <i>E. coli</i>					<b>0.552 (0.00)</b>	<b>0.335 (0.00)</b>
Tet <i>E. coli</i>					<b>0.550 (0.00)</b>	<b>0.486 (0.00)</b>
Virg <i>E. coli</i>					<b>0.719 (0.00)</b>	<b>0.719 (0.00)</b>
Total				<b>0.410 (0.00)</b>	<b>0.416 (0.00)</b>	<b>0.449 (0.00)</b>
<i>E. coli</i>				<b>0.529 (0.00)</b>	<b>0.563 (0.00)</b>	<b>0.391 (0.00)</b>
Fecal	-0.123 (0.26)	<b>0.355 (0.00)</b>	0.126 (0.21)			
R2A	-0.017 (0.87)	<b>0.243 (0.02)</b>	-0.006 (0.95)	<b>0.380 (0.00)</b>		

<sup>b</sup>**Bold** ( $P$ -value) shows  $P$ -value with significance  $< 0.05$ . <sup>a</sup>Blank cell indicates that the corresponding population measurement was not made in that year. Tables are created using linear regression charts. Relevant charts can be found at the end of the paper in appendix A.

Linear regression models (Table 3) showed that there was some positive and significant relationship,  $P < 0.05$ , between antibiotic resistant bacteria populations and commercial land use. However, the only year that these significant values were seen is in 2005. The table suggests a direct relationship between commercial land use and increase ARB counts. Significant values ( $P < 0.05$ ) are only seen in 2005.

**Table 3.** Summary of  $r^2$  and ( $P$ -value) resulting from linear regression models for commercial land use for all six years of study

Antibiotic Resistant Populations	2001	2002	2003	2004	2005	2006
Amp	-0.010 (0.92)					
Cipro	<sup>a</sup>	0.008 (0.94)	-0.185 (0.08)	0.198 (0.05)		
Ery		0.012 (0.91)	-0.046 (0.65)			
Strp	0.080 (0.42)					
Sulfa				0.086 (0.39)		
Tet	-0.024 (0.82)	0.111 (0.27)	0.054 (0.60)	0.148 (0.14)		
Virg				0.165 (0.10)		
Cipro coliform					<b>5.550 (0.00)<sup>b</sup></b>	0.011 (0.91)
Tet coliform					<b>0.403 (0.00)</b>	0.403 (0.22)
Virg coliform					<b>0.693 (0.00)</b>	0.159 (0.11)
Cipro <i>E. coli</i>					<b>0.610 (0.00)</b>	0.102 (0.31)
Tet <i>E. coli</i>					<b>0.610 (0.00)</b>	0.090 (0.37)
Virg <i>E. coli</i>					<b>0.729 (0.00)</b>	0.073 (0.47)
Total				0.107 (0.29)	<b>0.382 (0.00)</b>	0.173 (0.08)
<i>E. coli</i>				0.152 (0.13)	<b>0.616 (0.00)</b>	0.117 (0.24)
Fecal	0.101 (0.36)	0.174 (0.08)	-0.046 (0.65)			
R2A	0.075 (0.46)	0.059 (0.56)	-0.008 (0.94)	0.154 (0.12)		

<sup>b</sup>**Bold** ( $P$ -value) shows  $P$ -value with significance  $< 0.05$ . <sup>a</sup>Blank cell indicates that the corresponding population measurement was not made in that year. Tables are created using linear regression charts. Relevant charts can be found at the end of the paper in appendix A.

Linear regression models (Table 4) showed that there were inverse and significant relationship ( $P < 0.05$ ) between ARB populations and forested land use. There were more significant values in the years 2004 to 2006.

**Table 4.** Summary of  $r^2$  and ( $P$ -value) resulting from linear regression models for forested areas for all six years of study.

Antibiotic Resistant Populations	2001	2002	2003	2004	2005	2006
Amp	0.022 (0.83)					
Cipro	<sup>a</sup>	0.013 (0.90)	-0.019 (0.86)	<b>-0.367 (0.00)</b>		
Ery		0.040 (0.69)	-0.017 (0.86)			
Strp	-0.087 (0.39)					
Sulfa				-0.051 (0.62)		
Tet	0.026 (0.80)	-0.122 (0.22)	-0.072 (0.49)	<b>-0.353 (0.00)<sup>b</sup></b>		
Virg				<b>-0.286 (0.00)</b>		
Cipro coliform					<b>-0.393 (0.00)</b>	-0.123 (0.22)
Tet coliform					<b>-0.239 (0.02)</b>	0.059 (0.56)
Virg coliform					<b>-0.498 (0.00)</b>	<b>-0.207 (0.04)</b>
Cipro <i>E. coli</i>					<b>-0.420 (0.00)</b>	-0.052 (0.61)
Tet <i>E. coli</i>					<b>-0.423 (0.00)</b>	-0.102 (0.31)
Virg <i>E. coli</i>					<b>0.446 (0.00)</b>	-0.081 (0.42)
Total				<b>-0.273 (0.01)</b>	-0.360 (0.25)	-0.188 (0.06)
<i>E. coli</i>				<b>-0.303 (0.00)</b>	<b>-0.417 (0.00)</b>	0.170 (0.17)
Fecal	-0.021 (0.58)	-0.159 (0.11)	-0.111 (0.27)			
R2A	-0.065 (0.53)	-0.040 (0.69)	-0.107 (0.29)	-0.171 (0.09)		

<sup>b</sup>**Bold** ( $P$ -value) shows  $P$ -value with significance  $< 0.05$ . <sup>a</sup>Blank cell indicates that the corresponding population measurement was not made in that year. Tables are created using linear regression charts. Relevant charts can be found at the end of the paper in appendix A.

Linear regression models (Table 5) show that there is an inverse and significant relationship ( $P < 0.05$ ) between antibiotic resistant bacteria populations and farming land use. There were more significant values in the years 2004 to 2006. However, we were not able to determine if agricultural practices were row crop agriculture or animal agriculture

**Table 5.** Summary of  $r^2$  and ( $P$ -value) resulting from linear regression models for farming land use for all six years of study.

Antibiotic Resistant Populations	2001	2002	2003	2004	2005	2006
Amp	0.164 (0.10)					
Cipro		-0.044 (0.66)	<b>0.320 (0.00)</b>	-0.113 (0.26)		
Ery		-0.123 (0.22)	0.088 (0.39)			
Strp	0.055 (0.58)					
Sulfa				<b>-0.229 (0.02)</b>		
Tet	0.173 (0.09)	<b>-0.273 (0.01)</b>	-0.034 (0.74)	-0.065 (0.52)		
Virg				-0.112 (0.26)		
Cipro coliform					<b>-0.271 (0.01)</b>	0.061 (0.55)
Tet coliform					<b>-0.236 (0.02)</b>	0.053 (0.60)
Virg coliform					<b>-0.348 (0.00)</b>	<b>-0.312(0.00)</b>
Cipro <i>E. coli</i>					<b>-0.292 (0.00)</b>	<b>-0.310(0.00)</b>
Tet <i>E. coli</i>					<b>-0.284 (0.00)</b>	<b>-0.208(0.04)</b>
Virg <i>E. coli</i>					<b>0.442 (0.00)</b>	-0.177 (0.08)
Total				-0.164 (0.10)	-0.119 (0.25)	<b>-0.331(0.00)</b>
<i>E. coli</i>				<b>-0.235 (0.02)</b>	<b>-0.306 (0.00)</b>	<b>-0.245(0.01)</b>
Fecal	0.077 (0.48)	<b>-0.215 (0.03)</b>	0.039 (0.70)			
R2A	0.039 (0.70)	-0.168 (0.10)	0.134 (0.18)	<b>-0.261 (0.01)</b>		

<sup>b</sup>**Bold** ( $P$ -value) shows  $P$ -value with significance  $< 0.05$ . <sup>a</sup>Blank cell indicates that the corresponding population measurement was not made in that year. Tables are created using linear regression charts. Relevant charts can be found at the end of the paper in appendix A.

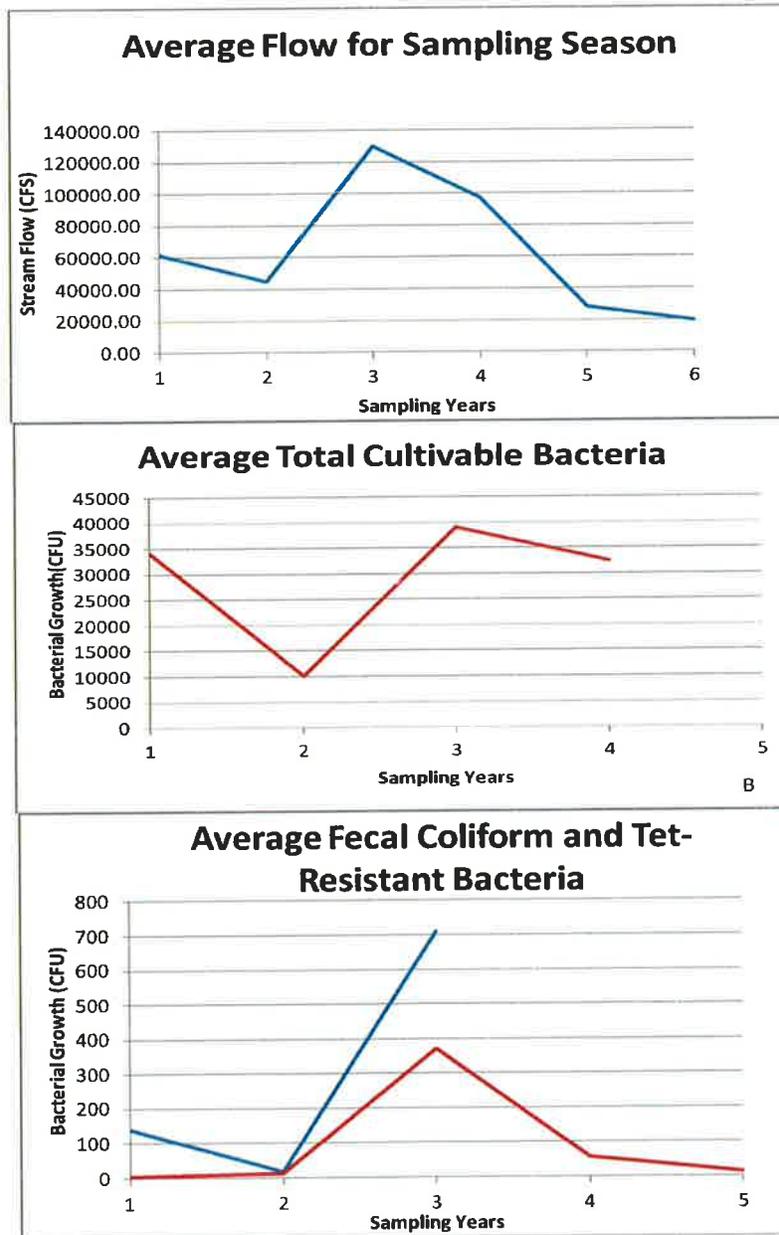
Linear regression models (Table 6) show that there is a significant relationship ( $P < 0.05$ ) between antibiotic resistant bacteria populations and wetlands. However, the relationships between them vary from year to year. There were more significant values in the years 2001 and 2003 to 2005.

**Table 6.** Summary of  $r^2$  and ( $P$ -value) resulting from linear regression models for wetlands for all six years of study.

Antibiotic Resistant Populations	2001	2002	2003	2004	2005	2006
Amp	<b>0.258 (0.01)<sup>b</sup></b>					
Cipro	<sup>a</sup>	-0.077 (0.44)	0.150 (0.15)	<b>0.326 (0.00)</b>		
Ery		0.156 (0.12)	0.024 (0.82)			
Strp	<b>0.192 (0.0)</b>					
Sulfa				-0.058 (0.56)		
Tet	0.130 (0.21)	-0.048 (0.63)	0.019 (0.85)	0.179 (0.07)		
Virg				<b>0.203 (0.04)</b>		
Cipro coliform					0.029 (0.78)	0.166 (0.10)
Tet coliform					0.031 (0.76)	0.049 (0.63)
Virg coliform					0.015 (0.89)	0.013 (0.90)
Cipro <i>E. coli</i>					0.082 (0.42)	-0.055 (0.58)
Tet <i>E. coli</i>					0.077 (0.45)	-0.119 (0.23)
Virg <i>E. coli</i>					0.627 (0.63)	-0.150 (0.13)
Total				<b>0.215 (0.03)</b>	<b>0.291 (0.00)</b>	0.120 (0.23)
<i>E. coli</i>				0.097 (0.33)	0.072 (0.48)	-0.152 (0.13)
Fecal	<b>0.268 (0.01)</b>	-0.105 (0.29)	<b>0.285 (0.00)</b>			
R2A	-0.017 (0.87)	-0.015 (0.88)	<b>0.298 (0.00)</b>	0.140 (0.16)		

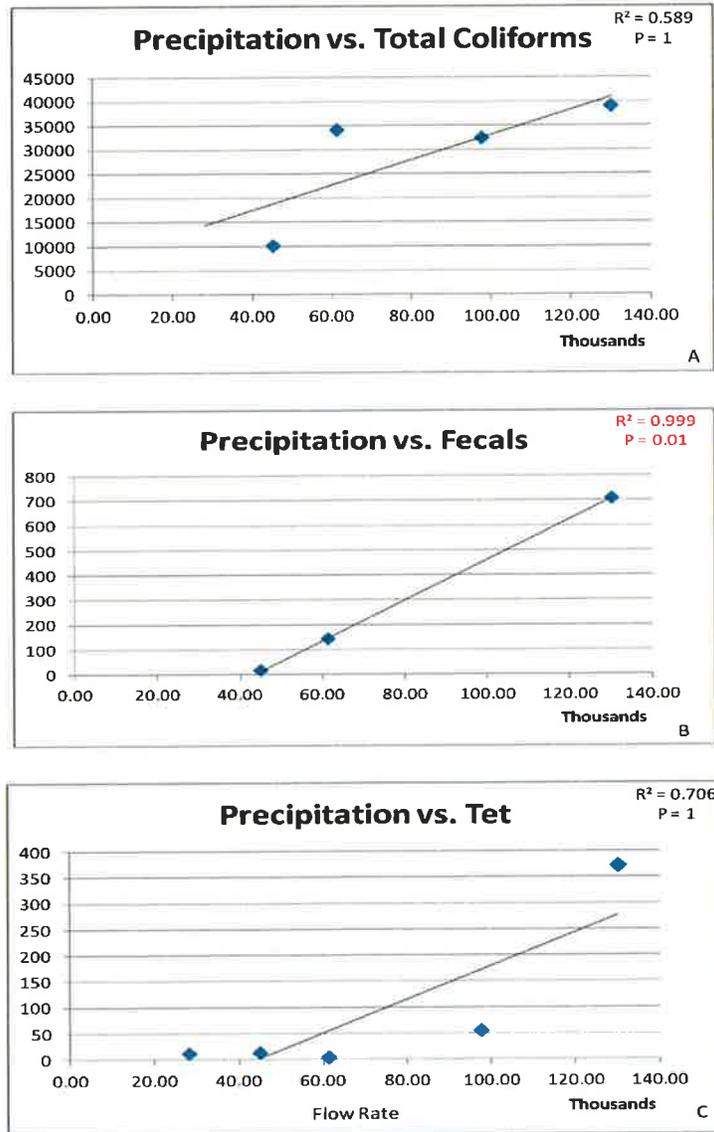
<sup>b</sup>**Bold** ( $P$ -value) shows  $P$ -value with significance  $< 0.05$ . <sup>a</sup>Blank cell indicates that the corresponding population measurement was not made in that year. Tables are created using linear regression charts. Relevant charts can be found at the end of the paper in appendix A.

Figure 10 shows a comparison of the Ohio river average flow rate and the river wide average of specific bacterial populations. The data indicate that as flow (reflecting the amount of precipitation in the basin) increased, so did the microbial population abundance. As the amount of rain decreased so did the average counts of bacteria in the river.



**Figure 10.** Comparison of river flow rate and average bacteria counts for six sampling years.

A scatter plot (Figure 11) was created to compare river flow rate with bacterial populations. These figures suggest a direct relationship between precipitation and average bacterial numbers. These figures indicate that as river flow increased so did the amount of bacterial growth and antibiotic resistant bacteria growth, but only the relationship between river flow and fecal coliforms was found to be statistically significant.



**Figure 11.** Total coliform (A), fecal coliform (B) and tetracycline resistant heterotrophic bacteria (C) versus average river flow rate.

## Discussion

Figures 2 to 5 show plot impact scores (IS) along the Ohio River for the sampling years 2003 to 2006. The figures show that impact scores tend to be high at the origin of the Ohio River (in Pittsburgh, PA) and dropping down by river mile 100. Then, impact scores start to rise again around river mile 200. During sampling seasons 2003 and 2005, higher IS value continued until around mile 500. In sampling season 2004, the peak continued until around river mile 650. In sampling season 2006, the peak was seen a little earlier around river mile 400. From there the IS peaks drop until river mile 900 in 2005 and river mile 850 in sampling season 2006 but not in sampling seasons 2003 and 2004.

These peaks and troughs correlated with areas of different land use. The peaks generally correlate well with highly urbanized and commercialized areas and the troughs correlate with areas of lower population. IS values generally start high around the origins of the Ohio River at Pittsburgh, PA. IS values often rise again near river mile 200 at Parkersburgh, WV. IS values often rise again around river mile 250 to 300, where the Kanawha River enters into the Ohio River, where the Guyandotte River enters into the Ohio River and at Huntington, WV. Higher IS scores were also often found around river mile 500, near Cincinnati, OH.

It is important to note that IS values are determined relative to other sites in the river during the same year, so direct comparison of IS values at a site from year to year is not supported. However, the data (figures 4-7), do show that, in the high flow years of 2003 and 2003, IS scores were much more positive than they were in the low flow years

of 2005 and 2006. This observation supports the hypothesis that precipitation and runoff are important factors in reducing water quality.

