# ANTIBIOTIC RESISTANCE PATTERNS IN MUNICIPAL WASTEWATER BACTERIA

by

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# Abstract

Antibiotics and pharmaceuticals are used to improve the quality of life worldwide. However, incomplete metabolism in humans has resulted in the release of large amounts of pharmaceutical drugs into municipal wastewater treatment plant. Past research has shown the release of antibiotic resistant organisms through wastewater effluents into streams and several studies have reported the occurrence of antibiotic resistant bacteria in major U.S. Rivers. Antibiotic resistant bacteria evolve and are selected by long-term environmental exposure to the low concentrations of antibiotics at the ng /L to $\mu$g/L range. Infections caused by antibiotic resistant organisms are difficult to treat. The aim of this study was to analyze antibiotic resistance patterns in selected wastewater bacteria that include fecal coliforms, *Escherichia coli* and enterococci. Microorganisms in municipal wastewater treatment plant influent, secondary clarifier effluent and disinfected effluent were plated in the presence of predetermined concentrations of selected antibiotics. These antibiotics included ciprofloxacin, sulfamethoxazole/ trimethoprim and vancomycin. The diversity of enterococci was further investigated with PCR analysis. Fecal coliforms, *E. coli* and enterococci were found to be resistant or highly resistant to one or more target antibiotics in the influent and secondary clarifier (SC) effluent. Biological treatment reduced the number of overall and resistant bacteria in the SC effluent sample. UV disinfection was generally very effective and eliminated all fecal indicator organisms.

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My Parents Venkat Reddy Nagulapally, Radha Nagulapally sister Sunitha and brother Gurunath have always supported me in my personal and professional career. I thank them for all their support to me.

# Dedication

I would like to dedicate my thesis in memory of my beloved brother Shobhan Nagulapally who was an inspiration to me to finish my masters program successfully. He was a brother, friend and role model for me. I always miss him in my entire life.

# CHAPTER 1 - INTRODUCTION

Antibiotics and pharmaceuticals are used to improve public health and quality of life worldwide. However, incomplete metabolism in humans has resulted in release of large amounts of pharmaceutical drugs into municipal wastewater treatment plants (WWTPs). Recent studies have shown the presence of low concentrations of antibiotics in WWTP effluents and surface waters (Giger et al, 2003; Golet et al, 2002; Hernando et al, 2006; Christian et al, 2003; Mulroy 2001). The prevalence of antibiotics in municipal wastewater and surface waters can lead to the development of antibiotic resistant bacteria due to long-term exposure to low concentrations of antibiotics in the ng /L to $\mu$g/L range (Gilliver et al, 1999; Khachatourians, 1998; Smith et al, 1999). A recent paper reported the release of antibiotic resistant organisms through wastewater effluents into streams (Gallert et al, 2005). Other studies have discussed the prevalence of antibiotic resistance bacteria in major U.S. Rivers (Ash et al. 2002).

The prevalence of infections caused by multiple antibiotic resistant organisms is increasing although there are advances in antibacterial therapy (Baquero 1997). Infections caused by antibiotic resistant bacteria are extremely hard to treat.

The aim of this study was to analyze the antibiotic resistance patterns in fecal bacteria (fecal coliforms, *Escherichia coli* and enterococci) collected from raw influent, secondary clarifier (SC) effluent and disinfected effluent wastewater. Fecal bacteria were tested for resistance against ciprofloxacin (CIP), sulfamethoxazole/trimethoprim (SXT) and vancomycin (VAN). Antibiotic resistant enterococci were identified through PCR to understand their diversity and resistance profiles in the wastewater plant.

# CHAPTER 2 - LITERATURE REVIEW

## 2.1 Water and Wastewater Constituents

Wastewater is a mixture of water and dissolved or suspended solids. Raw wastewater includes a variety of physical, chemical and biological constituents (Tchobanoglous et al. 2002). These are described below.

1. Physical characteristics

The physical characteristics of wastewater include total solids that are composed of floating, colloidal and settle able particles. Other important physical characteristics include turbidity, color, temperature, conductivity, density, specific weight and specific gravity.

2. Chemical characteristics

The chemical characteristics of wastewater are mainly divided into inorganic and organic. Inorganic constituents include nutrients, and metallic and non- metallic constituents. Organic chemical constituents are represented by Bio-Chemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) parameters.

3. Biological Characteristics

The biological characteristics of wastewater include pathogenic organisms of human and animal origin. Organisms present in wastewater include bacteria, fungi, algae, protozoa, and viruses

Among these characteristics, the biological characteristics are of fundamental importance because they include disease causing pathogenic bacteria of human origin. Potential infectious agents in untreated wastewater include bacteria, protozoa, helminths and viruses (Tchobanoglous et al. 2002).

## 2.2 Indicator Organisms for Fecal Contamination of Water

In early 1880's it was realized that pathogenic bacteria from human origin cause fecal contamination in water (Huber 1971). Fecal indicator organism are therefore, used as measures of surface water and wastewater quality. The Environmental Protection Agency (EPA) has noted that the occurrence of these fecal indicative in water is an indication of potential presence of pathogenic organisms capable of posing a threat to public health (EPA, 2006). Fecal indicator organisms include total coliforms, fecal coliforms, enterococci and *Escherichia coli* (Tchobanoglous et al. 2002). These organisms the natural inhabitants of gastrointestinal tracts in humans and warm-blooded animals and are discharged into wastewater treatment plants through human feces. Fecal indicators have also been found responsible for various diseases including cholera, typhoid, hepatitis, diarrhea and endocarditis (Gorbach et al. 1971; Gorbach et al. 1975; Sack et al. 1971; Aggarwal and Krawczynski 2000; Bajracharya et al, 2006).

### *2.2.1 Fecal coliforms*

Fecal coliforms are gram-negative bacteria that live in the digestive tract of warm-blooded animals and humans (Qasim, 1998). They are the indicators of potentially pathogenic bacteria from fecal origin (Asano, 1998). Fecal coliforms are excreted in the feces by humans and animals and ultimately reach wastewater treatment plants. Hence, a huge amount of fecal coliforms are observed in raw wastewater. The numbers of fecal coliform bacteria that have been reported in municipal wastewater influent and effluent samples are summarized in Table 2.1.

### *2.2.2 Escherichia coli*

*Escherichia coli* (*E.coli*) are used as indicators of microbiological quality of water. They are gram-negative bacteria and they are found naturally in both human and animal intestines. Usually *E. coli* plays a vital role in digestion and helps the body to absorb important vitamins from food. *E. coli* has several strains and most of these strains are human friendly but few like *E. coli* 0157:H7 are pathogenic to humans. Several intestinal and extra intestinal infections such as urinary tract infection, meningitis and diarrhea are caused by *E.coli* 0157:H7 (Sussman 1997; Cherubin et al, 1981). Commonly

observed *E. coli* numbers in municipal wastewater influent and effluent are summarized in Table 2.1.

**Table 2.1 Reported Bacterial Concentrations in Municipal Wastewater Treatment Plants**

| Bacteria Type (cfu*/ml) | Sampling Locations | | Reference |
|---|---|---|---|
| | Influent | Final Effluent | |
| Fecal coliforms | $5.0 \times 10^5$ | $1.4 \times 10^3$ | Gallert et al, 2005 |
| | $1 \times 10^4$ - $1.0 \times 10^6$ | - | Tchobanoglous et al, 2002 |
| *Escherichia coli* | $6.1 \times 10^4$ | $2.3 \times 10^2$ | Reinthaler et al, 2002 |
| Enterococci | $1.8 \times 10^4$ | $1.5 \times 10^2$ | Gallert et al, 2005 |
| | $1.0 \times 10^2$ - $1.0 \times 10^3$ | - | Tchobanoglous et al, 2002 |

* cfu = Colony forming units

### *2.2.3 Enterococci*

Enterococcus is a gram-positive bacterium commonly present in human intestines. Enterococci have been recognized as potentially pathogenic bacteria for humans for many years (Gilmore, 2002). The enterococci species *E. faecalis* and *E. faecium* have been identified as the most prevalent species responsible for clinical infections in humans (Gilmore 2002). Infections commonly caused by enterococci include endocarditis, bacteremia, urinary tract infections and intra-abdominal pelvic and soft tissue infections (Gilmore 2002). Many infecting strains originate in human intestines (Murray 1990). Table 2.1 shows the typical numbers of enterococci population found in influent and effluent wastewater streams in municipal wastewater treatment plants.

## 2.3 Emerging Contaminants of Concerns – Antibiotics in Wastewater

Antibiotics are used to treat a wide spectrum of bacterial infections. However, incomplete metabolism in humans has resulted in release of large amounts of antimicrobial compounds into wastewater treatment plants (WWTPs). Recent studies have discovered trace level concentrations of antibiotics in WWTP effluents and surface

waters (Kolpin et al. 2002; Christian et al. 2003; Miao et al. 2004; Koch et al. 2005; Close. 2007). Long-term exposure of microorganisms to low concentrations of antibiotics (ng/L to μg/L) in wastewater and surface water has the potential for the development of antibiotic resistance in these organisms (Gilliver et al. 1999; Khachatourians 1998; Smith et al. 1999).

## 2.4 Antibiotic Resistant Microorganisms

Antibiotic resistance is the ability of microorganisms to withstand the effects of antibiotics. The development and proliferation of antibiotic resistance in bacteria is of public health concern because a patient can develop an antibiotic resistant infection by contacting a resistant organism, or by having a resistant microbe emerge in the body as treatment with antibiotic begins (Lewis 1995).

In 1970, non-medical uses of antibiotics were questioned and antimicrobial agents were described as potential environmental contaminants and a threat to public health (Huber 1971). Since that time, several studies have reported the occurrence of antibiotic resistant organisms in environmental samples and advocated a global public health concern due to these bacteria (Pillai et al. 1997; Ash et al. 2002).

The important mechanisms by which microorganisms exhibit resistance to antibiotics include drug inactivation or modification, alteration of the target site, alteration in the metabolic pathway, and reduced drug accumulation (Katzung 2004). These mechanisms are described in more detail in the following paragraphs and shown in Figure 2.1.

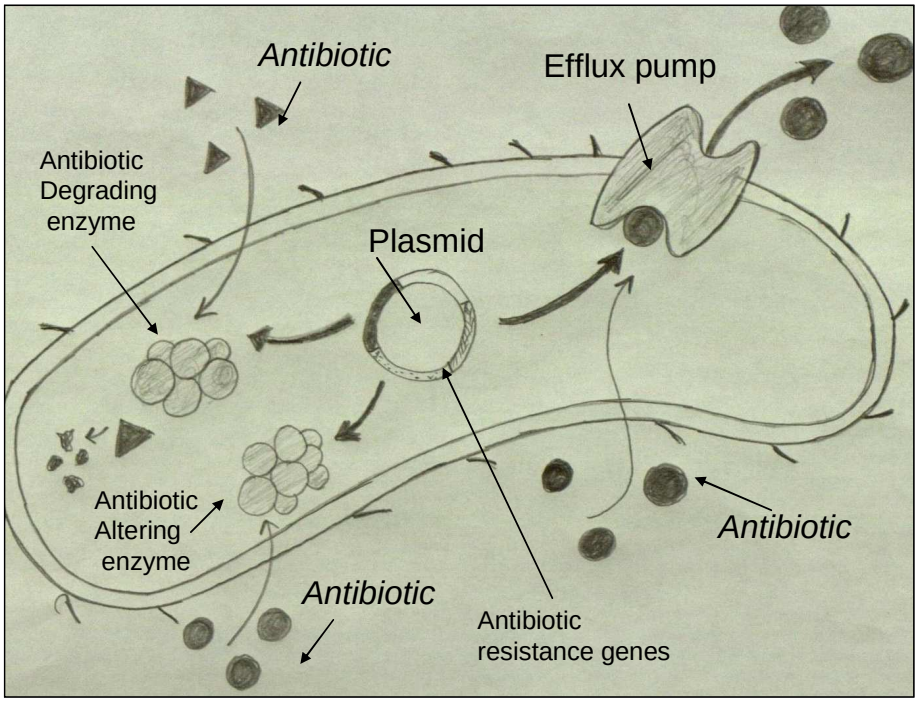
**Drug inactivation or modification**: Resistant bacteria synthesize and secret enzymes which affect the antimicrobial activity of the antibiotics. For example $\beta$-lactamases synthesized by antibiotic resistant bacteria hydrolyze the $\beta$-lactone ring of penicillin thereby inactivating the antibiotic (Katzung 2004).

**Alteration of target site**: Penicillin acts on bacteria by attaching to penicillin binding proteins (PBP), which are essential components for synthesis of bacterial cell wall. Bacteria develop resistance to penicillin either by the overproduction of PBPs or by synthesis of PBPs, which have low affinity to penicillins (Katzung 2004).

**Alteration of metabolic pathway**: Bacteria are able to modify their metabolic pathways in order to evade the action of antibiotics. For example, sulfonamides inhibit the synthesis of folic acid, and sulfanomide resistant bacteria develop alternate routes for synthesis of folic acid or derepress its synthesis (Katzung 2004).

**Reduced drug accumulation**: Bacteria developing resistance to antibiotics are able to reduce the uptake of the antibiotic by either altering the permeability of the drug or by enhancing active efflux of the drug (Katzung 2004).

**Figure 2.1 Antibiotic Resistance Mechanisms**

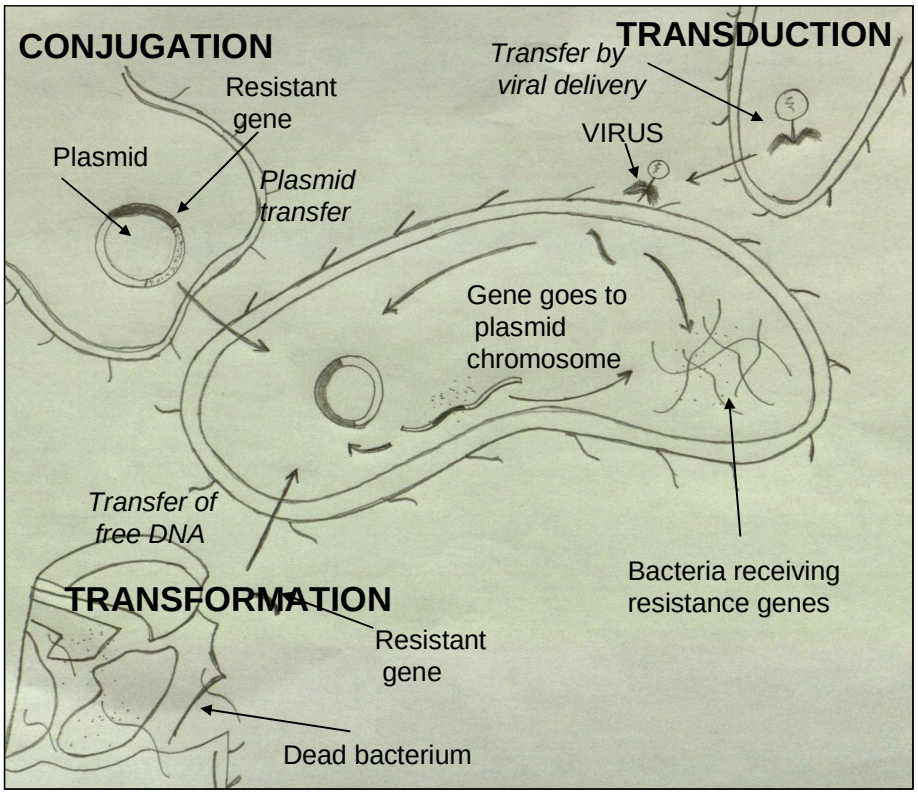



Yim, (2007)

Previously it was believed that resistance in bacteria was acquired by spontaneous mutation, which is called as primary resistance. The wide spread development of multiple antibiotic resistance in many species of bacteria led researchers to believe that another mechanism beyond spontaneous mutation was responsible for the acquisition of antibiotic resistance. The mechanism responsible for the development of resistance was through lateral or horizontal gene transfer. Horizontal gene transfer (HGT) has three possible mechanisms. Transduction, transformation and conjugation shown in Figure 2.2.

Transduction occurs when bacteria-specific viruses or bacteriophages transfer DNA between two closely related bacteria. Transformation is a process where parts of DNA are taken up by the bacteria from the external environment. This DNA present in the external environment is due to death of another bacterium. Conjugation occurs when there is direct cell-cell contact between two bacteria and transfer of small pieces of DNA called plasmids takes place (Yim 2007).

**Figure 2.2 Horizontal Gene Transfer**




Yim, (2007)

Recent studies have shown presence of antibiotic resistant bacteria in wastewater and surface waters. Gallert et al (2005), observed multi-resistant antibiotic fecal coliforms and enterococci in influent and effluent wastewater from treatment plants. Multiple anitibiotic resistant organisms have been observed in wastewater treatment plants across the world. More than 20 % of fecal coliforms were observed to be resistant to ampicillin, chloramphenicol, sulfanomide, tetracycline and streptomycin in one of the

treatment plant effluents in Finland (Niemi et al. 1983). Other studies across the world have revealed that fecal coliforms and *E. coli* in raw sewage were resistant to ampillicin, gentamycin, kanamycin, neomycin and streptomycin (Qureshi and Qureshi 1991).

Enterococci that were resistant to ciprofloxacin, sulfamethoxazole/trimethoprim and vancomycin at minimum inhibitory concentrations were also observed in raw and treated effluent wastewater (Gallert et al. 2005). *E. coli* were resistant to a wide range of antibiotics in raw and treated sewage (Hassani et al, 1992). Recent studies have identified fluoroquinolone resistant *E. coli* isolates in leukemia patients (Kern et al. 1994).

Antibiotic resistance provides a survival benefit to microorganisms and makes it difficult to eliminate the infections caused by them. Infections caused by antibiotic resistant bacteria are hard to treat. Hence, physicians have to prescribe higher dosage of alternative antibiotics to cure the infections. High doses have side effects and the potential to produce more antibiotic-resistant strains of bacteria. Hence, there is a need to study antibiotic resistance patterns in wastewater bacteria.

## 2.5 Target Antibiotics

Ciprofloxacin (CIP), sulfamethoxazole/trimethoprim (SXT) and vancomycin (VAN) were selected as the target antibiotics for this work. The rationale for choosing these compounds as target antibiotics was based on past work in KSU's environmental engineering laboratories (Koch et al. 2005; Close 2007) which had reported the occurrence of CIP, sulfamethoxazole (SMX) and azithromycin (AZI) in municipal wastewater treatment plants. This made it likely that the microbial biomass in these plants also included antibiotic resistant strains of bacteria.

Trimethoprim is used in combination with sulfamethoxazole and was added as a target antibiotic in this study. Vancomycin was selected because enterococci were resistant to many antibiotics and VAN is the only drug that is effective to treat the infections caused by resistant enterococcus bacteria (Wegener et al, 1999). These target antibiotics are described in further detail in the following sections.

### *2.5.1 Ciprofloxacin*

Ciprofloxacin is a widely prescribed antibacterial agent belonging to the fluoroquinoline group and is used to treat infections caused by gram-negative and gram-

positive bacteria (Katzung 2004). CIP is effective in treating patients suffering from cirrhosis (Hsieh et al., 1998). It is also used to treat urinary tract infections, skin and bone infections, gastrointestinal infections caused by multi-drug-resistant organisms, lower respiratory tract infections, febrile neutrophenia, and intra-abdominal infections (Davis et al, 1996). Table 2.2 summarizes the reported concentration of CIP in wastewater and surface waters.

**Table 2.2 Reported Antibiotic Concentrations Observed in Wastewater and Surface Water**

| Antibiotic | Location | Concentration | Reference |
|---|---|---|---|
| Ciprofloxacin | Plant Effluent | 36 – 106 ng/L | Golet et al, 2002 |
| Sulfamethoxazole | Plant Effluent | 6000 ng/L | Giger et al, 2003 |
| Trimethoprim | Plant Effluent | 154 ng/L | Hernando et al, 2005 |
| Ciprofloxacin | Surface Water | 12 ng/L | Christian et al, 2003 |
| Sulfamethoxazole | Surface Water | 40 - 200 ng/L | Christian et al, 2003 |
| Trimethoprim | Surface Water | 6 - 70 ng/L | Christian et al, 2003 |
| Vancomycin | Surface Water | 4.8 ng/L | Mulroy, 2001 |

### *2.5.2 Sulfamethoxazole/Trimethoprim*

Sulfamethoxazole is a sulfanomide group of antibiotic and trimethoprim is a synergist of the sulfonamide group (Katzung 2004). Trimethoprim is used in combination with other drugs. SXT is used to treat patients suffering from Wegener's granulomatosis, is a rare disease that primarily affects the upper respiratory tract, lungs and kidneys. This disease is characterized by inflammation in various tissues including blood vessels (Israel 2006). SXT is also used to treat the human immune deficiency virus (HIV) infection and pneumonia caused by *Pneumocystis carinii* (Carr et al. 1992). Concentrations of sulfamethoxazole and trimethoprim observed in wastewater effluent and surface waters are shown in Table 2.2.

