INVESTIGATION OF THE PRESENCE / ABSENCE OF VANCOMYCIN RESISTANT *ENTEROCOCCUS* IN THE RACCOON RIVER WATERSHED (A LONGITUDINAL STUDY)

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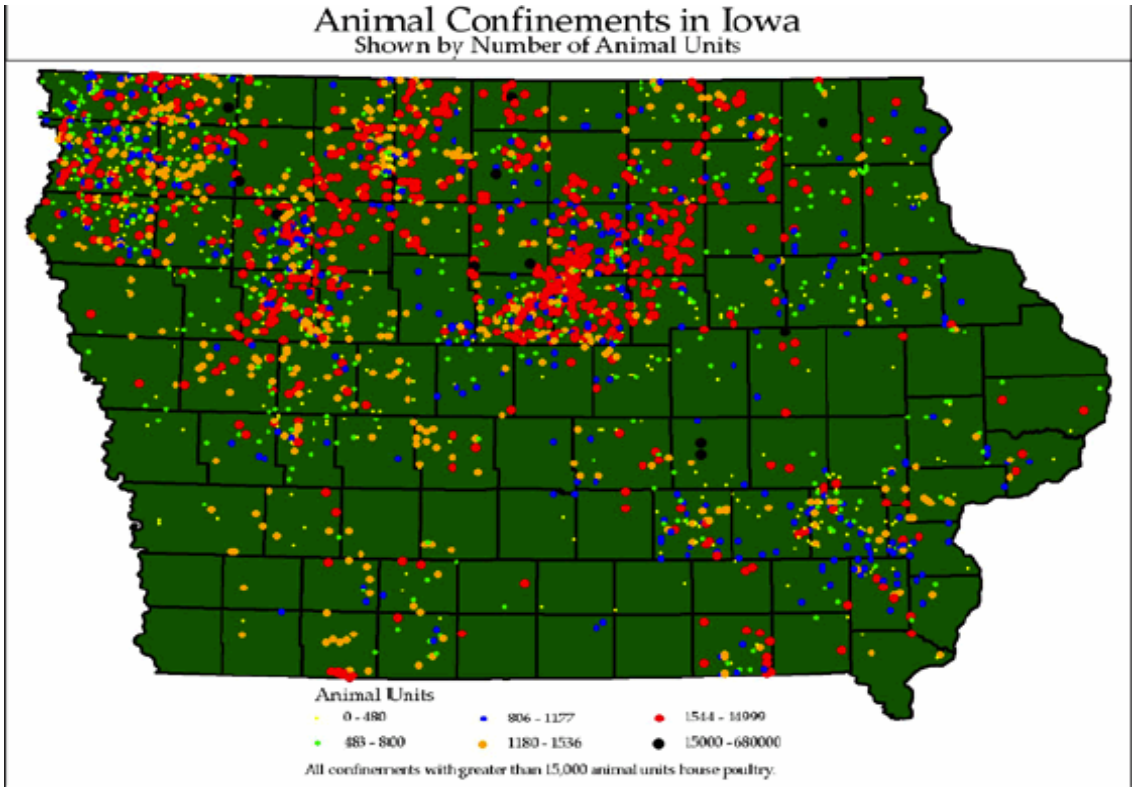
Background

The Raccoon River (RR) and its watershed have been widely monitored by the Des Moines Water Works (DMWW) since the 1950s. This monitoring has focused on bacterial and inorganic contamination (predominately nitrate). Since 2006, much of the monitoring has focused on the distribution of fecal contamination in the sub-watersheds of the RR.

Some of the common sources of fecal contamination of the watershed are: on-site livestock waste treatment processes, manure spraying systems, agriculture animal manure runoff, wildlife feces, human sewage treatment plant effluents, septic systems, storm water and other non-point source runoff.

Extensive beef cattle and poultry production occurs in the RR watershed. The livestock industry generates substantially more untreated waste than the human population in the watershed. Studies of microbial water contamination in the RR watershed, conducted by Dennis Hill of DMWW, indicate fecal concentrations increase in surface water following a significant rainfall. This documentation suggests agricultural runoff is potentially an important source of fecal pollution in source waters.