The CCA outputs (Figures 7 to 9) consistently showed that the largest environmental vectors were residential and commercial areas. This suggests that residential and commercial areas played the greatest role in affecting bacterial communities and may play a role in the increase of ARB populations in the river. The linear regression models reinforce the conclusions from the CCA outputs and also suggest that residential and commercial areas played a role in the increase of ARB populations (Tables 2 to 6). This could have been due to the fact that commercial and residential areas generally were areas of land with a high percentage of impermeable surfaces. Impermeable surfaces reduce the amount of percolation of surface water into the ground, and increase the amount of runoff into surrounding stream. (14, 26) Impermeable surfaces can also allow the accumulation of waste and in large rain events and can be washed directly into the water system.

In addition to impermeable surfaces, there are large populations in residential and commercial areas. Because there are larger populations in these areas, there is a high potential for fecal pollution and ARB populations finding their way into the Ohio River. The poor water quality could have been due to the large populations sometimes through inadequate, or sometimes absent, sanitary sewage handling and treatment systems. Also, commonly used water treatment methods are poor at removing antibiotics from wastewater before release to surface waters. So in an area with a large population there is an increased likelihood of the release of antibiotics and therefore an increased potential for the selection of resistant strains in the environment.

However, when examining these highly populated areas, there are other factors to consider. Industrialized areas are usually also found in these highly populated areas. Wastes from these areas have the potential to get into the river without proper treatment and may result in poor water quality. Industrial wastes such as heavy metals are also thought to contribute to antibiotic resistance. Studies have found cross resistance between metal resistant populations and antibiotic resistant populations, meaning that the bacterial cell uses the same mechanism for eliminating heavy metal to eliminate antibiotics (7, 10, 24). In addition, commercialized areas also have a high percentage of impermeable surfaces that could limit the amount of percolation of rain water into the soil and, therefore, could limit the breakdown of waste in the soil by bacteria before going back into the streams. All these factors may contribute to the observed numbers of ARB in the river (12).

The Canoco outputs and linear regression plots both showed a weak but positive correlation between commercial, residential, and sometimes wetland land use types and total heterotrophic bacteria, total coliform bacteria, and ARB. Both analyses suggest that residential, commercial, and wetland areas could be the source of ARB in the river. Data indicating that wetlands can sometimes be categorized with commercial and residential land use was somewhat surprising. However, because wetland play a role as a filter in a biological system, a wetland could filter the water for ARB, allow those cells to survive in reduced sediments and then serve as a long term source, particularly during high flow regimes (13, 31, 34).

Canoco outputs and linear regression plots showed a negative relationship between ARB and forested areas or agricultural areas. These data resulted in a surprising

negative correlation between agriculture, ARB, and coliform bacteria. It might be expected that agriculture can be a major contributor to ARB abundance because antibiotics are widely used in the different types of farming, livestock, and row crop type of farming (1, 17, 29, 30). Animal agriculture contributes to the amount of coliform bacteria introduced into the river, and, because antibiotics are used in the practice of livestock agriculture, both the antibiotics and the resistant cells can be introduced in the environment via animal feces (1, 17, 29, 30). Row crop farming can be expected to play a much smaller role in ARB introduction, except in those cases where animal feces are used as fertilizer.

Unfortunately, the land use files obtained from USGS used in this project did not distinguish between different types of farming. As a result, data on crop farming and livestock farming were reported together. Because these two categories were lumped together it is difficult to say exactly what role farm land plays on the impact of antibiotic resistant populations. Anecdotally, the vast majority of farmland use on the river watershed directly operable during sampling was relatively large crop lands. This type of agriculture may explain the correlations observed in this study.

The dynamic relationships between bacterial populations and environmental variables seen in this study may have been due to the amount of precipitation in the Ohio River basin. Due to the lack of rain during certain sampling years, runoff introduced into the river was limited. Those years tended to yield more low impact scores probably due to reduced runoff from impermeable surfaces. Sites with high impact scores in low flow years are likely being influenced by traceable point sources (i.e. not runoff).

Figures 10 and 11 suggest a trend between bacterial populations in the river and flow rate, which is a reflection of basin wide precipitation. The graphs suggest that, as the amount of rain increased, so did the amount of bacterial populations that were found in the river. The graphs also showed that, as the amount of rain decreased, so did the amount of bacterial populations in the river. This was expected because the increased amount of rain would increase the amount of runoff being introduced into the river, therefore limiting the environment's ability to filter water before going in the river. Figures 10 and 11 show a strong positive relationship between flow and bacterial populations; however, two of three populations compared did not have a statistically significant relationship. The only significant relationship seen was between flow and fecal coliform bacteria. The lack of significance between other bacterial population and flow may be due to the small amount of data points. Because there were six sampling seasons, there were only six possible data points available for this comparison, and not all bacterial population were enumerated in all six years. It seems clear from a preponderance of our data that precipitation has a major influence on the abundance of bacterial population in surface water. Long-term studies with fixed target populations may be needed before statistically valid relationships become apparent.

The Canoco outputs (Figures 7 to 9) show that total coliforms and total cultivable bacteria would not be good indicators of pollution as they did not deviate far from the origin, and thus do not appear to be influenced by environmental variables. However, the Canoco outputs show that ARB populations are influenced by different environmental factors and may be more useful as indicator of water quality. Similar results were found in studies conducted by Goni-Urriza et al (14) and Boon et.al (6) where they sampled

water around highly urbanized and populated areas and showed that ARB populations were more abundant in highly populated areas and can be a good indicator of the impact of land use on water quality.

Due to the emergence of new technologies, different sampling techniques were used from 2001 to 2004 than were used in sampling 2005 and 2006. Sampling seasons 2001 to 2004 were done using R2A agar and selected antibiotics. This method recovered a diverse group of bacteria that were found in the river but generated less significant relationships with land use type. The coliert and quanti-tray methods used in 2005 and 2006 selected for only coliform type bacteria but produced more significant relationships between the bacterial communities and environmental variables. This suggests that antibiotic resistant coliform (ARC) populations were more influenced by the environmental factors than other heterotrophic bacteria. This may mean that ARC populations are more sensitive indicators of environmental impact. This would also suggest that most antibiotic resistant populations are found in areas of large populations where people and animals are producing coliform type bacteria. This was also seen in the study conducted by Boon and Cattnach in Victoria, Australia, in 1999 (6), which showed that antibiotic resistance bacteria isolated from rivers and reservoirs around the urbanized city of Melbourne, Australia were impacted by sewage treatment facilities. Another study conducted by Goni-Urriza et al. in 2000 (14) showed that urban effluent from the city of Pamplona, Spain, also had an impact on ARB populations. This study showed that antibiotic resistance was higher in highly urbanized areas as compared to rural areas.

## Conclusions

This study showed that ARB populations in the Ohio River were influenced by the type of adjacent land use. However, the most prominent factor connecting land use to ARB abundance may be the amount of precipitation seen in the watershed. This study also showed that ARB population can be a useful indicator of water quality.

Even though the Canoco outputs were very sensitive and seemed to show that ARB populations were influenced by environmental variable, the type of influence was not stable from year to year. Canoco can be a useful tool to formulate a testable hypothesis but should not be interpreted as revealing cause and effect relationships.

Unlike some other methods used to measure water or environmental quality, the methods used here required no specialized training in taxonomy. The data were also collected in a relatively short time, within a few weeks, in each of the sampling seasons. To be able to identify the flora and fauna needed to perform a survey on the health of a stream would take years of specialized training. This specialization would be often outsourced, requiring lengthy time for data analysis and in turn a lot of money. However, the methods described here can be used to monitor large rivers because they are rapid, relatively inexpensive and can be performed by personnel trained in a limited time.

## Further Directions

Future analyses should include new land use/land cover data. The NLCD in this study was from the 1990s and land use has changed since the creation of the land use/land cover rasters and sampling times. Also a larger area of land use area should be taken into consideration to normalize the amount of mixing and amount of water

introduced into the river from its tributaries. More studies should also be conducted to determine the usefulness of ARB populations as indicators of water quality. More studies should also be conducted on how water quality in a river is affected by precipitation. A lack of precipitation may decrease ARB abundance in the river simply by removing the connection between land use and river (i.e. runoff). Therefore, research is needed to determine how to reduce contamination in times of high precipitation. More studies should also be done comparing the two different methods of sampling used in this study. Antibiotic resistant heterotrophic bacteria and antibiotic resistant coliform bacteria should be further compared to determine which one is a better measure of environmental quality.

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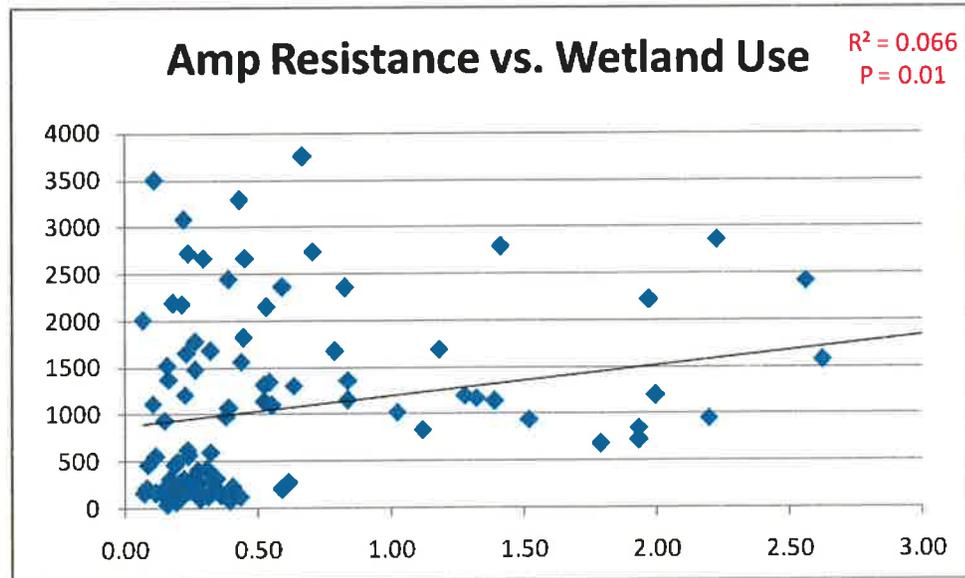
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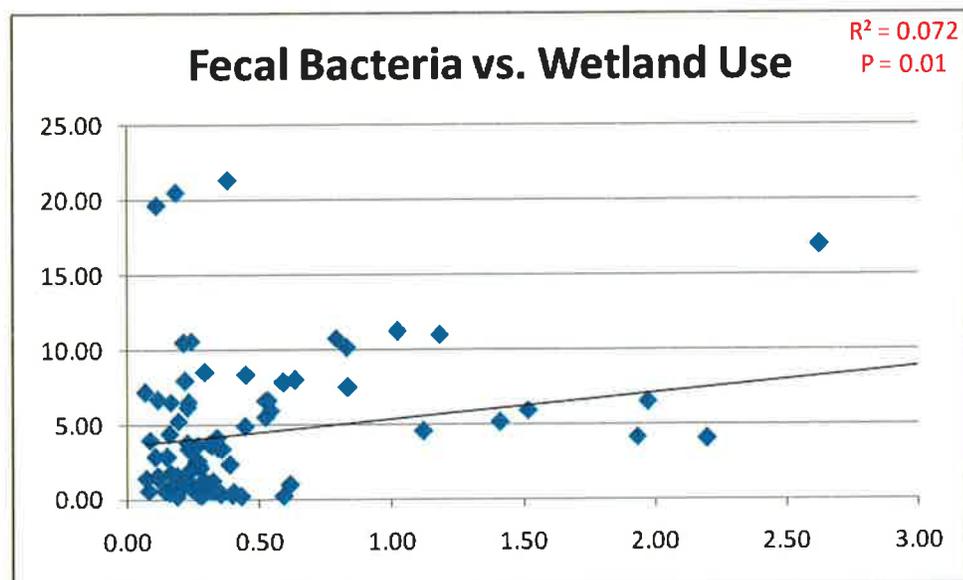
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**APPENDIX A – Significant Land Use/Microbe Relationships**  
Season 2001

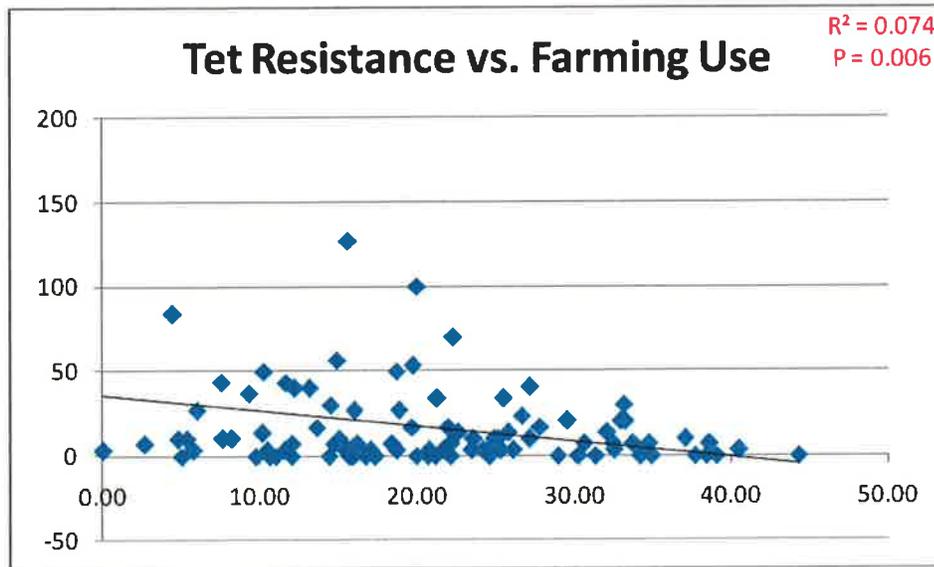


**Figure 12.** This figure shows a significant relationship between ampicillin resistant bacteria and wetland area. The y axis shows the colony forming units (CFU) of ampicillin resistant bacteria per ml of river water and the x axis shows the percentage of wetlands in the designated zone of influence.

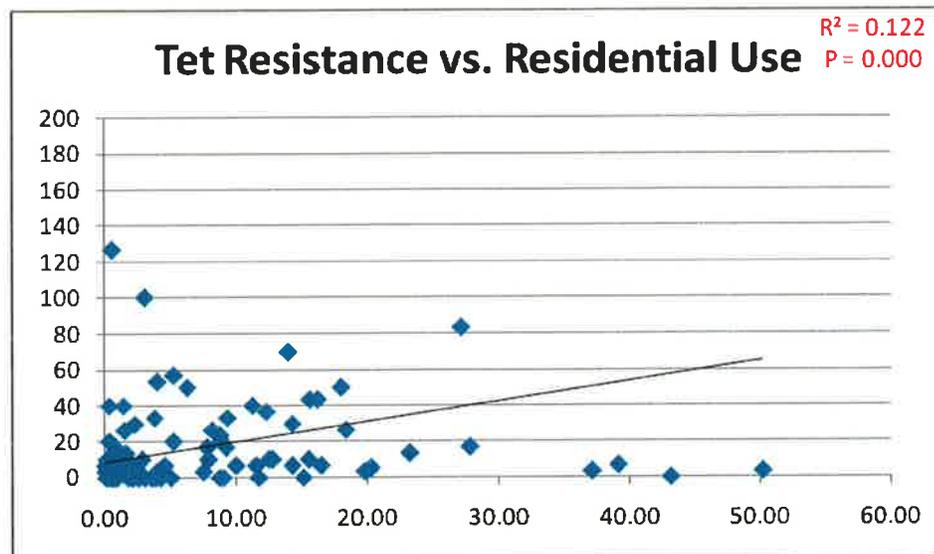


**Figure 13.** This figure shows a significant relationship between fecal coliforms and wetland area. The y axis shows the CFU of fecal coliform bacteria per ml of river water and the x axis shows the percentage of wetlands in the designated zone of influence.

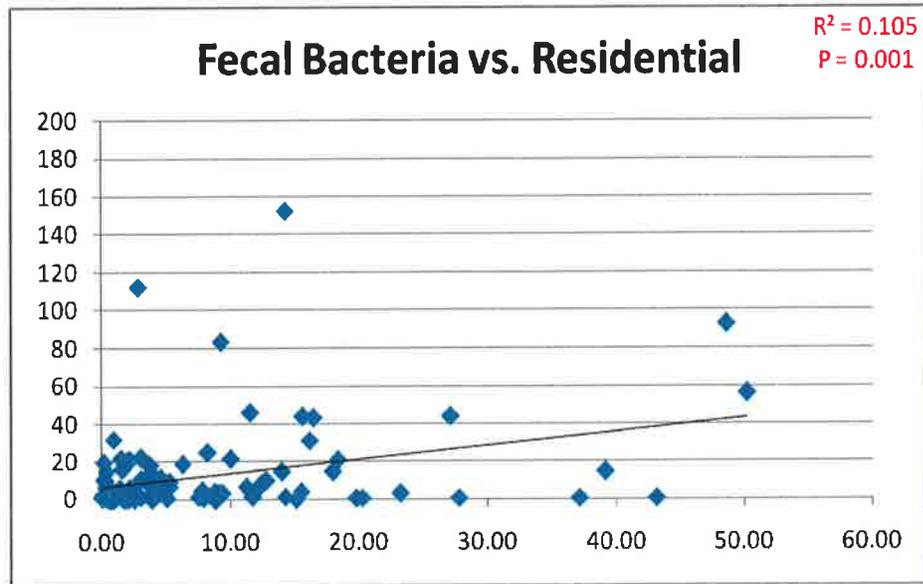
Season 2002



**Figure 14.** This figure shows a significant inverse relationship between tetracycline resistant bacteria and farm area. The y axis shows the CFU of tetracycline resistant bacteria per ml of river water and the x axis shows the percentage of farming in the designated zone of influence.