### *2.5.3 Vancomycin*

Vancomycin is a glycopeptide antimicrobial agent and is active against infections caused by mainly gram-positive bacteria (Bauer 2001). It is used as a "last resort" antibacterial agent. When treatment with other antibiotics has failed, antibiotic resistant enterococci can be treated with vancomycin. Vancomycin inhibits synthesis of a cell wall and acts synergistically with aminoglycosides for organisms such as enterococci (Briles et al. 2006). VAN is used to treat infections like pseudo membranous colitis and infections caused by susceptible organisms resistant to penicillin's (methicillin-resistant *staphlococcus aureus* and multiresistant *staphylococcus* epidermidis) (Gibson and Owen 1998; Nagarajan 1994). Table 2.2 shows the observed concentration of vancomycin in surface water.

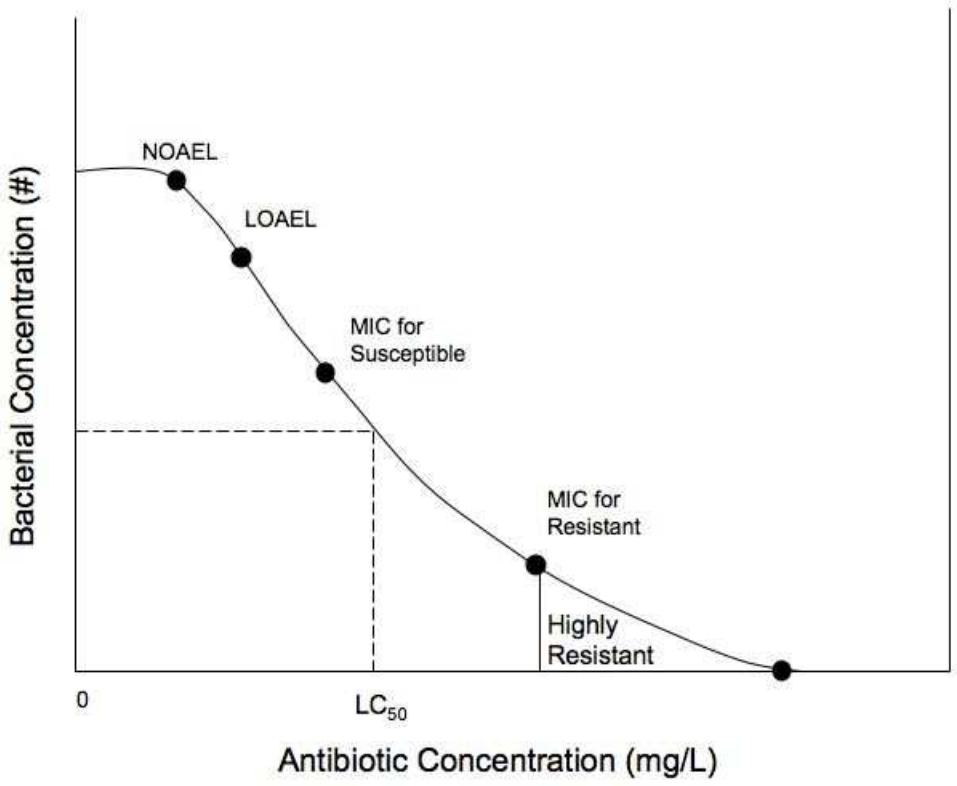
## 2.6 Minimum Inhibitory Concentration

Antibiotics, just like other toxic agents, exhibit a dose-response relationship in bacterial cultures. The response typically is mortality. Figure 2.3 illustrates a hypothetical dose response curve. Exposure to an antibiotic results in a reduction in observed bacterial concentration or population. The lowest concentration in a dose-response assay at which no bacteria are affected is called the 'no observed adverse effect level' or NOAEL. The lowest antibiotic concentration at which a significant decrease in the bacteria concentration is noted is considered to be the 'lowest observable adverse effect level' or LOAEL. As the antibiotic concentration is increased, the concentration resulting in a 50% kill of the bacterial population is described as the lethal concentration for 50% kill or $LC_{50}$. NOAEL, LOAEL and $LC_{50}$ depend on the type of bacteria, type of antibiotic and environmental conditions, including matrix chemistry.

Bacteria may be considered 'susceptible' to antibiotic resistance at a given concentration if a significant fraction survives exposure to the antibiotic at that concentration. These clinical concentrations for various antibiotics were described by Wu (1995) and are summarized in Table 2.3.The experimental protocol for determination minimum inhibitory concentration (MIC) was obtained from Andrews (2001). Bacteria susceptible to antibiotic resistance require a high dose of antibiotic for deactivation. Bacteria that grow at exposures lower than the susceptible level of an antibiotic are

considered potentially antibiotic resistant. The Minimum inhibitory concentration (MIC) is defined as the lowest concentration of antibiotic that inhibits the visible growth of bacteria (Andrews, 2001).

**Figure 2.3 Antibiotic Dose-Response Curve for Resistant Bacteria**




MIC = Minimum inhibitory concentration, NOAEL = Non-observable adverse effect level, LOAEL = Low-observable adverse effect level

Bacteria that are observed to grow at MIC level exposure are defined as antibiotic-resistant organisms while those that grow at even higher antibiotic exposures are considered highly resistant to the antibiotic. Bacteria that grow at exposures higher than the 'susceptible concentration' but lower than MIC are considered to have intermediate resistance to the antibiotic. MIC is important in the field of medicine to confirm the resistance of microorganisms to an antibiotic and to monitor the activity of

newly developed antibiotics. MICs for the target antibiotics used in this study are tabulated in Table 2.3.

**Table 2.3 Equivalent Minimum Inhibitory Concentration Breakpoints**

| Antibiotic | Susceptible Level (mg/L) | Resistant Level (mg/L) |
|---|---|---|
| Ciprofloxacin | < 1 | > 4 |
| Sulfamethoxazole/Trimethoprim | < 2/38 | > 8/152 |
| Vancomycin | < 8 | > 32 |

Wu, (1995)

## 2.7 Antibiotic Resistant Enterococci

The past few years have witnessed an increasing interest in the study of antibiotic resistant enterococci. Enterococci are the second or third most important bacterial genus responsible for hospital infections (Klare et al, 2003). *Enterococci* are intrinsically resistant to a wide range of antibiotics (Gilmore, 2002). Hence, this has always limited the choice of antibiotics against these organisms available for use. Nosocomial infections are caused by enterococci and, therefore, antibiotics have been used in greater frequency in hospitals. Murray (1999) has observed in his research that 12 species of *Enterococci* are pathogenic for humans including most common human isolates *Enterococcus faecalis* and *Enterococcus faecium*. *Enterococcus faecalis* causes 80% to 90% of human enterococcal infections, while *E. Faecium* accounts for a majority of the remainder (Moellering, 1992; Murray 1990; Schnell 1992)

Resistance in enterococci was developed by acquiring resistance genes on plasmids or transposons from other organisms or by spontaneous mutations (Gilmore, 2002). Resistant Enterococci were entering the environment through wastewater effluents and hospital wastewater.

# CHAPTER 3 - RESEARCH OBJECTIVES

The primary objective of the study was to analyze the antibiotic resistance patterns in municipal wastewater bacteria. Another objective of the study was enumeration and identification of cultured enterococci. Based on previous studies shown the prevalence of antibiotics in WWTP, it was hypothesized that:

1. Bacteria in WWTPs, specifically fecal coliforms, *E.coli* and enterococci include strains that are resistant to antibiotics such as ciprofloxacin, sulfamethoxazole/trimethoprim and vancomycin.
2. Activated sludge process is capable of removing antibiotic resistant bacteria from the aqueous phase.
3. UV disinfection effectively removes antibiotic resistant bacteria.
4. Biological treatment has effect on species diversity in the enterococcal population.
5. Exposure to high levels of sulfamethoxazole/trimethoprim and ciprofloxacin does not affect species diversity in the enterococcal population.

# CHAPTER 4 - MATERIALS AND METHODS

## 4.1 Wastewater Sampling

The municipal wastewater treatment plant selected for this study processes approximately 5 million gallons of water per day using a completely mixed activated sludge process (CMAS) with nitrification. CMAS is a biological process that utilizes microorganisms to transform the dissolved and particulate organic matter present in the wastewater. Carbonaceous organic matter in wastewater serves as an energy source for the production of new cells in a mixed population of microorganisms.

Preliminary treatment (Figure 4.1) at this plant consists of bar screening and aerated grit removal; the plant uses no primary clarifier. The wastewater treatment facility is operated with a hydraulic retention time (HRT) of 6 to 8 h and a sludge age or mean cell residence time (MCRT) ranging from of 4 days in summer and 7 days in winter. A portion of the settled biosolids from the activated sludge process is in recycled into the aerator of the activated sludge process through the help of splitter box. The excess biosolids produced during treatment are sent to the aerobic digesters for stabilization before disposal. Digesters are operated at a detention time of 107 days and the digested sludge is land-applied on agricultural fields. The secondary clarifier effluent is disinfected by allowing the water to flow through channels containing ultra violet (UV) lamps. UV disinfection destroys bacteria by disrupting their genetic material (Chang et al, 1985) and the treated final effluent is released into the receiving stream.

### *4.1.1 Sample Collection*

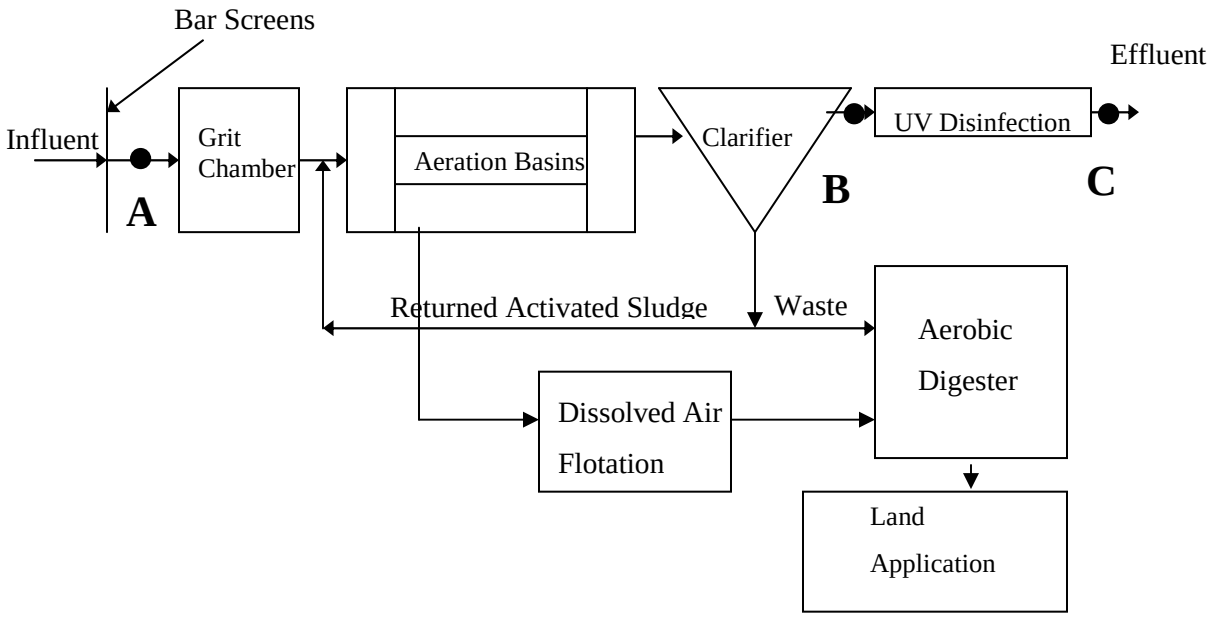
Wastewater samples were collected for the study from a nearby municipal wastewater treatment plants. The process schematic of this plant is illustrated in figure 4.1. Sampling was performed at various locations in the WWTP including the influent (raw sewage), secondary clarifier (SC) effluent and the final plant effluent after UV disinfection. Twenty-four hour composite samples were obtained at the influent and disinfected effluent sampling locations. The composite sampler (Sonford model TC-2, St. Paul Park, MN) utilized magnetic flow meters and removed a 250 ml sample from the screened influent flow after every 20,000 gallons of flow during a 24-hour sampling

period. Grab samples were collected from the secondary clarifier weir (after biological process) with a help of a container.

### *4.1.2 Sample Transport and Storage*

Samples were collected in 150 ml pre sterilized glass bottles and transported to the laboratory in a cooler. The cooler was filled with ice packs to preserve samples during transportation. Glass bottles used for the collection of samples were sterilized in an autoclave for 20 minutes using saturated steam under a pressure of 15 psi and a chamber temperature of at least $121^0$ C ($250^0$ C). Samples transported to the laboratory were stored at $4^0$ C in the refrigerator and analyzed on the day of collection.

**Figure 4.1 Process Flow Schematic of Municipal Wastewater Treatment Plant. Points A, B and C mark Sampling Locations**




## 4.2 Target Antibiotics

This work focused on four antibiotics. Two of these compounds (CIP and SMX) were selected because a pervious study conducted at the plant had observed trace levels of these pharmaceuticals in the influent and effluent stream. Trimethoprim was selected

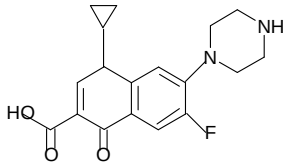
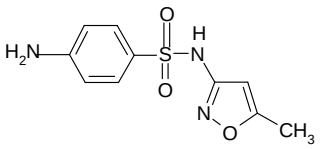
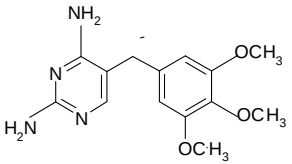
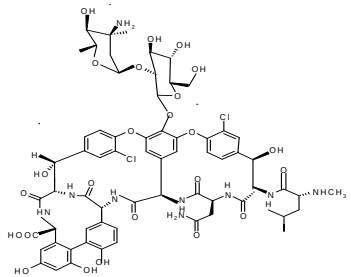
because it is used synergistically with SMX. VAN was also selected as a target antibiotic because it is used as “last resort” for the patients infected with gram-positive bacteria. Ciprofloxacin hydrochloride was purchased from ICN Biomedicals Inc (Irvine, CA) while vancomycin hydrochloride and sulfamethoxazole/trimethoprim were obtained from Sigma Aldrich (St. Louis, MO). Selected properties of the target antibiotics are tabulated in Table 4.1.

A stock solution of CIP was prepared by dissolving 100 mg of ciprofloxacin hydrochloride into 100 ml of distilled deionized water in a 250 ml volumetric flask. The antibiotic was allowed to dissolve completely for 5 min, transferred into a 250 ml serum bottle and labeled as CIP 100 mg/100 mL stock solution with the preparation date. The serum bottle was closed with Teflon lined rubber stopper, capped with aluminum crimp caps and stored at room temperature as recommended by the manufacturer.

A stock solution of SXT was prepared by dissolving 1900 mg of sulfamethoxazole and 100 mg of trimethoprim into 100 ml of DMSO in a 250 ml volumetric flask. The antibiotic was allowed to dissolve completely for 5 min and transferred into a 250 ml serum bottle labeled as SMT 100 mg/100 ml. The serum bottle was stoppered, capped and stored at room temperature as recommended by the manufacturer.

A stock solution of VAN was prepared by dissolving 100 mg of vancomycin in 100 ml of 1:1 ethyl alcohol and de-ionized distilled water in a 250 ml volumetric flask. The antibiotic was allowed to dissolve for 5 min and transferred into a serum bottle labeled VAN 100 mg/100 ml stock solution. The serum bottle was stoppered, capped and stored at 4°C in the refrigerator as recommended by the manufacturer.

**Table 4.1 Selected Properties of Target Antibiotics**

| **Antibiotic Type/ Properties** | **Ciprofloxacin** | **Sulfamethoxazole** | **Trimethoprim** | **Vancomycin** |
|---|---|---|---|---|
| Molecular structure |  |  |  |  |
| Empirical formula | $C_{17}H_{18}FN_3O_3$ | $C_{10}H_{11}N_3O_3S$ | $C_{14}H_{18}N_4O_3$ | $C_{66}H_{75}Cl_2N_{924}.HCl$ |
| Molecular weight | 331.4 gram/mole | 253.28 gram/mole | 290.32 gram/mole | 1485.71 gram/mole |
| Form | Powder | Powder | Powder | Powder |
| Class of antibiotics | Fluoroquinolone | Sulfonamide | Other Antimicrobial Agent | Glycopeptide antibiotic |
| Solvent | Water | Water | Dimethyl sulfoxide (DMSO) | Ethyl alcohol |
| Mode of action | Inhibits DNA gyrase | Inhibits para-amino benzoic acid (PABA) | Interferes with dihydrofolate reductase | Interferes with cell wall |
| Gram type | Gram-negative | Gram-negative | Gram-negative | Gram positive |

## 4.3 Bacterial Enumeration

The membrane filter technique was used to isolate discrete colonies of bacteria from the wastewater. This method was easy to perform with large volume of samples and results could be obtained rapidly compared to other methods (Hobbie et al. 1977). Enterococcus, fecal coliforms and *Escherichia coli* were the target organisms that were enumerated by the above-specified technique. The target organisms are fecal indicators of water quality and have the potential to develop antibiotic resistance.

### *4.3.1 Preparation of Selective Media Containing Antibiotics*

**mFC Agar**: Membrane fecal coliform (mFC) agar is a selective medium used to enumerate fecal coliforms. This media was purchased in powder form from Fisher Scientific (Hampton, NH). The media supports growth of fecal coliforms while inhibiting the growth of other organisms. The mFc agar solution was prepared by adding 52 grams of mFc agar powder to 1 L of distilled water in a 2 L conical flask. The solution was stirred to dissolve any clumps of agar. A 1% rosolic acid solution was prepared by adding 100 mg of rosolic acid (4-[bis (4-hydroxyphenyl) methylene]-2, 5-cyclohexadien-1-one) powder to 10 ml of 0.2N NaOH solution. Rosolic acid inhibits bacterial growth in general, except for fecal coliforms. Ten milliliters of the 1% solution of rosolic acid was added to 1 L of agar and the solution continuously stirred and heated for 10 min. The heated agar solution was allowed to cool to $50^{o}$ C. Precise amounts of the selected antibiotics (CIP, SMT, VAN) were added to the agar solution to achieve the predetermined antibiotic concentration in solution. Ten milliliters of agar solution were transferred into presterilized Petri dishes (Fisher Scientific, Hampton, NH) and the petri dishes with agar solution were allowed to dry for 24 h in a laminar flow hood. The dishes were stored in the refrigerator at 4°C until plating.

**KF streptococcus Agar:** KF streptococcus agar is a selective medium used to grow fecal enterococci bacteria. This medium was purchased in powder form from Fisher Scientific (Hampton, NH). KF agar supports the growth of fecal enterococci while inhibiting the growth of other organisms. KF agar solution was prepared by adding 76.5 grams of the agar to 1L of distilled water in a 2L conical flask**.** The solution was heated and stirred continuously for 5 minutes. The agar solution was thereafter cooled to $50^{o}$ C

and modified by adding 10 ml of 1% TTC (2,3,5 triphenyltetrazolium chloride). The 1% TTC (fisher Scientific) solution was prepared by adding 10 mg of TTC powder to 10 ml of distilled water. TTC was used as a redox indicator in agar solution to differentiate the bacteria. Predetermined amounts of the selected antibiotics (CIP, SXT, and VAN) were added to the agar solution. Ten milliliters of KF agar solution were transferred into each presterilized petri dish and the dishes were allowed to dry for 24 h and there after stored at $4^{o}$ C.

**Nutrient Agar with MUG Medium:** MUG (4-methyl umbrelliferyl $\beta$-D gluconoride) media (Fisher Scientific, Hampton, NH) was used for the detection and enumeration of *Escherichia coli* in wastewater sample. Approximately, 23.1 grams of nutrient agar in 1 L distilled water was boiled to dissolve completely. The agar solution was sterilized at $121^{o}$-$124^{o}$ C for 15 minutes and allowed to cool to $50^{o}$ C. Precise amounts of the selected antibiotics (CIP, SXT, and VAN) were added to the nutrient agar and the solution was stirred for few seconds to ensure that antibiotics were completely distributed in the solution. Ten millimeters of nutrient agar was transferred into each pre-sterilized Petri dish. The dishes were allowed to dry for 24 h and stored at $4^{o}$ C.