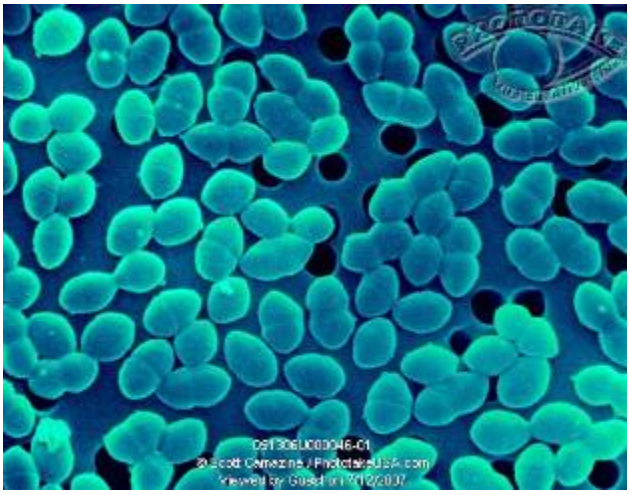


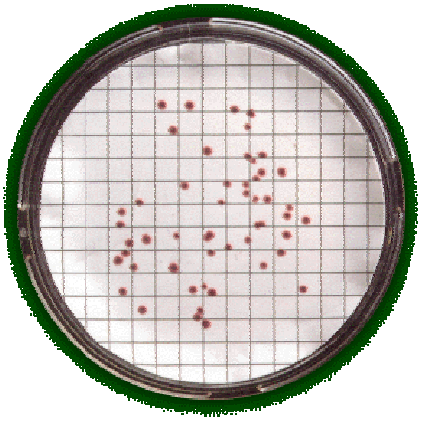
Introduction

The occurrence of fecal bacteria in water is one of the single most important indicators of potential public health hazards relating to infectious diseases. Both drinking water and recreational water quality standards rely on total counts of bacteria as indicators for the

potential presence of fecal contamination. Two of the organisms most commonly used as indicators of fecal contamination are *Enterococcus* and coliform bacteria, especially *E.coli*. The drinking water industry commonly uses coliform bacteria as indicators of water quality and fecal contamination. However, according to studies conducted by the Environmental Protection Agency (USEPA) *Enterococcus* bacteria have a greater correlation with swimming-associated gastrointestinal illness in both fresh and brackish waters than fecal coliform. Therefore, in 2004 *Enterococcus* took the place of fecal coliform as the new federal standard for water quality at public beaches. *Enterococcus* is believed to provide a higher correlation than fecal coliform with many of the human pathogens often found in sewage (1).

Enterococcus Bacteria





Enterococcus bacteria cultured on mE agar for 48 hours at 35°C.

Description

The genus *Enterococcus* was first classified in 1984. Prior to 1984 members of this genus were classified as Group D Streptococcus. Enterococci belong to the phylum *Firmicutes* and are gram-positive cocci. They occur in pairs and are difficult to distinguish from Streptococci by microscopy. The distinction between *Enterococcus* and *Streptococcus* was made possible by DNA analysis (2). Enterococci are known to survive in harsh environments and reside almost everywhere. They are characterized by their ability to grow in 6.5% sodium chloride, at low and elevated temperatures (10^{O}C and 45^{O}C) and at elevated pH (9.5).

Natural Habitats

Enterococci can be found in soil, food, water, animals, birds and insects. In humans and other warm blooded animals enterococci inhabit the gastrointestinal tract and the genitourinary tract. As a result, they are found in the feces and urine of warm blooded mammals including man.

Significance

Enterococci are considered benign in their normal habitat (gastrointestinal tract of warm blooded mammals). Outside their natural habitat, surface waters for example, enterococci are considered pathogenic, causing urinary tract and wound infections and life-threatening diseases such as bacteraemia (bacteria in the blood stream), endocarditis (inflammation of the inner layer of the heart and heart valve), and

meningitis (infection of the fluid in the spinal cord and the fluid that surrounds the brain). Enterococci may infect open wounds and skin ulcers where they can potentially be the root of infections associated with recreational waters. USEPA has suggested that a single fresh water sample should not exceed 61 enterococci organisms per 100mL, or a geometric mean of five samples should not exceed 33 enterococci organisms. (3)

Two species of *Enterococcus* which are commonly isolated from the gastrointestinal tract of humans are, *Enterococcus faecalis* ("pertaining to feces"), and *Enterococcus faecium* ("of Feces") (4). *E. faecalis* is the more prevalent of the two species and has been linked to ~80% of all previously mentioned infections (5). Both organisms are significant to the medical field and are responsible for up to 10% of all hospital-acquired infections. These infections are spread through ingestion or contact with contaminated food or water.