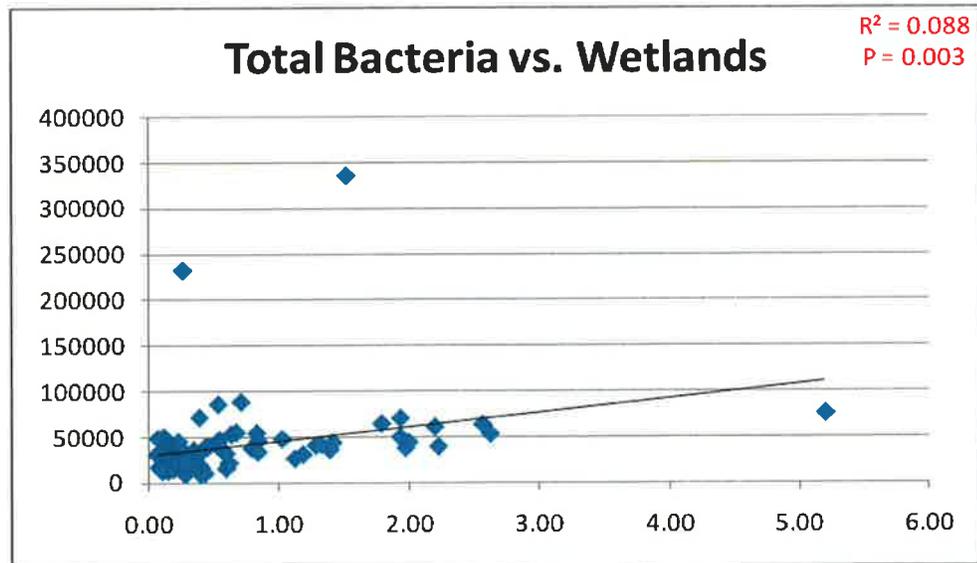


**Figure 15.** This figure shows a significant relationship between tetracycline resistant bacteria and residential land use. The y axis shows the CFU of tetracycline resistant bacteria per ml of river water and the x axis shows the percentage of residential land use in the designated zone of influence.

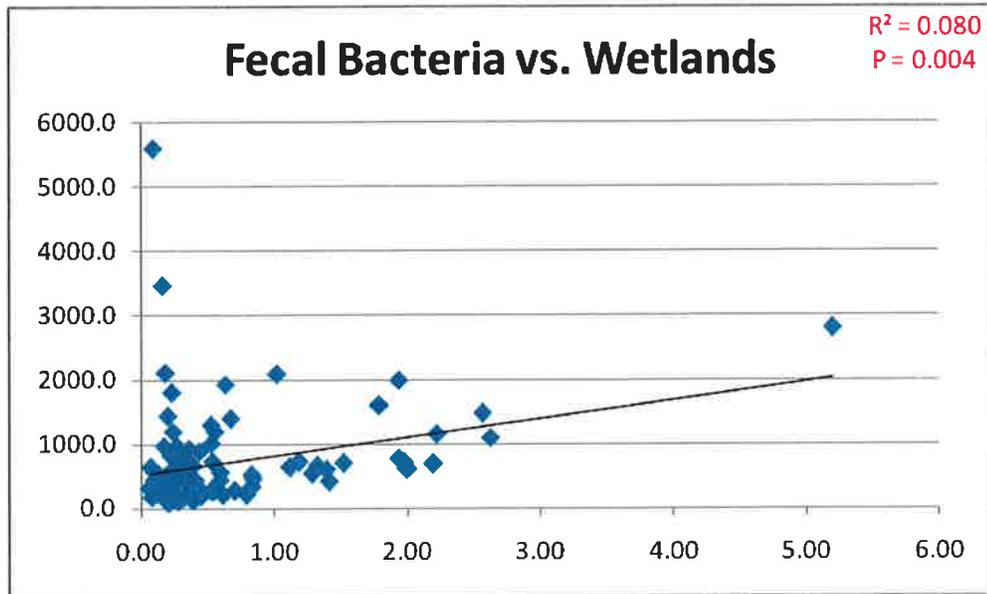


**Figure 16.** This figure shows a significant relationship between fecal coliform bacteria and residential land use. The y axis shows the CFU of fecal coliform bacteria per ml of river water and the x axis shows the percentage of residential land use in the designated zone of influence.

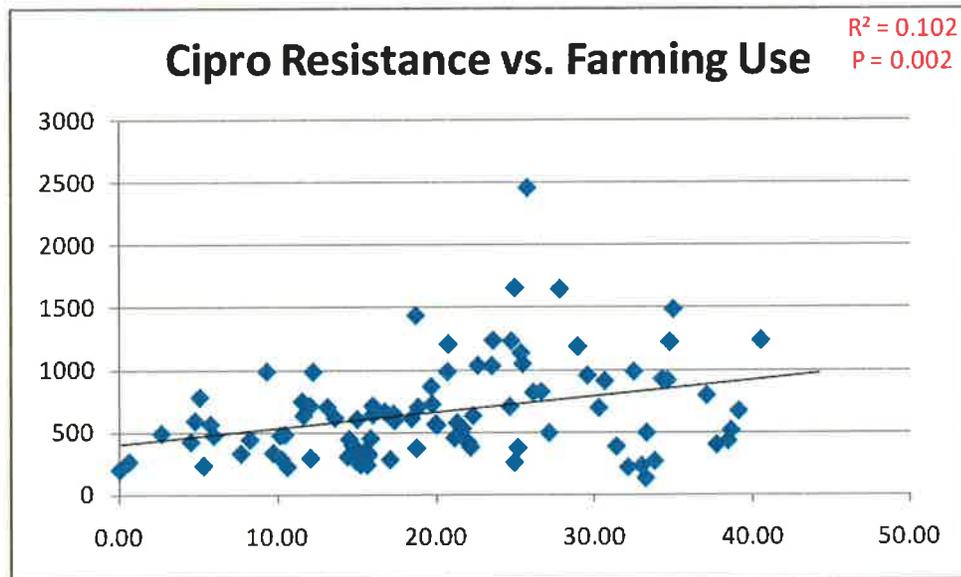
Season 2003



**Figure 17.** This figure shows a significant relationship between total cultivable bacteria and wetlands. The y axis shows the CFU of total cultivable bacteria per ml of river water and the x axis shows the percentage of wetlands in the designated zone of influence.

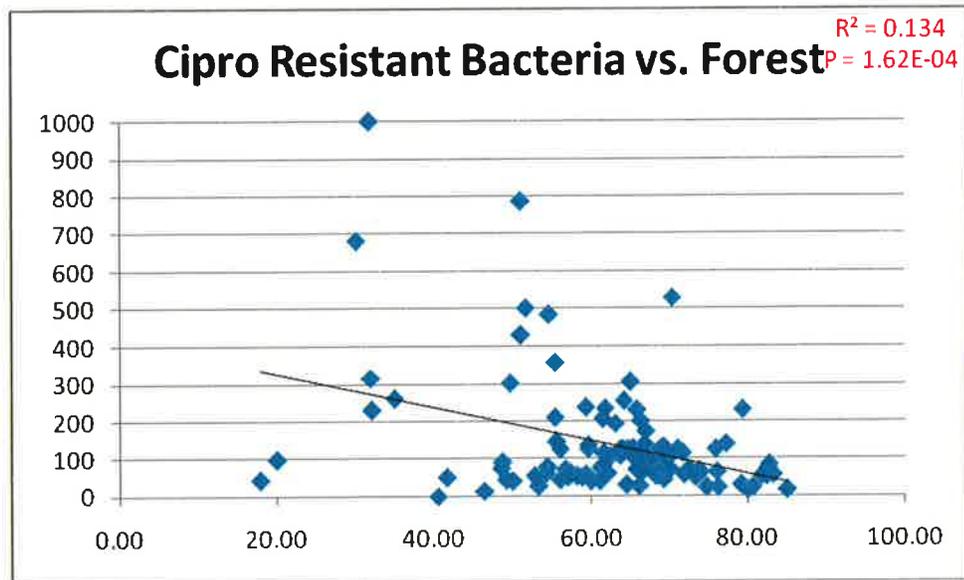


**Figure 18.** This figure shows a significant relationship between fecal coliform bacteria and wetland area. The y axis shows the CFU of fecal coliforms per ml of river water and the x axis shows the percentage of wetlands in the designated zone of influence.

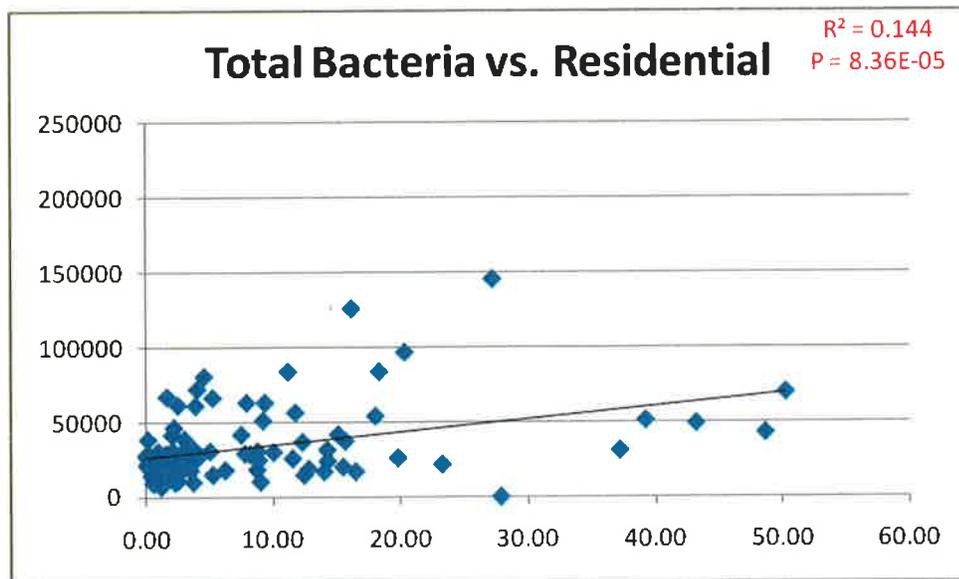


**Figure 19.** This figure shows a significant relationship between cirpofloxacin resistant bacteria and farming. The y axis shows the CFU of ciprofloxacin resistant bacteria per ml of river water and the x axis shows the percentage of farm use in the designated zone of influence.

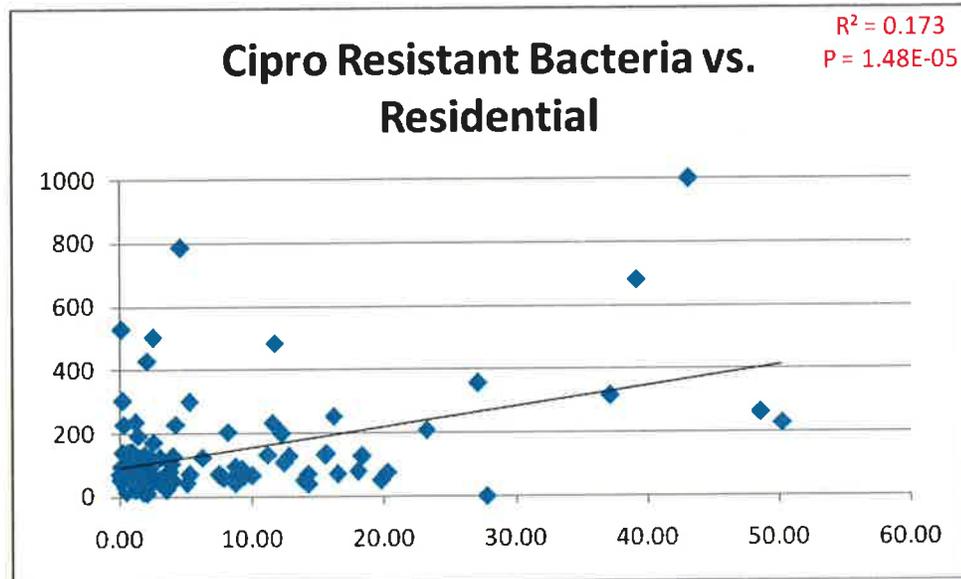
Season 2004



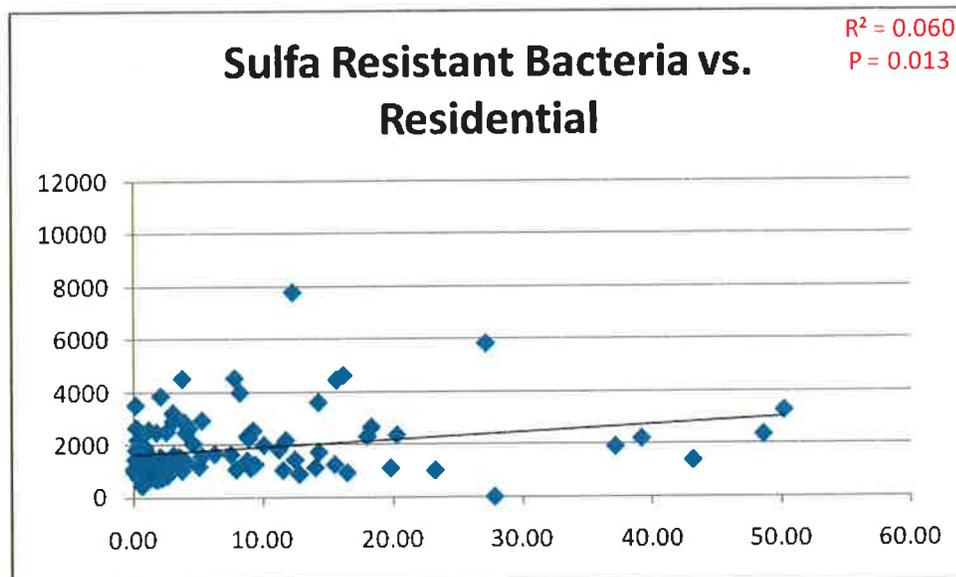
**Figure 20.** This figure shows a significant inverse relationship between ciprofloxacin resistant bacteria and forested area. The y axis shows the CFU of ciprofloxacin resistant bacteria per ml of river water and the x axis shows the percentage of forested land in the designated zone of influence.



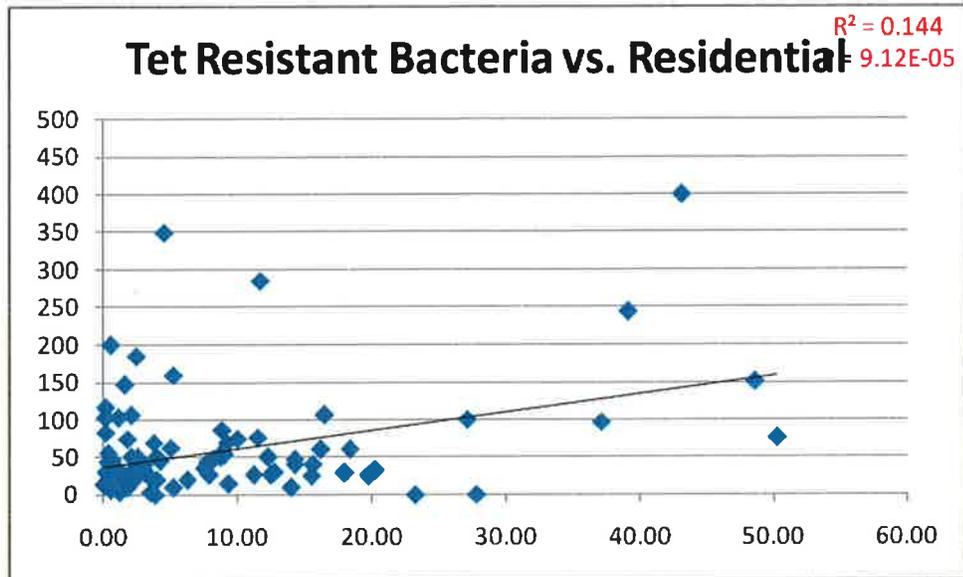
**Figure 21.** This figure shows a significant relationship between total cultivable bacteria and residential land use. The y axis shows the CFU of total cultivable bacteria per ml of river water and the x axis shows the percentage of residential land use in the designated zone of influence.



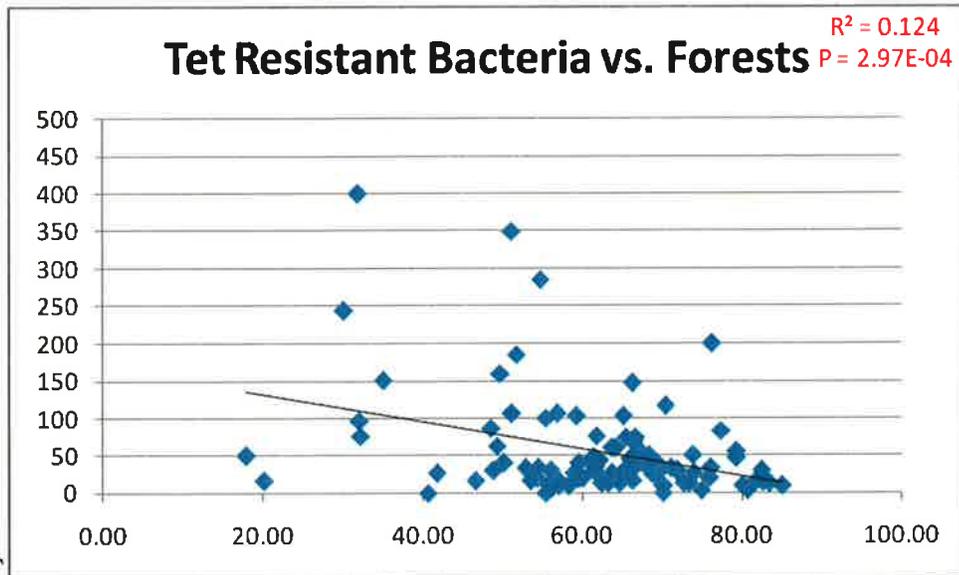
**Figure 22.** This figure shows a significant relationship between ciprofloxacin resistant bacteria and residential land use. The y axis shows the CFU of ciprofloxacin resistant bacteria per ml of river water and the x axis shows the percentage of residential land use in the designated zone of influence.