### *5.3.2 Membrane Filtration Technique*

Individual bacteria are difficult to count because of their small size. Direct counts of bacteria are possible under the microscope but it requires a lot of time and expertise. One of the easiest methods to count bacterial colonies in water is by spreading a sample over a wide area of culture media and counting the colonies that grow on it. When the bacteria are spread on the media, each bacterial cell in the original sample produces a single colony of daughter cells. However, this approach has difficulties if the solution has a large number of bacteria because the number of colonies produced can overlap one another on the petri dish. Such challenges are usually overcome by using serial dilution techniques (Sahm and Washington 1991).

Samples collected from the WWTP were diluted in Benzer dilution fluid (BDF). The BDF solution consisted of 7 grams of NaCl and 1 gram of Trypticase soy agar mixed in 1 L of distilled water. BDF solution was sterilized in the autoclave and used for dilution of wastewater samples. Five pre-sterilized test tubes were filled with 9 ml of

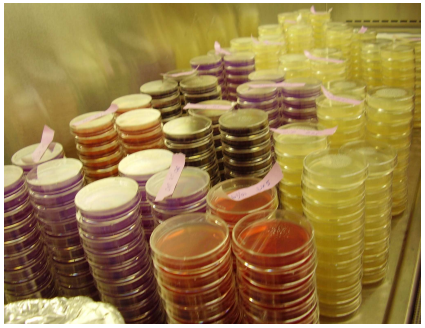
BDF solution. Sample bottles were shaken in order to evenly distribute the bacteria present in the wastewater. One-milliliter aliquot of the sample was transferred into the first tube and mixed to produce a 1/10 dilution. One mL of diluted sample from this first tube was transferred to the second tube and mixed to produce a 1/100 dilution and the process was repeated until the 1/1000, $1/10^4$, $1/10^5$ dilutions had been prepared in the five tubes. Aliquots were transferred using sterilized pipettes. The plates were labeled with sampling location, date and sample number. The samples were filtered using a 0.45 $\mu$m, 47 mm, diameter, cellulosic white grid filter (Fishers scientific, Hampton, NH) placed on fa 3-prong filter holder (Figure 4.2b). Approximately 25 ml of distilled water was first added to wet the filter paper. Precise volumes of diluted samples were then transferred on to the filter (Figure 4.2c)

A vacuum pump system was used to expedite the passage of water through the filter while bacteria were collected on the membranes. Filters were removed, carefully and transferred with the help of sterilized forceps on to the appropriate selective media plates (Figure 4.2d). All inoculated agar plates were incubated in a temperature controlled shaker an (Environ Shaker 3597, Lab-Line Instruments Inc IL) at temperatures and time as shown in Table 4.5.

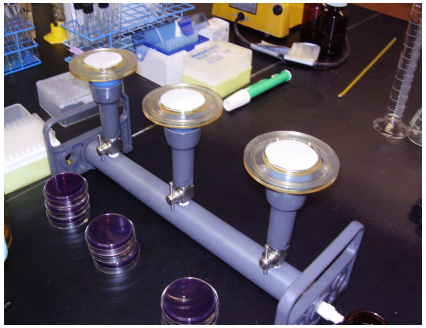
**Table 4.2 Incubation Time and Incubation Temperatures for Target Organisms**

| **Type of Bacteria** | **Culture Medium** | **Incubation Temperature** | **Incubation Time** |
|---|---|---|---|
| Fecal coliforms | mFC agar | $44.5^o$ C | 24 h |
| Fecal enterococcus | KF agar | $35^o$ C | 48 h |
| *Escherichia coli* | Nutrient MUG agar | $37^o$ C | 2 – 4 h |

**Figure 4.2 Bacterial Enumeration (a) Culture Media on Petri Dishes; (b) Filter Holder; (c) Sample Filtration; (d) Transfer of Filter Membrane on Culture Media; (e) Incubation; (f) Fecal Coliforms on Filter Membrane; (g) Dark Field Quebec Colony Counter; (h) *E. coli* under UV Light**

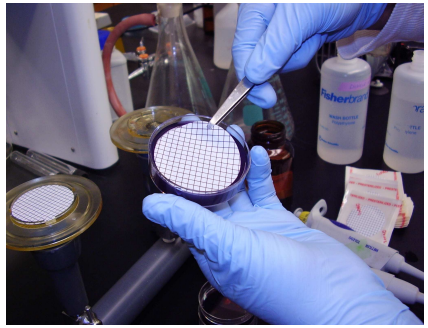


(a)



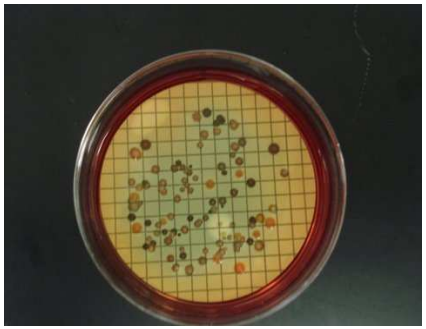
(b)

(c)



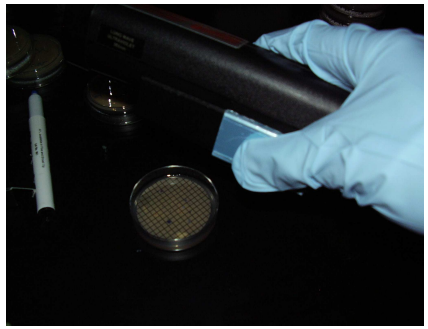
(d)

(e)



(f)

(g)



(h)

Presumptive enterococcal colonies were sub-cultured on trypticase soy agar (TSA; Becton Dickinson, MA, USA), incubated at 37$^{o}$C for 24 h and stored at 4$^{o}$C for further analysis.

## 4.4 Identification of Enterococci

Multiplex or single polymerase chain reactions (PCR) were used to identify enterococci at species level. PCR is an approach, which allows the amplification of a short, well-defined part of a deoxyribonucleic acid (DNA) strand into millions. This can be a single gene, just a part of a gene. DNA is double-stranded and is measured in complementary DNA building blocks (nucleic acids) called base pairs (bp). PCR can amplify only short DNA fragments, usually up to 10-kilo base pairs (1000 bp).

PCR requires following basic components:

- Template DNA, which contains the region of the DNA fragment to be amplified;
- Forward and Reverse primers, which determine the beginning and end of the region to be amplified;
- DNA polymerase, which synthesizes a DNA copy of the region to be amplified;
- Nucleotides, from which the DNA polymerase builds the new DNA; and
- Buffer, which provides a suitable chemical environment for the DNA polymerase

The PCR reaction is carried out in small reaction tubes (0.2-0.5 ml volumes) inserted into a thermal cycler. This machine heats and cools the reaction tubes within it to the precise temperature required for each step of the reaction. A heated lid is placed on top of the reaction tubes to prevent evaporation of the reaction mixture. PCR is an especially valuable tool because the reaction is highly specific, easily automated, and capable of amplifying minute amounts of sample.

### *4.4.1 Multiplex Polymerase Chain Reaction*

Species-level identification was performed using multiplex PCR for four common enterococcus species: *E. faecalis, E. faecium, E. casseliflavus* and *E. gallinarum.* Briefly,

the multiplex PCR was performed with described primers (Table 4.6) targeted at the D-alanine-D-alanine ligase *(ddl*) genes of *E. faecalis* and *E. faecium* (Dutka-Malen et al. 1995), the *vanC1* gene of *E. gallinarum,* and the *vanC2/C3* gene of *E. casseliflavus* (Kariyama et al. 2000) in a final volume of 25 μL consisting of 23 μL of master mix (10x buffer, 1.5 mM of $MgCl_2$, 0.25 mM each deoxynucleotide triphosphate (dATP, dCTP, dGTP,and dTTP), 1.2 U of Taq DNA polymerase) and 2.0 μL of template DNA. Overnight bacterial cultures on LB agar were used to extract the DNA. For DNA template, 1-2 colonies of pure culture were suspended in a mixture of 25 $\mu$L of $ddH_2O$ and 25 $\mu$L of chelex in micro centrifuge tubes (Fisher Scientific, Fairlawn, NJ). Tubes were boiled for 10 min at $100^{o}$ C. The microcentrifuge tubes were centrifuged (Centrifuge 5415 D, Eppendorf, Westbury, NY) at 7,200 rpm for 1 min. The supernatant obtained from centrifuging contained the template DNA.

**Table 4.3 Multiplex PCR Primers used for Detection of Vancomycin Resistance Enterococci**

| **Primer Name** | **Positive Control** | **Sequence 5'→ 3'** | **Primer Conc. (pM)** | **Product (bp)** |
|---|---|---|---|---|
| *E. gallinarum* | ATCC 49579 | GGTATCAAGGAAACCTC<br>CTTCCGCCATCATAGCT | 2.5 | 822 |
| *E. casseliflavus* | ATCC 25788 | CGGGGAAGATGGAGTAT<br>CGCAGGGACGGTGATTTT | 2.5 | 484 |
| *E. faecalis* | ATCC 19433 | ATCAAGTACAGTTAGTCTTTATTAG<br>ACGATTCAAAGCTAACTGAATCAGT | 5.0 | 941 |
| *E. faecium* | ATCC 19434 | GGATTAGATACCCTGGTAGTCC<br>TCGTTGCGGGACTTAACCCAAC | 1.25 | 658 |
| 16S rDNA | - | GGATTAGATACCCTGGTAGTCC<br>TCGTTGCGGGACTTAACCCAAC | 2.5 | 320 |

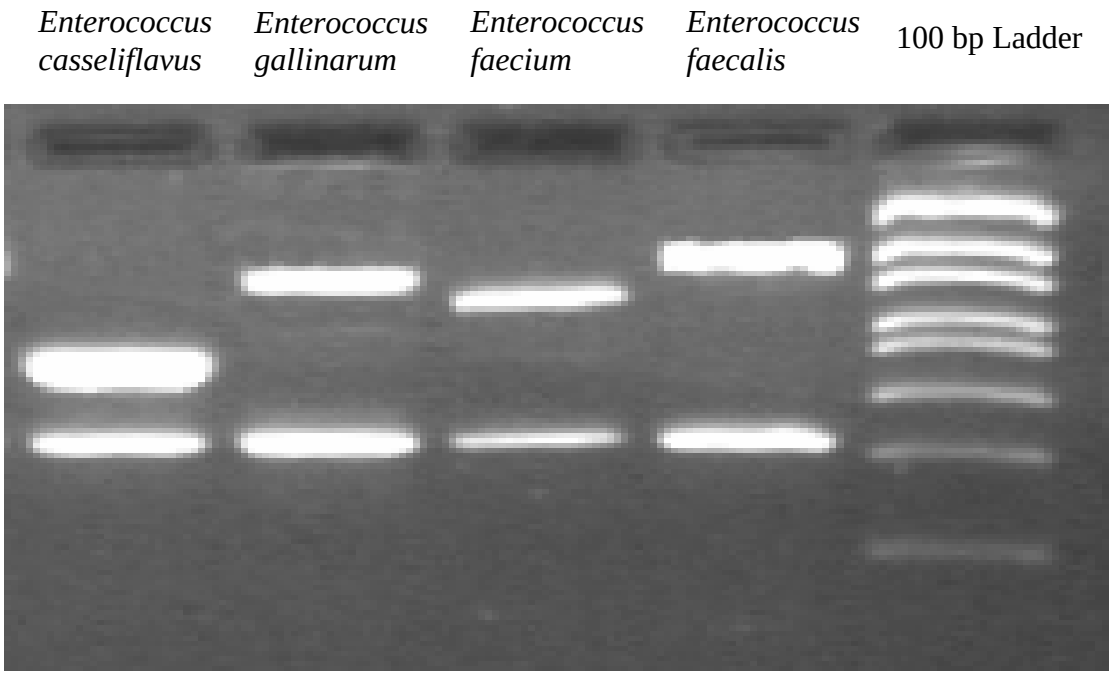
Amplification was conducted using a Peltier Thermal Cycler (MJ Research, Waltham, MA, USA) using the process described previously (Kariyama et al. 2000).

Fifteen microliters of PCR product was electrophoresed on a 3.0% agarose gel (Fisher Scientific, Fairlawn, NJ) containing 0.05% of ethidium bromide and visualized under UV light using the Bio-Rad Gel Doc imaging system (Bio-Rad Laboratories, Hercules, CA). Control strains consisting of *E. faecalis* ATCC 19433, *E. faecium* ATCC 19434, *E. gallinarum* ATCC 49579 and *E. casseliflavus* ATCC 25788 were included with each PCR assay. *E. mundtii* ATCC 43186 was used as negative control.

### *4.4.2 Single Polymerase Chain Reaction*

For unidentified isolates, the *sodA* gene encoding the manganese-dependent superoxidase dismutase was amplified by single PCR using sodA degenerate primers: *sodA* forward d1 (5'-CCITAYICITAYGAYGCIYTIGARCC-3') and *sodA* reverse d2 (5'-ARRTARTAIGCRTGYTCCCAIACRTC-3') (Poyart et al, 2000). For screening of *SodA* gene, 1-2 colonies of pure culture from LB agar (Becton Dickinson, MA) was suspended in a mixture of 25 $\mu$L of dd$H_2O$ and 25 $\mu$L of chelex in microcentrifuge tubes. The suspension was boiled for 10 min, and then centrifuged for 5 min at 7,200 rpm to extract DNA. Two $\mu$L of extracted DNA was used as a template for PCR. The master mix contained: 100 nM of each primer, 400 µM dNTPs, 3mM $MgCl_2$ and 0.5 unit *Taq* polymerase (all from Promega, Madison, WI) in a final volume of 25 $\mu$L. Amplification was conducted using a Peltier Thermal Cycler (MJ Research, Waltham, MA) with the program described previously (Poyart et al, 2000). PCR products were purified using the GFX PCR DNA and gel band purification kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and visualized under UV light (Figure 4.3) on a 1.0% agarose gel (Fisher Scientific, Fairlawn, NJ) with 0.05% of ethidium bromide. Sequencing analysis of the *sodA* gene (480 bp) wasperformed using an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) at the Kansas State University DNA Sequencing Facility using the same *sodA* degenerate primers used for PCR. The sequences were compared with the sequences in the National Center for Biotechnology Information GenBank database by using Basic Local Alignment and Search Tool (BLAST) (Altschul et al. 1990). Sequences were manually aligned and edited with Codoncode Aligner Version 1.3.4 (CodonCode Corporation, Dedham, MA).

**Figure 4.3 Gel Picture for Multiplex Polymerase Chain Reaction**




## 4.5 Statistical Analysis

The enumerated bacteria data in wastewater was analyzed using one-way analysis of variance (ANOVA) in a completely randomized experimental design. Means were compared using the least square means (LSMEANS) procedure (p value = 0.05) of a general linear model (PROC GLM). The comparison of influent and SC effluent samples for prevalence and diversity of enterococci were evaluated using chi- square analysis of contingency tables and Fisher's exact test (p value = 0.05). The SAS software (SAS Institute 2003) statistical software used to conduct statistical analysis.

# CHAPTER 5 - RESULTS AND DISCUSSION

This section describes the results obtained from preliminary and final experiments conducted to evaluate the prevalence of antibiotic resistant bacteria in municipal wastewater treatment systems. Preliminary experiments were performed using duplicate samples of raw influent and secondary clarifier (SC) effluent collected during summer (July-August). Final experiments were performed using triplicate samples collected from raw influent, SC effluent and disinfected effluent during winter (December) and spring (March). The average bacterial counts observed in duplicate and triplicate samples are illustrated in figures and discussed in this section. Controls represent enumeration of samples in media with no antibiotics.

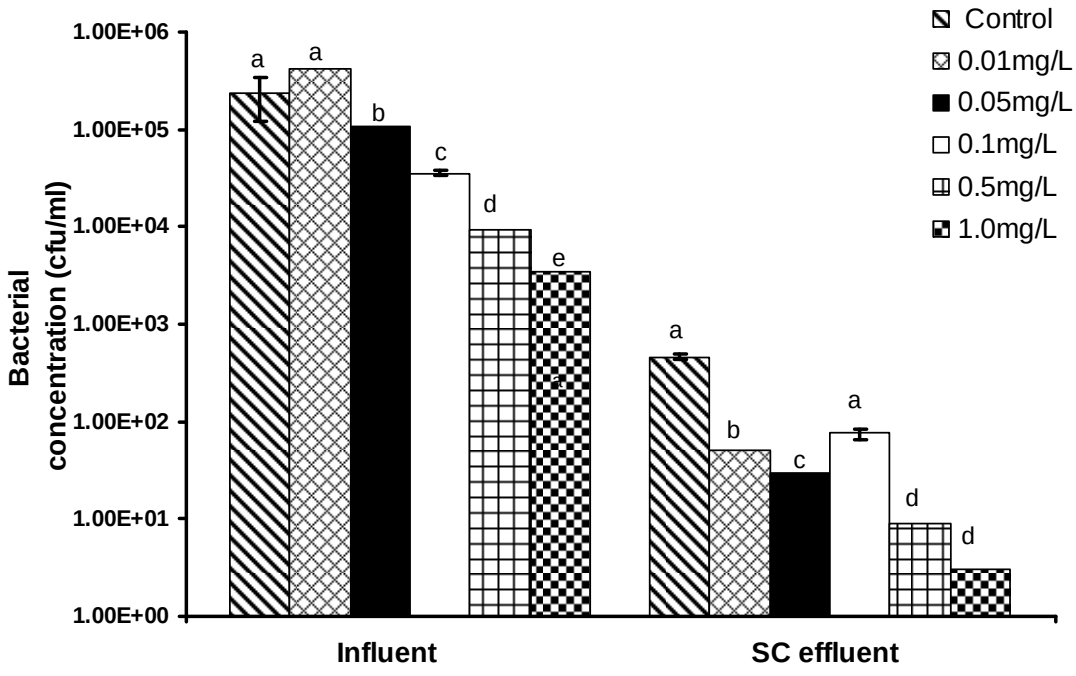
## 5.1 Antibiotic Resistant Bacteria in Summer Samples

The bacterial enumeration conducted in summer was performed with duplicate samples. The influent wastewater temperature during the sampling period was 26°C. Samples were collected from the influent and the SC effluent. Fecal coliforms and enterococcus bacteria were grown in media containing ciprofloxacin (CIP), sulfamthoxazole/trimethoprim (SXT) and vancomycin (VAN) at concentrations below the minimum inhibitory concentration (MIC) levels specified by Wu (1995). Lower concentrations were evaluated to determine the no observable adverse effect level (NOAEL) and the lowest observed adverse effect level (LOAEL) of the pharmaceutical agent on the target wastewater organisms.

### *5.1.1 Fecal Coliforms*

Figure 5.1 illustrates the impact of various concentrations of CIP on the growth of fecal coliforms obtained from the raw influent and SC effluent samples. The raw wastewater was found to contain approximately $2.33 \times 10^5$ cfu/mL fecal coliforms in the 24-hr composite samples (control samples in Figure 5.1). The activated sludge wastewater treatment process resulted in a reduction in the aqueous fecal coliform concentration to approximately, $4.65 \times 10^2$ cfu/mL (2 log removal) in the SC effluent.

**Figure 5.1 Impact of Ciprofloxacin Concentration on Fecal Coliforms in Influent and Secondary Clarifier Effluent Samples**




Similar alphabets represent no significant difference within dataset

Fecal coliforms in the influent and SC effluent wastewater samples were exposed to five different concentrations of CIP - 0.01, 0.05, 0.1, 0.5 and 1.0 mg/L. The fecal coliform population remained unaffected when exposed to CIP 0.01 mg/L; hence this dose was considered NOAEL under the experimental conditions. CIP concentration of 0.05 mg/L had a significant (p value = 0.0475) impact on the growth of fecal coliforms in the raw wastewater and was, therefore, considered to represent LOAEL for this evaluation. The CIP concentration of 0.05 mg/L reduced the bacterial count to $1.08 \times 10^5$ cfu/mL. The $LC_{50}$ for CIP exposure to fecal coliforms in the influent wastewater sample was estimated to be at a value of < 0.05 mg/L. Fecal coliform numbers at higher antibiotic exposures were reduced to approximately $3.55 \times 10^4$, $9.25 \times 10^3$, and $3.4 \times 10^3$ cfu/mL at CIP concentrations of 0.1, 0.5 and 1.0 mg/L, respectively. SC effluent samples exposed to CIP 0.01 mg/L showed approximately 50 cfu/mL indicating a $LC_{50}$ value of < 0.01 mg/L in the SC effluent and LOAEL of 0.01 mg/L. It is possible that matrix effects

were reduced in the relatively cleaner effluent sample causing the antibiotic to be a more potent bactericide with a lower $LC_{50}$ value. When exposed to CIP 0.05 mg/L, CIP 0.1 mg/L, CIP 0.5 mg/L and CIP 1.0 mg/L, the SC effluent fecal coliform concentrations were further reduced to approximately 30 , 75, 9.0 and 3.0 cfu/mL, respectively.