The concern the medical field faces with *Enterococcus* is two-fold. First, and of greatest concern, is the ability of *Enterococcus* to acquire resistance to antibiotics. *Enterococcus* is hardy and has a natural or innate resistance to many antibiotics. Antibiotic resistance is the potential of a microorganism to resist and endure the effects of an antibiotic. In addition through exchange of genetic information, *Enterococcus* is able to acquire resistance genes that allow it to be multiple-resistant (resistant to multiple antibiotics). Thus after many exposures and transfers, a bacterium has the potential to become multi-resistant to several antibiotics. Second is the virulence of

Enterococci. *Enterococci* generally have a limited potential for causing disease because they do not naturally produce any known toxins. What makes them pathogenic is their ability to obtain and express virulence factors. Virulence is the strength of its pathogenicity (the ability to cause disease or illness to the host of a microorganism)(6). Again, *Enterococcus* acquires the genes responsible of virulence either by mutation or plasmid swapping (7). A third concern is the selective pressure exerted by exposure of these organisms to antibiotics in their environment. As human and animal medicine relies heavily on antimicrobial drugs, the selective pressure driving antimicrobial resistance is increased.

Enterococci are one of the most drug resistant bacteria. Enterococci may even be resistant to the "last resort" drug vancomycin. Since Enterococci emerged as a significant cause of human infection, the number of vancomycin-resistance infections has increased. This resistance could potentially prove fatal to human beings. Therefore *Enterococcus* in the Raccoon River watershed, a primary source for Des Moines Water Treatment Plant, should be identified and characterized and the occurrence and distribution of the organism should be assessed.