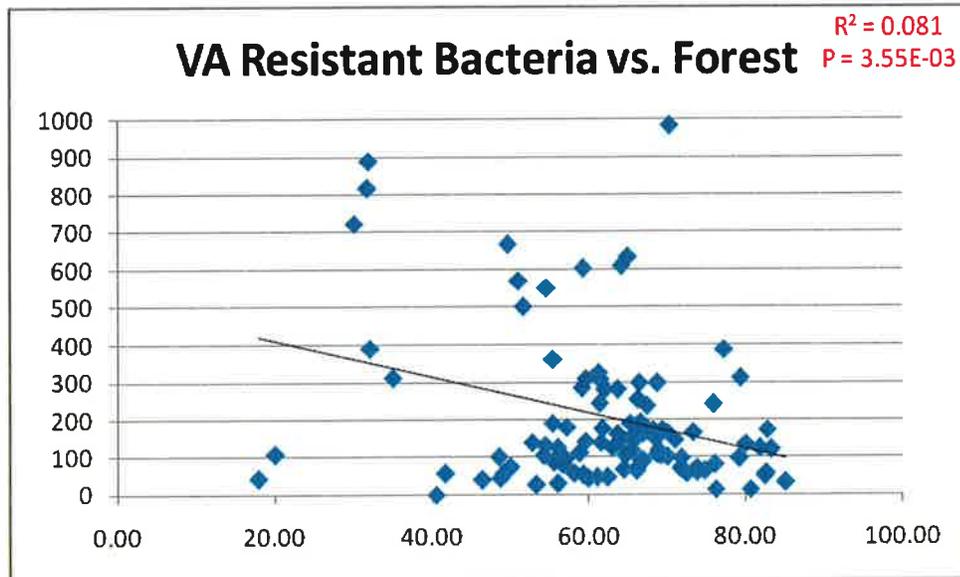
**Figure 23.** This figure shows a significant relationship between sulfamethazole resistant bacteria and residential land use. The y axis shows the CFU of sulfamethazole resistant bacteria per ml of river water and the x axis shows the percentage of residential land use in the designated zone of influence.



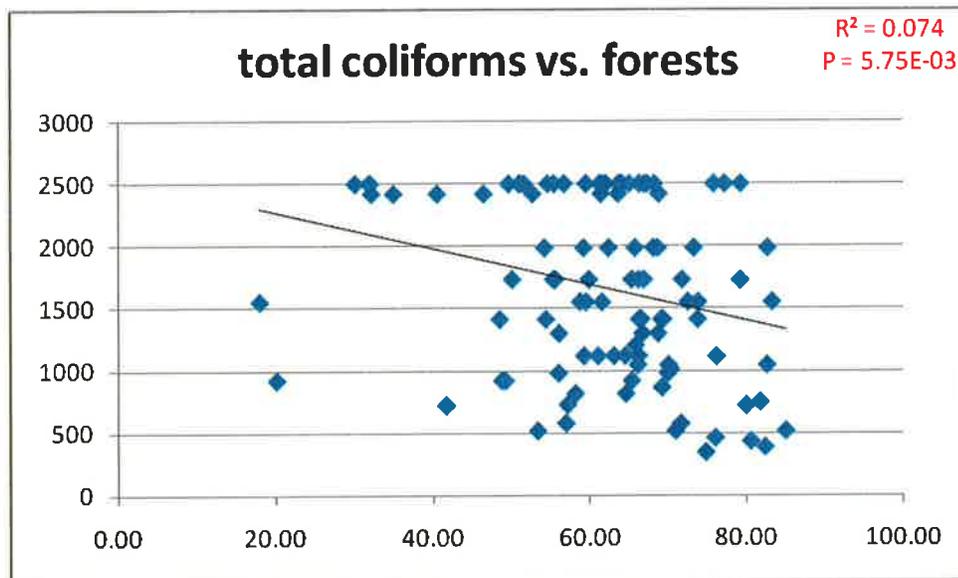
**Figure 24.** This figure shows a significant relationship between tetracycline resistant bacteria and residential land use. The y axis shows the CFU of tetracycline resistant bacteria per ml of river water and the x axis shows the percentage of residential land use.



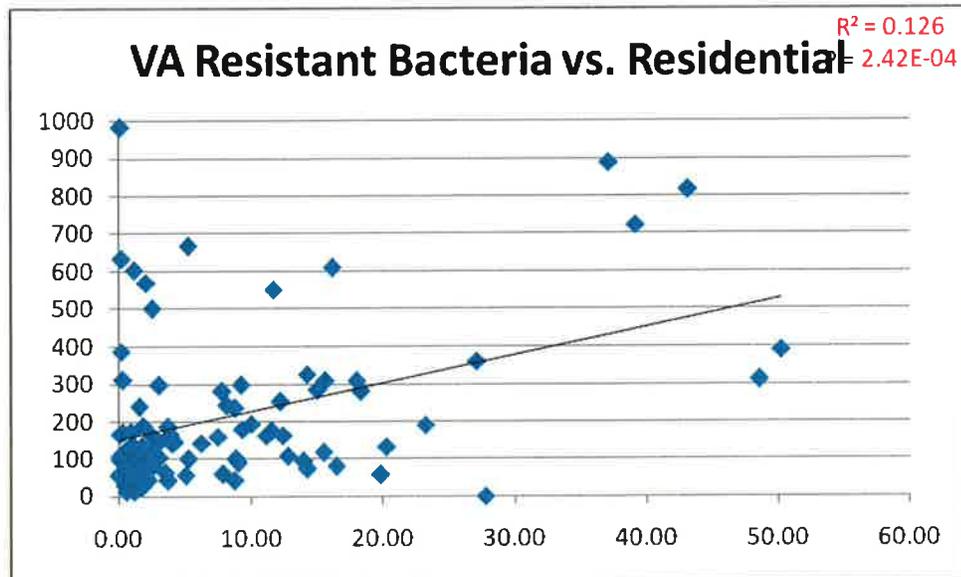
**Figure 25.** This figure shows a significant inverse relationship between tetracycline resistant bacteria and forest area. The y axis shows the CFU of tetracycline resistant bacteria per ml of river water and the x axis shows the percentage of forested land in the designated zone of influence.



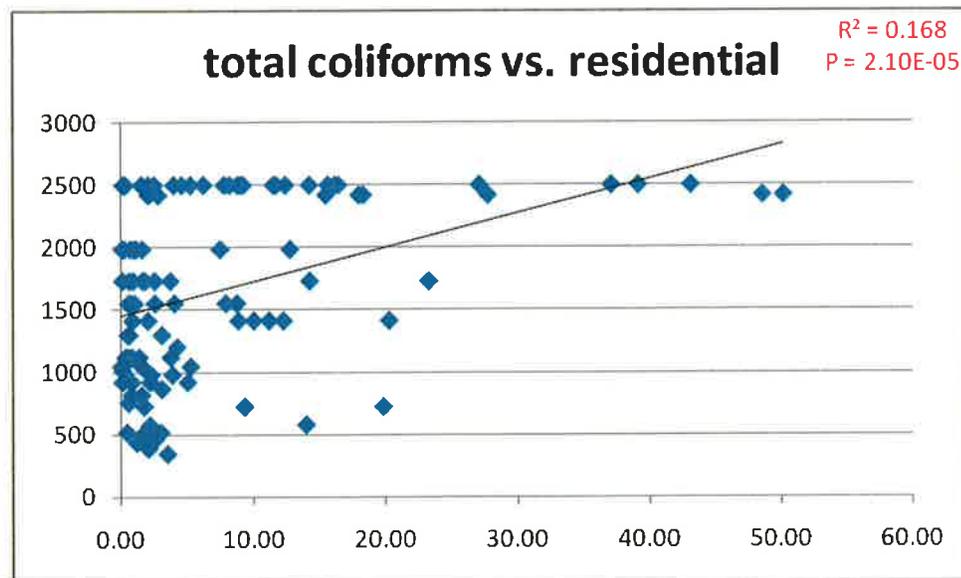
**Figure 26.** This figure shows a significant inverse relationship between virginiamycin resistant bacteria and forest area. The y axis shows the CFU of virginiamycin resistant bacteria per ml of river water and the x axis shows the percentage of forested land in the designated zone of influence.



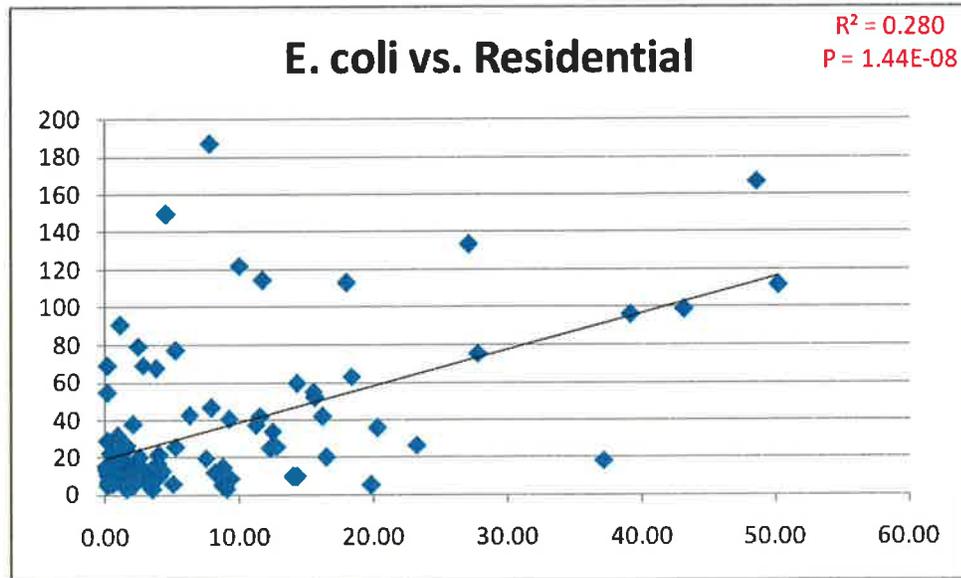
**Figure 27.** This figure shows a significant inverse relationship between total coliform bacteria and forest area. The y axis shows the Most Probable Number (MPN) of total coliform bacteria per 100 ml of river water and the x axis shows the percentage of forested land in the designated zone of influence.



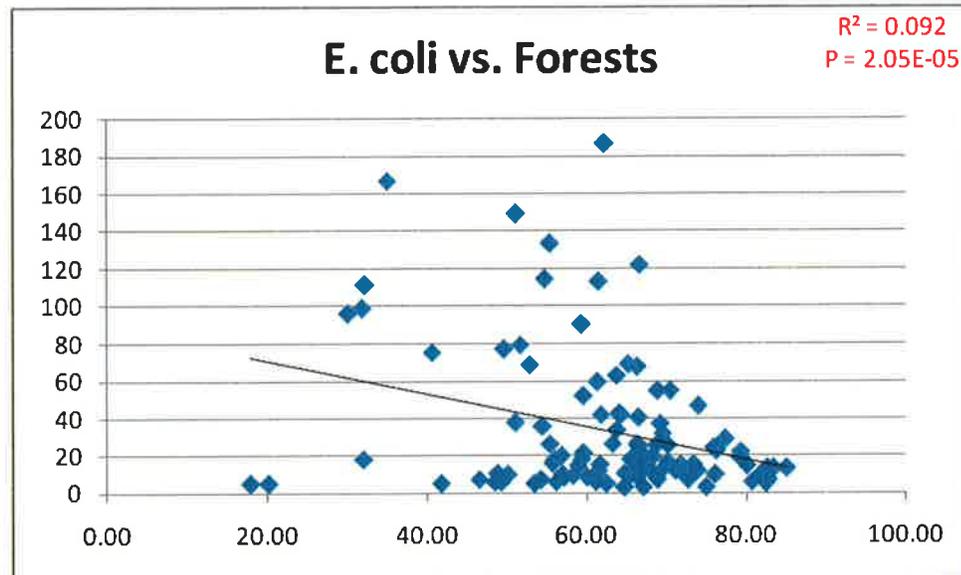
**Figure 28.** This figure shows a significant relationship between virginiamycin resistant bacteria and residential land use. The y axis shows the CFU of virginiamycin resistant bacteria per ml of river water and the x axis shows the percentage of residential land use in the designated zone of influence.



**Figure 29.** This figure shows a significant relationship between total coliform bacteria and residential land use. The y axis shows the MPN of total coliform bacteria per 100 ml of river water and the x axis shows the percentage of residential land use in the designated zone of influence.

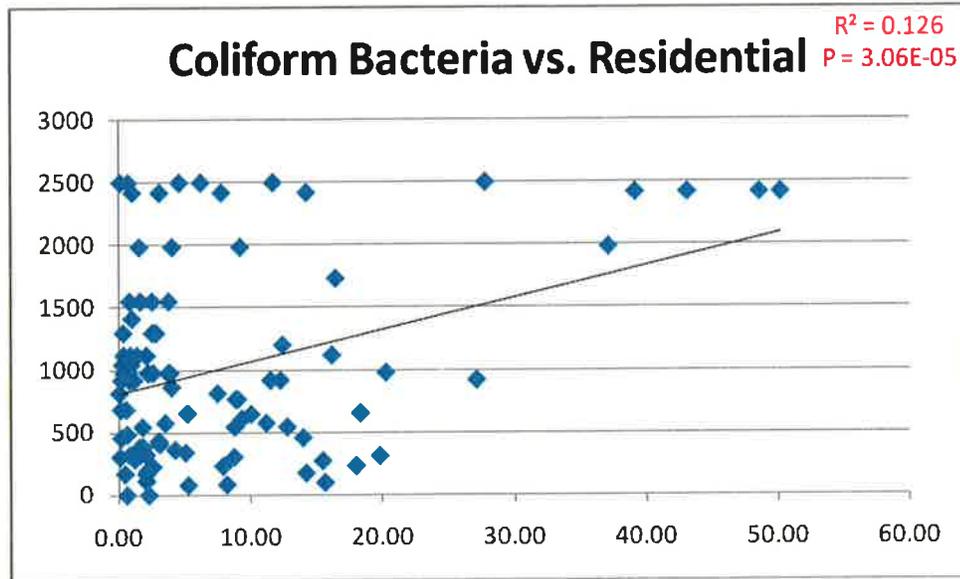


**Figure 30.** This figure shows a significant relationship between *E. coli* and residential land use. The y axis shows the MPN of *E. coli* per 100 ml of river water and the x axis shows the percentage of residential land use in the designated zone of influence.

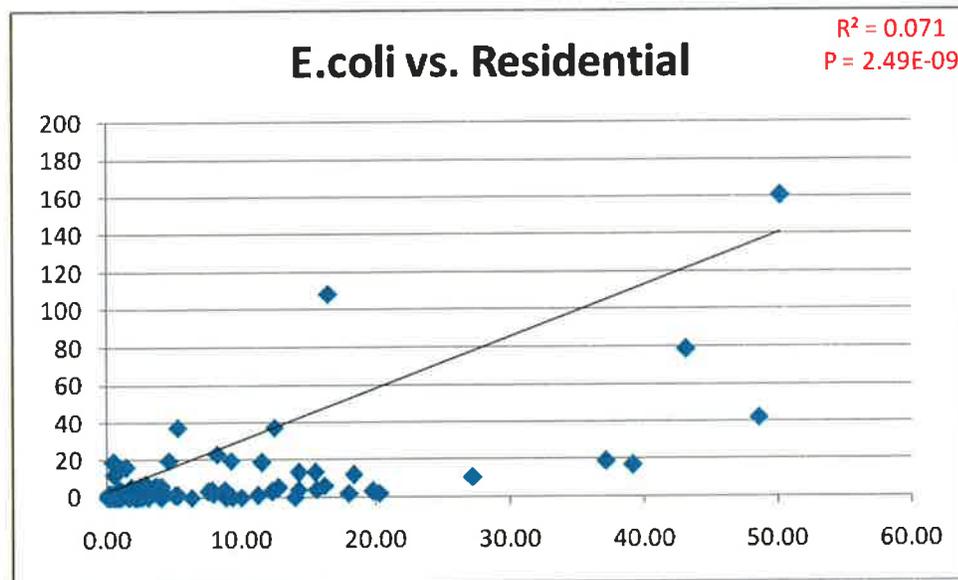


**Figure 31.** This figure shows a significant inverse relationship between *E. coli* and forest area. The y axis shows the MPN of *E. coli* per 100 ml of river water and the x axis shows the percentage of forested land in the designated zone of influence.