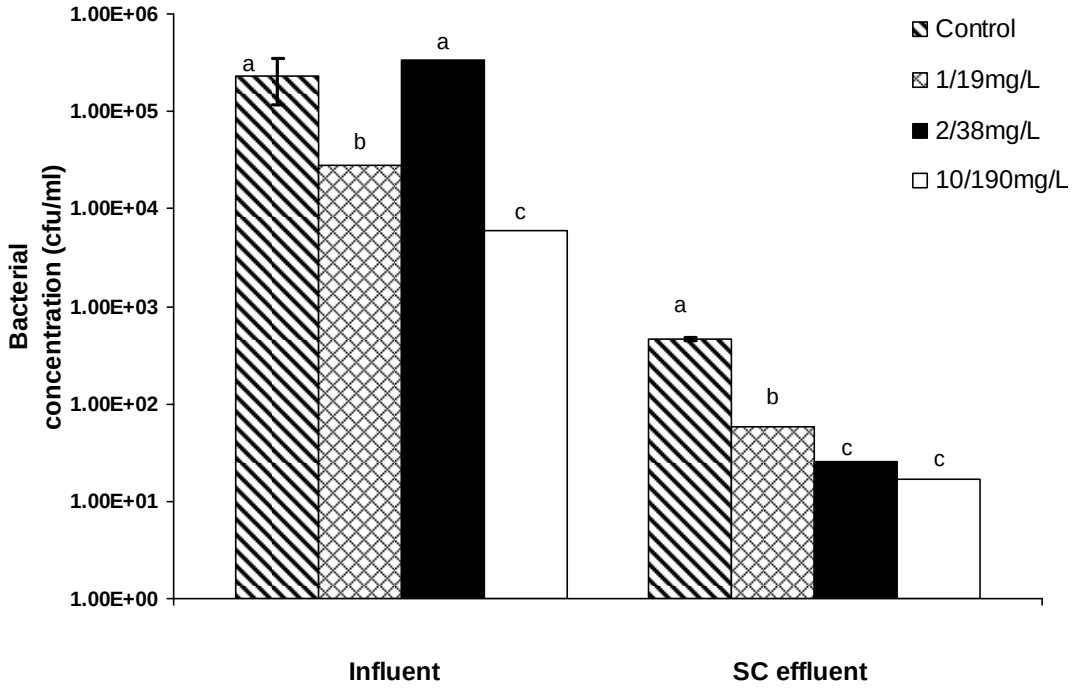
Fecal coliform survivability in the presence of SXT was also evaluated in summer samples. Figure 5.2 illustrates the impact of various SXT concentrations on the growth of fecal coliforms obtained from the raw wastewater and SC effluent. Fecal coliforms cultured from influent and SC effluent samples were exposed to three different levels of SXT – a combination of 1 mg/L trimethoprim and 19 mg/L sulfamethoxazole (1/19 mg/L SXT), 2/38 mg/L and 10/190 mg/L. In influent samples, the SXT level of 1/19 mg/L showed significant (p value = 0.0285) impact on the growth of fecal coliforms and was, therefore, considered to represent the LOAEL. Data obtained for SXT concentration of 2/38 mg/L showed no impact on the growth of fecal coliforms, but was considered an outlier.

The $LC_{50}$ for SXT exposure to fecal coliforms in the influent sample was expected to occur between 2/38 mg/L and 10/190 mg/L. Exposure to 10/190 mg/L of SXT (>MIC) reduced the fecal coliform count from $2.33 \times 10^5$ cfu/mL to $5.9 \times 10^3$ cfu/mL. The bacteria that survived exposure to SXT 10/190 mg/L were considered to be resistant to the antibiotic. In SC effluent samples, even the lowest level of SXT exposure (1/19 mg/L) produced a large reduction in the coliform population. This level of SXT resulted in a 75% kill of bacteria. Thus the $LC_{50}$ value for the SC effluent was < 1/19 mg/L indicating greater potency of the antibiotic in the 'cleaner' effluent sample. Exposing the fecal coliforms in the SC effluent to SXT levels of 2/38 mg/L and 10/190 mg/L reduced the bacterial populations to 30 and 18 cfu/mL, respectively.

The trends shown in Figures 5.1 and 5.2 illustrate that exposure to CIP and SXT had a significant impact on fecal coliforms, especially at the higher antibiotic exposures evaluated. All CIP concentrations studied in the summer were below the MIC value of 32 mg/L and showed fecal coliform survivals ranging from 100 to 2% with increasing CIP exposure. In the SC effluent samples, the bacterial survivability ranged from 20 to 2% at the CIP concentrations evaluated. Although the activated sludge process was successful in a 2-log removal of fecal coliforms, a significant amount of bacteria were still able to

grow at various CIP concentrations. Three percent of the fecal coliforms in the influent sample were found to be highly resistant to SXT at 10/190 mg/L (> MIC level). Although fecal coliform numbers were significantly reduced after biological treatment, the fraction of SXT-resistant organisms increased from 3 % to 7 % in the SC effluent sample.

**Figure 5.2 Impact of Sulfamethoxazole/Trimethoprim Concentration on Fecal Coliform in Influent and Secondary Clarifier Effluent Samples**




Similar alphabets represent no significant difference within dataset.

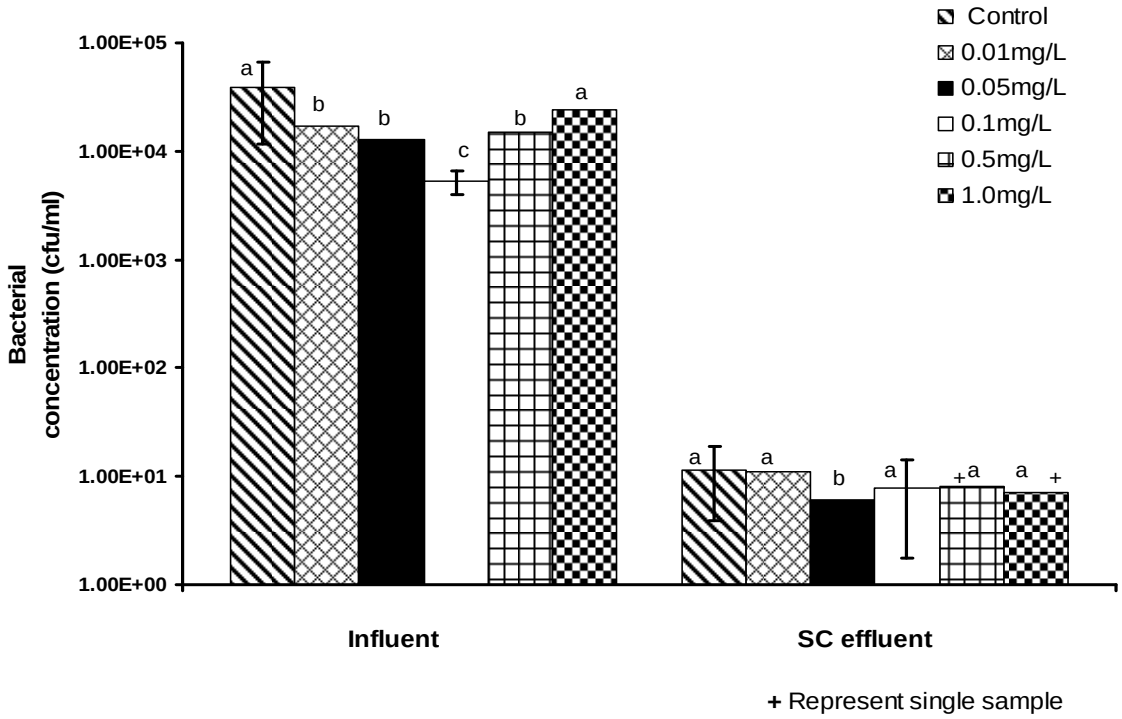
### *5.1.2 Enterococci*

The 24-hour composite samples of raw wastewater collected in the summer were found to contain approximately 3.9 x $10^4$ cfu/mL of enterococci bacteria (control samples in Figure 5.3). Figure 5.3 illustrates the effects of CIP exposure on the growth of enterococci obtained from influent and SC effluent samples. The biological treatment was

very efficient in reducing enterococci concentrations. A 3-log removal was observed with SC effluent enterococci concentrations of approximately 12 cfu/mL.

Enterococci were exposed to the same concentrations of CIP as fecal coliforms. Although exposure to a CIP concentration of 0.01 mg/L reduced the enterococci count in the influent sample by more than 50% to 1.7 x $10^4$ cfu/mL, exposure to a significantly higher level of CIP (1.0 mg/L) showed a higher bacterial count (2.4 x $10^4$ cfu/mL). The lack of a dose-response relationship for the range of CIP concentrations evaluated suggested that these antibiotic levels had no impact on the enterococci population in the influent samples. Although activated sludge treatment produced a 99.9% reduction in enterococci numbers, no significant impact of CIP was observed up to the level evaluated.

**Figure 5.3 Impact of Ciprofloxacin Concentration on Enterococci in Influent and Secondary Clarifier Effluent Samples**

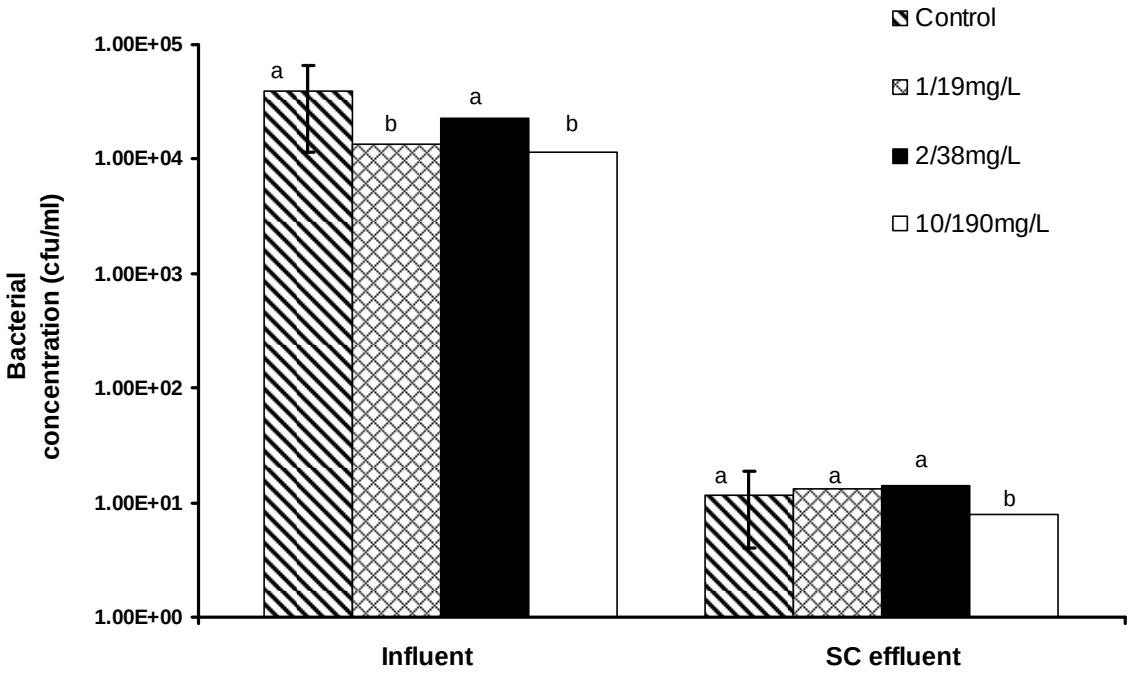



Similar alphabets represent no significant difference within dataset.

Analogous to what was seen for CIP exposure; no dose-response trends were observed for enterococci bacteria in the presence of SXT. Enterococci in influent and SC

effluent samples appeared to remain unaffected by the presence of SXT. In fact, nearly 100% of the enterococci population in the raw wastewater and SC effluent samples appeared to be highly resistant to SXT at 10/190 mg/L (> MIC level).

**Figure 5.4 Impact of sulfamethoxazole/Trimethoprim Concentration on Enterococci in Influent and Secondary Clarifier Effluent Samples**

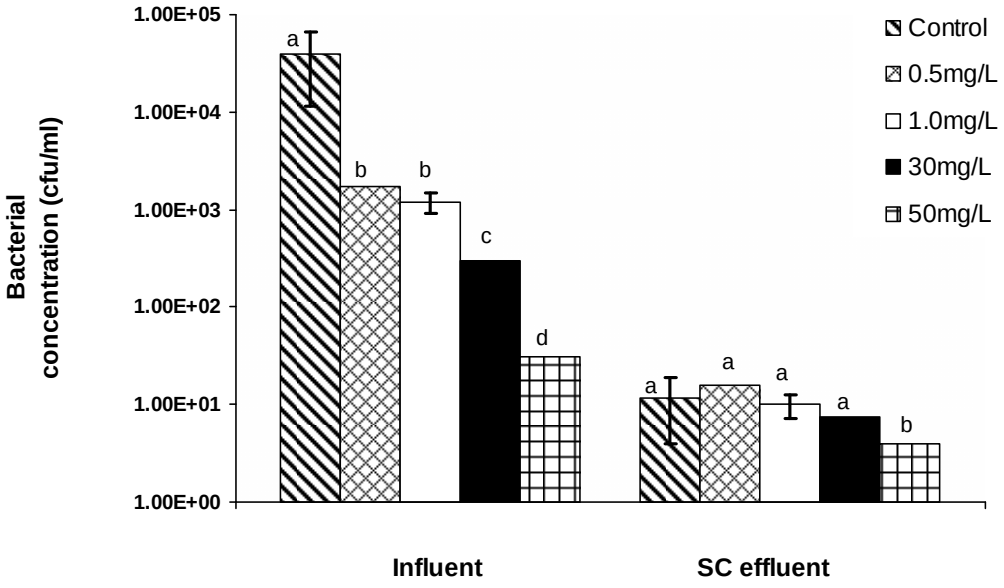



Similar alphabets represent no significant difference within dataset.

The enterococci bacteria in influent and SC effluent samples were also exposed to various concentrations of vancomycin. Figure 5.5 illustrates the impact of VAN on the growth of enterococci obtained from the raw wastewater and SC effluent samples. Vancomycin had a significant impact on the growth of enterococci in the influent even at the lowest exposure level of 0.5 mg/L. The VAN concentration of 0.5 mg/L reduced enterococci numbers to $1.75 \times 10^3$ cfu/mL producing significant difference (p value = 0.0070) therefore, was considered as LOAEL for this evaluation of 0.5 mg/L. The $LC_{50}$ value was determined to be < 0.5 mg/L. Enterococci numbers at higher VAN exposures were reduced to $1.19 \times 10^3$ cfu/mL and $3.0 \times 10^2$ cfu/mL at 1.0 mg/L and 30 mg/L,

respectively. Thirty-one colony-forming units of enterococci per milliliter of influent were considered to be highly resistant to VAN at 50 mg/L (> MIC level). In SC effluent samples, however, no significant (p value = 0.3505) impact of VAN exposure was noted for doses of 0.5, 1.0, 30 and 50 mg/L. Hence, 0.5 mg/L was considered as NOAEL for this evaluation. Approximately 4.0 cfu/mL of enterococci were found to be highly resistant to VAN at 50 mg/L (> MIC level).

**Figure 5.5 Impact of Vancomycin Concentration on Enterococci in Influent and Secondary Clarifier Effluent Samples**




Similar alphabets represent no significant difference within dataset.

Figures 5.3 to 5.5 illustrate that although the enterococci population showed no susceptibility to CIP and SXT, VAN was significantly more lethal to these organisms even at the lowest concentration studied. Although toxic effects were noted for VAN, some organisms were able to survive even the largest level of exposure evaluated. About 1% of enterococci in the raw influent were highly resistant to VAN above the MIC level. Similarly, approximately 37 to 65% of enterococci in the SC effluent were resistant to VAN at MIC level.

## 5.2 Antibiotic Resistant Bacteria in Winter Samples

Bacterial enumeration conducted in the winter season was performed with triplicate samples. The influent wastewater temperature during the sampling period was 12°C. A similar array of antibiotics was used to evaluate the resistance patterns in wastewater organisms as in summer. An additional sampling point (the disinfected effluent) and another target organism (*Escherichia coli*) were included in the winter sampling protocol. Bacteria in the wastewater samples were also enumerated at higher antibiotic exposures (above MIC level) to probe for the presence of highly resistant organisms.

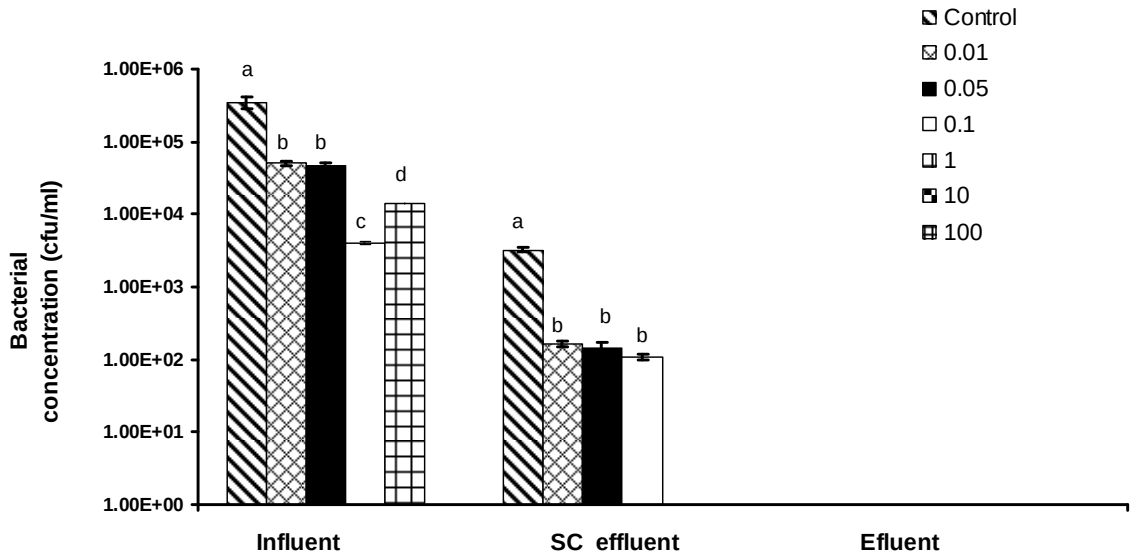
### *5.2.1 Fecal Coliforms*

The raw wastewater was found to contain $3.50 \times 10^5$ cfu/mL fecal coliforms in winter, a number that was comparable to what was observed in the summer. The activated sludge process produced a 2-log removal of the aqueous phase fecal coliform concentration resulting in a population of approximately $3.233 \times 10^3$ cfu/mL in the SC effluent. The disinfection process was effective with no fecal coliforms discharged into the receiving stream in the winter.

Fecal coliforms collected from raw influent, SC effluent and the disinfected effluent were exposed to CIP concentrations similar to those tested in the summer. However, two higher concentrations (10 mg/L and 100 mg/L) were also evaluated. Figure 5.6 illustrates the impact of various concentrations of CIP on fecal coliform growth in the raw influent and SC effluent samples. No growth was observed at 10 and 100 mg/L CIP exposures to fecal coliforms in the influent, SC effluent or disinfected effluent.

The fecal coliforms exposed to lower concentrations of CIP (< MIC level) showed a similar trend as the summer results. LOAEL for this evaluation was 0.01 mg/L and exhibited a significant difference (p value <0.0001) from the control. $LC_{50}$ was < 0.01 mg/L in the influent and SC effluent samples for this evaluation. No clear dose-response relationship was observed in influent and effluent samples for CIP exposures of 1.0 mg/L or less.