Materials and Methods

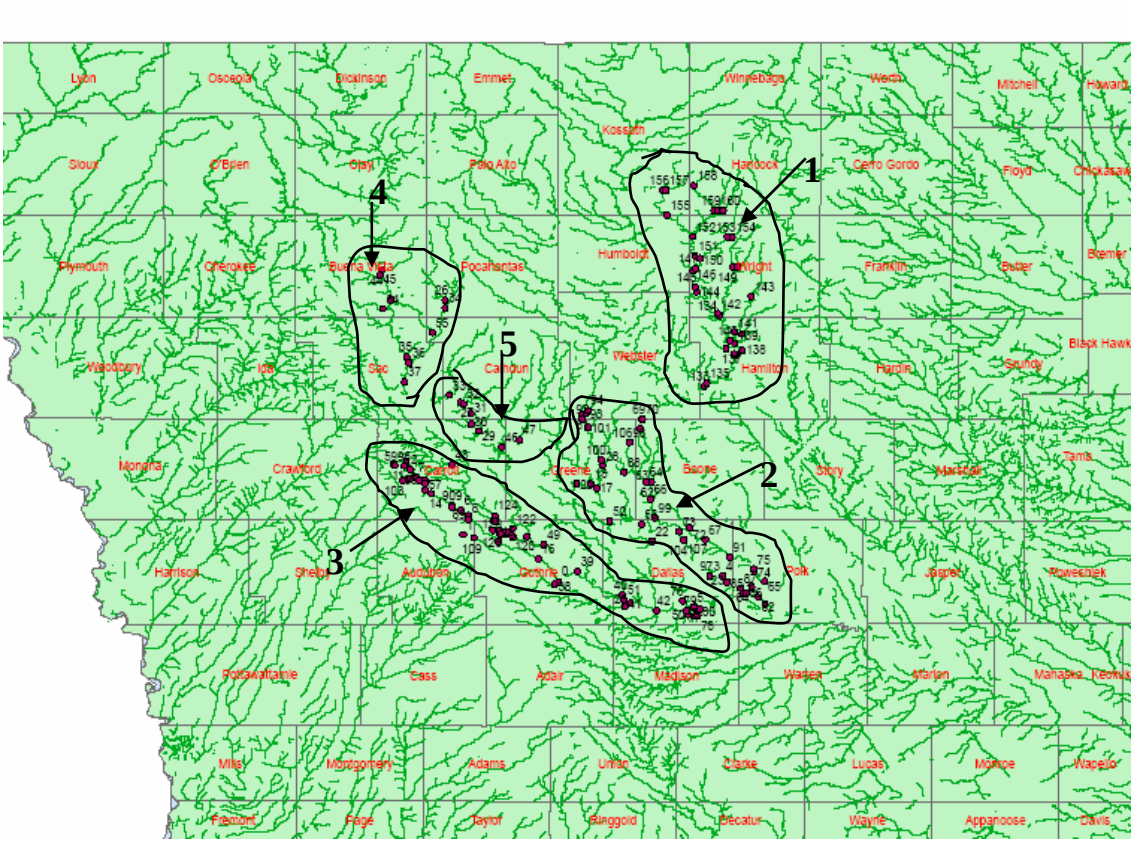
Samples

Ongoing watershed studies involving Agriculture's Clean Water Alliance (ACWA), Iowa Soybean Association (ISA), Iowa Department of Natural Resources (IDNR) and DMWW

provided samples for this study. The ACWA provided DMWW with 43 samples from selected sites in the RR watershed bi-weekly from April through August for anion and fecal coliform analysis. Twenty-six of these 43 samples were sub-sampled for *Enterococcus* analysis. ISA provided DMWW with 30 samples from selected sites in the Boone River watershed for analysis on a bi-weekly basis from April through August of 2007 for anion and fecal coliform analysis. These samples were sub-sampled for *Enterococcus* analysis. ISA also supplied DMWW with 15-20 samples from selected sites in the RR and Beaver Creek watersheds on a weekly basis for bacteria and anion analysis. These 15-20 samples were chosen from a pool of 100 sites in the watersheds based on previous results. Therefore, the sample sites changed weekly. All samples were sub-sampled for *Enterococcus* and estrogen-mimicking compounds. The estrogen mimicking compounds will not be discussed in this paper since the focus of this study is *Enterococcus*.

The samples sites were grouped into 5 distinguishable sets (Clusters) based on geographical proximity (see Figure 1). Cluster 1 consisted of samples from the Boone River. Cluster 2 consisted of samples from Beaver Creek and Walnut Creek. Cluster 3 consisted of samples from the South Raccoon River, including Brushy Creek. Cluster 4 consisted of samples from the North Raccoon River including Indian and Cedar Creeks. Cluster 5 consisted of samples from the North Raccoon River including Elk Run and Lake Creek.

Figure 1: Collection Sites Grouped into Sample Sets (Clusters)



The data presented at this time encompasses 603 *Enterococcus* samples from the collection period April 11 through July 5 of 2007.

Distribution of samples

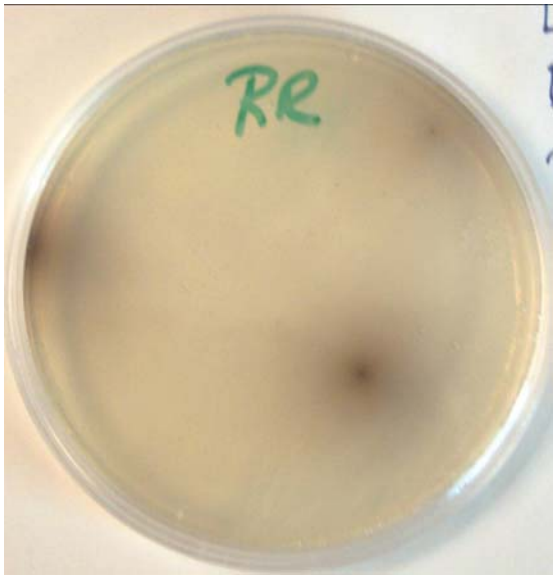
	Cluster 1 Site #	Cluster 2 Site #	Cluster 3 Site #	Cluster 4 Site #	Cluster 5 Site #
Sample site & number of samples collected at each site	BR1 (6)	20 (1)	42A (2)	2 (2)	10 (20
	BR2 (8)	21 (2)	A (9)	3 (2)	11 (6)
	BR3 (7)	24 (10)	33 (12)	4 (2)	12 (6)
	BR4 (7)	23 (2)	28AA (1)	5 (6)	13 (1)
	BR5 (7)	25 (7)	WRP4 (1)	6 (2)	14 (6)
	BR6 (7)	45 (1)	UR1(2)	7 (6)	17 (6)
	BR7 (8)	46 (2)	C8-19 (1)	8 (2)	19 (7)
	BR8 (7	70A (6)	26 (7)	9 (7)	2A (2)
	BR9 (7)	40B (3)	37 (8)		4A (2)
	BR10 (9)	BC10B (5)	38 (1)		14a (6)
Total sites in cluster	30 Sites	45 Sites	75 Sites	7 Sites	10 Sites
Sample type	213 *Enterococcus* Samples	147 *Enterococcus* Samples	145 *Enterococcus* Samples	29 *Enterococcus* Samples	38 *Enterococcus* Samples
Site area	Boone River	Beaver and Walnut Creek	South Raccoon and Brushy Creek	North Raccoon including Cedar and Indian Creek	North Raccoon including Elk Run and Lake Creek