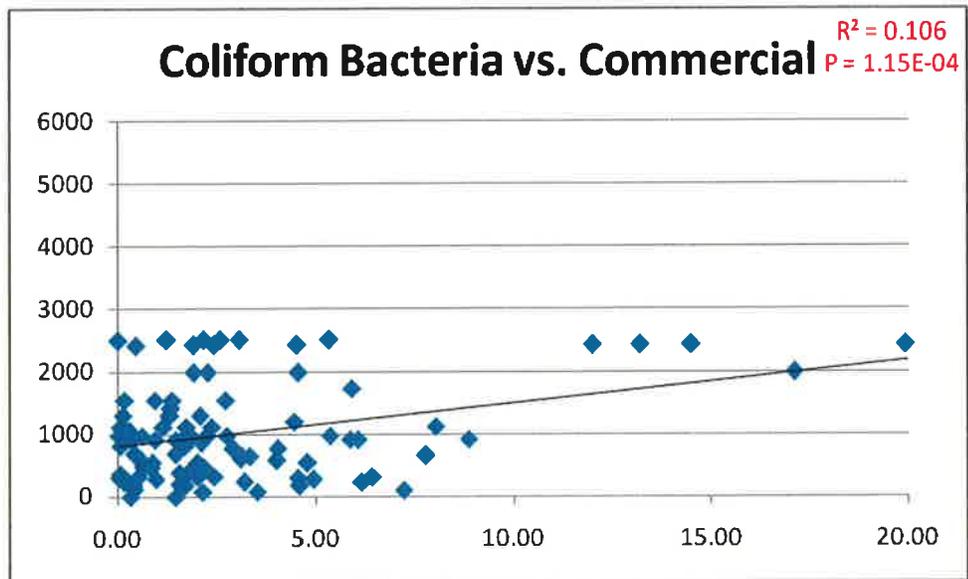
Season 2005



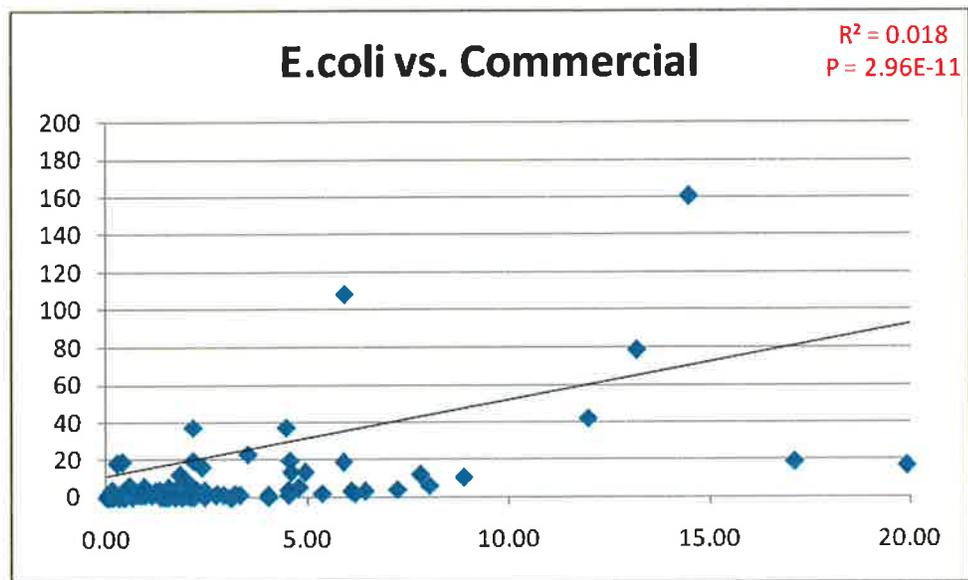
**Figure 32.** This figure shows a significant relationship between coliform bacteria and residential land use. The y axis shows the MPN of total coliform bacteria per 100 ml of river water, and the x axis shows the percentage of residential land use in the designated zone of influence.



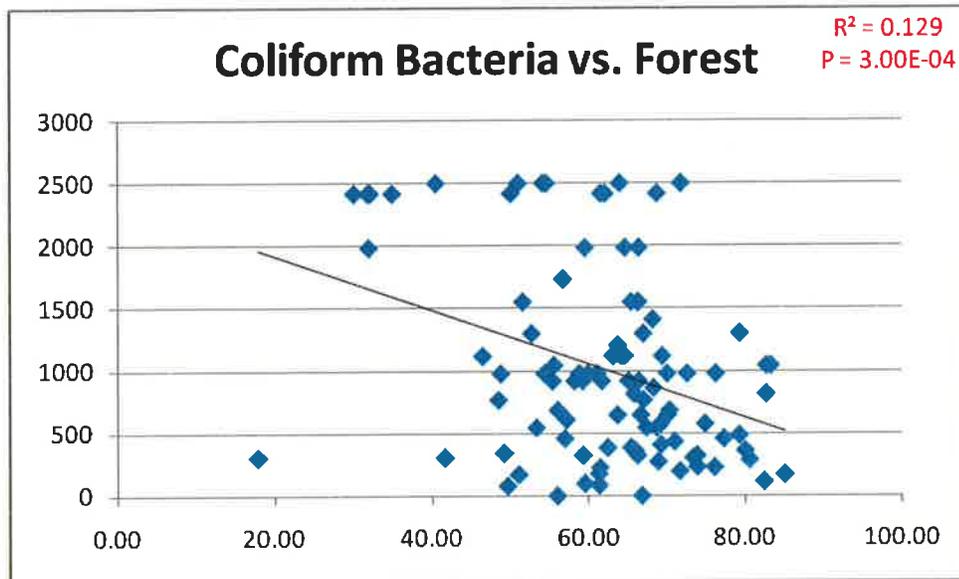
**Figure 33.** This figure shows a significant relationship between *E. coli* and residential land use. The y axis shows the MPN of *E. coli* per 100 ml of river water and the x axis shows the percentage of residential land use in the designated zone of influence.



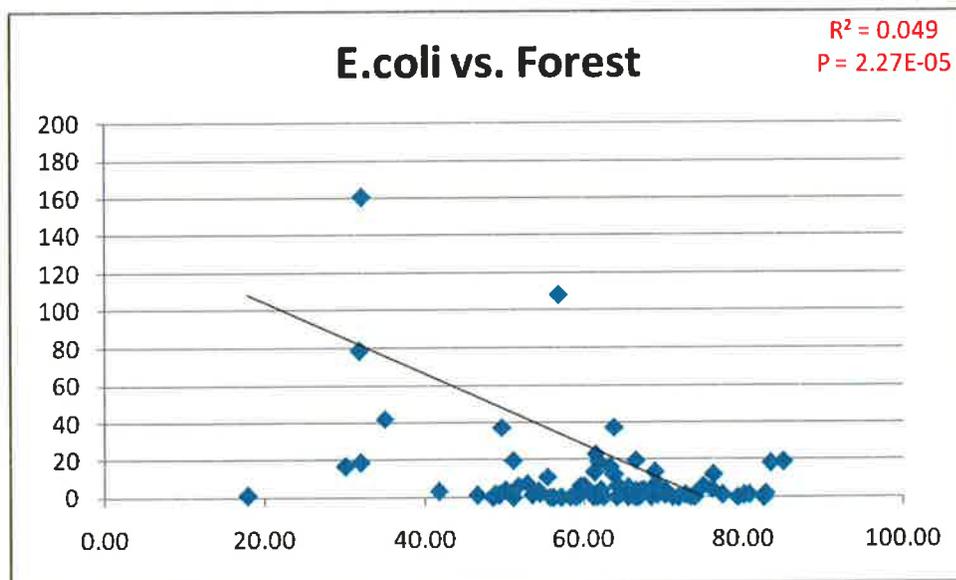
**Figure 34.** This figure shows a significant relationship between total coliform bacteria and commercial land use. The y axis shows the MPN of total coliform bacteria per 100 ml of river water, and the x axis shows the percentage of commercial land use in the designated zone of influence.



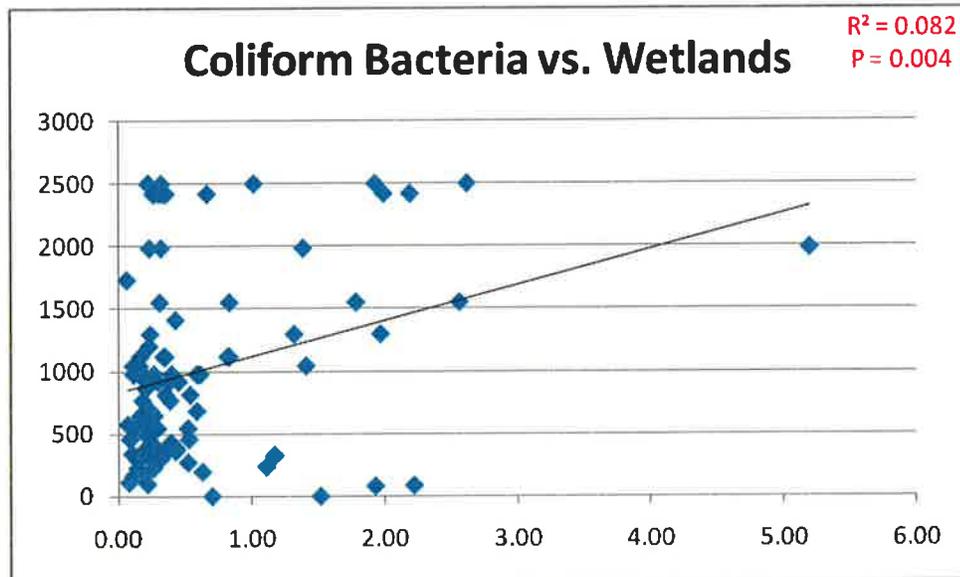
**Figure 35.** This figure shows a significant relationship between *E. coli* and commercial land use. The y axis shows the MPN of *E. coli* per 100 ml of river water and the x axis shows the percentage of commercial land use in the designated zone of influence.



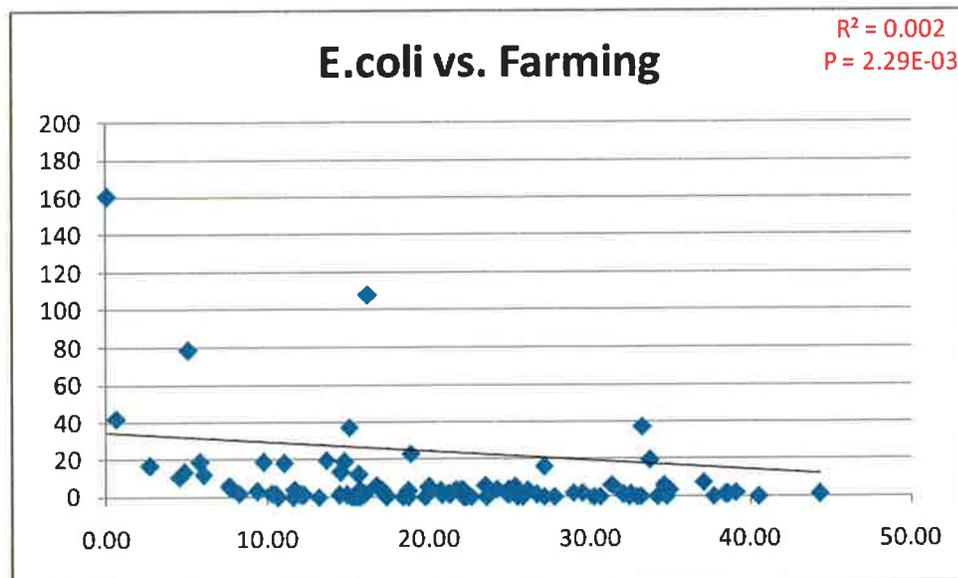
**Figure 36.** This figure shows a significant inverse relationship between total coliform bacteria and forest area. The y axis shows the MPN of total coliforms per 100 ml of river water, and the x axis shows the percentage of forested land in the designated zone of influence.



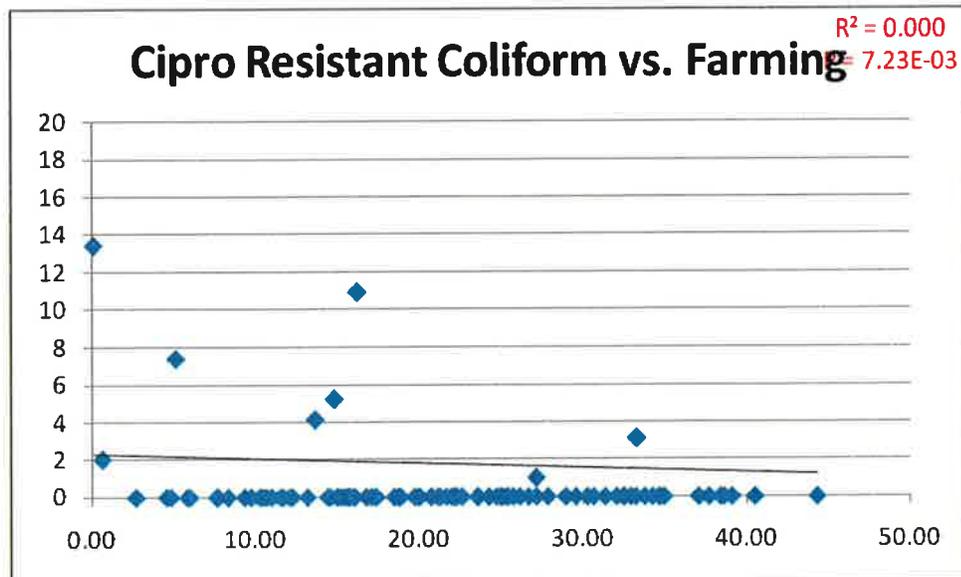
**Figure 37.** This figure shows a significant inverse relationship between *E. coli* and forest area. The y axis shows the MPN of *E. coli* per 100 ml of river water and the x axis shows the percentage of forested land in the designated zone of influence.



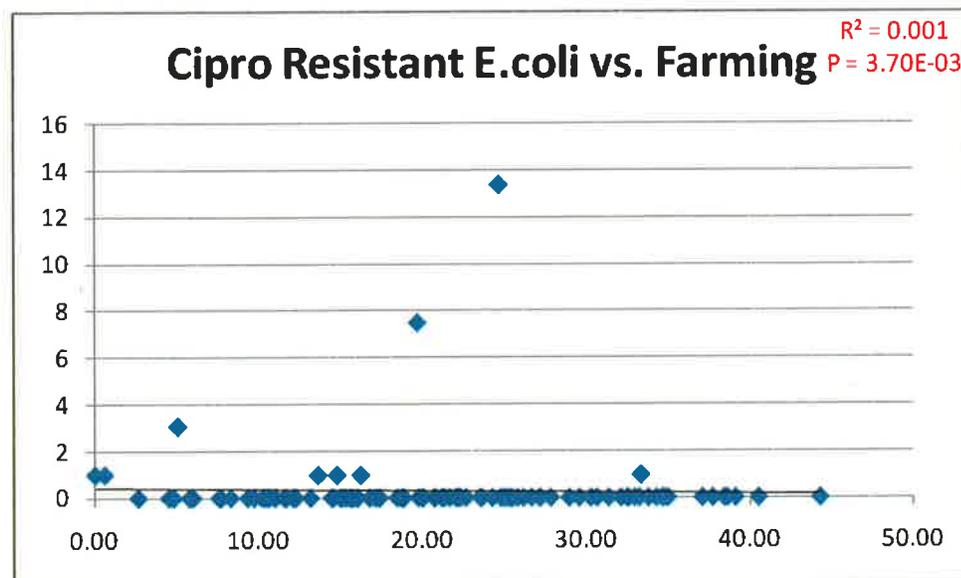
**Figure 38.** This figure shows a significant relationship between total coliform bacteria and wetlands. The y axis shows the MPN of total coliform bacteria per 100 ml of river water, and the x axis shows the percentage of wetlands in the designated zone of influence.



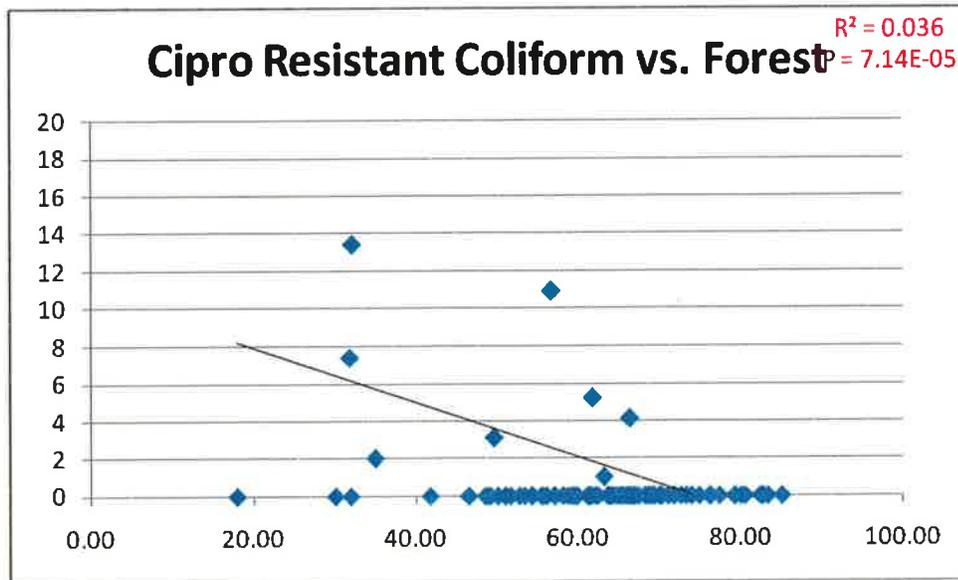
**Figure 39.** This figure shows a significant inverse relationship between *E. coli* and farming. The y axis shows the MPN of *E. coli* per 100 ml of river water and the x axis shows the percentage of farm land in the designated zone of influence.



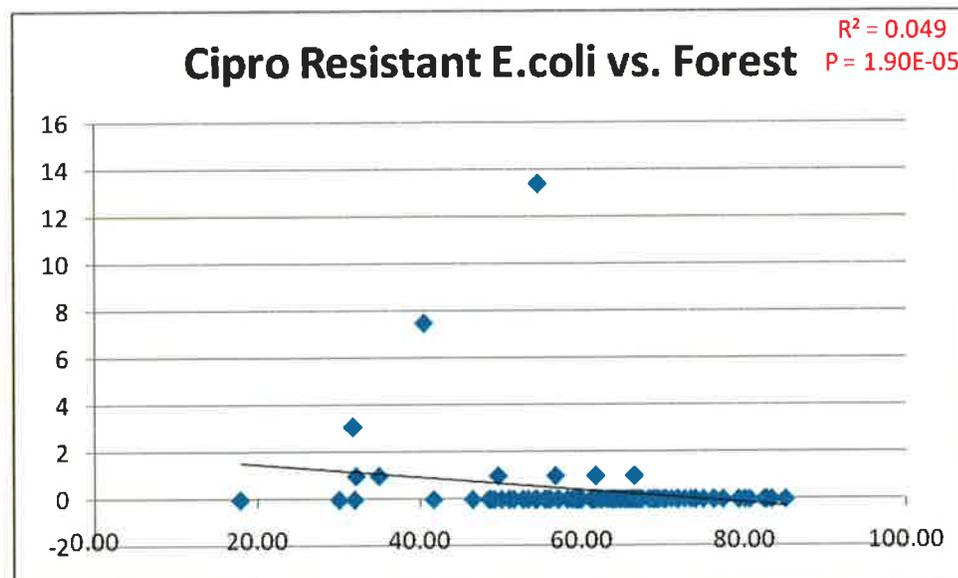
**Figure 40.** This figure shows a significant inverse relationship between ciprofloxacin resistant coliform bacteria and farming. The y axis shows the MPN of ciprofloxacin resistance coliforms per 100 ml of river water and the x axis shows the percentage of farmland of river waterand in the designated zone of influence.