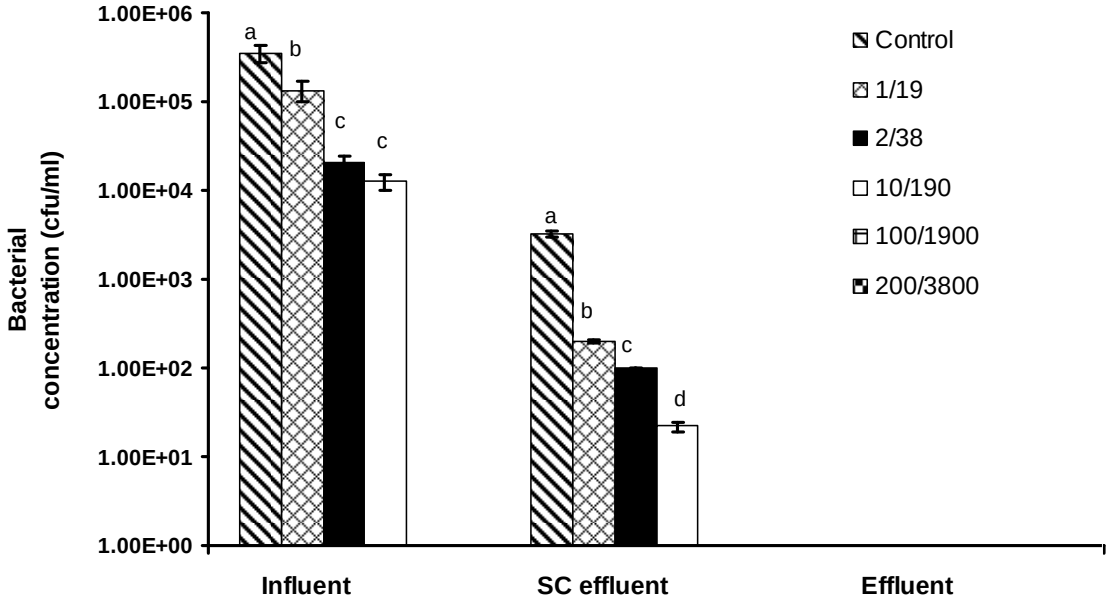
**Figure 5.6 Impact of Ciprofloxacin Concentration on Fecal Coliform in Influent, Secondary Clarifier Effluent and Disinfected Effluent Samples**




Similar alphabets represent no significant difference within dataset.

In addition to SXT concentrations of 1/19, 2/38, and 10/190, the fecal coliforms in winter samples were also exposed to higher SXT levels of 100/1900 mg/L and 200/3800 mg/L. These concentrations were above the MIC level of SXT. Figure 5.7 illustrates the impact of various levels of SXT exposure on the growth of fecal coliforms during the winter sampling. There was a significant reduction in the number of colony forming units observed (p value <0.001) when fecal coliforms were exposed to SXT 1/19; this dose was, therefore, considered the LOAEL for this evaluation. The $LC_{50}$ value for SXT in both samples was < 1/19 mg/L. The two highest exposures of SXT completely inhibited bacterial growth. Fecal coliform counts were 2.52 $x10^5$ cfu/mL and 2.2 $x10^2$ cfu/mL in the raw and SC effluent samples, respectively, at an exposure of 10/190 mg/L SXT (> MIC level). Thus, approximately 4% of fecal coliforms in the influent and 0.7% in the SC effluent were deemed to be highly resistant to SXT.

**Figure 5.7 Impact of Sulfamethoxazole/Trimethoprim Concentration on Fecal Coliform in Influent, Secondary Clarifier Effluent and Disinfected Effluent Samples**




Similar alphabets represent no significant difference within dataset.

### *5.2.2 Enterococci*

Influent wastewater samples collected in winter were found to contain approximately 1.4 x $10^4$ cfu/mL of enterococci bacteria. However, identification of enterococci by polymerase chain reaction (PCR) in follow-up experiments revealed that approximately 11% of the organisms enumerated as enterococci were actually lactococcus *bacilli*. Hence the enterococci numbers shown in the following figures represent a slight overestimation of the true enterococci numbers in the winter samples.

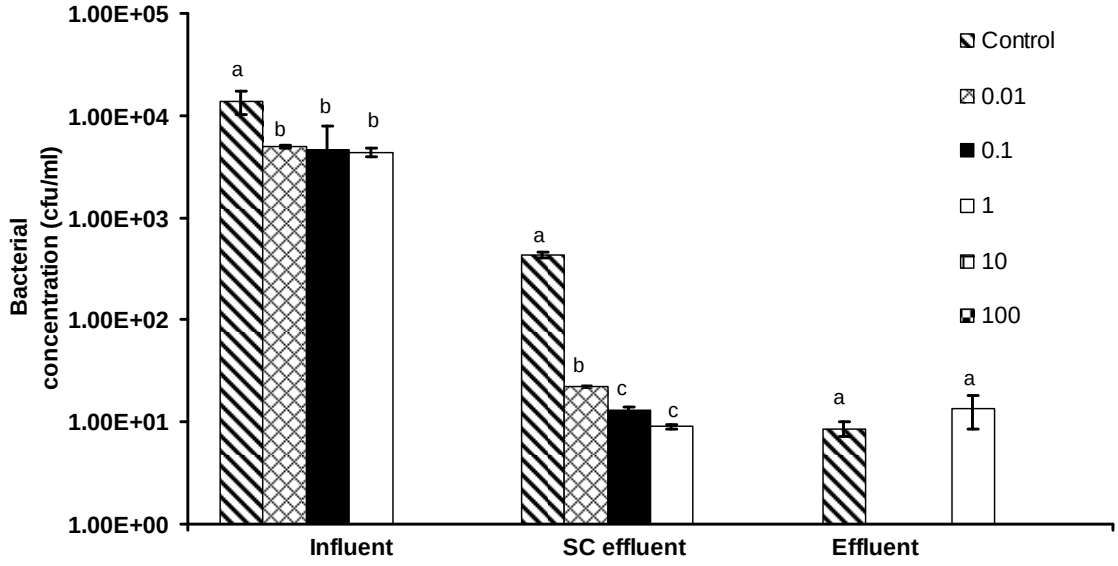
Biological treatment produced a 97% reduction of aqueous-phase enterococci resulting in an SC effluent concentration of 4.3 x$10^3$ cfu/mL. UV-disinfection did not completely remove all enterococci in winter. The final plant effluent was observed to contain approximately 0.070x $10^2$ cfu/mL of these bacteria.

Figure 5.8 illustrates the impact of CIP exposure on the growth of enterococci in influent, SC effluent and disinfected effluent samples. Although CIP levels of 10 and 100

mg/L completely inhibited the growth of enterococci, no dose-response relationship was observed in influent samples for CIP exposures of 1.0 mg/L or less. CIP appeared to have an impact on the enterococci in the SC effluent sample where the antibiotic produced nearly a 2-log deactivation of the bacteria. Although some bacteria escaped destruction in the UV-system, the final effluent did not appear to contain any CIP resistant enterococci.

Enterococci survivability in the presence of SXT was also evaluated in the winter but no growth was noticed in the presence of even the lowest SXT level of 1/19 mg/L. Enterococci population multiply less rapidly than fecal coliforms and *E.coli* and have been shown to be more sensitive to antibiotics in at lower temperatures (Miescier and Cabelli 1982; Martinez et al., 2002).

**Figure 5.8 Impact of Ciprofloxacin Concentration on Enterococci in Influent, Secondary Clarifier Effluent and Disinfected Effluent Samples**

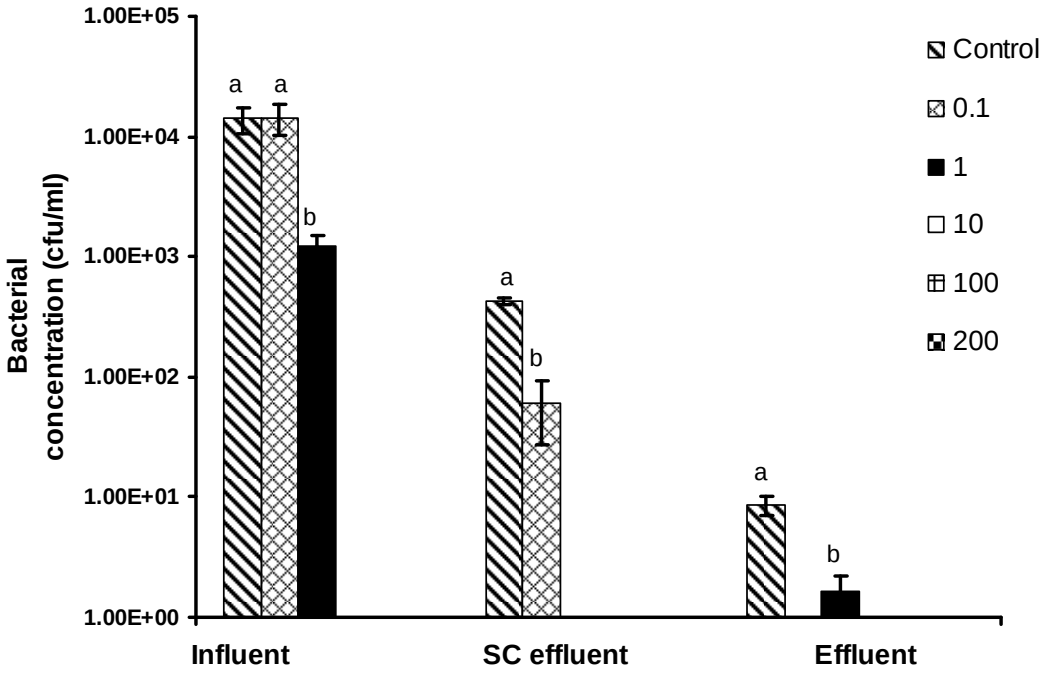



Similar alphabets represent no significant difference within dataset.

Enterococci bacteria in the influent, SC effluent and disinfected effluent samples were also exposed to various concentrations of VAN (Figure 5.9). VAN concentrations of 10, 100 and 200 mg/L showed complete kill of enterococci in all samples. The LOAEL for VAN in the influent sample was 1.0 mg/L as there was a significant difference in the bacterial population (p value < 0.01) compared to the control sample; 0.1 mg/L showed

no significant difference (p value = 0.8567) and was considered as NOAEL for this evaluation. No VAN-resistant enterococci were observed in any wastewater sample collected during winter.

**Figure 5.9 Impact of Vancomycin Concentration on Enterococci in Influent, Secondary Clarifier Effluent and Disinfected Effluent Samples**




Similar alphabets represent no significant difference within dataset.

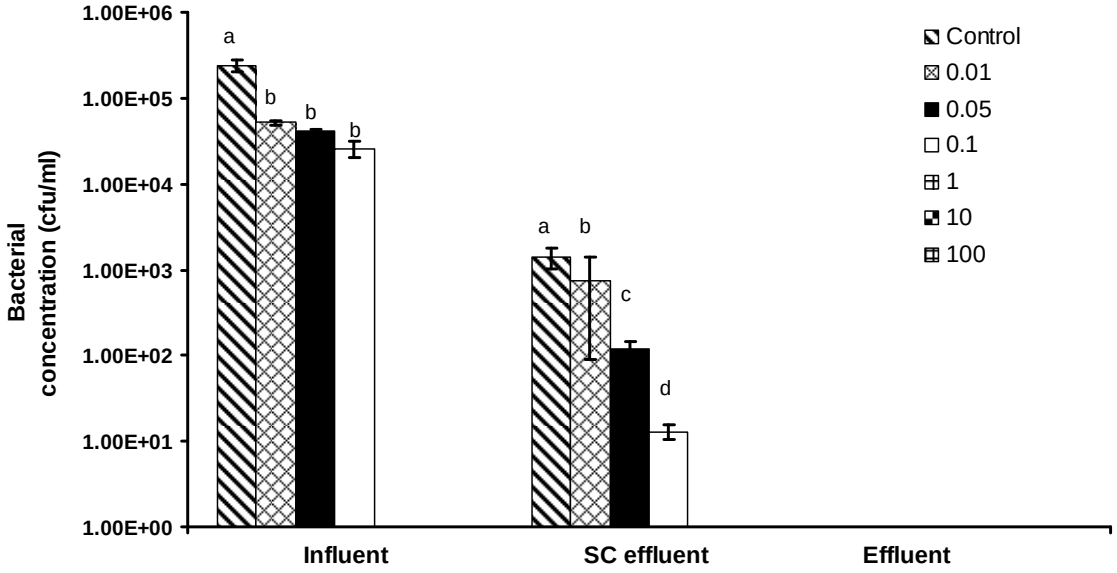
### *5.2.3 E. coli*

Raw wastewater collected in winter was found to contain approximately 2.4 x $10^5$ cfu/mL *E. coli*. Figure 5.10 illustrates the effects of CIP exposure on the growth of *E. coli* obtained from influent and SC effluent samples. The activated sludge process reduced the *E. coli* count to 1.4 x $10^3$ cfu/mL (2-log removal) and UV disinfection was successful in destroying all *E. coli* in the effluent wastewater.

The *E. coli* enumerated from the raw influent had a survivability of 5.17 x $10^4$ cfu/mL when exposed to 0.01 mg/L CIP. This number was significantly different from the control (p value <0.001) and indicated the LOAEL; $LC_{50}$ for this evaluation was <

0.01 mg/L. A dose-response relationship was observed when *E. coli* in the influent and SC effluent samples were exposed to CIP concentrations ranging from 0.01 to 0.1 mg/L. Higher doses of 10 and 100 mg/L CIP completely inhibited the growth of these organisms in influent and SC effluent samples.

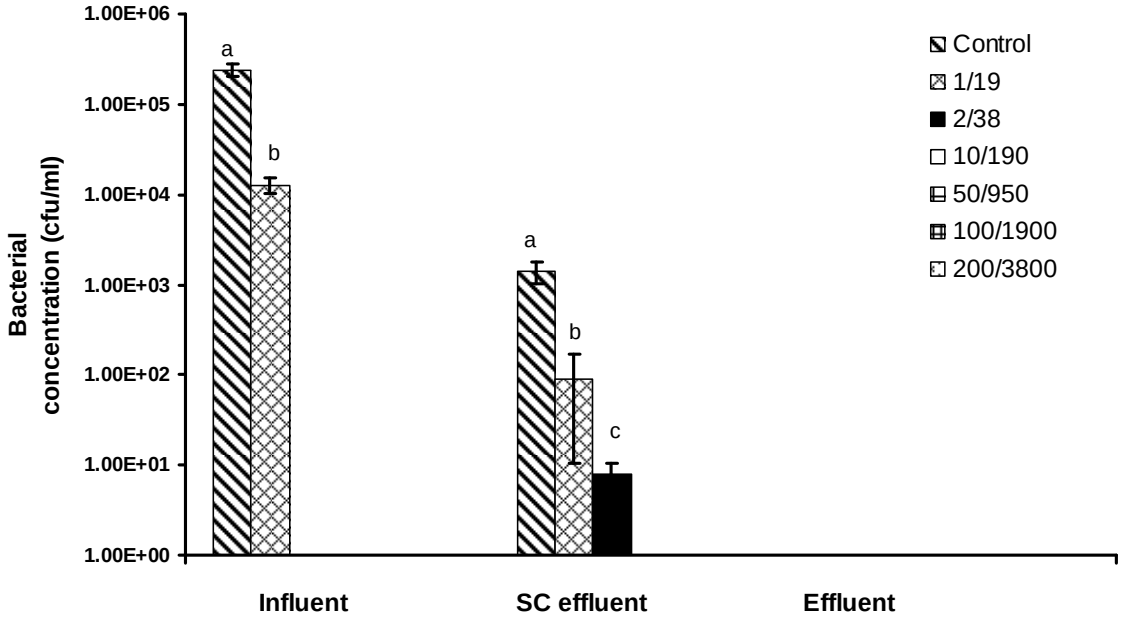
**Figure 5.10 Impact of Ciprofloxacin Concentration on *E. coli* in Influent, Secondary Clarifier Effluent and Disinfected Effluent Samples**




Similar alphabets represent no significant difference within dataset.

*E. coli* obtained from the raw wastewater were exposed to various concentrations of SXT as shown in Figure 5.11. Exposure to SXT 1/19 mg/L reduced the viable *E. coli* count in the influent to 1.27 x $10^4$ cfu/mL. SXT concentration of 1/19 mg/L was considered as LOAEL for this evaluation because the bacterial population at this dose was significantly different from the control (p value <0.001). No *E. coli* growth in influent samples was observed at SXT exposures of 2/38 mg/L and above. In the SC effluent sample, however, some *E. coli* survival was noted at the SXT level of 2/38 mg/L.

**Figure 5.11 Impact of Sulfamethoxazole/Trimethoprim Concentration on *E. coli* in Influent, Secondary Clarifier Effluent and Disinfected Effluent Samples**




Similar alphabets represent no significant difference within dataset.

## 5.3 Antibiotic Resistant Bacteria in Spring Samples

Bacterial enumeration of wastewater samples collected in the spring season was performed using triplicate samples. The influent wastewater temperature during the sampling period was 17°C. Additional concentrations of CIP 4.0 mg/L, SXT 8/152 mg/L and VAN 32 mg/L were incorporated in the spring experiments to investigate the presence of resistant organisms at the MIC level.

### *5.3.1 Fecal Coliforms*

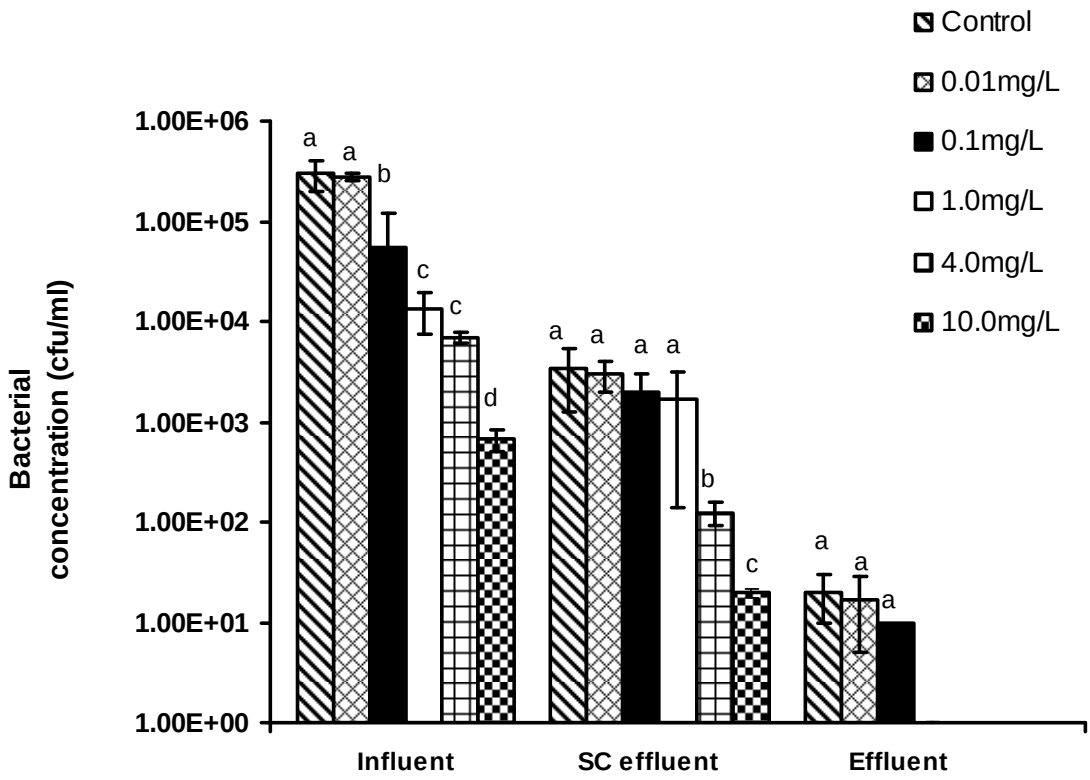
Influent wastewater collected in spring was found to contain a fecal coliform concentration of approximately $3.0 \times 10^5$ cfu/mL (Figure 5.12). This number was similar to the fecal coliform counts seen in summer and winter. Biological wastewater treatment reduced the fecal coliforms by an order of two resulting in $3.33 \times 10^3$ cfu/mL in the SC effluent. UV disinfection further reduced the bacteria concentration in the final effluent sample to 20 cfu/mL. No toxic effects were noted and no significant reduction in

bacterial counts was observed (p-value = 0.5777) in sample at CIP concentration of 0.01 mg/L. Hence 0.01 mg/L was considered the NOAEL in the influent samples. In the SC effluent, the NOAEL was significantly higher (p-value = 0.1152) at a concentration of 1.0 mg/L. Fecal coliforms growth in the influent and SC effluent samples were significantly impacted (p-value <0.0001, p-value = 0.067) at CIP 0.1 mg/L and CIP 4.0 mg/L; these concentrations were, therefore, considered as LOAEL for these evaluations. $LC_{50}$ was estimated to be at a CIP concentration less than 0.1 mg/L.