Analysis

DMWW submitted the water samples to Des Moines University (DMU). All samples were centrifuged and an aliquot was taken out and flash-frozen awaiting estrogen

analyses. The other aliquot was used to screen for *Enterococcus*. In order to screen out false positive enterococci identification, three successive *Enterococcus* isolation procedures were preformed.

1. Enterococcosel cell agar containing sodium azide was inoculated with the pellet from each of the centrifuged samples. Black/brown football shaped colonies were formed if the samples were positive for *Enterococcus*.



2. Tubes containing 6.5% sodium chloride in tryptic soy broth were inoculated with a single *Enterococcus* black colony from each positive sample and incubated. Growth appeared as turbidity if the isolate was halotolerant.

3. *Enterococcus* agar was inoculated with the positive high salt positive organism samples and incubated. Pinkish red colonies appeared on the *Enterococcus* agar if the samples were positive.

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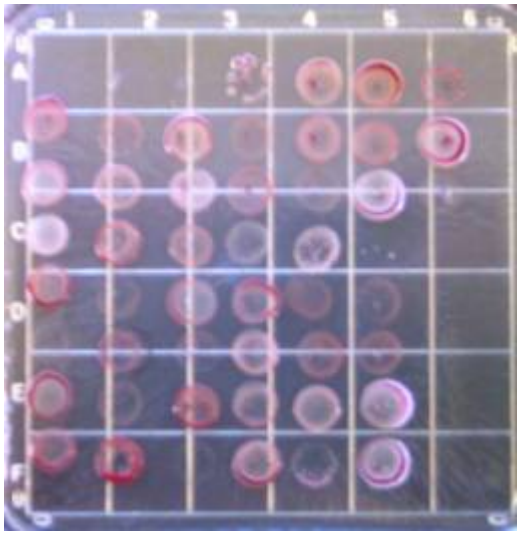
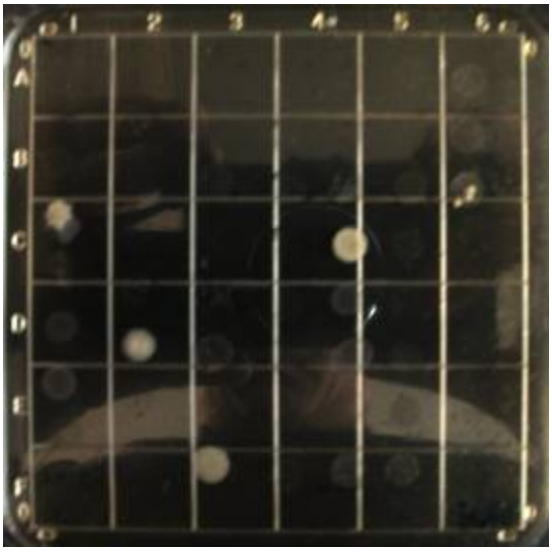