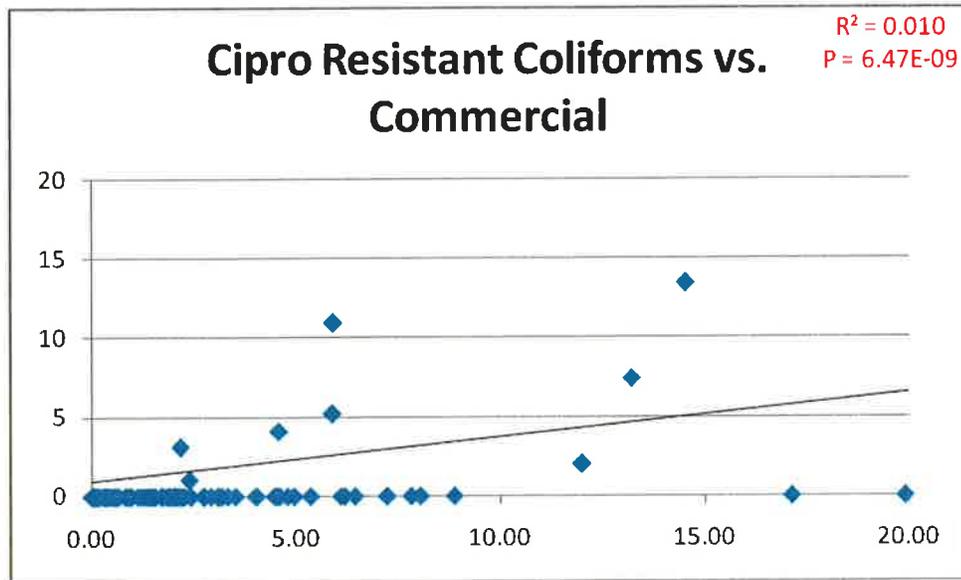
**Figure 41.** This figure shows a significant inverse relationship between ciprofloxacin resistant *E. coli* and forest area. The y axis shows the MPN of resistant *E. coli* per 100 ml of river water and the x axis shows the percentage of farmland of river waterand in the designated zone of influence.



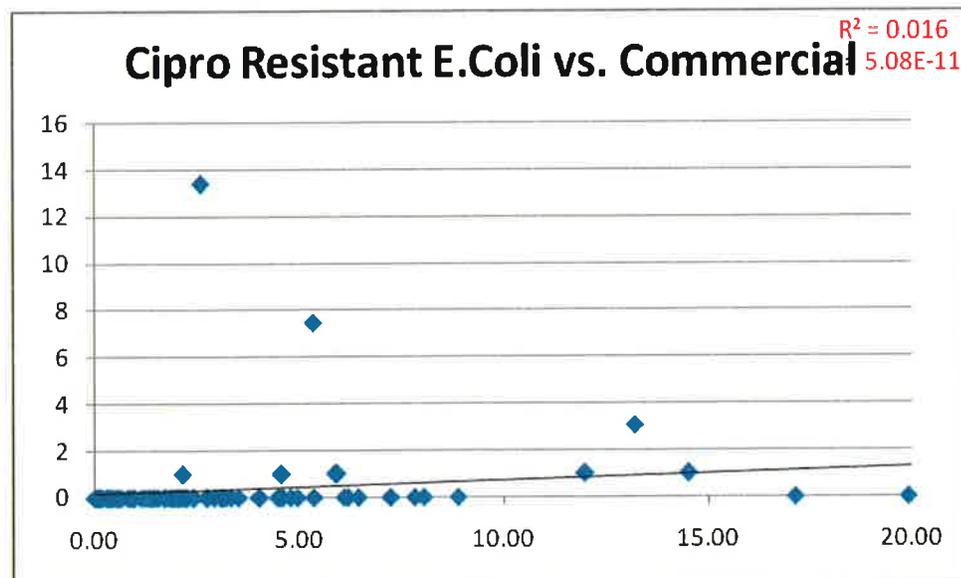
**Figure 42.** This figure shows a significant inverse relationship between ciprofloxacin resistant total coliform bacteria and forest area. The y axis shows the MPN of resistant coliforms per 100 ml of river water, and the x axis shows the percentage of forested area in the designated zone of influence.



**Figure 43.** This figure shows a significant inverse relationship between ciprofloxacin resistant *E. coli* and forest area. The y axis shows the MPN of resistant *E. coli* per 100 ml of river water and the x axis shows the percentage of forested land in the designated zone of influence.

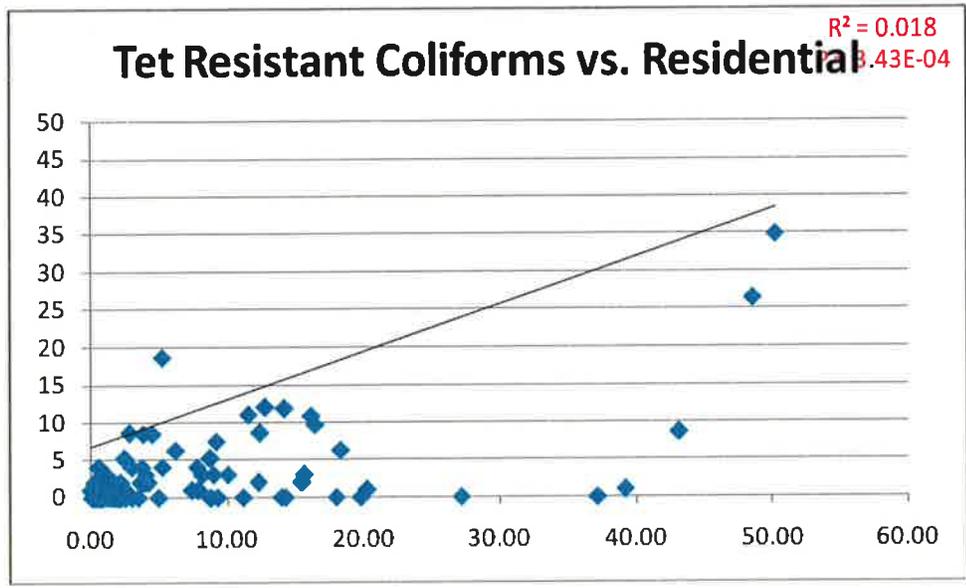


**Figure 44.** This figure shows a significant relationship between ciprofloxacin resistant total coliform bacteria and commercial land use. The y axis shows the MPN of ciprofloxacin resistant total coliforms per 100 ml of river water, and the x axis shows the percentage of commercial land use in the designated zone of influence.

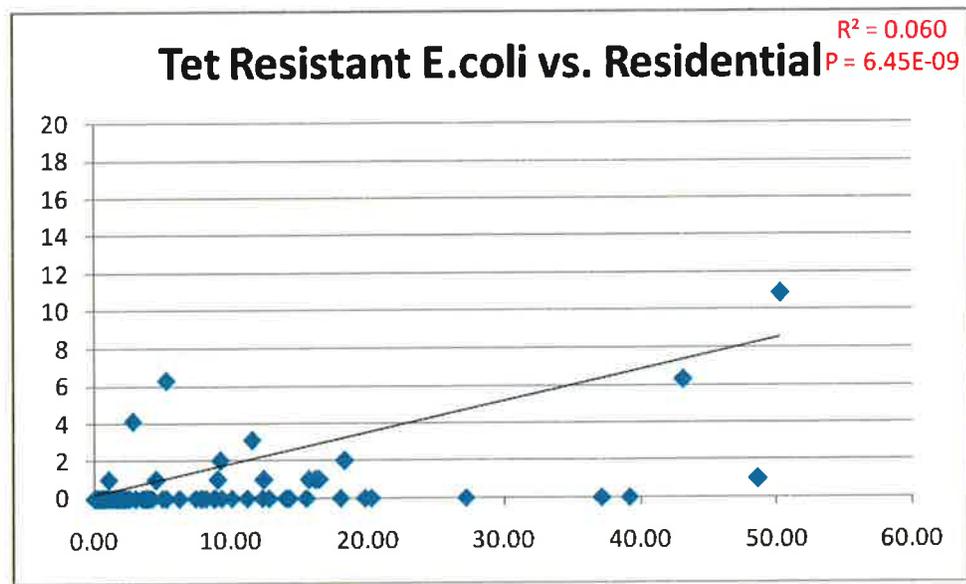


**Figure 45.** This figure shows a significant relationship between ciprofloxacin resistant *E. coli* and commercial land use. The y axis shows the MPN of ciprofloxacin resistant *E. coli* per 100 ml of river water and the x axis shows the percentage of commercial land use in the designated zone of influence.

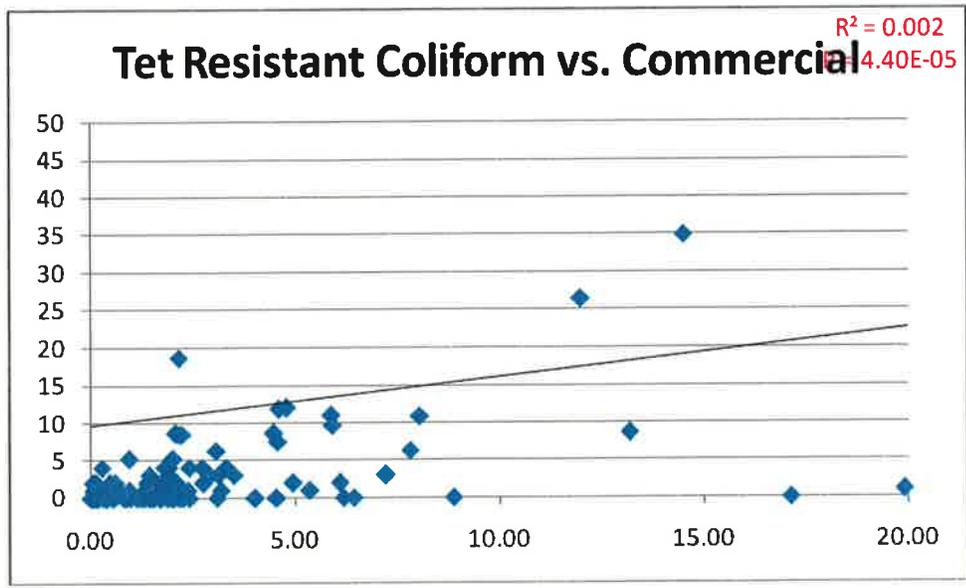




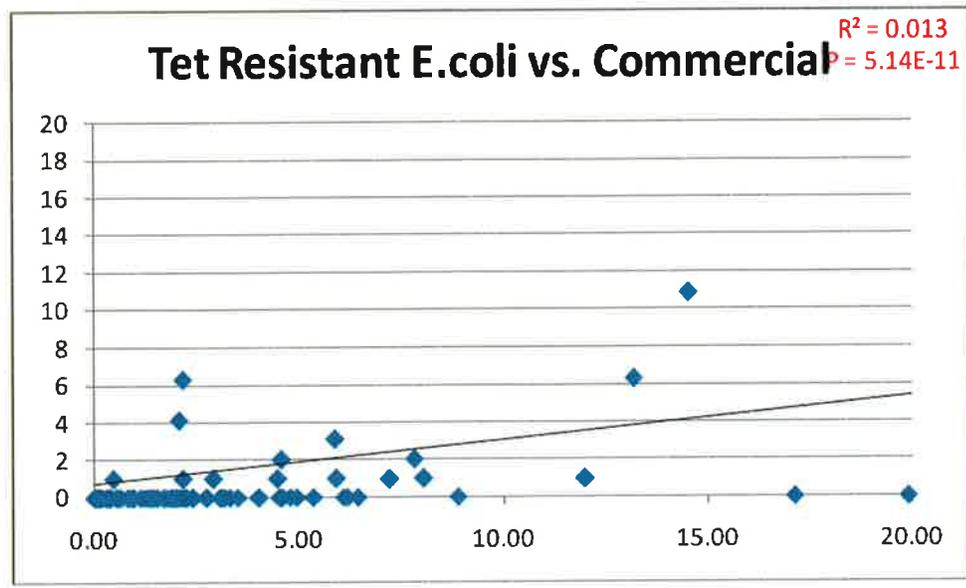
**Figure 48.** This figure shows a significant relationship between tetracycline resistant total coliform bacteria and residential land use. The y axis shows the MPN of tetracycline resistant coliforms per 100 ml of river water, and the x axis shows the percentage of residential land use in the designated zone of influence.



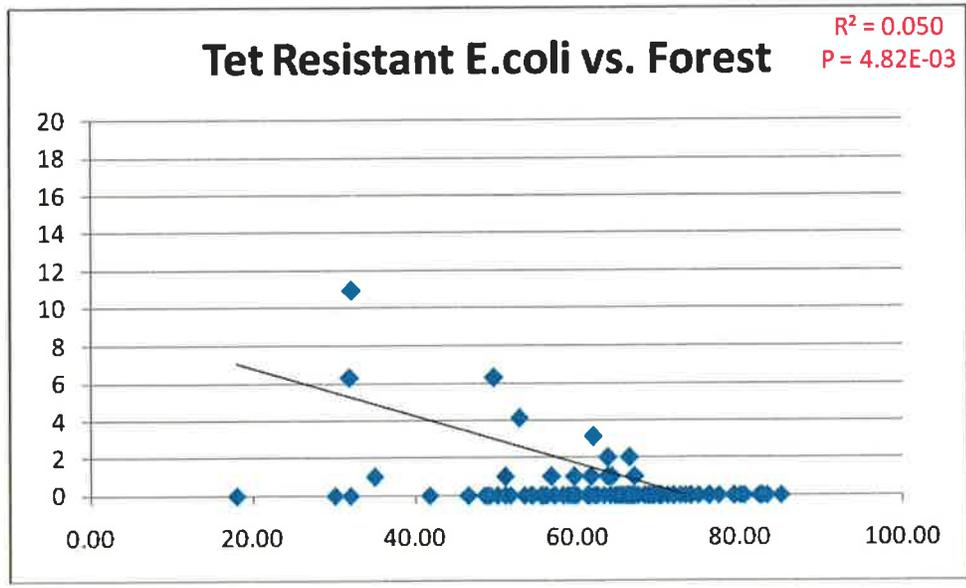
**Figure 49.** This figure shows a significant relationship between tetracycline resistant *E. coli* and residential land use. The y axis shows the MPN of tetracycline resistant *E. coli* per 100 ml of river water and the x axis shows the percentage of residential land use in the designated zone of influence.



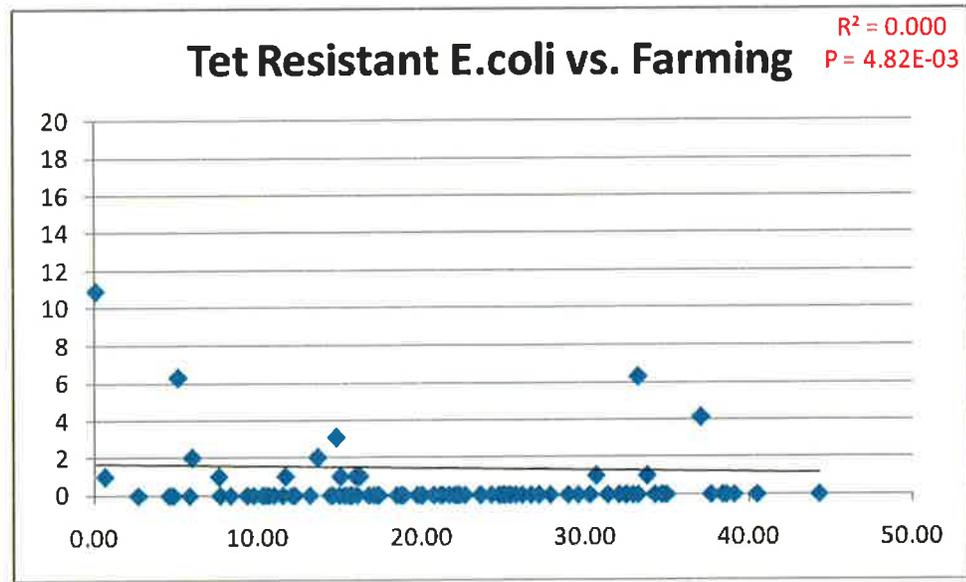
**Figure 50.** This figure shows a significant relationship between tetracycline resistant total coliform bacteria and commercial land use. The y axis shows the MPN of tetracycline resistant coliforms per 100 ml of river water, and the x axis shows the percentage of commercial land use in the designated zone of influence.



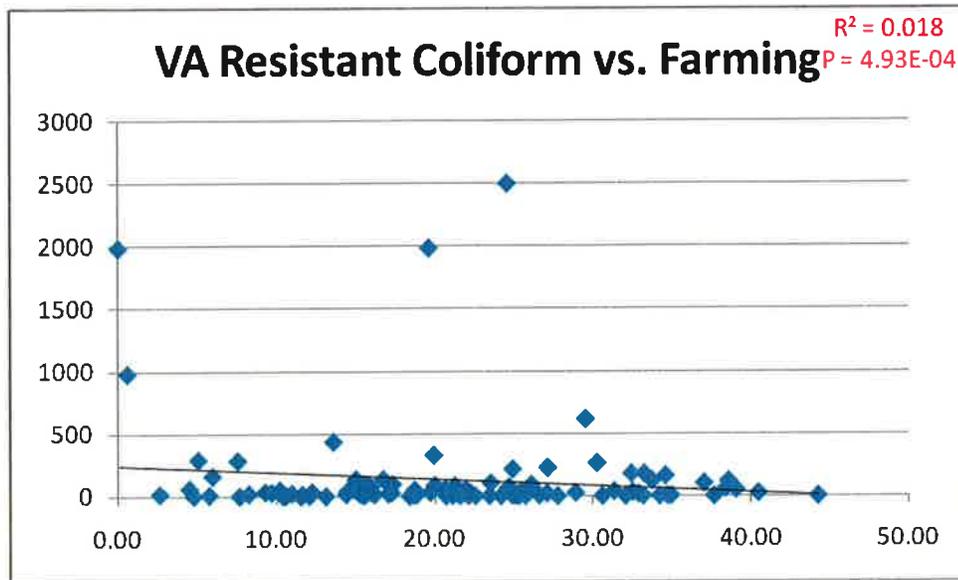
**Figure 51.** This figure shows a significant relationship between tetracycline resistant *E. coli* and commercial land use. The y axis shows the MPN of tetracycline resistant *E. coli* per 100 ml of river water, and the x axis shows the percentage of commercial land use in the designated zone of influence.