Exposure to higher CIP concentrations resulted in a steady decline in microbial survivability. Approximately, 3% ($4.67 \times 10^2$ cfu/mL) of fecal coliforms in the raw influent were found to be resistant to CIP at the MIC level of 4 mg/L. No growth was observed at CIP concentrations greater than 4 mg/L. Organisms enumerated from the SC effluent showed a similar trend. Approximately 3% of the fecal coliform population ($1.0 \times 10^2$ cfu/mL) in the SC effluent was resistant to CIP at the MIC level. No resistant fecal coliforms were found in the disinfected effluent although 83% and 50% of the bacteria were potentially resistant to CIP at concentrations of 0.01 mg/L and 0.1 mg/L, respectively.

Fecal coliforms enumerated in spring were also exposed to SXT for antibiotic resistance analysis (Figure 5.13). The influent revealed (p value = 0.0203) a LOAEL value of 1/19 mg/L SXT while NOAEL concentration was observed in the SC effluent and final effluent. Approximately $1.1 \times 10^3$ cfu/mL (0.04%) of fecal coliforms in the influent sample were resistant to SXT at a concentration of 8/152 mg/L. In the SC effluent, the resistant fraction was significantly higher – 14% or $4.67 \times 10^2$ cfu/mL. Both influent and SC effluent samples revealed the presence of fecal coliforms that were highly resistant to SXT (at a level of 10/190 mg/L). No fecal coliform survival was noted in any sample at the SXT concentration of 50/950 mg/L. No SXT resistant organisms were observed in the disinfected effluent.

**Figure 5.12 Impact of Ciprofloxacin Concentration on Fecal Coliform in Influent, Secondary Clarifier Effluent and Disinfected Effluent Samples**



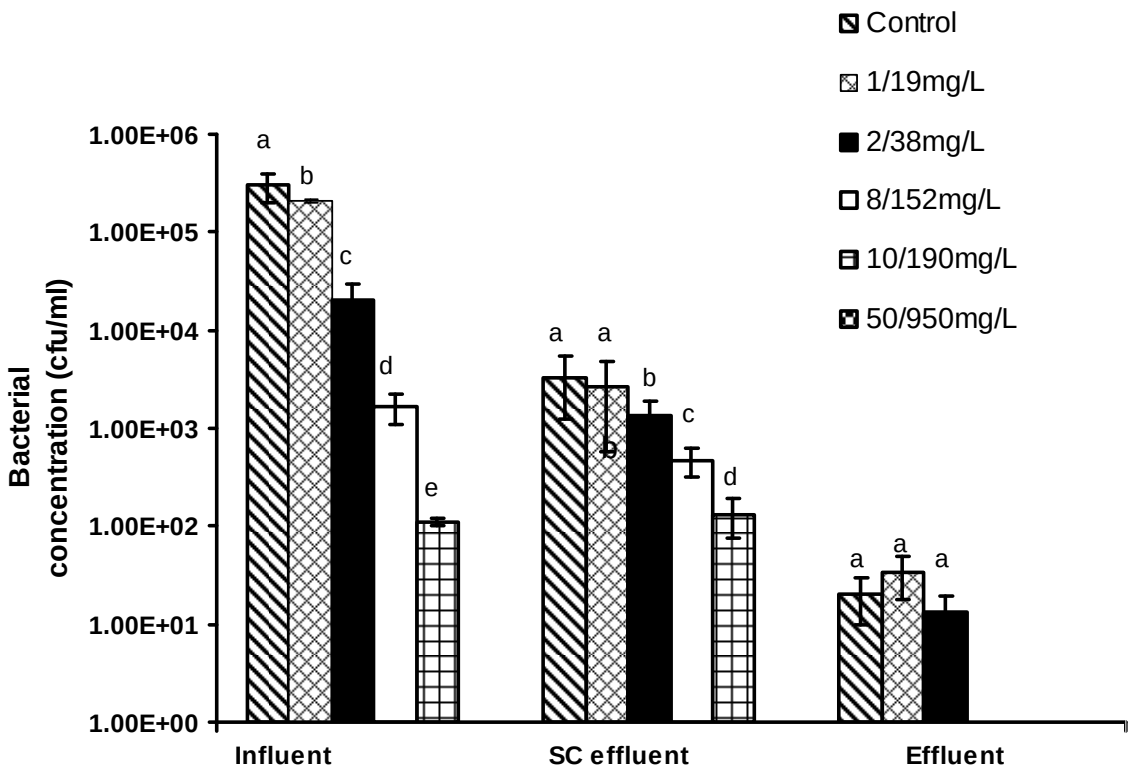

Similar alphabets represent no significant difference within dataset.

### *5.3.1 Enterococci*

The population of enterococci observed in the raw wastewater samples during spring sampling was approximately 1.33 x $10^4$ cfu/mL. However identification of enterococci by polymerase chain reaction in follow-up experiments showed that approximately 30% of the organisms enumerated as enterococci were actually *lactococcus bacilli*. Thus the data shown in the following figures represent an overestimation of enterococci numbers. Figure 5.14 illustrates the impact of various concentrations of CIP on the growth of these organisms in the raw wastewater, SC effluent and disinfected effluent samples. Biological treatment produced an SC effluent enterococci concentration of approximately 7.5 x $10^2$ cfu/mL, representing a 2-log

reduction. UV disinfection killed all enterococci in the stream exiting the wastewater treatment facility.

**Figure 5.13 Impact of Sulfamthoxazole/Trimethoprim Concentration on Fecal Coliform in Influent, Secondary Clarifier Effluent and Disinfected Effluent Samples**

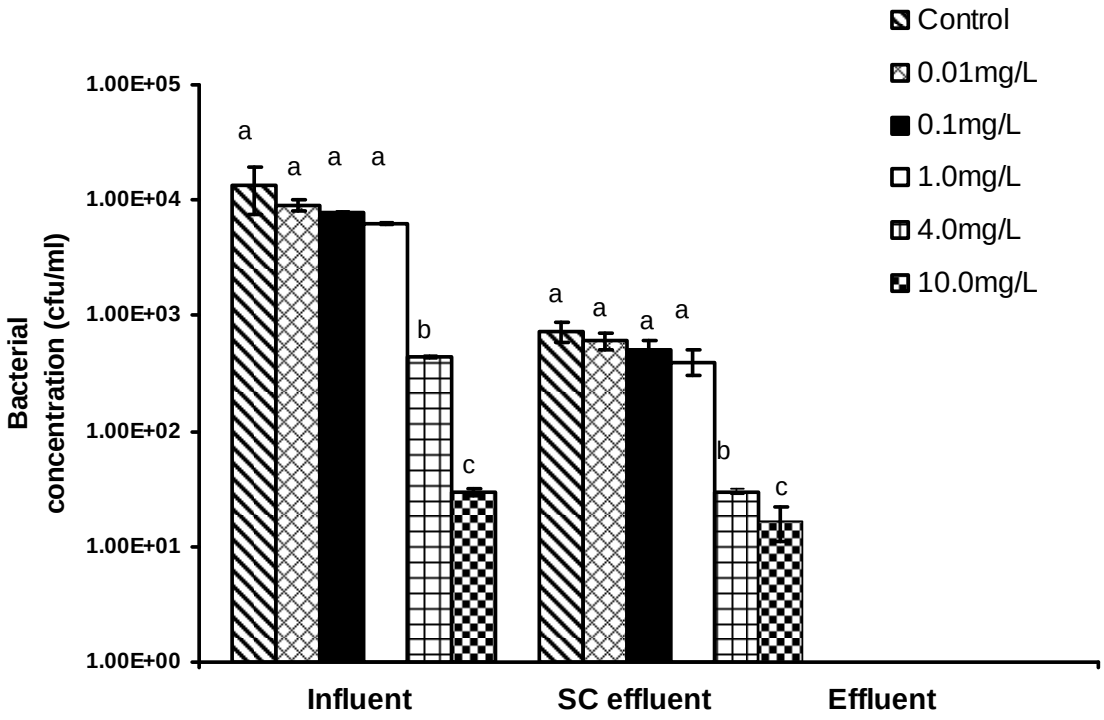



Similar alphabets represent no significant difference within dataset.

Exposure to 0.01 mg/L CIP appeared to have a significant impact (p value = 0.0467) on the concentration of enterococci. Thus, a concentration of 0.01 mg/L represented the LOAEL for this experiment. The enterococci population was reduced to 6.2 x $10^3$ cfu/mL (46%) at a CIP concentration of 1.0 mg/L indicating an $LC_{50}$ in the influent matrix of < 1.0 mg/L. In the SC effluent, an exposure to 1.0 mg/L of CIP reduced the microbial population to 5.0 x $10^2$ cfu/mL representing 68% of the population in control samples. A population of 4.3 x $10^3$ cfu/mL or approximately 3.3% of the enterococci in the raw wastewater was resistant to CIP at the MIC level of 4 mg/L. Approximately 30 cfu/mL (or 0.2%) were highly resistant to CIP at 10 mg/L. Enterococci

enumerated from the SC effluent sample showed similar characteristics at exposures of 1.0 mg/L, 4.0 mg/L and 10 mg/L CIP. Approximately 0.4% (30 cfu/mL) of the enterococci in the SC effluent were resistant to CIP at the MIC level and 0.2% (17 cfu/mL) were highly resistant at 10 mg/L CIP.

**Figure 5.14 Impact of Ciprofloxacin Concentration on Enterococci in Influent, Secondary Clarifier Effluent and Disinfected Effluent Samples**

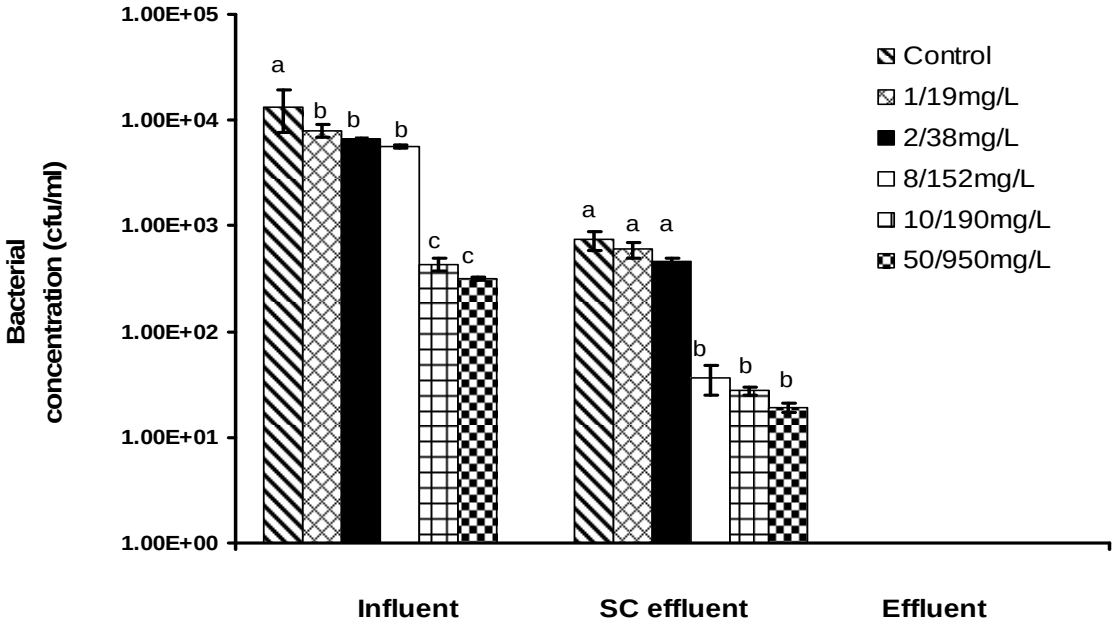



Similar alphabets represent no significant difference within dataset.

Exposure to SXT at a dose of 1/19 mg/L had no impact on the enterococci population in the raw or SC effluent samples (Figure 5.15). However, higher concentrations showed an increasing lethal response on the bacterial population. Approximately 5.67 x $10^3$ cfu/mL (42%) of the enterococci in the influent and 0.37 x $10^2$ cfu/mL (5%) in the SC effluent were resistant to SXT exposure at the MIC level of 8/152 mg/L. Furthermore, 4.33 x $10^2$ cfu/mL (3.3%) and 3.17 x $10^2$ cfu/mL (2.4%) of the enterococci population in the influent was characterized as highly resistant to SXT at

concentrations of 10/190 mg/L and 50/950 mg/L, respectively. Enterococci populations in the SC effluent showed similar characteristics. Approximately 5% (37 cfu/mL) of the SC effluent population was resistant to SXT at the MIC level and 3.7% (27 cfu/mL) and 2.5% (19 cfu/mL) were highly resistant at SXT concentrations of 10/190 and 50/950 mg/L, respectively.

**Figure 5.15 Impact of Sulfamethoxazole/Trimethoprim Concentration on Enterococci in Influent, Secondary Clarifier Effluent and Disinfected Effluent Samples**

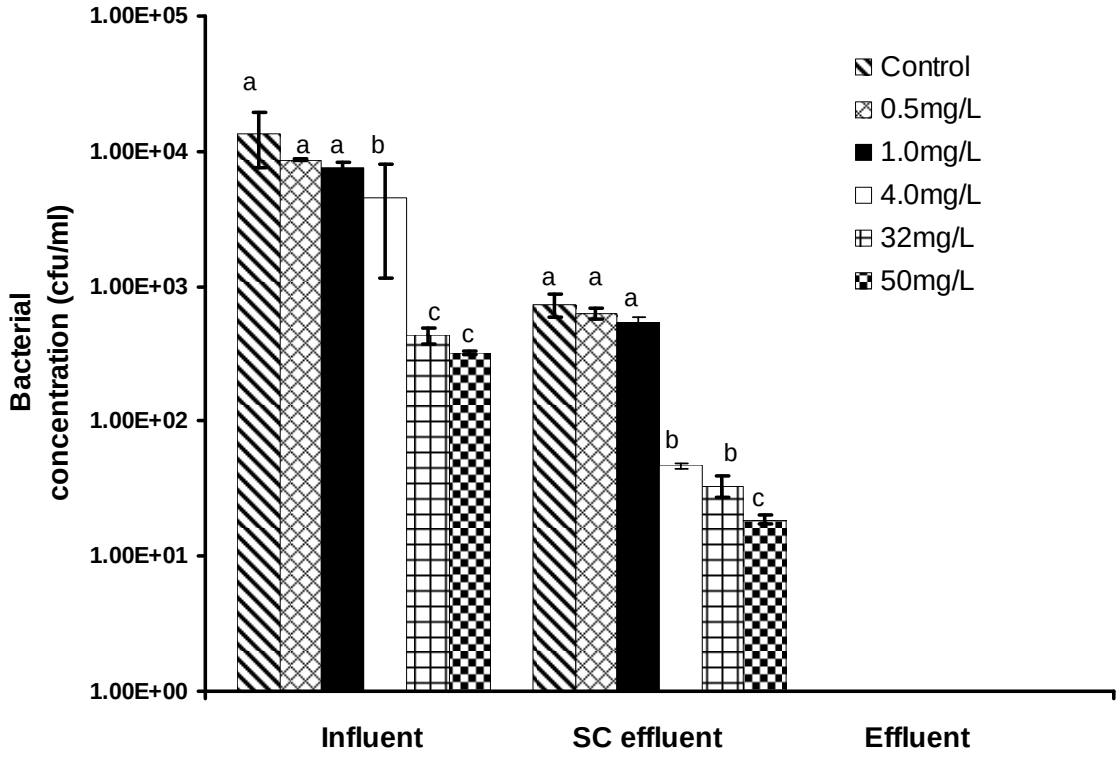



Similar alphabets represent no significant difference within dataset.

Vancomycin resistant enterococci were also observed in the influent and SC effluent samples collected in spring (Figure 5.16). Approximately 3% ($4.33 \times 10^2$ cfu/mL) and 2% ($3.2 \times 10^2$ cfu/mL) of the enterococci population in the influent appeared to be resistant to VAN at the MIC level of 32 mg/L and highly resistant at 50 mg/L, respectively. Biological treatment was efficient in reducing the enterococci numbers but resistance patterns were similar even after the treatment process. Approximately 4.5% (33 cfu/mL) and 2.5% (19 cfu/mL) of the enterococci population in

the SC effluent was resistant to VAN at the MIC level of 32 mg/L and highly resistant at 50 mg/L, respectively.

**Figure 5.16 Impact of Vancomycin Concentration on Enterococci in Influent, Secondary Clarifier Effluent and Disinfected Effluent Samples**

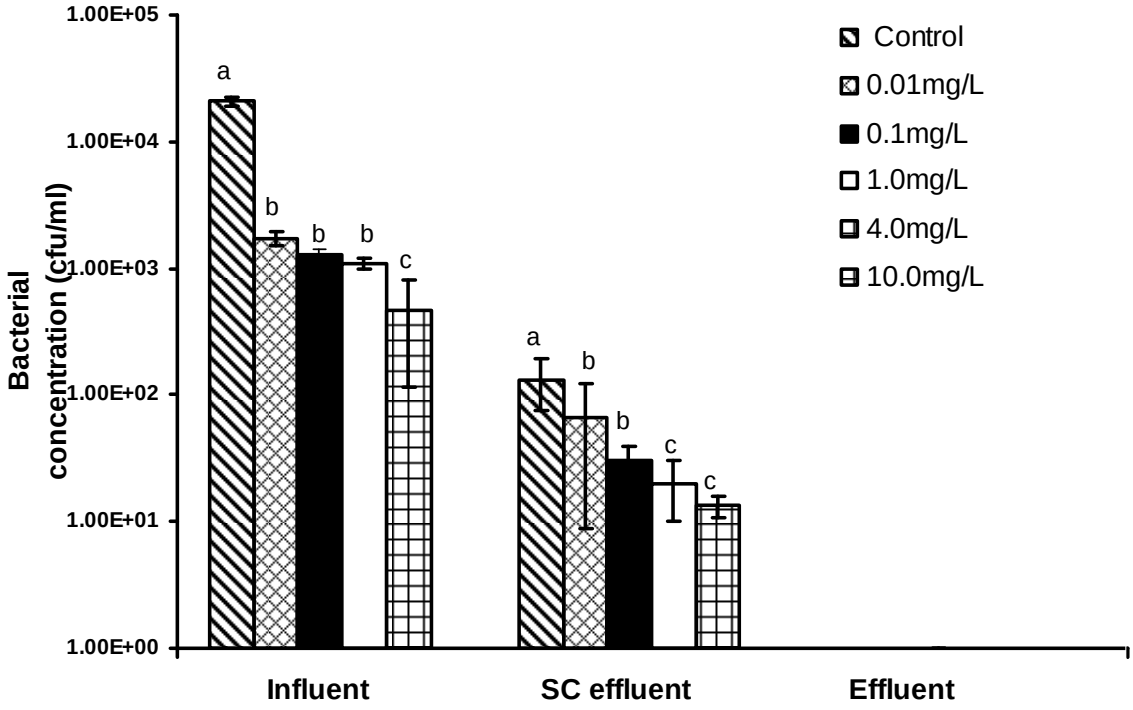



Similar alphabets represent no significant difference within dataset.

### *5.3.3 E. coli*

The raw wastewater collected in spring was found to contain an *E. coli* concentration of approximately 2.0 x $10^4$ cfu/mL (Figure 5.17). The activated sludge process reduced the *E. coli* concentration by 2 logs resulting in a population of 1.33 x $10^2$ cfu/mL. Hundred percent kill of *E. coli* was achieved by UV disinfection. *E. coli* growth in the influent sample was suppressed (p value <0.0001) even at the lowest CIP exposure of 0.01 mg/L resulting in a reduction in *E. coli* numbers to 1.73 x $10^3$ cfu/mL. Hence 0.01 mg/L concentration was considered the LOAEL in the influent matrix. $LC_{50}$ for this evaluation was < 0.01 mg/L.