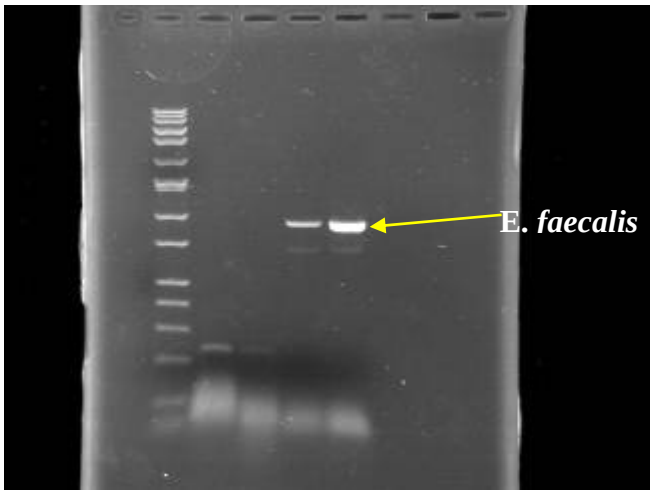
Confirmation of vancomycin resistance employed Tryptic Soy Agar containing 6ug/ml of vancomycin. Growth on this medium indicated resistance to vancomycin.



For detailed procedural instructions on *Enterococcus* isolation refer to Appendix A on page 20.

The vancomycin resistant samples were then examined by polymerase chain reaction (PCR) with primers and electropherosis to determine the species of *Enterococcus* (*Enterococcus faecalis* or *Enterococcus faecium*). The vancomycin resistant genes of interest (Van A, B, C1 or C2) were subsequently determined by PCR. For detailed procedural instructions on PCR, see Appendix B on pages 21 - 21.

Photo: *E. faecalis* detected by means of PCR and electropherosis.



RESULTS

For this study, an acceptable level of *Enterococcus* contamination of 104 colony forming units per 100 milliliters of water was assumed (this value was from an online reference). A 15mL water sample was plated and a level of concern of 16 or more colonies was determined to be the marker for this study. The 15 ml was concentrated by centrifugation and plated in pour plates.

Enterococcus Results

A total of 603 samples were analyzed for *Enterococcus*. Ninety-three (15%) of the 603 samples were negative for *Enterococcus*. 290 (48%) of the 603 samples contained between 1 and 15 colonies of *Enterococcus*. These colonies were considered to be below the level of concern (16 colonies). 220 (36%) of the 603 samples contained 16 colonies of *Enterococcus* or greater. Thus, 36% of all the Raccoon River Watershed samples analyzed were determined to be above the level of concern (16 colony forming units) for *Enterococcus*. Further analysis established that 30 (13%) of the samples that were determined to be above the level of concern for *Enterococcus* were vancomycin-resistant and 24 (8%) of the samples below the established level of concern were vancomycin-resistant.

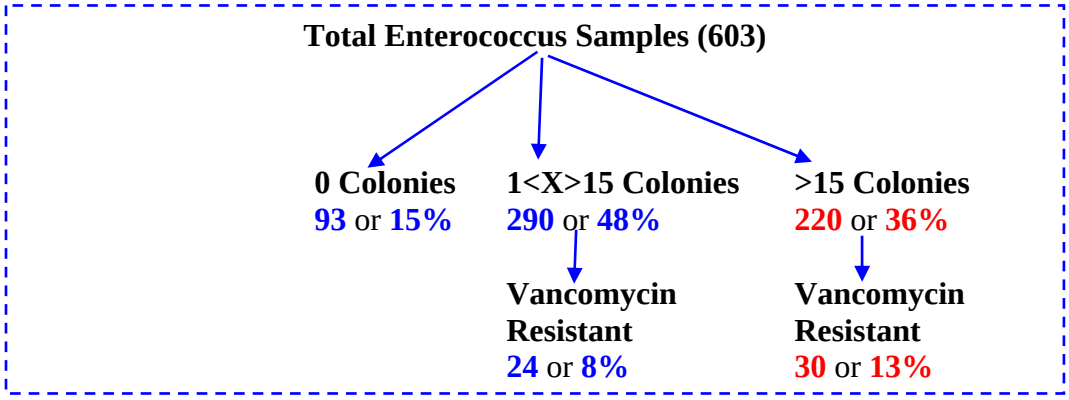


Figure 2: The cluster distribution of samples containing 16 colonies or greater of Enterococcus:

	Cluster 1	**Cluster 2**	**Cluster 3**	**Cluster 4**	**Cluster 5**
	Boone River	Beaver and Walnut Creek	South Raccoon River and Brushy Creek	North Raccoon River Cedar and Indian Creek	North Raccoon River Including Elk Run and Lake Creek
Number of Enterococcus samples above level of concern	**70** or **33%**	**46** or **32%**	**63** or **43%**	**9** or **31%**	**7** or **18%**