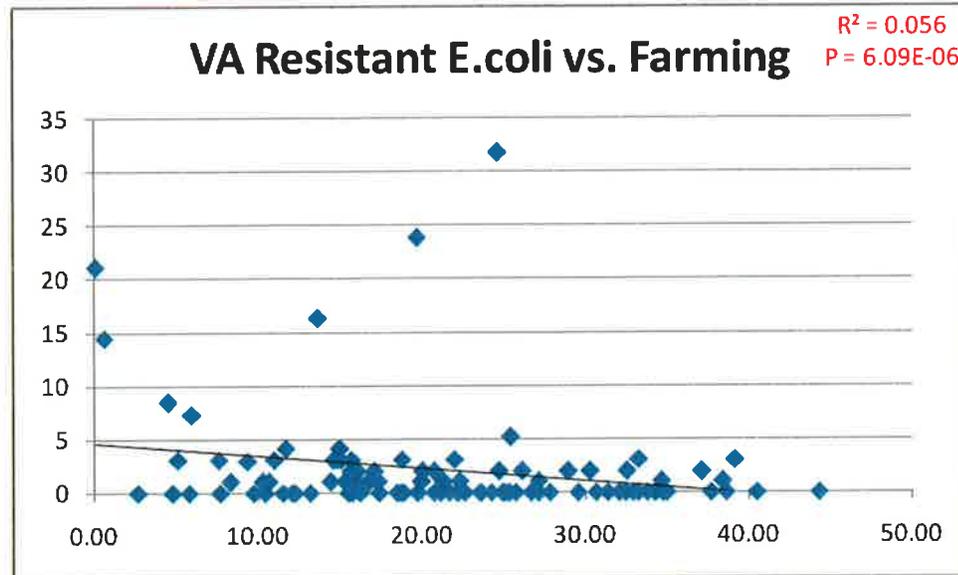
**Figure 52.** This figure shows a significant inverse relationship between tetracycline resistant *E. coli* and forest area. The y axis shows the MPN of tetracycline resistant *E. coli* per 100 ml of river water, and the x axis shows the percentage of forested land in the designated zone of influence.



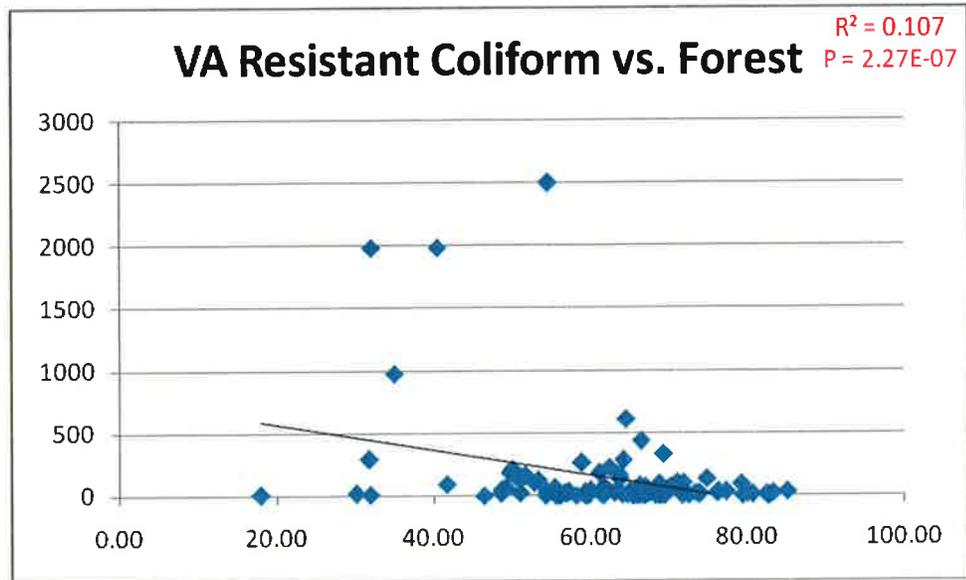
**Figure 53.** This figure shows a significant inverse relationship between tetracycline resistant *E. coli* and farmland of river water. The y axis shows the MPN of tetracycline resistant *E. coli* per 100 ml of river water, and the x axis shows the percentage of farmland of river water in the designated zone of influence.



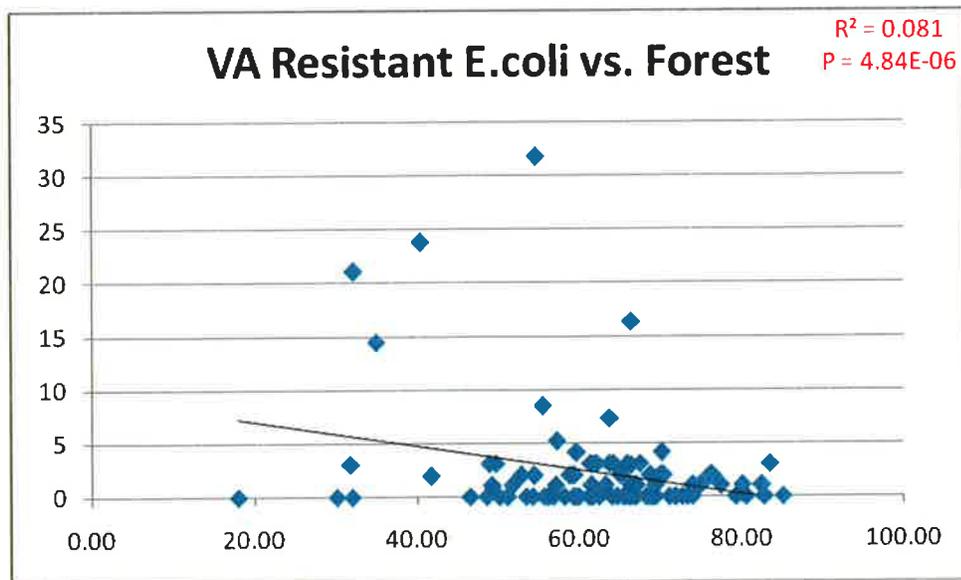
**Figure 54.** This figure shows a significant inverse relationship between virginiamycin resistant total coliform bacteria and farml of river waterand. The y axis shows the MPN of virginiamycin resistant coliforms per 100 ml of river water, and the x axis shows the percentage of farml of river waterand in the designated zone of influence.



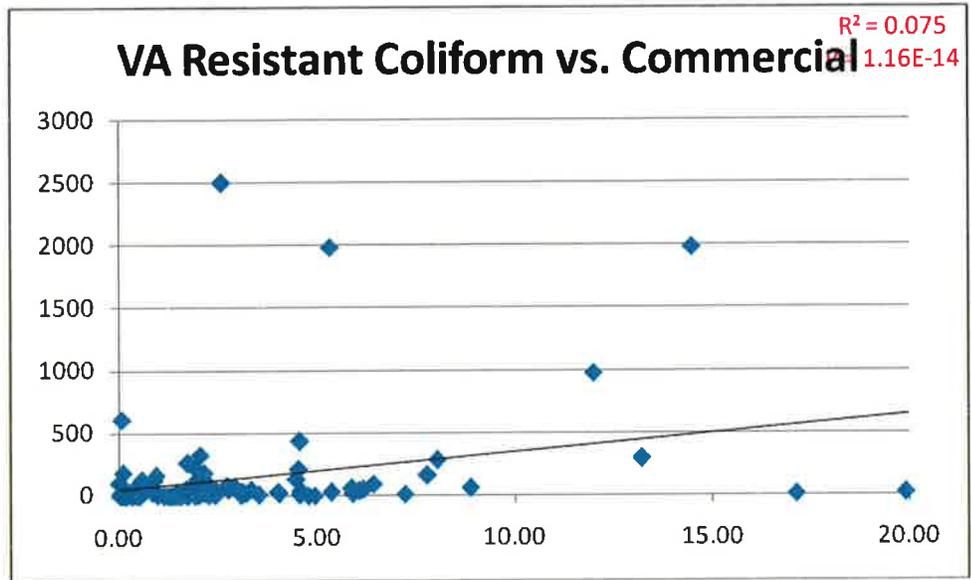
**Figure 55.** This figure shows a significant inverse relationship between virginiamycin resistant *E. coli* and farml of river waterand. The y axis shows the MPN of virginiamycin resistant *E. coli* per 100 ml of river water and the x axis shows the percentage of farml of river waterand in the designated zone of influence.



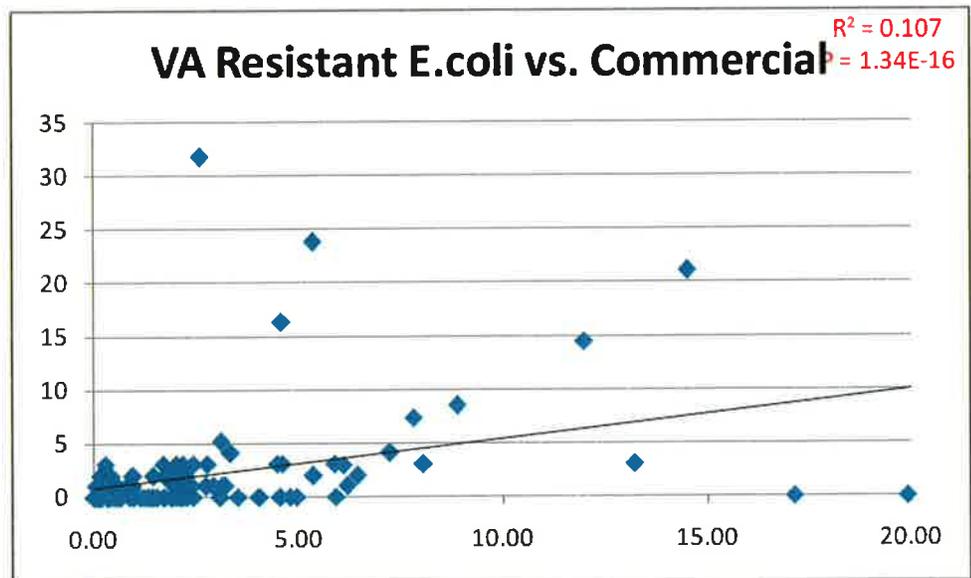
**Figure 56.** This figure shows a significant inverse relationship between virginiamycin resistant total coliform bacteria and forest area. The y axis shows the MPN of virginiamycin resistant coliforms per 100 ml of river water, and the x axis shows the percentage of forested land in the designated zone of influence.



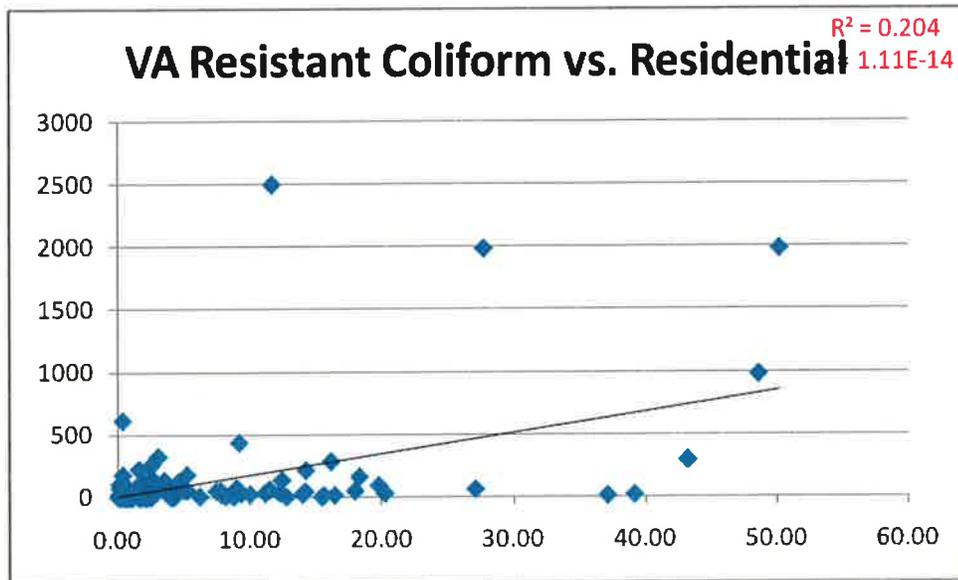
**Figure 57.** This figure shows a significant inverse relationship between virginiamycin resistant *E. coli* and forests. The y axis shows the MPN of virginiamycin resistant *E. coli* per 100 ml of river water and the x axis shows the percentage of forested land in the designated zone of influence.



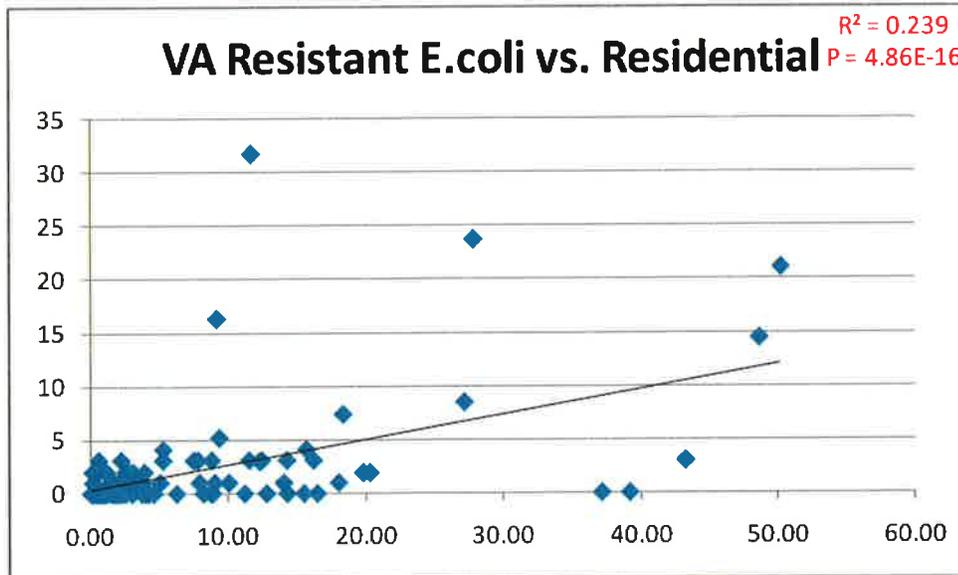
**Figure 58.** This figure shows a significant relationship between virginiamycin resistant total coliform bacteria and commercial land use. The y axis shows the MPN of virginiamycin resistant coliforms per 100 ml of river water, and the x axis shows the percentage of commercial land use in the designated zone of influence.



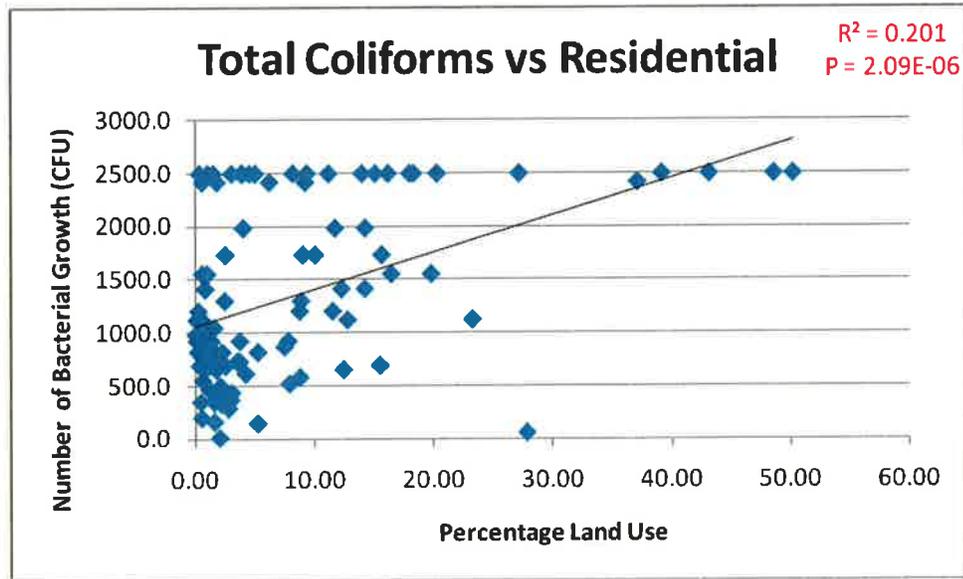
**Figure 59.** This figure shows a significant relationship between virginiamycin resistant *E. coli* and commercial land use. The y axis shows the MPN of virginiamycin resistant *E. coli* per 100 ml of river water, and the x axis shows the percentage of commercial land use in the designated zone of influence.



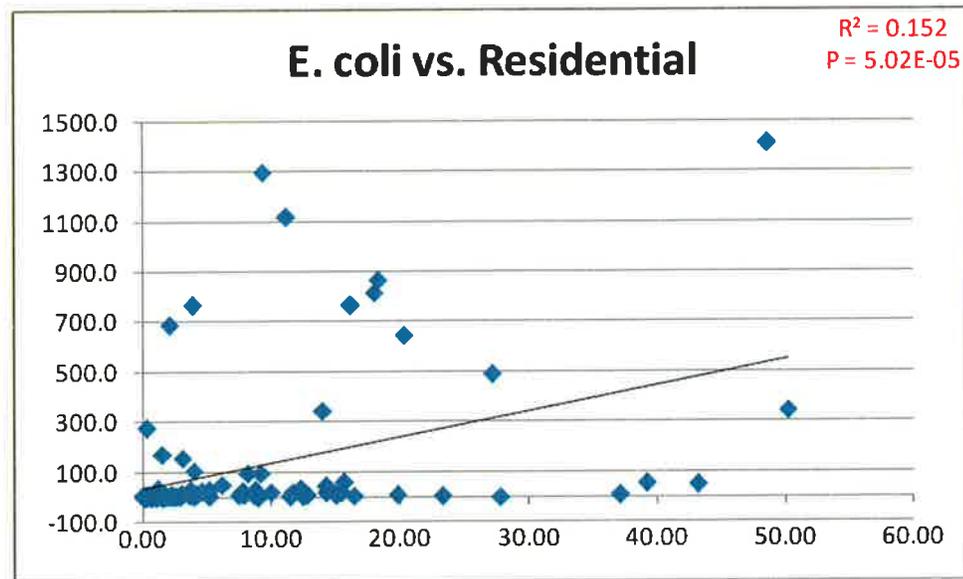
**Figure 60.** This figure shows a significant relationship between virginiamycin resistant total coliform bacteria and residential land use. The y axis shows the MPN of virginiamycin resistant coliforms per 100 ml of river water, and the x axis shows the percentage of residential land use in the designated zone of influence.