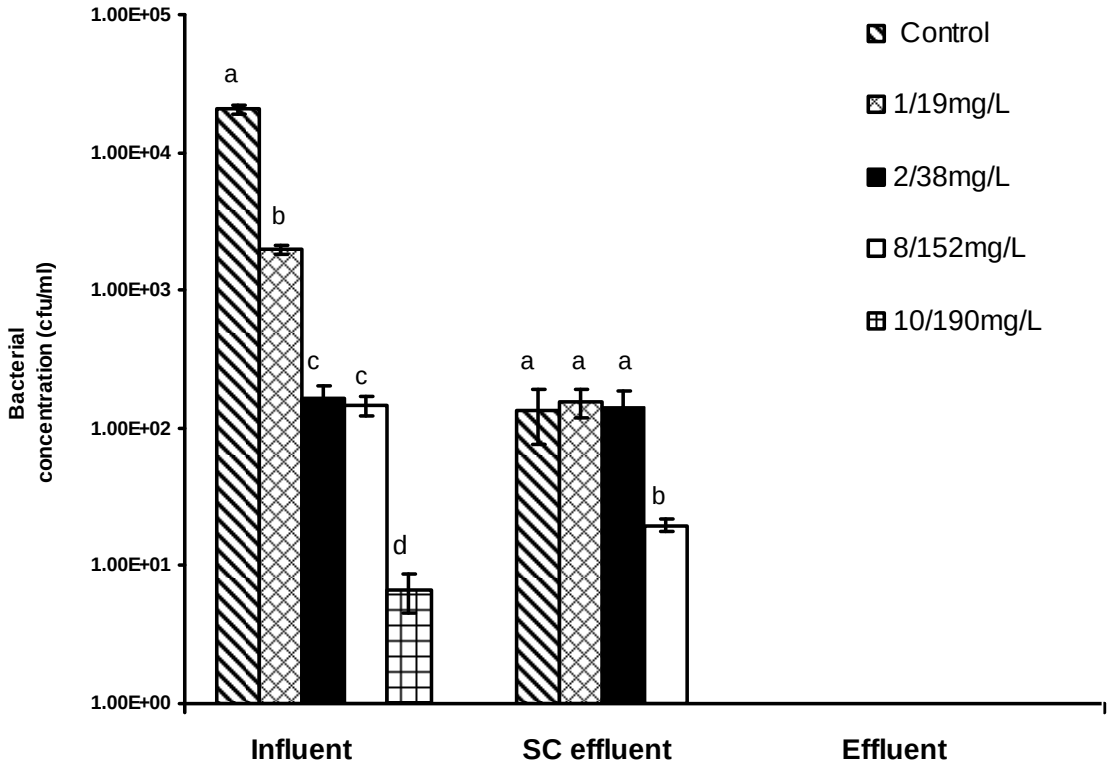
**Figure 5.17 Impact of Ciprofloxacin Concentration on *E. coli* in Influent, Secondary Clarifier Effluent and Disinfected Effluent Samples**




Similar alphabets represent no significant difference within dataset.

Exposure to higher doses of CIP resulted in a decline in survivability. Approximately 3% (5 cfu/mL) of *E. coli* in the influent were resistant to CIP at the MIC level of 4 mg/L. No growth was observed at CIP concentrations of > 4 mg/L. *E. coli* enumerated from the SC effluent showed similar trends. Approximately 10% ($0.13 \times 10^2$ cfu/mL) of *E. coli* in the influent were resistant to CIP at the MIC level of 4 mg/L. Exposure to SXT at concentrations of 1/19 mg/L and 2/38 mg/L showed significant responses on the *E. coli* population in the influent, but not in the SC effluent sample. Approximately, $1.47 \times 10^2$ (0.72%) of *E. coli* in the influent and 20 cfu/mL (15%) in the SC effluent were resistant to SXT at the MIC level of 8/152 mg/L. Approximately 7 cfu/mL *E. coli* in the raw wastewater were highly resistant to SXT at 10/190 mg/L.

**Figure 5.18 Impact of Sulfamthoxazole/Trimethoprim Concentration on *E. coli* in Influent, Secondary Clarifier Effluent and Disinfected Effluent Samples**




Similar alphabets represent no significant difference within dataset.

## 5.5 Seasonal Variations in Antibiotic Resistance Patterns

The seasons in which samples were collected appeared to have some impact on NOAEL, and LOAEL concentrations for different target organisms. Fecal coliforms collected in influent exposed to CIP showed a similar NOAEL concentration of 0.01 mg/L in the summer and spring, although no NOAEL was observed in the winter (Table 5.1). LOAEL for CIP in the case of fecal coliforms was similar in the influent and SC effluent sample. Seasons also appeared to have an impact on $LC_{50}$ levels. $LC_{50}$ was lower in summer compared to spring in both influent and SC effluent samples. On the other hand *E. coli* and enterococci showed no significant difference in the NOAEL and LOAEL for CIP during the different seasons. $LC_{50}$ of enterococci was lower in summer compared to spring season.

Fecal coliforms, *E. coli* and enterococci showed similar LOAEL values for SXT in influent and SC effluent samples collected in summer, winter and spring (Table 5.2). $LC_{50}$ values for fecal coliforms increased from 1/19 mg/L in summer to 2/38 mg/L in spring. *E. coli* and enterococci bacteria also showed higher $LC_{50}$ values in spring compared to summer and winter samples. $LC_{50}$ values for *E. coli* and enterococci in the SC effluent were as high as 8/152 mg/L. VAN had a LOAEL of 1.0 mg/L and 0.1 mg/L for enterococci in influent and SC effluent respectively during winter (Table 5.3). However, in spring samples, LOAEL was observed at 0.5 mg/L VAN.

The various reasons for the different trends of NOAEL and LOAEL can be explained by relating the bacterial susceptibility to temperature variations. McMahon et al (2007) reported that under low temperatures lower MICs or low concentration of antibiotics would be efficient in complete susceptibility of bacteria. As the temperature, increases the concentration of antibiotic required for the susceptibility would increase.

Antibiotic resistant patterns observed in this study are summarized in Table 5.4. Fecal coliforms, *E. coli* and enterococci were resistant or highly resistant to one or more target antibiotics in the influent and SC effluent. Approximately 6.67 x $10^2$ cfu/mL (0.22 %) of fecal coliforms were highly resistant to CIP, while 1.1 x$10^2$ cfu/mL (0.03 %) were highly resistant to SXT in the influent sample in spring. After the biological treatment, the number of fecal coliform bacteria were reduced in the SC effluent sample but the percentage of bacteria that were resistant to CIP and SXT increased. Approximately 1.23 x $10^2$ cfu/mL (3.69 %) of fecal coliforms in the SC effluent were resistant to CIP. Fecal coliforms in influent and SC effluent were highly resistant to SXT in all the seasons.

**Table 5.1 Seasonal Variations in Antibiotic Resistance Patterns in Response to Exposure to Ciprofloxacin**

| | | Fecal coliforms | | | *Escherichia coli* | | | Enterococci | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | **NOAEL** | **LOAEL** | **$LC_{50}$** | **NOAEL** | **LOAEL** | **$LC_{50}$** | **NOAEL** | **LOAEL** | **$LC_{50}$** |
| **Influent** | **SPR** | 0.01 (P=0.5777) | 0.1 (P<0.0001) | < 0.1 | NO | 0.01 (P<0.0001) | < 0.01 | NO | 0.01 (P<0.0001) | 0.1 to 1.0 |
| | **WIN** | NO | 0.01 (P<0.0001) | < 0.01 | NO | 0.01 (P<0.0001) | < 0.01 | NO | 0.01 (P<0.0001) | < 0.01 |
| | **SUM** | 0.01 (P=0677) | 0.05 (P=0475) | < 0.05 | NO | NO | NO | 1.0 (P =2733) | NO | < 1.0 |
| **SC Effluent** | **SPR** | 0.1 (P=0.1152) | 1.0 (P<0.0054) | 1 to 4 | 0.01 (P=0.0824) | 0.1 (P=0.0152) | 0.01-0.1 | NO | 0.01 (P<0.0001) | 1 to 4 |
| | **WIN** | NO | 0.01 (P<0.0001) | < 0.01 | NO | 0.01 (P<0.0001) | 0.01 to 0.0 5 | NO | 0.01 (P<0.0001) | < 0.01 |
| | **SUM** | NO | 0.01 (P<0.0001) | < 0.01 | NO | NO | > 1.0 | 1.0 (P=0.7152) | NO | < 1.0 |

NO = Not observed, SPR= Spring, WIN= Winter, SUM= Summer

**Table 5.2 Seasonal Variations in Antibiotic Resistance Patterns in Response to Exposure to Sulfamethoxazole/Trimethoprim**

| | | Fecal coliforms | | | *Escherichia coli* | | | Enterococci | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | **NOAEL** | **LOAEL** | **$LC_{50}$** | **NOAEL** | **LOAEL** | **$LC_{50}$** | **NOAEL** | **LOAEL** | **$LC_{50}$** |
| **Influent** | **SPR** | NO | 1/19 (P=0.0203) | 1/19 -2/38 | NO | 1/19 (P<0.0001) | < 1/19 | NO | 1/19 (P=0.0320) | 1/19-2/38 |
| | **WIN** | NO | 1/19 (P<0.0001) | < 1/19 | NO | 1/19 (P<0.0001) | < 1/19 | NO | NO | NO |
| | **SUM** | 2/38 (P=0.2195) | 10/190 (P=0.0189) | < 1/19 | NO | NO | NO | 10/190 (P=0.1785) | NO | < 1/19 |
| **SC Effluent** | **SPR** | 1/19 (P=0.5182) | 2/38 (P=0.0077) | < 1/19 | 2/38 (P=0.5027) | 8/152 (P=0.027) | <8/152 | 1/19 (P=0.0784) | 2/38 (P=0.0021) | 2/38-8/190 |
| | **WIN** | NO | 1/19 (P<0.0001) | < 1/19 | NO | 1/19 (P<0.0001) | < 1/19 | NO | NO | NO |
| | **SUM** | NO | 1/19 (P<0.0001) | < 1/19 | NO | NO | NO | 10/190 (P=0.4826) | NO | < 2/38 |

NO = Not observed, SPR= Spring, WIN= Winter, SUM= Summer

**Table 5.3 Seasonal Variations in Antibiotic Resistance Patterns in Response to Exposure to Vancomycin Concentration to Enterococci**

| Enterococci | | | | |
|---|---|---|---|---|
| | | **NOAEL** | **LOAEL** | **$LC_{50}$** |
| **Influent** | **SPR** | 0.5 (P=0.0600) | 1.0 (P=0.0268) | 1 – 4 |
| | **WIN** | 0.1 (P=0.8567) | 1.0 (P<0.0001) | < 1.0 |
| | **SUM** | NO | 0.5 (P=0.0070) | NO |
| **SC Effluent** | **SPR** | NO | 0.5 (P<0.0001) | 1 – 4 |
| | **WIN** | NO | 0.1 (P<0.0001) | < 0.1 |
| | **SUM** | 50 (P=0.0979) | NO | NO |

NO = Not observed, SPR= Spring, WIN= Winter, SUM= Summer

Approximately 4.67 x $10^2$ cfu/mL (3.5%) of *E. coli* in the influent showed resistance to CIP while 13 cfu/mL (1.733%) were resistant in the SC effluent. About 7 cfu/mL (0.05%) of *E. coli* in the raw wastewater were highly resistant to SXT. No SXT resistant *E. coli* were observed in the samples collected from the influent and SC effluent in other seasons. CIP resistant enterococci were observed in the influent in spring; 14.8% (30 cfu/mL) were highly resistant to CIP. However, the percentage of CIP resistant enterococci was reduced to 0.48% (16 cfu/mL) in the SC effluent sample. SXT resistant enterococci were observed in the influent sample although 4.33 x$10^2$ cfu/mL and 0.27 cfu/mL enterococci in the SC effluent were resistant to the antibiotic.

The above results show that wastewater treatment plants are potential sources for the antibiotic resistant organisms in surface waters. UV disinfection was found to work very efficiently at the wastewater treatment plant studied. The disinfected effluent samples were never positive for antibiotic resistant organisms.

**Table 5.4 Antibiotic Resistant Patterns in Target Organisms**

| | | **CIP (MIC = 4 mg/L)** | | | | | | **SXT (MIC = 8/152 mg/L)** | | | | | | **VAN (MIC = 32 mg/L)** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **Fecal coliforms (cfu/mL)** | | ***E. coli* (cfu/mL)** | | **Enterococci (cfu/mL)** | | **Fecal coliforms (cfu/mL)** | | ***E. coli* (cfu/mL)** | | **Enterococci (cfu/mL)** | | **Enterococci (cfu/mL)** | |
| | | **R (4)** | **HR (10** | **R (4)** | **HR (10)** | **R (4)** | **HR (10)** | **R (8/152)** | **HR (10/190)** | **R (8/152)** | **HR (10/190)** | **R (8/150)** | **HR (10/190)** | **R (32)** | **HR (50)** |
| **Influent** | **SPR** | 6970 | 667 | 467 | 0 | 433 | 30 | 1670 | 110 | 146 | 7 | 5667 | 433 | 0 | 0 |
| | **WIN** | ND | 0 | ND | 0 | ND | 0 | ND | 12700 | ND | 0 | ND | ND | ND | ND |
| | **SUM** | ND | ND | ND | ND | ND | ND | ND | 5900 | ND | 0 | ND | 11500 | 0 | 0 |
| **SC Effluent** | **SPR** | 120 | 100 | 13 | 0 | 30 | 17 | 467 | 134 | 20 | 0 | 36 | 27 | 0 | 0 |
| | **WIN** | ND | 0 | ND | 0 | ND | ND | ND | 22 | ND | ND | ND | ND | 0 | 0 |
| | **SUM** | ND | ND | ND | ND | ND | ND | ND | 17 | ND | ND | ND | 8 | ND | 0 |

MIC = Minimum inhibitory concentration, R = Resistant, HR = Highly resistant, SUM = Summer, WIN = Winter, SPR = Spring

ND = Not detected

## 5.4 Identification of Antibiotic Resistant Enterococci

A total of 231 out of 259 (89.1%) enterococcal isolates were identified to species level by multiplex or single polymerase chain reaction (PCR). The prevalence of various *Enterococcus* spp. isolated from influent and SC effluent during winter and spring is summarized in Table 5.5. The most frequently identified species was *E. faecalis* (72.7%), followed by *E. casseliflavus* (12.1%), *E. faecium* (9.9%), and *E. hirae* (3.0%). The prevalence of *E. faecalis* is attributed to the human waste in municipal wastewater and the fact that *E. faecalis* is an important part of the normal gastro-intestinal microbial community in humans and domestic animals.

In winter, there were no significant differences in the prevalence of *E. faecalis* (p-value 0.2429), *E. casseliflavus* (p-value 0.6793), *E. hirae* (p-value 0.4988), and *E. raffinosus* (p-value 0.6730) in influent and SC effluent samples. Likewise, there were no significant differences in prevalence of *E. faecalis* (p-value 0.2567), *E. casseliflavus* (p-value 0.5003), and *E. faecium* (p-value 0.3982) in influent and SC effluent samples in spring. None of the enterococci were resistant to tested antibiotics in the winter season. However, some spring isolates from influent and SC effluent were found resistant to CIP and SXT. Overall, no significant difference in prevalence of *E. faecalis* (p-value 0.7065) was observed in winter and spring.

**Table 5.5 Diversity of Selected Enterococci (Control and Highly resistant) from Influent and Secondary Clarifier Effluent Sample**

| | Winter | | | | | | | | Spring | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Influent | | | | SC Effluent | | | | Influent | | | | SC Effluent | | | |
| | Ctrl | CIP | SXT | VAN | Ctrl | CIP | SXT | VAN | Ctrl | CIP | SXT | VAN | Ctrl | CIP | SXT | VAN |
| *E. faecalis* | 22 | 0 | 0 | 0 | 14 | 0 | 0 | 0 | 13 | 28 | 20 | 0 | 19 | 28 | 24 | 0 |
| *E. faecium* | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 8 | 2 | 2 | 0 | 1 | 7 | 1 | 0 |
| *E. casseliflavus* | 7 | 0 | 0 | 0 | 7 | 0 | 0 | 0 | 8 | 0 | 0 | 0 | 2 | 0 | 4 | 0 |
| *E. gallinarum* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| *E. hirae* | 3 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *E. raffinosus* | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total | 34 | 0 | 0 | 0 | 28 | 0 | 0 | 0 | 30 | 30 | 22 | 0 | 22 | 35 | 30 | 0 |

# CHAPTER 6 - SUMMARY AND CONCLUSONS

Antibiotic resistance patterns were analyzed for municipal wastewater bacteria. The microorganisms studied included fecal coliforms, *Escherichia coli* and enterococci. Wastewater samples were collected in summer, winter and spring. Bacteria in municipal wastewater treatment plant influent, secondary clarifier effluent and disinfected effluent were plated in the presence of predetermined concentrations of selected antibiotics. The antibiotics included for the study were ciprofloxacin, sulfamethoxazole/trimethoprim and vancomycin. The diversity of enterococci was further investigated using PCR analysis.

Several conclusions may be derived from this work. These include:

- Significant numbers of fecal coliforms, *E. coli* and enterococci were found to be resistant or highly resistant to CIP and SXT in the influent and SC effluent.
- Approximately 6.6 x $10^2$ cfu/mL (0.22%) of fecal coliforms were highly resistant to CIP, while 1.1 x $10^2$ cfu/mL (0.03 %) were highly resistant to SXT in the influent sample collected in spring. In SC effluent samples 1.23 x $10^2$ cfu/mL (3.69 %) fecal coliforms were resistant to CIP. Fecal coliforms in influent and SC effluent were highly resistant to SXT in all seasons.
- Approximately 4.67 x $10^2$ cfu/mL (3.5%) *E. coli* in the influent showed resistance to CIP while 0.13 x $10^2$ cfu/mL (1.73%) were resistant in the SC effluent in spring. *E.coli* were also resistant to SXT and approximately 1.46 x $10^2$ cfu/mL (0.70%) were observed in the influent sample in spring.
- Approximately 4.33 x $10^2$ cfu/mL (0.03%) and 30 cfu/mL enterococci were resistant and highly resistant to CIP in the influent sample. Approximately 30 cfu/mL (4.10%) and 17 cfu/mL (2.31%) enterococci population collected from SC effluent were resistant and highly resistant collected in spring. Approximately 5.66 x $10^3$ cfu/mL of enterococci were resistant and 4.33 x $10^2$ cfu/mL were highly resistant to SXT in influent sample collected in summer. Enterococci that

were isolated from the SC effluent sample were also resistant and highly resistant to SXT.

- *E. faecalis* constituted the largest fraction of the enterococci population in the wastewater samples; no significant difference was observed in the prevalence of *E. faecalis* (p value 0.7065) in winter and spring.
- No significant difference in diversity of enterococcal species in influent and SC effluent was observed in winter and spring seasons.

The UV disinfection process in the treatment plant appeared to be efficient in reducing the number of resistant bacteria in the final effluent. The final effluent released into the surface water was free from resistant organisms.