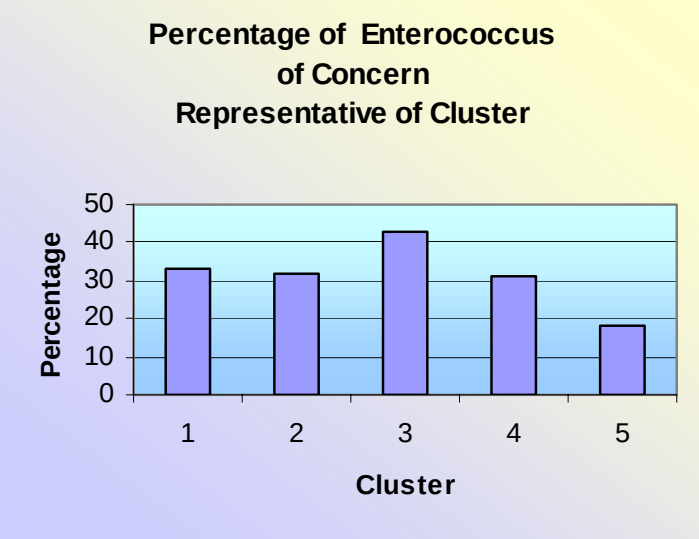
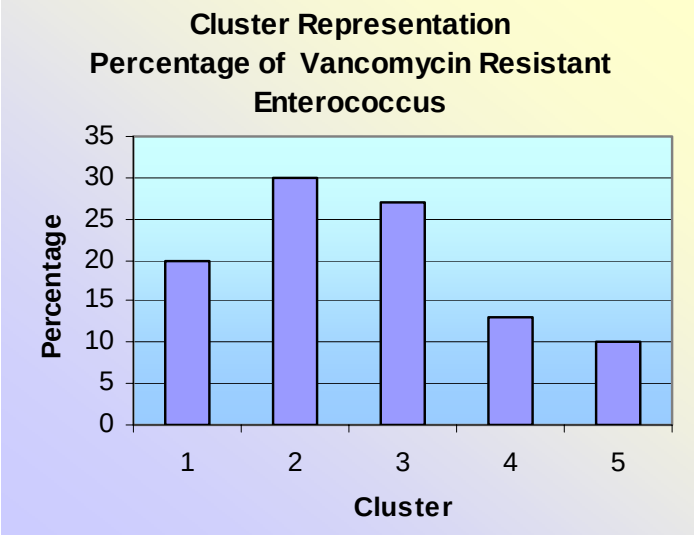


Figure 3: The cluster distribution of samples containing 16 colonies or greater of vancomycin-resistant Enterococcus:

	Cluster 1	**Cluster 2**	**Cluster 3**	**Cluster 4**	**Cluster 5**
	Boone River	Beaver and Walnut Creek	South Raccoon River and Brushy Creek	North Raccoon River Cedar and Indian Creek	North Raccoon River Including Elk Run and Lake Creek
Number of Vancomycin resistant Enterococcus	**6** or **20%**	**9** or **30%**	**8** or **27%**	**4** or **13%**	**3** or **10%**



DISCUSSION

Only half of all samples collected were analyzed for *Enterococcus* and vancomycin resistance at the time of this report. Therefore, conclusions can be drawn only for the data presented. It is clear that vancomycin-resistant enterococci were present in the

Raccoon River watershed at the time of this study. *Enterococcus* densities above the level of concern were highest in cluster 3, vancomycin resistance was highest in clusters 2 and 3.

Based on these results, the authors felt that Brushy Creek would be good a candidate for further investigation. Notable concentrations of *E.coli* and estrogen have been detected in Brushy Creek in the past. Upon completion of all analyses, a final report will be written. The final report will include any possible correlation between *E.coli*, estrogen-mimicking compounds and *Enterococcus*.

Future Direction

There were two areas of study still in progress at the time of this report and not addressed in detail here. The first is the evaluation of *Enterococcus* by PCR and electropherosis. The PCR technique will determine which gene (Van A, B, C1 or C2) is responsible for the vancomycin resistance. The second area of study still under investigation is the correlation between estrogen and *Enterococcus* levels with regards to rainfall, elevation, industry and agricultural practices.

Once all data is finalized and compiled, the intent will be to deliver this information to water treatment and health care professionals.

Two potential benefits for health care professionals are:

1. Identification of the genus and species of *Enterococcus*, and the genes that are responsible for resistance, could enable health care professionals to choose alternative antibiotics to treat resistant organisms.
2. Species identification could provide an understanding why some enterococci acquire resistant genes. These resistant genes enable the *Enterococcus* to express virulence, thus making them pathogenic.

Three potential benefits for water treatment professionals are:

1. Identification of the cluster location and concentration of *Enterococcus* could guide water treatment professionals when choosing the most effective mitigation strategy, i.e. physical removal during treatment or prevention of upstream contamination.
2. Knowledge of the correlations between *Enterococcus*, *E.coli* and estrogen could further enable water treatment personal to pin point the source of contamination and choose the suitable remediation techniques.
3. Because of its longevity, *Enterococcus* is believed to provide a higher correlation than fecal coliform with many of the human pathogens often found in sewage (1). *Enterococcus* may be a more suitable indicator organism than *E. coli* for watershed characterization.

Appendix A:

Isolation of Enterococcus to screen out false positives

1. Collect 14 mL source water sample
2. Centrifuge 5 minutes at 30,000 rpm (pelleting suspended solids)
3. Pour off 7 – 9 mL supernatant (into separate 14 mL tube) to within 2 mL of pellet
    a. This supernatant will be used for the YES screen
    b. Freeze (-80) samples
4. Make enterococcosel agar (Contains sodium azide)
5. Add 10 mL of media to the 2 mL of water sample containing the pellet and resuspend
6. Pour plates
7. Incubate 2 days at $37^{O}C$
8. Count black/brown colonies (colonies will be brown and football shaped)
9. Cut one black colony out
10. Put black colony into 1 mL high salt (6.5%) tryptic soy broth
    a. Sodium chloride is added to pre-made tryptic soy broth at concentration of 6.5%
11. Incubate 2 days at $37^{O}C$
12. Spot 4 uL of sample on vancomycin resistant agar (6 ug/ml vancomycin into tryptic soy agar) and on Enterococcus agar
13. Incubate overnight at $37^{O}C$
    a. Colonies will turn pink or red on Enterococcus agar if positive. Growth will appear on vancomycin resistant agar if organism is resistant to vancomycin

Appendix B:

PCR and PCR sample preparation

The polymerase chain reaction (PCR) is a biochemistry and molecular biology technique for exponentially amplifying a fragment of DNA, via enzymatic replication, without using a living organism (such as *E. coli* or yeast). PCR can be used for amplification of a single or a few copies of a piece of DNA across several orders of magnitude, generating millions or more copies of the DNA piece. (8)

Each gene listed below has a specific primer set designed to identify the gene. The identification is confirmed by PCR and electropherosis (running of DNA samples after PCR)

The sizes for identification of each PCR product are listed below:

	Base pairs (weight of product)	
Van A	983	
Van B	500	phenotypic classes defined by the
Van C1	800	level of resistance to vancomycin
Van C2		
Faecum	658	species of Enterococcus
Faecalis	941	

Streak Enterococcus vancomycin resistant organism onto growth plate

1. Incubate overnight at $37^{O}C$
2. Select an isolated colony from growth plate add that to 10ul of Lyse-n-Go and run program
3. After program is complete you have your template DNA.
4. To set a PCR reaction add the following to PCR tube:
    a. 1 uL forward primer
    b. 1 uL reverse primer
    c. 5 uL template prepared from Lyse-N-Go step
    d. 12.5 uL master mix (nucleotide)
    e. 5.5 uL sterile water to a total volumn of 25 uL
5. Run PCR program: 3-4 hours
6. After completion of PCR program run a gel (Electrophoresis)
    a. Prepare a 1% agarose gel

a. To a seperate tube add 2 uL of bromphenol blue and 15 uL of pcr product.
b. Add the contents of the above tube to a well in the gel
c. Add 10 uL ladder (Molecular weight standards)
d. Run 45 minutes at 100 volts
e. After running stain with Ethidium Bromide and using a uv box identify.

The lower molecular weight DNA moves through the gel quicker then the higher molecular weight DNA. Therefore, the higher base pairs will come off last. The first stage of electropherosis determines the species of Enterococcus and the second stage determined gene causing the resistance

References:

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