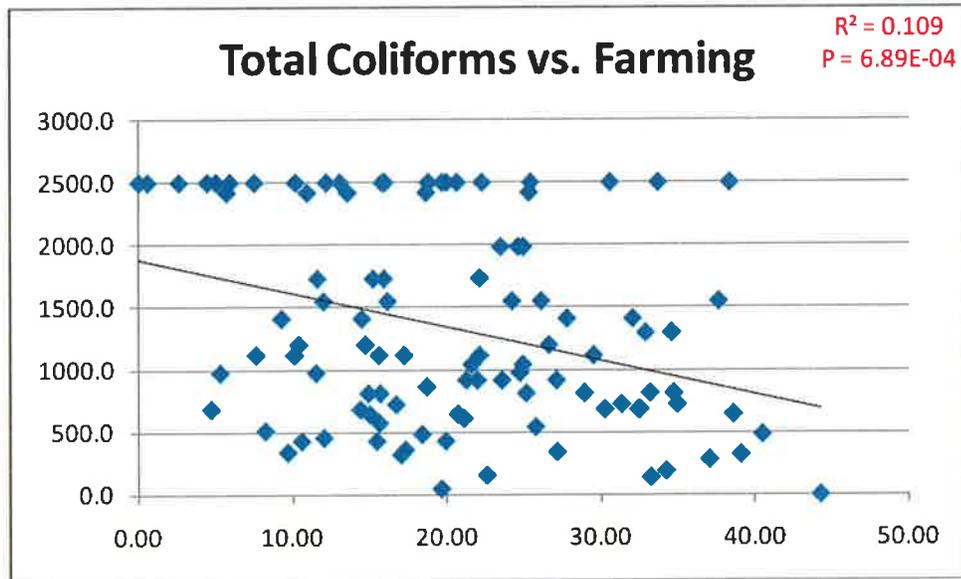
**Figure 61.** This figure shows a significant relationship between virginiamycin resistant *E. coli* and residential land use. The y axis shows the MPN of virginiamycin resistant *E. coli* per 100 ml of river water, and the x axis shows the percentage of residential land use in the designated zone of influence.



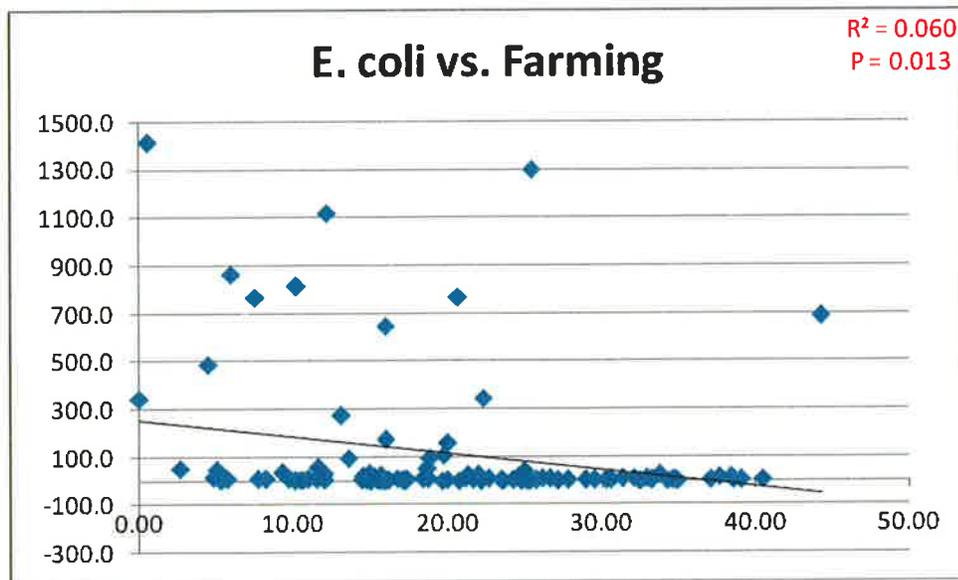
**Figure 62.** This figure shows a significant relationship between coliform bacteria and residential land use. The y axis shows the MPN of total coliform bacteria per 100 ml of river water, and the x axis shows the percentage of residential land use in the designated zone of influence.



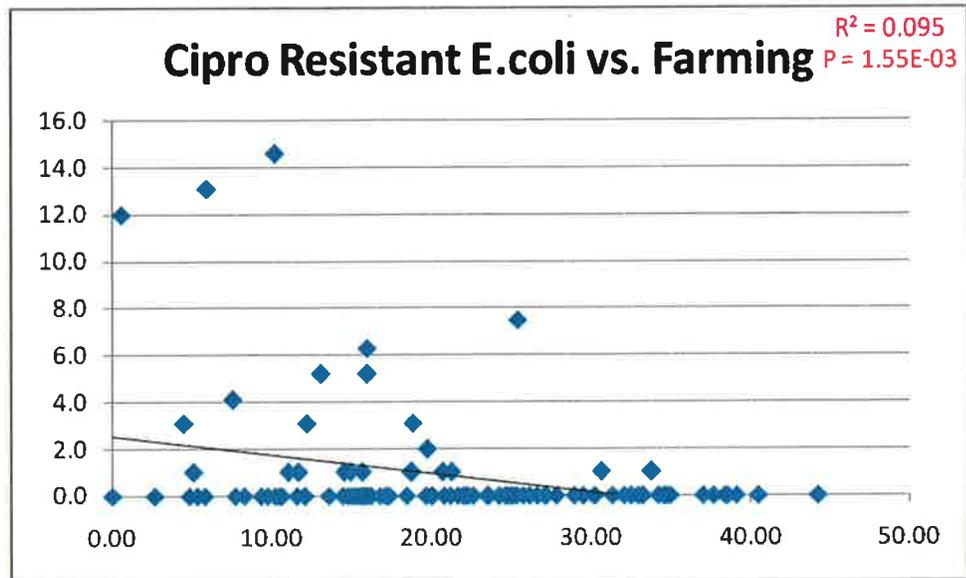
**Figure 63.** This figure shows a significant relationship between *E. coli* and residential land use. The y axis shows the MPN of *E. coli* per 100 ml of river water, and the x axis shows the percentage of residential land use in the designated zone of influence.



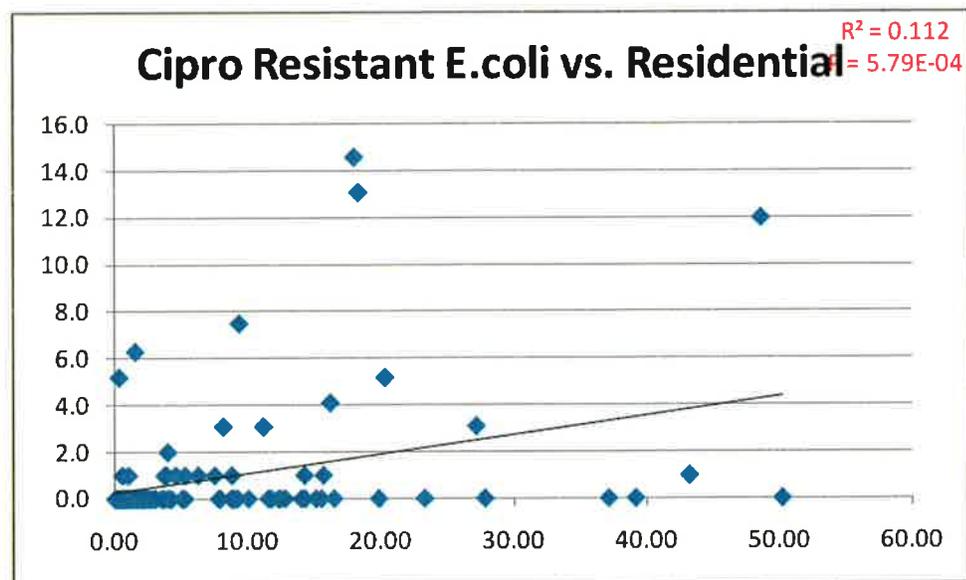
**Figure 64.** This figure shows a significant inverse relationship between coliform bacteria and farmland of river water. The y axis shows the MPN of total coliforms per 100 ml of river water, and the x axis shows the percentage of farmland of river water in the designated zone of influence.



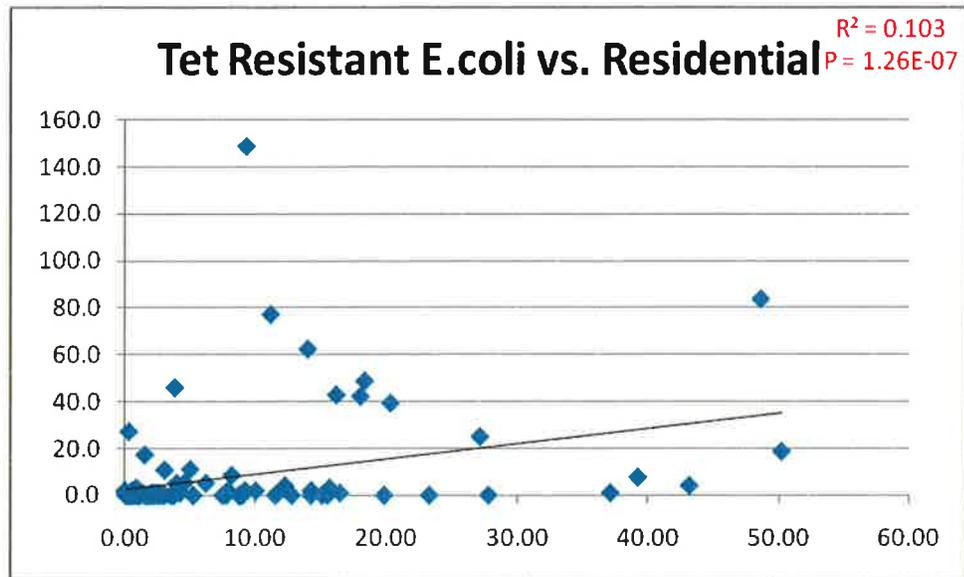
**Figure 65.** This figure shows a significant inverse relationship between *E. coli* and farming. The y axis shows the MPN of *E. coli* per 100 ml of river water, and the x axis shows the percentage of farmland of river water in the designated zone of influence.



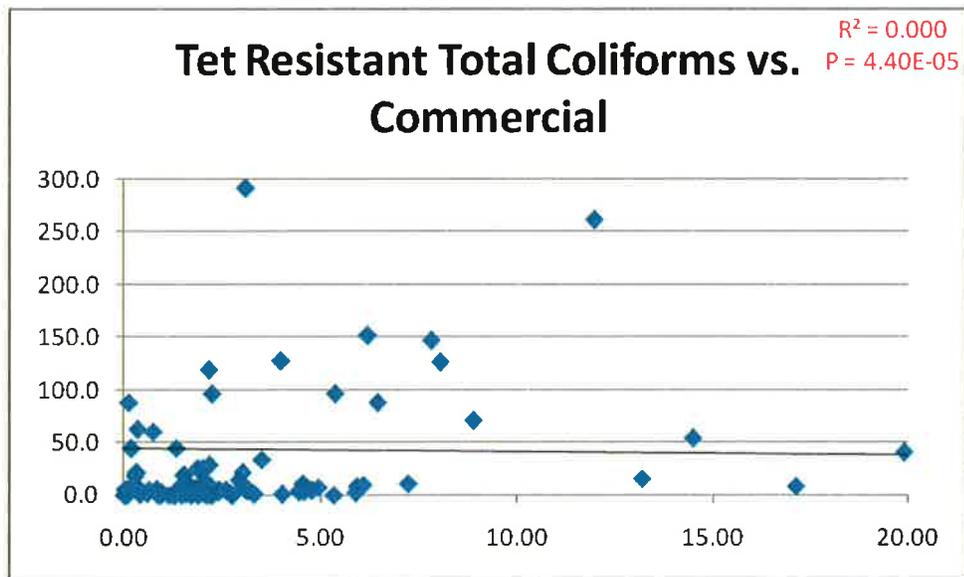
**Figure 66.** This figure shows a significant inverse relationship between ciprofloxacin resistant *E. coli* and farming. The y axis shows the MPN of ciprofloxacin resistant *E. coli* per 100 ml of river water, and the x axis shows the percentage of farmland of river water and in the designated zone of influence.



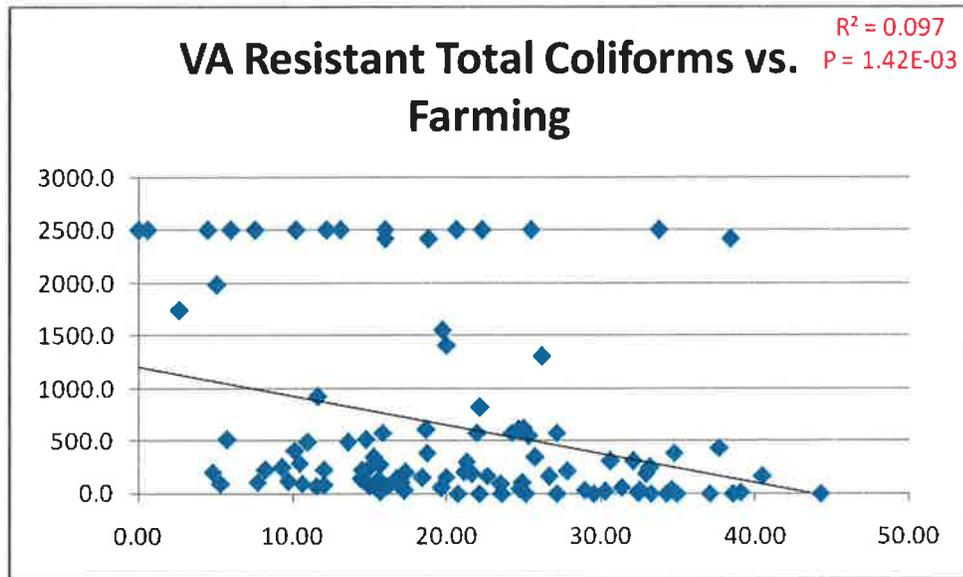
**Figure 67.** This figure shows a significant relationship between ciprofloxacin resistant *E. coli* and residential land use. The y axis shows the MPN of ciprofloxacin resistant *E. coli* per 100 ml of river water, and the x axis shows the percentage of residential land use in the designated zone of influence.



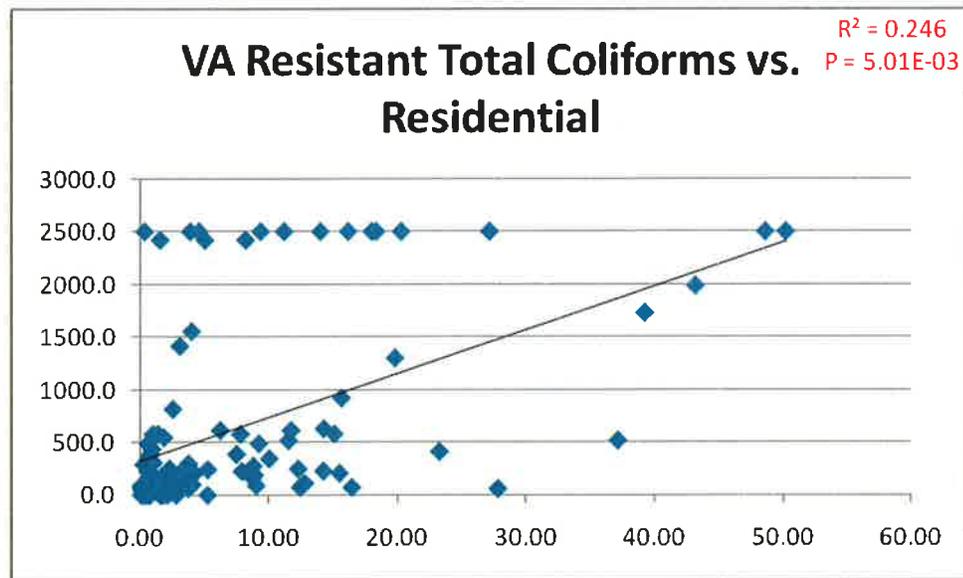
**Figure 68.** This figure shows a significant relationship between tetracycline resistant *E. coli* and residential land use. The y axis shows the MPN of tetracycline resistant *E. coli* per 100 ml of river water, and the x axis shows the percentage of residential land use in the designated zone of influence.



**Figure 69.** This figure shows a significant inverse relationship between tetracycline resistant total coliform bacteria and commercial land use. The y axis shows the MPN of tetracycline resistant coliforms per 100 ml of river water, and the x axis shows the percentage of commercial land use in the designated zone of influence.



**Figure 70.** This figure shows a significant inverse relationship between virginiamycin resistant total coliform bacteria and farmland of river waterland. The y axis shows the MPN of virginiamycin resistant coliforms per 100 ml of river water, and the x axis shows the percentage of farmland of river waterland in the designated zone of influence.



**Figure 71.** This figure shows a significant relationship between virginiamycin resistant total coliform bacteria and residential land use. The y axis shows the MPN of virginiamycin resistant coliforms per 100 ml of river water, and the x axis shows the percentage of residential land use in the designated zone of influence.