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# Appendix A

**Table A.1: Impact of Ciprofloxacin Concentration on Fecal Coliform in Influent and Secondary Clarifier Effluent Samples Collected in Summer**

| Antibiotic Conc. (mg/L) | Influent (cfu/mL) | Influent (cfu/mL) | Influent (cfu/mL) | Influent (cfu/mL) | Average | STDEV | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | Average | STDEV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 350000 | 300000 | 100000 | 180000 | 232500 | 113541.475 | 480 | 450 | 28 | 25 | 245.75 | 253.467 |
| 0.01 | 390000 | 450000 | NA | NA | 420000 | - | 51 | 50 | NA | NA | 50.5 | - |
| 0.05 | 106000 | 110000 | NA | NA | 108000 | - | 31 | 28 | NA | NA | 29.5 | - |
| 0.1 | 35000 | 33000 | 39000 | 35000 | 35500 | 2516.611 | 84 | 82 | 73 | 63 | 75.5 | 9.609 |
| 0.5 | 9500 | 9000 | NA | NA | 9250 | - | 8 | 10 | NA | NA | 9 | - |
| 1 | 3800 | 3000 | NA | NA | 3400 | - | 1 | 5 | NA | NA | 3 | - |

**Table A.2: Impact of Sulfamethoxazole/trimethoprim Concentration on Fecal Coliform in Influent and Secondary Clarifier Effluent Samples Collected in Summer**

| Antibiotic Conc. (mg/L) | Influent (cfu/mL) | Influent (cfu/mL) | Influent (cfu/mL) | Influent (cfu/mL) | Average | STDEV | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | Average | STDEV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 100000 | 180000 | 350000 | 300000 | 232500 | 113541.475 | 28 | 25 | 480 | 450 | 245.75 | 253.467 |
| 1/19 | 30000 | 27000 | NA | NA | 28500 | - | 60 | 55 | NA | NA | 57.5 | - |
| 2/38 | 300000 | 360000 | NA | NA | 330000 | - | 29 | 22 | NA | NA | 25.5 | - |
| 10/190 | 6000 | 5800 | NA | NA | 5900 | - | 19 | 15 | NA | NA | 17 | - |

**Table A.3: Impact of Ciprofloxacin Concentration on Enterococci in Influent and Secondary Clarifier Effluent Samples Collected in Summer**

| Antibiotic Conc. (mg/L) | Influent (cfu/mL) | Influent (cfu/mL) | Influent (cfu/mL) | Influent (cfu/mL) | Average | STDEV | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | Average | STDEV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 15000 | 17000 | 70000 | 55000 | 39250 | 27548.442 | 2 | 20 | 14 | 10 | 11.5 | 7.5498 |
| 0.01 | 19000 | 15000 | NA | NA | 17000 | - | 11 | 9 | NA | NA | 11 | - |
| 0.05 | 14000 | 12000 | NA | NA | 13000 | - | 6 | 6 | NA | NA | 6 | - |
| 0.1 | 5500 | 5000 | 3000 | 3100 | 5250 | 1287.1156 | 0 | 15 | 8 | 7.8 | 7.9 | 6.128 |
| 0.5 | 13000 | 17000 | NA | NA | 15000 | - | 4 | 8 | NA | NA | 8 | - |
| 1 | 22000 | 26000 | NA | NA | 24000 | - | 20 | 7 | NA | NA | 7 | - |

**Table A.4: Impact of Sulfamethoxazole/Trimethoprim Concentration on Enterococci in Influent and Secondary Clarifier Effluent Samples Collected in Summer**

| Antibiotic Conc. (mg/L) | Influent (cfu/mL) | Influent (cfu/mL) | Influent (cfu/mL) | Influent (cfu/mL) | AVG | STDEV | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | Average | STDEV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 15000 | 17000 | 70000 | 55000 | 39250 | 27548.44 | 14 | 10 | 2 | 20 | 11.5 | 7.5498 |
| 1/19 | 15000 | 12000 | NA | NA | 13500 | - | 13 | 11 | NA | NA | 13 | - |
| 2/38 | 23000 | 22000 | NA | NA | 22500 | - | 14 | 12 | NA | NA | 14 | - |
| 10/190 | 13000 | 10000 | NA | NA | 11500 | - | 8 | 8 | NA | NA | 8 | - |

**Table A.5: Impact of Vancomycin Concentration on Enterococci in Influent and Secondary Clarifier Effluent Samples Collected in Summer**

| Antibiotic Conc. (mg/L) | Influent (cfu/mL) | Influent (cfu/mL) | Influent (cfu/mL) | Influent (cfu/mL) | AVG | STDEV | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | AVG | STDEV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 15000 | 17000 | 70000 | 55000 | 39250 | 27548.44 | 2 | 20 | 14 | 10 | 11.5 | 7.5498 |
| 0.5 | 1500 | 2000 | NA | NA | 1750 | - | 15 | 16 | NA | NA | 15.5 | - |
| 1 | 1390 | 1450 | 1000 | 900 | 1185 | 275.4995 | 10 | 8 | 8 | 14 | 10 | 2.8284 |
| 30 | 200 | 400 | NA | NA | 0 | - | 8 | 7 | NA | NA | 7.5 | - |
| 50 | 25 | 36 | NA | NA | 30.5 | - | 3 | 5 | NA | NA | 4 | - |

**Table A.6: Impact of Ciprofloxacin Concentration on Fecal Coliform in Influent, Secondary Clarifier Effluent and Disinfected Effluent Samples Collected in Winter**

| Antibiotic Conc. (mg/L) | Influent (cfu/mL) | Influent (cfu/mL) | Influent (cfu/mL) | AVG | STDEV | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | AVG | STDEV | Effluent (cfu/mL) | Effluent (cfu/mL) | Effluent (cfu/mL) | AVG | STDEV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 300000 | 430000 | 320000 | 350000 | 70000 | 3000 | 3200 | 3500 | 3233.4 | 251.661 | 0 | 0 | 0 | 0 | 0 |
| 0.01 | 55000 | 50000 | 48000 | 51000 | 3605.551 | 165 | 178 | 150 | 164.4 | 14.0118 | 0 | 0 | 0 | 0 | 0 |
| 0.05 | 50000 | 43000 | 48000 | 47000 | 3605.551 | 170 | 140 | 120 | 143.4 | 25.166 | 0 | 0 | 0 | 0 | 0 |
| 0.1 | 20000 | 28000 | 25000 | 24334 | 4041.451 | 120 | 100 | 110 | 110 | 10 | 0 | 0 | 0 | 0 | 0 |
| 1 | 10000 | 18000 | 15000 | 14334 | 4041.451 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table A.7: Impact of Sulfamethoxazole/Trimethoprim Concentration on Fecal Coliform in Influent, Secondary Clarifier Effluent and Disinfected Effluent Samples Collected in Winter**

| Antibiotic Conc. (mg/L) | Influent (cfu/mL) | Influent (cfu/mL) | Influent (cfu/mL) | AVG | STDEV | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | AVG | STDEV | Effluent (cfu/mL) | Effluent (cfu/mL) | Effluent (cfu/mL) | AVG | STDEV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 300000 | 430000 | 320000 | 350000 | 70000 | 3000 | 3200 | 3500 | 3233.4 | 251.66 | 0 | 0 | 0 | 0 | 0 |
| 1/19 | 100000 | 170000 | 130000 | 133334 | 35118.84 | 200 | 210 | 190 | 200 | 10 | 0 | 0 | 0 | 0 | 0 |
| 2/38 | 20000 | 25000 | 18000 | 21000 | 3605.55 | 100 | 97 | 101 | 99.4 | 2.08166 | 0 | 0 | 0 | 0 | 0 |
| 10/190 | 10000 | 15000 | 13000 | 12667 | 2516.61 | 20 | 21 | 25 | 22 | 2.645 | 0 | 0 | 0 | 0 | 0 |
| 100/1900 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 |
| 200/3800 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 | 0 |

**Table A.8: Impact of Ciprofloxacin Concentration on Enterococci in Influent, Secondary Clarifier Effluent and Disinfected Effluent Samples Collected in Winter**

| Antibiotic Conc. (mg/L) | Influent (cfu/mL) | Influent (cfu/mL) | Influent (cfu/mL) | AVG | STDEV | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | AVG | STDEV | Effluent (cfu/mL) | Effluent (cfu/mL) | Effluent (cfu/mL) | AVG | STDEV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 10000 | 15000 | 17000 | 14000 | 3605.55 | 400 | 420 | 470 | 430 | 26.45 | 10 | 9 | 7 | 8.7 | 1.52 |
| 0.01 | 5000 | 5200 | 4800 | 5000 | 200 | 20 | 23 | 24 | 22.33 | 0.838 | 0 | 0 | 0 | 0 | 0 |
| 0.1 | 0 | 4500 | 4700 | 3066.7 | 2657.69 | 0 | 20 | 19 | 13 | 3.78 | 0 | 0 | 0 | 0 | 0 |
| 1 | 4000 | 4200 | 4900 | 4366.7 | 472.58 | 10 | 8 | 9 | 9 | 0.577 | 10 | 11 | 19 | 13.4 | 4.93 |
| 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table A.9: Impact of Vancomycin Concentration on Enterococci in Influent, Secondary Clarifier Effluent and Disinfected Effluent Samples Collected in Winter**

| Antibiotic Conc. (mg/L) | Influent (cfu/mL) | Influent (cfu/mL) | Influent (cfu/mL) | AVG | STDEV | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | AVG | STDEV | Effluent (cfu/mL) | Effluent (cfu/mL) | Effluent (cfu/mL) | AVG | STDEV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 10000 | 15000 | 17000 | 14000 | 3605.5 | 400 | 420 | 470 | 430 | 26.45 | 10 | 9 | 7 | 8.7 | 1.52 |
| 0.1 | 10000 | 18000 | 15000 | 14333.4 | 4041.4 | 100 | 70 | 9 | 59.7 | 32.64 | 0 | 0 | 0 | 0 | 0 |
| 1 | 1000 | 1200 | 1500 | 1233.4 | 251.68 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 2 | 1.7 | 0.57 |
| 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 200 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table A.10**: **Impact of Ciprofloxacin Concentration on *E.coli* in Influent, Secondary Clarifier Effluent and Disinfected Effluent Samples Collected in Winter**

| Antibiotic Conc. (mg/L) | Influent (cfu/mL) | Influent (cfu/mL) | Influent (cfu/mL) | AVG | STDEV | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | AVG | STDEV | Effluent (cfu/mL) | Effluent (cfu/mL) | Effluent (cfu/mL) | AVG | STDEV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 200000 | 280000 | 240000 | 240000 | 40000 | 1000 | 1500 | 1700 | 1400 | 360.6 | 0 | 0 | 0 | 0 | 0 |
| 0.01 | 50000 | 56000 | 49000 | 51666.7 | 3785.93 | 0 | 1200 | 1000 | 733.3 | 642.91 | 0 | 0 | 0 | 0 | 0 |
| 0.05 | 40000 | 44000 | 39000 | 41000 | 2645.7 | 100 | 110 | 150 | 120 | 26.45 | 0 | 0 | 0 | 0 | 0 |
| 0.1 | 20000 | 25000 | 31000 | 25333.4 | 5507.6 | 10 | 14 | 15 | 13 | 2.64 | 0 | 0 | 0 | 0 | 0 |
| 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table A.11 Impact of Sulfamethoxazole/Trimethoprim Concentration on *E.coli* in Influent, Secondary Clarifier Effluent and Disinfected Effluent Samples Collected in Winter**

| Antibiotic Conc. (mg/L) | Influent (cfu/mL) | Influent (cfu/mL) | Influent (cfu/mL) | AVG | STDEV | SC Effluent | SC Effluent | SC Effluent | AVG | STDEV | Effluent (cfu/mL) | Effluent (cfu/mL) | Effluent (cfu/mL) | AVG | STDEV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 200000 | 280000 | 240000 | 240000 | 40000 | 1000 | 1500 | 1700 | 1400 | 360.55 | 0 | 0 | 0 | 0 | 0 |
| 1/19 | 10000 | 15000 | 13000 | 12666.7 | 2516.6 | 0 | 120 | 150 | 90 | 79.37 | 0 | 0 | 0 | 0 | 0 |
| 2/38 | 0 | 0 | 0 | 0 | 0 | 10 | 9 | 5 | 8 | 2.645 | 0 | 0 | 0 | 0 | 0 |
| 10/190 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 50/950 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 100/1900 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 200/3800 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table A.12 Impact of Ciprofloxacin Concentration on Fecal Coliform in Influent, Secondary Clarifier Effluent and Disinfected Effluent Samples Collected in Spring**

| Antibiotic Conc. (mg/L) | Influent (cfu/mL) | Influent (cfu/mL) | Influent (cfu/mL) | AVG | STDEV | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | AVG | STDEV | Effluent (cfu/mL) | Effluent (cfu/mL) | Effluent (cfu/mL) | AVG | STDEV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 200000 | 400000 | 300000 | 300000 | 100000 | 1000 | 5000 | 4000 | 3333.4 | 2081.66 | 10 | 30 | 20 | 20 | 10 |
| 0.01 | 300000 | 280000 | 250000 | 276666.7 | 25166.11 | 3000 | 2000 | 4000 | 3000 | 1000 | 10 | 10 | 30 | 16.7 | 11.7 |
| 0.1 | 18000 | 15000 | 130000 | 54333.4 | 65546.42 | 2000 | 3000 | 1000 | 2000 | 1000 | 10 | 10 | 10 | 10 | 0 |
| 1 | 10000 | 20000 | 10000 | 13333.4 | 5773.50 | 3000 | 2000 | 0 | 1666.7 | 1527.52 | 0 | 0 | 0 | 0 | 0 |
| 4 | 7700 | 7200 | 6000 | 6966.7 | 873.689 | 100 | 110 | 160 | 123.4 | 32.14 | 0 | 0 | 0 | 0 | 0 |
| 10 | 500 | 800 | 700 | 666.7 | 152.75 | 22 | 9 | 8 | 99.7 | 2.081 | 0 | 0 | 0 | 0 | 0 |

**Table A.13 Impact of Sulfamethoxazole/Trimethoprim Concentration on Fecal Coliform in Influent, Secondary Clarifier Effluent and Disinfected Effluent Samples Collected in Spring**

| Antibiotic Conc. (mg/L) | Influent (cfu/mL) | Influent (cfu/mL) | Influent (cfu/mL) | AVG | STDEV | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | AVG | STDEV | Effluent (cfu/mL) | Effluent (cfu/mL) | Effluent (cfu/mL)) | AVG | STDEV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 200000 | 400000 | 300000 | 300000 | 100000 | 1000 | 5000 | 4000 | 3333.4 | 2081.665 | 10 | 30 | 20 | 20 | 10 |
| 1/19 | 200000 | 220000 | 210000 | 210000 | 10000 | 2000 | 1000 | 5000 | 2666.7 | 2081.665 | 30 | 20 | 50 | 33.4 | 15.27 |
| 2/38 | 20000 | 10000 | 30000 | 20000 | 10000 | 1000 | 2000 | 1000 | 1333.4 | 577.350 | 10 | 20 | 10 | 13.4 | 5.77 |
| 8/152 | 1000 | 2000 | 2000 | 1666.7 | 577.35 | 600 | 500 | 300 | 466.7 | 152.75 | 0 | 0 | 0 | 0 | 0 |
| 10/190 | 100 | 110 | 120 | 110 | 10 | 100 | 100 | 200 | 133.4 | 57.73 | 0 | 0 | 0 | 0 | 0 |
| 50/950 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table A.14: Impact of Ciprofloxacin Concentration on Enterococci in Influent, Secondary Clarifier Effluent and Disinfected Effluent Samples Collected in Spring**

| Antibiotic Conc. (mg/L) | Influent (cfu/mL) | Influent (cfu/mL) | Influent (cfu/mL) | AVG | STDEV | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | AVG | STDEV | Effluent (cfu/mL) | Effluent (cfu/mL) | Effluent (cfu/mL) | AVG | STDEV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 10000 | 20000 | 10000 | 13333.4 | 5773.50 | 600 | 700 | 900 | 733.4 | 152.75 | 0 | 0 | 0 | 0 | 0 |
| 0.01 | 8000 | 9000 | 10000 | 9000 | 1000 | 500 | 600 | 700 | 600 | 100 | 0 | 0 | 0 | 0 | 0 |
| 0.1 | 8000 | 7000 | 8000 | 7666.7 | 577.35 | 500 | 600 | 400 | 500 | 100 | 0 | 0 | 0 | 0 | 0 |
| 1 | 6200 | 6500 | 6000 | 6233.4 | 251.66 | 400 | 300 | 500 | 400 | 100 | 0 | 0 | 0 | 0 | 0 |
| 4 | 420 | 450 | 430 | 433.4 | 15.27 | 30 | 28 | 27 | 30 | 1.52 | 0 | 0 | 0 | 0 | 0 |
| 10 | 32 | 30 | 27 | 29.7 | 2.5 | 20 | 10 | 20 | 16.7 | 5.77 | 0 | 0 | 0 | 0 | 0 |

**Table A.15 Impact of Sulfamethoxazole/Trimethoprim Concentration on Enterococci in Influent, Secondary Clarifier Effluent and Disinfected Effluent Samples Collected in Spring**

| Antibiotic Conc. (mg/L) | Influent (cfu/mL) | Influent (cfu/mL) | Influent (cfu/mL) | AVG | STDEV | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | AVG | STDEV | Effluent (cfu/mL) | Effluent (cfu/mL) | Effluent (cfu/mL) | AVG | STDEV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 10000 | 20000 | 10000 | 13333.4 | 5773.5 | 600 | 700 | 900 | 733.4 | 152.75 | 0 | 0 | 0 | 0 | 0 |
| 1/19 | 8000 | 7000 | 9000 | 8000 | 1000 | 500 | 600 | 700 | 600 | 100 | 0 | 0 | 0 | 0 | 0 |
| 2/38 | 6500 | 6800 | 6700 | 6666.7 | 152.752 | 450 | 490 | 420 | 453.4 | 35.11 | 0 | 0 | 0 | 0 | 0 |
| 8/152 | 5900 | 5500 | 5600 | 5666.7 | 208.166 | 30 | 30 | 50 | 36.7 | 11.547 | 0 | 0 | 0 | 0 | 0 |
| 10/190 | 400 | 500 | 400 | 433.4 | 57.7 | 28 | 29 | 25 | 27.4 | 2.08 | 0 | 0 | 0 | 0 | 0 |

**Table A.16: Impact of Vancomycin Concentration on Enterococci in Influent, Secondary Clarifier Effluent and Disinfected Effluent Samples Collected in Spring**

| Antibiotic Conc. (mg/L) | Influent (cfu/mL) | Influent (cfu/mL) | Influent (cfu/mL) | AVG | STDEV | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | AVG | STDEV | Effluent (cfu/mL) | Effluent (cfu/mL) | Effluent (cfu/mL) | AVG | STDEV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 10000 | 20000 | 10000 | 13333.4 | 5773.50 | 600 | 700 | 900 | 733.4 | 152.7 | 0 | 0 | 0 | 0 | 0 |
| 0.5 | 8800 | 8500 | 8700 | 8666.6 | 152.7 | 600 | 700 | 600 | 633.4 | 57.7 | 0 | 0 | 0 | 0 | 0 |
| 1 | 7000 | 8000 | 8000 | 7666.7 | 577.35 | 500 | 500 | 600 | 533.4 | 57.7 | 0 | 0 | 0 | 0 | 0 |
| 4 | 6600 | 6500 | 610 | 4570 | 3429.8 | 48 | 47 | 45 | 46.7 | 1.527 | 0 | 0 | 0 | 0 | 0 |
| 32 | 400 | 500 | 400 | 433.4 | 57.73 | 30 | 40 | 30 | 33.4 | 5.77 | 0 | 0 | 0 | 0 | 0 |
| 50 | 320 | 330 | 310 | 320 | 10 | 19 | 20 | 17 | 18.7 | 1.52 | 0 | 0 | 0 | 0 | 0 |

**Table A.17: Impact of Ciprofloxacin Concentration on *E.coli* in Influent, Secondary Clarifier Effluent and Disinfected Effluent Samples Collected in Spring**

| Antibiotic Conc. (mg/L) | Influent (cfu/mL) | Influent (cfu/mL) | Influent (cfu/mL) | AVG | STDEV | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | AVG | STDEV | Effluent (fu/mL) | Effluent (cfu/mL) | Effluent (cfu/mL) | AVG | STDEV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 22000 | 21000 | 19000 | 20666.7 | 1527.52 | 100 | 200 | 100 | 133.4 | 57.73 | 0 | 0 | 0 | 0 | 0 |
| 0.01 | 1500 | 1900 | 1800 | 1733.4 | 208.2 | 100 | 100 | 0 | 66.7 | 57.73 | 0 | 0 | 0 | 0 | 0 |
| 0.1 | 1300 | 1200 | 1400 | 1300 | 100 | 40 | 30 | 20 | 30 | 10 | 0 | 0 | 0 | 0 | 0 |
| 1 | 1200 | 1100 | 1000 | 1100 | 100 | 30 | 20 | 10 | 20 | 10 | 0 | 0 | 0 | 0 | 0 |
| 4 | 100 | 800 | 500 | 466.7 | 351.18 | 16 | 13 | 11 | 13.4 | 2.51 | 0 | 0 | 0 | 0 | 0 |
| 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table A.18: Impact of Sulfamethoxazole/Trimethoprim Concentration on *E.coli* in Influent, Secondary Clarifier Effluent and Disinfected Effluent Samples Collected in Spring**

| Antibiotic Conc. (mg/L) | Influent (cfu/mL) | Influent (cfu/mL) | Influent (cfu/mL) | AVG | STDEV | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | SC Effluent (cfu/mL) | AVG | STDEV | Effluent (cfu/mL) | Effluent (cfu/mL) | Effluent (cfu/mL) | AVG | STDEV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 22000 | 21000 | 19000 | 20666.7 | 1527.52 | 100 | 200 | 100 | 133.4 | 57.7350 | 0 | 0 | 0 | 0 | 0 |
| 1/19 | 2100 | 2000 | 1800 | 1966.7 | 152.752 | 120 | 150 | 190 | 153.4 | 35.118 | 0 | 0 | 0 | 0 | 0 |
| 2/38 | 190 | 180 | 120 | 163.4 | 37.859 | 180 | 100 | 150 | 143.4 | 40.414 | 0 | 0 | 0 | 0 | 0 |
| 8/152 | 170 | 150 | 120 | 146.7 | 25.166 | 18 | 19 | 22 | 19.7 | 2.08166 | 0 | 0 | 0 | 0 | 0 |
| 10/190 | 5 | 6 | 9 | 6.667 | 2.0816 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 50/950 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |