OCCURRENCE AND RELEASE OF ANTIBIOTIC RESISTANT BACTERIA AND ANTIBIOTIC RESISTANT GENES IN WASTEWATER UTILITIES

By

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ABSTRACT

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Antibiotics 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 plants. The objectives of this study were: (1) to quantify the occurrence and release of antibiotic resistant genes (ARGs) and antibiotic resistant bacteria (ARB) into the environment through the effluent and biosolids of different wastewater treatment utilities including an MBR (Membrane Biological Reactor) utility, conventional utilities and multiple sludge treatment processes, and (2) to quantify antibiotic resistance gene levels in manure, biosolids and soil samples. Tetracycline and sulfonamide resistance genes (Tet-W, Tet-O and Sul-I) along with tetracycline and sulfonamide resistant bacteria were quantified in all the samples. Advance wastewater treatment (MBR) and advance biosolids treatment (Lime stabilization and anaerobic digestion) was effective in reducing the number of antibiotic resistant bacteria and antibiotic resistant genes. The concentrations of tetracycline and sulfonamide resistance genes found in biosolids are less than concentrations found in manure samples.

I would like to dedicate my thesis to my beloved brother Mr. Ahsan Munir who inspired me and inculcated strength in me to finish my masters program successfully. He is a lovely brother, a friend and a role model for me. I always admire him for his hardwork, intelligence and his love for humanity.

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CHAPTER 1

LITERATURE REVIEW

Antibiotic Resistant Bacteria: A human health threat

The emergence of antibiotic resistance bacteria and their resistant genes is becoming a major global health issue. Antibiotics are used throughout the world to help improve the quality of health. Antibiotics have long been considered the "magic bullet" that would end infectious disease. Bacteria have adapted defenses against these antibiotics and continue to develop new resistances, even as we develop new antibiotics.

Our environment is greatly impacted by the presence of antibiotic resistant bacteria and genes which is of great concern for the public health. According to WHO (World health organization) report, in U.S. alone million of people acquire infection due to antibiotic resistant pathogens every year and thousand of them die due to it (*WHO Annual Report,2000*). In recent years, much attention has been given to the increase in antibiotic resistance. As more microbial species and strains become resistant, many diseases have become difficult to treat, a phenomenon frequently endorsed to both indiscriminate and inappropriate use of antibiotics in human medicine.

The use of numerous antimicrobial agents, in particular antibiotics as treatments in animal, human, and plant health maintenance, is a worldwide practice. Large amounts of antibiotics are released into municipal wastewater due to incomplete metabolism in humans and finally find their way into different natural environmental compartment. Different studies have shown the presence of antibiotics in WWTP effluents and also in the surface waters (Christian et al, 2003; Golet et al, 2002). Long term bacterial exposure to even low concentration of antibiotics in the water and wastewater streams lead to the development of antibiotic resistance bacteria. However, the use of antibiotics and antimicrobials in raising animals has also contributed significantly to the pool of antibiotic resistant organisms globally and antibiotic resistant bacteria are now found in large numbers in virtually every ecosystem on earth. Antibiotic usage provides selective pressure that result in emergence of antibiotic resistant bacteria and resistance genes. While some resistant bacteria are found naturally in the environment, pathogens and nonpathogens are released into the environment in several ways, contributing to a web of resistance that includes humans, animals, and the environment, essentially the biosphere.

Antibiotics in the Environment

Antibiotic classes of compounds frequently used in agriculture include tetracycline, aminoglycosides, cephalosporin, macrolides, and fluoroquinolones, and sulfonamides (Christian et al. 2003). Antibiotic medicines have been shown to be released to soils and to persist in the environment. A study group indicated the potential for a range of veterinary medicines to be taken up from soil by plants used for human (Boxall et al. 2006). Different studies have been conducted to determine the presence of antibiotics in the soil, biosolids and manure samples. Indeed, tetracycline concentrations in the range of several hundred micrograms per kilogram have been detected in soil some months after manure application (Kummerer et al. 2004).

Along with inappropriate use of antibiotics in human medicine, higher practice of growth promoters in the agricultural industry has given rise to bacterial resistant. Intensive animal production involves giving livestock animals' large quantities of antibiotics to promote growth and prevent infection. These uses promote the selection of antibiotic resistance in bacterial populations. Bacitracin, chlortetracycline, erythromycin, lincomycin, neomycin, oxytetracycline, penicillin, streptomycin, tylosin or virginiamycins are the common antibiotics added in feed to improve the growth of swine (Khachatourians et al. 1998). Antibiotics used in both veterinary and human medicine are: penicillins, cephalosporins, tetracyclines, chloramphenicols, aminoglycosides, spectinomycin, lincosamide, macrolides, nitrofuranes, nitroimidazoles, sulfonamides, trimethoprim, polymyxins and quinolones (Teuber et al. 2001). In a study based in China, determination of three classes of commonly used veterinary antibiotics including five sulfonamides, three tetracyclines and one macrolide in swine wastewater was conducted (Ben et al. 2008). Different antibiotics detected in animal manure and biosolids are listed in Table 1.1 and 1.2 respectively.

Antibiotics detected	Reference
Tetracycline,	Aga et al. 2003, Ben et al. 2008, Campagnolo et al. 2002,
Oxytetracycline	Uslu et al. 2008
Sulfonamide	Ben et al. 2008, Campagnolo et al. 2002
Monensin	Dolliver et al.2008
Macrolide (Tylosin)	Kumar et al. 2004, Campagnolo et al. 2002, Ben et al. 2008
Lincosamide (lincomycin)	Sengelov et al.2003
β-Lactam	Campagnolo et al. 2002
Fluoroquinolon	Campagnolo et al. 2002

 Table 1.1: List of different types of antibiotics detected in animal manure

Antibiotics detected	References
Tetracycline	Spongberg et al. 2008, Lindberg et al. 2005
Sulfonamide, Trimethoprim	Okudo et al. 2009, Gbel et al. 2005, Lindberg et al. 2005
Macrolide	McArdell et al. 2003, Spongberg et al. 2008, Okudo et al.
Lincosamide(Clindamycin)	2009, Gbel et al. 2005, X1a et al. 2005, Lindberg et al. 2005
	Spongberg et al. 2008
Fluoroquinolone,	Sponghorg et al. 2008, Olgudo et al. 2000, Vie et al. 2005
Ciprofloxacin	Golet et al. 2002, Lindberg et al. 2005
Triclosan	Xia et al. 2005
Penicillin	Lindberg et al. 2005
Cephalosporin	Lindberg et al. 2005
Nitroimidazole	Lindberg et al. 2005

Table 1.2: List of different types of antibiotics detected in Biosolids

Sources of Resistance in the Environment

Resistance genes exist naturally in the environment owing to a range of selective pressures in nature (Allen et al. 2010). Originally antibiotic resistance limited to clinically isolated strains which cause epidemic disease was only an issue but in recent years, antibiotic resistant among bacteria is found from every environment on earth. Surprisingly, environmental bacteria harbour antibiotic resistance genes in regions independent of human activities (Allen et al. 2010). Resistance developing in nonpathogenic organisms found in humans, animals, and the environment can serve as a source from which pathogens can acquire genes conferring resistance, and in turn, they can become resistant by acquiring genes from pathogens discharged into the environment, e.g. via wastewater sewage or agricultural runoff (Levy, 1997). Physical forces (wind and water (runoff, leaching)) and biological forces (human, animals, insects and birds) cause widespread propagation of antibiotic resistance genes throughout many environments (Allen et al. 2010).

Resistant microorganisms can be found naturally in all environments, but most of the resistance is associated with anthropogenic impacts of either agricultural or direct human impact (Levy, 2002). Wastewater treatment plants are considered to be a major source of occurrence and propagation of the antibiotic resistant bacteria and their genes. In addition to use in humans, antibiotics are added to animal feed to treat infections, and as growth promoters. Once resistant organisms are spread into the environment, they pose a health risk if they colonize or spread resistance genes to bacteria that colonize humans.

Agricultural impacts: Land application of manure is one of the most common methods of utilization of animal waste. It has been estimated that greater than 90% of the poultry manure generated in the U.S. is mainly applied to agricultural lands as fertilizer (Moore et al. 2005). Runoff from manure application is increasingly being recognized as a serious environmental problem. Runoff from poultry manure consists of microorganisms, heavy metals, and antibiotic residues. The types of soil, rainfall amount, and method of manure application have a large impact on the fate of bacteria in manure applied to land. Runoff after the rainfall event was found to contain large numbers of bacteria (Heinonen-Tanski et al. 2001). The organisms in runoff may be associated with increased antibiotic resistance in the aquatic environment.

Human waste impacts: In addition to the effects of agricultural uses of antibiotics, human have significant impact on the occurrence of antibiotic resistance in the

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environment. Antibiotic use in humans can lead to resistance in the environment via discharge of domestic sewage, hospital wastewater, and/or industrial pollution. Antibiotic resistant organisms from the human gastrointestinal tract, as well as unabsorbed antibiotics, can enter the environment via sewage. Hospital wastewaters having higher concentration of antibiotics have shown higher impact on incidence of antibiotic resistance (Reinthaler et al. 2003). Humans have applied additional selective pressure for antibiotic resistance genes because of the large quantities of antibiotics produced, consumed and applied in medicine and agriculture (Allen et al. 2010). Both the resistant microorganisms and antibiotic residues are excreted, entering the sewage system. Our environment is generally not safe from contamination with untreated sewage; breaches occur frequently where leakage or overflow into groundwater or natural waters occurs (Harwood et al. 2001). Raw domestic sewage contains high numbers of antibiotic resistant bacteria (Pruden et al. 2006). 80.5% of fecal samples from healthy people have been found to contain antibiotic resistant organisms (Reinthaler et al. 2003).

Although sewage treatment processes reduce the numbers of bacteria in wastewater, the effluent will still generally contain large numbers of both resistant and susceptible bacteria (Auerbach et al. 2007). In one of the study, decrease in VRE (Vancomycin resistant enterococci) was observed from 16% in untreated wastewater to 12.5% at the final effluent (Schwartz et al. 2003).

Industrial pollution also influences the occurrence of antibiotic resistance, with pharmaceutical plants yielding a particularly strong effect. High levels of multiple resistant *Acinetobacter* were found in pharmaceutical plant effluents (Guardabassi et al. 1998). Thus, many studies have shown the presence of resistant organisms throughout the world. However, the evidence suggests that human and agricultural activity have a great impact on the levels of resistant organisms in all environments.

Occurrence in the Environment

Antibiotic Resistant Bacteria and Antibiotic Resistant Genes in Natural Waters: Several research studies have reported the occurrence of antibiotic resistant organisms in environmental samples and advocated a global public health concern due to these bacteria. Ash et al. (2002) have studied the prevalence of antibiotic resistance of gram negative bacteria in major rivers of United States. Studies have shown that highest ARB and ARGs were observe in hospital biofilms, followed by activated sludge of municipal sewage, then surface water and then drinking water (Schwartz et al. 2003). According to Peak et al. (2007), antibiotic use affects distribution of resistance genes in associated regions. Resistant bacteria can also be found in high numbers in lakes. In a study of two Spanish lakes, 71% of isolates were resistant to at least one antibiotic including erythromycin (31.1%), tetracycline (17.8%), chloramphenicol (22.2%), and penicillin (68.9%) (Alvero, 1987). Populations especially in rural areas, rely on untreated groundwater for their water supplies. Few studies have been done to determine the antibiotic resistance of isolates from groundwater. Unfortunately, agricultural applications of manure can affect groundwater supplies. Chee-Sanford et al. (2001) were able to show that tetracycline resistant enterococci could be isolated from groundwater underneath swine farms. In West Virginia, coliforms in groundwater were found to have high levels of resistance (McKeon et al. 1995).

Antibiotic Resistant Bacteria and Antibiotic Resistant Genes in Wastewater and Biosolids: Bacterial populations which are resistant to one or more antibiotics and their resistant genes have been found in wastewater samples, biosolids, and animal manure (Pruden et al. 2006; Schwartz et al. 2003). Biosolid samples seem to contain a high concentration of antibiotic resistance bacteria as studied by Brooks et al. (2007) in contrast to the concentration in groundwater reported by Chee-Sanford et al. (2001). According to Szczepanowski et al. (2009) antibiotic resistant bacteria can disseminate their resistance among members of the endogenous microbial community, once they reach the wastewater treatment plants.

Antibiotic Resistant Bacteria and Antibiotic Resistant Genes in Soil Ecosystems and Manure: Soils can contain high numbers of antibiotic resistant bacteria. Published studies have shown the occurrence of antibiotic resistance among soil bacteria (D'costa et al., 2006). These numbers are generally higher in regions affected by pollution or agriculture, but there are unaffected areas that contain high levels as well, perhaps from natural production of antibiotics by soil bacteria. Tropical soils have been found to contain antibiotic resistant *Rhizobium*, even in the absence of pollution (Wiener et al. 1998). *Pseudomonas aeruginosa* isolated from various soils in Spain were resistant to many antibiotics and had higher levels of resistance than isolates from nearby surface waters (Marques et al. 1979).

Increased resistance has been found in soils after application of manure. Studies have reported higher levels of resistance in *Pseudomonas* and *Bacillus* isolates after the application of pig manure (Jensen et al. 2001). In Norway, fields that were without antibiotic application for 10 years nevertheless had high levels of resistant organisms. Resistance in organic soil was 72% and resistance in sandy soil was 74%, including

resistance to chloramphenicol, tetracycline, ampicillin, and streptomycin (Bronstad et al. 1996).

Objectives

The overall objective of the study was to evaluate the development and release of antibiotic resistant bacteria and antibiotic resistant genes in the wastewater utilities. The first specific goal was to analyze the antibiotic resistance patterns in microorganisms in samples collected from raw influent, secondary clarifier (SC) effluent and disinfected effluent from different wastewater treatment plants (presented in Ch. 2). Biosolids, manure and soil samples were analyzed for antibiotic resistant bacteria and antibiotic resistance gene concentration and the effect of land application of manure and biosolids (presented in Ch. 3). Bacteria were tested for resistance against tetracycline and sulfonamide. Antibiotic resistant genes were also quantified using Q-PCR for tetracycline and sulfonamide resistance genes (Tet-W, Tet-O and Sul-I).

CHAPTER 2

Munir M., K. Wong, and I. Xagoraraki. 2010. Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. Water Research (published) <u>doi:10.1016/j.watres.2010.08.033</u>

RELEASE OF ANTIBIOTIC RESISTANT BACTERIA AND GENES IN THE EFFLUENT AND BIOSOLIDS OF FIVE WASTEWATER UTILITIES IN MICHIGAN

Abstract

The purpose of this study was to quantify the occurence and release of antibiotic resistant genes (ARGs) and antibiotic resistant bacteria (ARB) into the environment through the effluent and biosolids of different wastewater treatment utilities including an MBR (Membrane Biological Reactor) utility, conventional utilities (Activated Sludge, Oxidative Ditch and Rotatory Biological Contactors-RBCs) and multiple sludge treatment processes (Dewatering, Gravity Thickening, Anaerobic Digestion and Lime Stabilization). Samples of raw wastewater, pre- and post- disinfected effluents, and biosolids were monitored for tetracycline resistant genes (*tetW* and *tetO*) and sulfonamide resistant gene (*sul-1*) and tetracycline and sulfonamide resistant bacteria. ARGs and ARB concentrations in the final effluent were found to be in the range of ND(non-detectable)copies/100mL and 5.00×10^2 -6.10×10⁵ CFU/100mL respectively. 2.33×10^{6} Concentrations of ARGs (tetW and tetO) and 16s rRNA gene in the MBR effluent were observed to be 1-3 log less, compared to conventional treatment utilities. Significantly higher removals of ARGs and ARB were observed in the MBR facility (range of removal: 2.57 to 7.06 logs) compared to that in conventional treatment plants (range of removal: 2.37-4.56 logs) (p<0.05). Disinfection (Chlorination and UV) processes did not contribute in significant reduction of ARGs and ARB (p>0.05). In biosolids, ARGs and ARB concentrations were found to be in the range of 5.61×10^{6} - 4.32×10^{9} copies/g and 3.17×10^4 - 1.85×10^9 CFU/g, respectively. Significant differences (p<0.05) were observed in concentrations of ARGs (except *tetW*) and ARB between the advanced biosolid treatment methods (i.e., anaerobic digestion and lime stabilization) and the conventional dewatering and gravity thickening methods.

Keywords: Antibiotic resistant genes, Antibiotic resistant bacteria, Tetracycline, Sulfonamide, Wastewater treatment, Biosolids, Effluent

Introduction

The escalating problem of emergence of antibiotic resistant bacteria and their resistant genes is becoming a major global health issue (Levy, 2002; Chee-Sanford et al., 2001). The use of numerous antimicrobial agents as treatments in animal, human, and plant health maintenance, is a worldwide practice providing both desirable and undesirable consequences. Links have been found to exist between antibiotic use and the emergence of antibiotic resistant bacterial pathogens (Aminov et al., 2001; Levy, 2002; Peak et al., 2007; Seveno et al., 2002). Studies have proven increase in antibiotic resistance strains that belong to pathogenic bacteria (Blasco et al., 2008) and over the years, nearly every bacterial pathogen has developed resistance to one or more clinical antibiotics (Todar, 2008).

The general observation published in different studies is that the environmental compartments which are most directly impacted by human or agricultural activities showed higher concentrations of antibiotic -resistant bacteria and antibiotic -resistant genes (Pruden et al., 2006; Chee-Sanford et al., 2001). Large amounts of antibiotics are released into municipal wastewater due to incomplete metabolism in humans or due to disposal of unused antibiotics (Nagulapally et al., 2009), which finally find their ways

into different natural environmental compartments. Antibiotic resistant genes and antibiotic resistant bacteria have been detected in wastewater samples (Zhang et al., 2009; Auerbach et al., 2007; Brooks et al., 2007; Pruden et al., 2006; Reinthaler et al., 2003). Also, the release of antibiotic resistant organisms through wastewater effluents into streams has been previously reported (Gallert et al., 2005; Iwane et al., 2001). Iwane and their colleagues reported approximately 8% and 6.7% of tetracycline resistant bacteria to be found in the pre- and post- chlorinated samples of a wastewater treatment plant respectively and then close to discharge location in the river water, similar percentages of bacteria were found to be resistant to tetracycline (Iwane et al., 2001). In addition, biosolids samples were reported to contain a high concentration of antibiotic resistant bacteria (Brooks et al., 2007). Also, the role of wastewater treatment plants in reducing the load of antibiotic resistant bacteria present in raw sewage is not well known (Rijal et al. 2009). However, it has been suggested that certain conditions within the wastewater treatment plants might increase the number of antibiotic resistant bacteria during the treatment process (Silva et al. 2006; Reinthaler et al. 2003). To the best of our knowledge, comparisons between different wastewater and biosolids treatment processes have not been studied so far.

The objective of this study was to quantify the release of antibiotic resistant genes (ARGs) and antibiotic resistant bacteria (ARB) in the effluent and biosolids of wastewater treatment plants (WWTPs). This is the first study that surveys the release of ARGs and ARB into the environment through the effluent and biosolids of different wastewater treatment utilities including an MBR (Membrane Biological Reactor), conventional wastewater utilities and multiple sludge treatment processes. This study has attempted to provide comparisons between different wastewater treatment processes and biosolid treatment processes along with the comparison of release loads of ARGs and ARB in the environment through the effluent and biosolids. In this study, samples of raw wastewater, effluent and biosolids were monitored for tetracycline and sulfonamide resistant bacteria, tetracycline resistant genes (tetW and tetO) and sulfonamide resistant gene (sull) using quantitative polymerase chain reaction (qPCR) assays and conventional heterotrophic plate count methods. Tetracycline and sulfonamide resistance genes (tetW, tetO and sull) were chosen in this study because tetracycline and sulfonamide are the most commonly used antibiotics in human and veterinary medicine (Boxall et al., 2003; Chopra and Roberts, 2001). In addition, quantitative detection systems already exist for this class of genes (Pei et al. 2006; Aminov et al. 2001). TetW and tetO genes are common in intestinal and rumen environments (Aminov at al. 2001) and have been cited as being promiscuous in their ability to spread among and across populations (Pei et al. 2006; Smith et al., 2004; Billington et al., 2002). Sull gene is also one of the most commonly detected sulfonamide resistant genes in the environment (Pei et al. 2006).

Materials and Methods

Sample Collection: Samples of raw wastewater, effluent prior to disinfection, and final effluent after disinfection were collected from five different WWTPs located in Michigan (U.S.A.). Biosolid samples were also collected from the same treatment plants. Characteristics of the different WWTPs based on wastewater treatment processes, disinfection methods and sludge treatment methods are given in Tables 2.1 and 2.2. Two or three sampling events were conducted from each of these treatment plants starting from December 2008 till October 2009. Samples were kept in ice and were transported to

the Water Quality Laboratory at Michigan State University (East Lansing, U.S.A.) for immediate processing.

Sample Processing: Bacteria in the effluent samples were concentrated by filtration with 0.45 μ m HA filters (Millipore, Billerica, MA). The volume of effluent samples filtered was 1 liter. The filters were collected in a 50ml tubes and 50ml Phosphate Buffer Water (PBW) was added in each tube containing a filter. The tubes were then vortexed for 5 minutes to allow the biomass layer on the filters to mix with water. For influent raw samples, 50mL sample volumes were directly collected into the tubes. All the tubes were then centrifuged for 20 minutes at 4500rpm to concentrate the sample down to 2ml. Supernatant was discarded and the concentrates were stored at -80 °C until DNA extraction was performed for molecular analysis. Biosolid samples were directly stored at -80 °C. The volume of all the samples initially collected for processing was taken into account when calculating the final concentrations.

DNA Extraction: DNA was extracted from the concentrated samples using MagNA pure Compact DNA extraction machine (Roche) following the protocol in the manufacturer's manual. Before DNA extraction, a lysis step was carried out with the samples using Lysis Buffer and Proteinase K solution and the mixture was then placed in the heating block at 65° C for 30 minutes. The lysed samples were used for DNA extraction and the extracts were stored in a freezer at -20 $^{\circ}$ C.

Quantification: Real-time Polymerase Chain reaction was used for quantification of two tetracycline ARGs (*tetW* and *tetO*) and one sulfonamide ARGs (*sul1*) using the SYBR

Green approach. The primers and the probes along with the annealing temperatures used for the tetracycline- and sulfonamide- resistant genes were previously developed (Aminov et al., 2001; Pei et al., 2006). The Eubacterial 16s rRNA genes were quantified according to the protocol described by Suzuki et al. (2001) using a TaqMan QPCR method. All QPCR analyses were performed using a Roche Light Cycler 1.5. QPCR reactions were performed with a temperature program of 15 min at 95 °C (initial denaturing), followed by 50 cycles of 15 sec at 95 °C; 30 sec at the annealing temperature (given in Table 2.3) followed by a melting curve stage with temperature ramping from 60 to 95 °C and a final cooling for 30 sec at 40 °C. The primer sequences used for quantification of antibiotic -resistant genes and 16s rRNA genes are summarised in Table 2.3.

Standard Curves: Positive controls were used to construct the standards by transforming gene bearing plasmids into the *E. coli* using TOPO Cloning kit (InvitrogenTM). Biosolids sample were taken from a wastewater treatment plant (East Lansing, MI) at different times and were analysed for the presence of antibiotic resistant genes by PCR and Gel electrophoresis. PCR reaction was performed with initial denaturation at 94 °C for 5 min, followed by 25 cycles of 94 °C for 30 sec, annealing for 30 sec at the annealing temperature (Table 2.3), extension at 72 °C for 30 sec and a final extension step at 72 °C for 7 min. Fresh PCR product from the samples with confirmed presence of the target gene was mixed with the cloning solution containing the vector. This mixture was then transformed into the competent *E.coli* cells followed by growth of these cells on media.

Culture suspension was prepared using the transformed colonies, screened by PCR again to verify cloning of the target gene. Plasmid was extracted according to the QlAprepTM Spin Miniprep Kit (QIAGEN). The concentration of the purified plasmid DNA was determined using NanoDrop spectrophotometer (NanoDrop[®] ND-1000, Wilmington, DE). Standards with different range of concentrations were prepared by serial dilutions of purified plasmid extracts. Absolute quantification was done using QPCR. The CT value (threshold cycle) in the quantification graphs for each respective concentration was used to finally generate the standard curve.

Culture Method: The conventional approach of heterotrophic plate count (HPC) method was used to evaluate the concentration of antibiotic resistant bacteria in the samples. The analysis was done within 24-48 hrs of sample collection. The concentration of resistant microorganisms was determined by plating samples on media amended with two different antibiotics: (1) tetracycline,16µg/mL (Sigma Aldrich) and (2) sulfonamide, 50.4µg/mL (sulfamethoxazole, Sigma Aldrich). R2A plating media (Difco Laboratories, Franklin Lakes, NJ) were used and each antibiotic was individually amended into the media along with antifungal additive cyclohexamide, 200µg/mL (Sigma Aldrich). The samples were serially diluted and 0.1mL of the dilution was used for spread plating. Plates were incubated for 2 days at 37 °C and then for a period of 5 days at 27 °C (Brooks et al.,2007). Total hetrotrophic culturable bacterial population was determined by plating samples on media without antibiotics.

Statistical Analysis: Student t-test was used to conduct the statistical analysis of the results (i.e., for comparison of concentration means). The null hypothesis which is the

concentration of ARGs (or ARB) was not different between different samples was rejected at a p-value less than or equal to 0.05.

Estimation of overall release: Estimation of the ARGs and ARB released into the environment was conducted based on the discharge through the effluent and biosolids of all the WWTPs and the concentrations measured in this study. Information about average daily discharge rates of the effluent and the biosolids produced was obtained from the managers of all the five WWTPs (personal communication). To compare the daily release loads of ARGs (or ARB) from effluent and biosolids, number of copies (or CFU) were calculated using equations (1), (2) and (3), respectively. Release loads from individual WWTPs were calculated and averaged. Contribution of effluent and biosolids in the release of ARGs (or ARB) was then calculated using equations (4) and (5) respectively.

$$IL = C_{in} \times Q_{in} \tag{1}$$

$$RL_{eff} = C_{eff} \times Q_{eff}$$
(2)

$$RL_{biosolid} = C_{biosolid} \times Q_{biosolid}$$
(3)

$$F_{ARG (eff)} or F_{ARB (eff)} = \frac{\text{RL}_{eff} (\text{copies/day})}{\text{IL} (\text{copies/day})}$$
(4)

$$F_{ARG (biosolid)} or F_{ARB (biosolid)} = \frac{\text{RL}_{\text{biosolid}} (\text{copies/day})}{\text{IL} (\text{copies/day})}$$
(5)

where,

IL = Number of copies (or CFU) per day in the influent,

RL_{eff}, RL_{biosolid} = Release load (copies or CFU) released per day through effluent and biosolids respectively,

C_{in}, C_{eff}, C_{biosolid} = Concentration of ARGs or ARB in influent, effluent and biosolids respectively,

 $Q_{in} = Inflow rate,$

Q_{eff}, Q_{biosolid} = Outflow rate of effluent and biosolids respectively,

FARG (eff) or FARB (eff) = Fraction of contribution of ARGs or ARB through effluent,

 $F_{ARG (biosolid)}$ or $F_{ARB (biosolid)}$ = Fraction of contribution of ARGs or ARB through biosolids

Results

Overall Concentrations of ARGs and ARB in Wastewater Treatment Plants:

Concentrations of ARGs and ARB found in this study are presented in Table 2.4 and 2.5 respectively. Variations among different WWTPs in the raw influent concentration for different genes are expected because of different locations and related human activities. Also wastewater treatment plants receive inflow from a wide variety of sources beyond human population including industrial, hospital and animal waste.

Overall, the trends observed in concentration ranges at different sampling points from all the wastewater treatment plants are: *raw influent* > *pre-disinfected effluent* >

post-disinfected effluent (Figs. 2.1 and 2.2). The concentration ranges of raw influent and biosolids had no significant difference (p>0.05) for both *tetO* and *sul1* genes (Fig. 2.1-b and 2.1-c). However, higher concentration of *tetW* genes were observed in biosolids (Fig. 2.1-a) compared to concentrations in raw samples (p<0.05). Significantly higher (p<0.05) concentration of tetracycline resistant bacteria were observed in biosolids as compared to raw samples (Fig. 2.2-a). However, sulfonamide resistant bacteria show no significant difference (p>0.05) between biosolids and raw (Fig. 2.2-b).

ARGs and ARB in Effluent:

Concentration of ARGs (*tetW* and *tetO*) and 16s rRNA gene in the effluent from a MBR (Membrane Biological Reactor) utility were 1-3 log less compared to conventional treatment utilities, but no significant differences (p>0.05) could be drawn using t-test analysis due to smaller sampling events at the MBR facility.

Similarly, no significant difference (p>0.05) was observed for ARB among different utilities.

ARGs and ARB removals:

Log removal values were calculated based on concentrations of ARGs and ARB in the raw influent samples and the final effluent samples and are shown in figures 2.5 and 2.6, respectively. Among different WWTPs, the highest removals of *tetW*, *tetO* and 16s rRNA genes were observed in the Traverse City WWTP which is a MBR facility with a UV disinfection process (Fig. 2.3-a, 2.3-b and 2.3-d). The highest removals of *sul1* genes were observed in activated sludge wastewater utilities (Lansing and East Lansing) (Fig. 2.3-c). Significant difference (p<0.05) was observed in the log removals between conventional methods and MBR for *tetW*, *tetO* and 16s rRNA genes. Findings in this study show that the MBR facility provided the highest removal efficiency for most of the ARGs from the wastewater stream.

For tetracycline resistant bacteria, the highest removal was detected by activated sludge process (Fig. 2.4-a) whereas for sulfonamide resistant bacteria, highest removal was observed in the MBR utility (Fig. 2.4-b). However, there was no significant difference observed in log removals for antibiotic resistant bacteria (p>0.05) between conventional methods and MBR.

Overall disinfection did not prove to have significant contribution to ARGs and ARB reduction (Fig. 2.3 and 2.4). Very little change in concentrations of ARGs and ARB was observed between pre- and post- disinfected effluents from all treatment plants. Also, the statistical t-test between concentrations of ARGs in pre- and post-disinfected effluent does not show a significant difference between UV and chlorination disinfection process (p>0.05).

Normalization of the concentration of ARGs with that of total 16s rRNA genes, showed a reduction in ratio from the raw to the effluent samples for both the *tetW* and *tetO* genes, suggesting that there is a better reduction in concentrations of tetracycline - resistant genes compared to that of total 16s rRNA genes during the wastewater treatment process (Fig.2.5). However, for *sul1* genes, the ratio with 16s rRNA genes remained the same throughout the treatment process. Also the concentrations of ARB normalized with the total hetrotrophic culturable bacterial count showed approximately same ratios throughout the treatment (Fig. 2.6).

ARGs and ARB in Biosolids:

High concentrations of ARGs and ARB have been found in the biosolid samples. Significant difference (p<0.05) was observed in concentrations of both *tetO* and *sul1* genes in biosolids samples between the advanced treatment methods (anaerobic digestion and lime stabilization) and the conventional treatment methods (dewatering and gravity thickening) (Fig. 2.7). For *tetW* gene, the concentration was found to be lowest in the lime-stabilized biosolid samples $(1.75 \times 10^7 - 1.85 \times 10^8 \text{ copies/g})$ but there was no significant difference (p>0.05) observed between the advanced and conventional treatment methods. Also there was no significant difference (p>0.05) observed and traditonal treatment processes.

Both ARB and hetrotrophic culturable bacterial concentrations in biosolids were also observed to be significantly (p<0.05) different between the advanced and the conventional sludge treatment methods (Fig. 2.8). Overall, results of this study showed that the advanced sludge treatment methods provide better reduction of ARGs and ARB.

Comparison of ARGs and ARB release in Effluent and Biosolids:

Release loads of biosolids were observed to be significantly higher than the effluent loads for all the ARGs and ARB analysed (p<0.05) showing biosolids to have higher contribution in the release of the ARGs and ARB in the environment relative to effluent (Fig. 2.9). Asumming steady flow for all the treatment plants, F_{ARG} (eff) $(1.37 \times 10^{-6} - 9.29 \times 10^{-4})$ and F_{ARB} (eff) $(6.38 \times 10^{-6} - 2.27 \times 10^{-3})$ were much lower than F_{ARG} (biosolid). $(2.09 \times 10^{-3} - 1.15 \times 10^{+1})$ and F_{ARB} (biosolid), $(3.81 \times 10^{-3} - 6.38 \times 10^{+1})$,

which indicates the majority of ARGs and ARB coming into the WWTP would eventually present in the sludge rather than effluent.

Discussion

This study documents the occurence of ARGs and ARB at different points in multiple conventional WWTPs and an MBR facility in Michigan. Tables 2.4 and 2.5 illustrate reported ranges of ARGs and ARB presented in different published studies along with a summary of concentration ranges detected in this study. We observed that eventhough the concentrations of ARGs and ARB in raw wastewater are significantly reduced with wastewater treatment, high concentration are discharged into the effluent. Discharge of final effluent from wastewater treatment plants, still contaminated with ARGs and ARB, is a potential route for entry of ARGs and ARB into the natural environment. It was reported in the literature that percentages of antibiotic resistance in a treated wastewater effluent were found to be mostly higher than the percentages from a wastewater treatment plant (Iwane et al., 2001).

It has been reported that the wastewater treatment process can have an influence on antibiotic resistance through selective pressures and can lead to increase in concentrations of antibiotic resistant bacteria (Y. Zhang et al., 2009, Silva et al. 2006; Reinthaler et al. 2003). Wastewater has been said to stimulate horizontal gene transfer among microbial species (Aminov et al., 2001; Lorenz and Wackemagel, 1994). Therefore, wastewater treatment plants could increase the antibiotic resistance of surviving bacteria, and serve as important reservoirs for the spread of antibiotic resistance to opportunistic pathogens if the treatment processes were not effective. However, in our study we observed significant reduction in the concentration of ARGs and ARB. Similar findings have also been reported by Rijal et al. (2009) which supports the reduction of antibiotic resistant fecal coliform bacteria in a wastewater treatment facility. Differences in removals of ARGs and ARB were found in this study from different wastewater treatment utilities which might be attributed to multiple selective pressures in the environment. In our study, advanced wastewater treatment in an MBR utility was observed to provide better treatment efficiency (range of overall log removal of ARGs and ARB: 2.57 to 7.06) compared to other treatment techniques (range of overall log removal of ARGs and ARB: 2.37 to 4.56). Based on the observed low standard deviations in log removals for all the WWTPs, it is likely to observe similar log reductions if more sampling was done.

Very little change was observed in concentrations of ARGs and ARB between pre- and post-disinfected effluents, therefore the disinfection process did not prove to contribute much in the ARGs and ARB reduction. This was stated by a previous study (Auerbach et al. 2007). Several studies have found that chlorination selects for ARB (Murray et al., 1984; Armstrong et al., 1982), while some other studies demonstrated that disinfection does not select ARB but instead induces the development of antibiotic resistance (Rutala et al., 1997; Murray et al., 1984). However, the mechanism involved in chlorine-induced antibiotic resistance in bacteria is still unknown (Xi et al., 2009). Additional study is needed to understand the effect of disinfection on concentration of ARGs and ARB in wastewater treatment plants.

High concentrations of ARGs and ARB were detected in the biosolids samples which may potentially spread in the natural soil environment via agricultural land application of biosolids. The concentrations of ARB detected in our study observed to be within the range of the previously published concentration of $6.78 \times 10^5 - 4.46 \times 10^8$ CFU/g in biosolids (Brook et al., 2007) and were consistent with the range reported by other research studies (Auerbach et al., 2007; T. Zhang et al., 2009). In this study, advanced biosolids treatment methods (anaerobic digestion and lime stabilization) were found to significantly reduce the ARGs and ARB concentrations in the biosolids as compared to simple dewatering and gravity thickening.

It was found that the *tetW* and *tetO* gene concentrations were lower than *sul1* gene concentration in different samples which was similar to previous observations (Pei et al., 2006). Concentrations of bacteria (CFU/g or CFU/mL) were mostly found to be 1-2 log smaller than concentrations of their respective resistant genes (copies/g or copies/mL) in same samples because not all bacteria are cultivable.

Human exposure to ARGs and ARB, which might be pathogenic in nature, could occur in number of ways. The water environment is considered to play an important part in providing a medium for the transfer of the resistant genes and resistant bacteria to the environment (Baquero et al., 2008; Iwane et al., 2001). Wastewater treatment plants hold an important place in the elimination or the spread of antibiotic -resistant microbes as the treatment systems and their operational conditions might influence the fate of resistant bacteria or resistant genes (Iwane et al., 2001). Although, treated effluents with trace amount of ARGs and ARB from the treatment plants discharged into rivers or streams can add to the contamination of the environment, comparison of release loads of ARGs and ARB calculated in this study, showed that biosolids application seems to be a major source of entry of ARGs and ARB into the natural environment from WWTPs. However, the extent of human exposure to ARGs and ARB is still not well examined. Future studies on human exposure to these resistant contaminants are needed. These may include the ability of bacterial species to survive in the soil and aquatic environment, the biological fitness of the resistance genes they carry, the opportunities to reach new hosts, and the ability of bacterial species to colonize and/or transfer resistance genes.

Conclusions

Wastewater utilities seem to be a potential sources of emerging tetracycline and sulfornamide resistant genes and -bacteria in our environment. All raw influent, effluent and biosolid samples analyzed in this study were found to contain high concentrations of tetracycline and sulfornamide resistant genes and bacteria. The concentration levels of ARGs and ARB in raw sewage were found be much higher than their respective concentations in treated effluent. The concentrations of these resistant microbes and genes were observed to decline several orders of magnitude in the treated effluent. No significant difference in concentrations of both ARGs and ARB was observed in predisinfected and post-disinfected effluents. Significant difference (p<0.05) was observed in the log removals for the *tetW*, *tetO* and 16s rRNA genes between conventional wastewater utilities and an MBR facility. The MBR facility provided the highest removal efficiency for most of the ARGs from the wastewater stream.

Comparisons of concentrations of ARGs and ARB in biosolids and raw influent samples showed that in the case of lime stabilization, concentrations of different ARGs and ARB in biosolids samples appeared to be less, compared to that in the influent raw samples. Significant difference (p<0.05) was observed in concentration of ARGs (*tetO*
and *sul1*), and ARB in biosolids samples between the advanced treatment methods (anaerobic digestion and lime stabilization) and the conventional dewatering and gravity thickening methods. Daily release loads of ARGs and ARB in the environment were found to be higher through biosolids relative to effluents.

Acknowledgements

We would like to thank the managers of all the wastewater treatment plants for providing the samples and information needed for this study. Also, we would like to acknowledge sampling assistance and help provided by Frederick J. Simmons and Arun Kumar.

Tables and Figures

Table 2.1: Wastewater Treatment Characteristics

	EAST	IMLAY	ROMEO	TRAVERSE	LANSING	
	LANSING			CITY		
Wastewater	Activated	Oxidation	Rotating	Membrane	Activated	
treatment	Sludge	Ditch	Biological	Biological	Sludge	
process (Biological	(AS)	(OD)	Contactors (RBCs)	Reactor (MBR)	(AS)	
treatment)						
Capacity	18.8 MGD	0.9 MGD	2.1 MGD	17.0 MGD	37.0 MGD	
Average	13.4 MGD	0.4 MGD	0.8 MGD	8.5 MGD	20.0 MGD	
flow						
Discharge	14.1 MGD	0.02 MGD	0.8 MGD	4.0 MGD	19.0 MGD	
Rate						
Disinfection	Chlorine	Ultra-Violet	Chlorine	Ultra-Violet	Ultra-Violet	
	(Cl)	(UV)	(Cl)	(UV)	(UV)	

MGD-Millions gallon per day

	EAST LANSING	IMLAY	ROMEO	TRAVERS E CITY	LANSING
Sludge treatment	Dewatering (No Anaerobic Digestion)	Gravity Thickening (No Anaerobic Digestion)	Anaerobic Digestion	Anaerobic Digestion	Lime Stabilization
Disposal of sludge	Landfill	Agricultura l land	Agricultural land	Agricultural land	Agricultural land
Disposal rate (dry tons per year)	3596	118	125	850	4380
% solid	18.05%	1.49%	7.98%	4.85%	9.20%

Table 2.2: Biosolids Treatment Characteristics.

Table 2.3: Primers and Probes used in the study.

Target	Primers	Sequences	Annealing temperature (°C)		Amplicon Size (bp)	References	
		(5'-3')	PCR	Q-PCR			
Tet-W	tet(W)-FV	GAGAGCCTGCTATATGCCAGC	64	60	168	Aminov et al. 2001	
	tet(W)-RV	GGGCGTATCCACAATGTTAAC					
Tet-O	tet(O)-FW	ACGGARAGTTTATTGTATACC	60	50	171	Aminov et al. 2001	
	tet(O)-RV	TGGCGTATCTATAATGTTGAC					
Sul-I	sul(I)-FW	CGCACCGGAAACATCGCTGCAC	55.9	55	163	Pei et al. 2006	
	sul(I)-RW	TGAAGTTCCGCCGCAAGGCTCG					
Racteria	BACT1369F	CGGTGAATACGTTCYCGG	56	55	143	Suzuki et al. 2001	
165	PROK1492R	GGWTACCTTGTTACGACTT	50	55	110		
rRNA	TM1389F	CTTGTACACACCGCCCGTC					
	(Probe)						

Type of WWTP	Type of Sludge treatme nt	Antibiotic resistant genes detected	Raw influent (copies/mL)	Pre- disinfected effluent (copies/mL)	Post- disinfected effluent (copies/mL)	Biosolids (copies/g)	References
AS	AnD,	Tet-Q*	$10^{7.2} - 10^9$	-	$10^{3.8} - 10^{6.2}$	$10^{8.4} - 10^{9}$ a	Auerbach et al.
UV, Cl	GrT	Tet-G*	$10^{6.4}$ - $10^{7.8}$	-	$10^{4.2}$ - $10^{5.9}$	$10^{8.5}$ - $10^{9.2}$ a	2007
AS	AnD	Tet C	$10^{8.13} - 10^{8.3}$	$10^{5.36} - 10^{5.57}$	ND- 10 ^{4.12}	$10^{8.49}$ - $10^{8.97}$	Zhang et al.
Cl		Tet A	$10^{7.78}$ - $10^{8.2}$	$10^{4.38}$ - $10^{4.81}$	ND- 10 ^{4.33}	$10^{8.09a}$ -10 ^{9.11}	2009a
AS, OD,	DeW,	Tet-W	$10^{5.37} - 10^{7.4}$	$10^{0.37}$ - $10^{4.03}$	ND- 10 ^{3.63}	$10^{5.37} - 10^{7.4}$	This Study
MBR	AnD,	Tet-O	$10^{5.51}$ - $10^{7.61}$	ND- 10 ^{3.96}	ND- 10 ^{3.96}	$10^{6.8}$ - $10^{9.24}$	
UV, Cl	LS	Sul-I	$10^{5.46}$ - $10^{7.54}$	$10^{2.98}$ - $10^{4.78}$	$10^{4.37}$ -10 ^{6.75}	$10^{6.75}$ - $10^{9.4}$	

Table 2.4: Reported concentrations of ARGs in different samples of WWTPs detected by Quantitative PCR Method.

Note: ^a Units are expressed as copies/mL; ND=non-detectable

*= data approximated from the published graphs;

Tet=tetracycline -resistant gene, Sul=Sulfonamide -resistant gene;

Wastewater treatment type: AS=Activated Sludge process; OD=Oxidative ditch; RBCs= Rotatory Biological Contactors; MBR= Membrane Biological Reactors;

Disinfection type: UV=Ultraviolet radiation disinfection; Cl=Chlorination disinfection;

Biosolid treatment: DeW=Dewatering; GrT=Gravity Thickening; AnD=Anaerobic Digestion; LS=Lime Stabilization

Type of WWTP	Type of Sludge treatment	Antibiotic targeted	Raw influent (CFU/mL)	Pre- disinfected effluent (CFU/mL)	Post- disinfected effluent (CFU/mL)	Biosolids (CFU/g)	References
AS Cl	DeW	24 different antibiotics	10 ^{3.9} -10 ^{5.45}	-	$10^{0.78} - 10^{3.15}$	-	Reinthaler et al. 2003
_	AnD	Ampicillin, cephalothin, ciprofloxacin , tetracycline	-	-	-	10 ^{5.8} -10 ^{10.95*}	Brooks et al. 2007
AS, OD, RBCs, MBR UV, Cl	DeW, GrT, AnD, LS	Tetracycline -resistant Sulfonamide -resistant	$10^{4.18} - 10^{5.36}$ $10^{5.23} - 10^{7.08}$	$10^{1.18} - 10^{2.73}$ $10^{2.18} - 10^{4.03}$	$10^{0.7} - 10^{2.48}$ $10^{2.02} - 10^{3.79}$	$10^{4.5} - 10^{9.07}$ $10^{6.09} - 10^{9.27}$	This Study

Table 2.5: Reported concentrations of ARB in different samples detected by Plating (HPC) Method.

*= data approximated from the published graphs;

Wastewater treatment type: AS=Activated Sludge process; OD=Oxidative ditch; RBCs= Rotatory Biological Contactors; MBR= Membrane Biological Reactors;

Disinfection type: UV=Ultraviolet radiation disinfection; Cl=Chlorination disinfection;

Biosolid treatment: DeW=Dewatering; GrT=Gravity Thickening; AnD=Anaerobic Digestion; LS=Lime Stabilization



Figure. 2.1: Log concentration (copies/100mL) of tetracycline resistant genes (tetW, tetO), sulfonamide resistant gene (sul1) and 16s rRNA gene abundance at different sampling points of all the five wastewater utilities. Note: n=no. of samples, X-axis labels indicate different sampling points, Rectangular boxes indicate the interquartile range of the data, Median value is indicated by the horizontal line inside the box, Small circles'o' represent the mean values.



Figure. 2.1 (Cont'd)



Figure. 2.1 (Cont'd)





Figure. 2.1 (Cont'd)



Figure. 2.2: Log concentration (number of CFU/100mL) of tetracycline resistant bacteria, sulfonamide resistant bacteria and total heterotrophic plate count at different sampling points of all the five wastewater utilities. Note: n=no. of samples, X-axis labels indicate different sampling points, Rectangular boxes indicate the interquartile range of the data, Median value is indicated by the horizontal line inside the box, Small circles'o' represent the mean values.



Sulfonamide resistant bacteria

Figure. 2.2 (Cont'd)



Total heterotrophic plate count

Figure. 2.2 (Cont'd)



Figure. 2.3: Log removals of tetracycline resistant gene (tetW, tetO), sulfonamide resistant gene (sul1) and 16s rRNA gene abundance from wastewater sample of different wastewater utilities. Error bars indicate standard deviation around mean values.

Abbreviations: OX=Oxidative ditch; RBCs= Rotatory Biological Contactors; AS=Activated Sludge process; MBR= Membrane Biological Reactors; Cl=Chlorination disinfection; UV=Ultraviolet radiation disinfection; n=no. of sampling events.



Figure. 2.3 (Cont'd)



Figure. 2.3 (Cont'd)

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Figure. 2.3 (Cont'd)



Figure. 2.4: Log removals of tetracycline resistant bacteria, sulfonamide resistant bacteria and total heterotrophic plate count from wastewater sample of different wastewater utilities. Error bars indicate standard deviation around mean values. Abbreviations: OX=Oxidative ditch; RBCs= Rotatory Biological Contactors; AS=Activated Sludge process; MBR= Membrane Biological Reactors; Cl=Chlorination disinfection; UV=Ultraviolet radiation disinfection; n=no. of sampling events.



Figure. 2.4 (Cont'd)



Figure. 2.4 (Cont'd)



Fraction of Tet-W antibiotic resistant genes with respect to total 16srRNA genes



X-axis labels indicate sampling points, Rectangular boxes indicate the interquartile range of the data, Median value is indicated by the horizontal line inside the box, Small circles represent the mean values.



Fraction of Tet-O antibiotic resistant genes with respect to total 16srRNA genes

Figure. 2.5 (Cont'd)



Fraction of Sul-I antibiotic resistant genes with respect to total 16srRNA genes

Figure. 2.5 (Cont'd)





X-axis labels indicate sampling points, Rectangular boxes indicate the interquartile range of the data, Median value is indicated by the horizontal line inside the box, Small circles represent the mean values.



Figure. 2.6 (Cont'd)



Figure. 2.7: Log concentration of tetracycline resistant gene (tetW, tetO), sulfonamide resistant gene (sul1) and 16s rRNA gene abundance in biosolids sample of different wastewater utilities by real time PCR.

Sludge treatment processes include: DeW=Dewatering; GrT=Gravity Thickening; AnD=Anaerobic Digestion; LS=Lime Stabilization. n=no. of sampling events, X-axis labels indicate type of treatment process, Rectangular boxes indicate the interquartile range of the data, Median value is indicated by the horizontal line inside the box, Small circles represent the mean values.



Figure. 2.7 (Cont'd)

•







16srRNA

Figure. 2.7 (Cont'd)



Tetracycline resistant bacteria

Figure. 2.8: Log concentration (CFU/g) of tetracycline resistant bacteria, sulfonamide resistant bacteria and also total heterotrophic plate count in biosolids sample of different wastewater treatment utilities.

Sludge treatment processes include: DeW=Dewatering; GrT=Gravity Thickening; AnD=Anaerobic Digestion; LS=Lime Stabilization. n=no. of sampling events, X-axis labels indicate type of treatment process, Rectangular boxes indicate the interquartile range of the data, Median value is indicated by the horizontal line inside the box, Small circles represent the mean values.



Sulfonamide resistant bacteria

Figure. 2.8 (Cont'd)



Total Heterotrophic Plate Count

Figure. 2.8 (Cont'd)



Figure. 2.9: Release of copies or CFU of ARGs (Tet-W, Tet-O, sul1) and ARB (Tet R2A and Sul R2A) respectively through Effluent and Biosolids into the environment on a daily basis from the WWTPs. Error bars indicate standard deviation around mean values from all WWTPs.

CHAPTER 3

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LEVELS OF ANTIBIOTIC RESISTANCE GENES IN MANURE, BIOSOLIDS AND FERTILIZED SOIL

Abstract

Increasing antibiotic resistance genes in the environment may pose threat to public health. In this study, tetracycline and sulfonamide resistance genes (Tet-W, Tet-O and Sul-I) were quantified in 24 manure samples from 3 farms and 18 biosolids samples from 7 different wastewater treatment plants (WWTPs) using Quantitative Polymerase Chain Reaction (Q-PCR) methods. Concentrations of Tet-W and Tet-O genes were observed to be significantly higher (p < 0.05) in manure than in biosolids samples. The background soil samples showed significantly lower concentration of the above genes compared to manure and biosolids. Lime stabilized biosolids showed significantly (p<0.05) lower concentration of antibiotic resistance genes compared to other biosolids treatment methods. Elevated levels of antibiotic resistance genes (Tet-W, Tet-O and Sul-I) was observed in the amended soil samples after the land application of manure or biosolids (Site A) monitored for a period of about four months. However, at another site (Site B) no significant increase (p>0.05) in concentration of antibiotic resistance genes was observed after biosolids application on soil. Even though the concentration of ARGs in manure was statistically higher than that in biosolids, when they were applied on land, the contribution to the soil depended upon the background soil concentration and the soil characteristics. Further study of multiple soil samples in various locations is needed.

Introduction

Our environment is greatly impacted by the presence of antibiotic- resistance bacteria and genes. An emerging threat to public and environmental health has been reported due to the growing evidence of increasing antibiotic resistance both in benign and pathogenic bacteria (Knapp et al., 2010; Blasco et al., 2008). Understanding the source of antibiotic- resistance genes is of great importance as human exposure to these microbial contaminants can occur in number of ways (Snary et al., 2004). Land application of animal manure, or biosolids produced from wastewater treatment plants, can be one of the major activities responsible for introduction of antibiotic resistance bacteria and genes in the environment.

The use of antibiotics for the treatment of humans, animals, and plants and also as growth promoters in the agriculture industry is a universal practice. Multiple antibiotic classes of compounds are frequently used in agriculture, veterinary and human medicine including tetracycline, aminoglycosides, cephalosporin, macrolides, and fluoroquinolones, and sulfonamides (Ben et al., 2008; Kumar et al., 2005b; Christian et al., 2003; Teuber, 2001; Khachatourian, 1998). Correlation has been reported between the antibiotic use and the increase in emergence of antibiotic resistance bacterial pathogens (Heuer and Smalla, 2007; Aarestrup, 2005; Levy, 2002; Seveno et al., 2002; Nwosu, 2001; Aminov et al., 2001; Witte, 1999).

In the United States, about 180 million dry tons of livestock and poultry waste are produced annually, which is a potential source of antibiotic resistance bacteria and genes
into the environment (Chee-Sanford et al., 2001). Also approximately 5.6 million dry tons of biosolids are generated annually in United States (National Research Council, 2002), which may be another potential source of antibiotic resistance bacteria and genes. Usage of manure and biosolids in agriculture is considered a way of maintaining or restoring soil quality, due to their fertilizing properties. Manure application on soils can be a major route for distribution of antibiotic resistance genes in the environment (Schmiddt et al., 2006) as it leads to introduction of both residues of antibiotics and bacteria carrying the resistance genes (Heuer and Smalla, 2007; Boxall et al., 2004). According to Kummerer (2004), little is known regarding the effects of antibiotics on resistance levels of environmental bacteria in manure and also the fate of these bacteria and their genes introduced into the soil. Published studies have shown the occurrence of antibiotic resistance among soil bacteria (D'costa et al., 2006; Resenfield et al., 2004; Harris and Woodbine, 1967).

In this study, two classes of antibiotics were selected. Tetracycline, which is one of the most commonly used antibiotics, along with sulfonamide that was recently grouped into a 'high priority' category of veterinary medicines (Boxall et al., 2003). Their occurrence in the environment is considered to be high and have been reported to be detected in animal manure (Ben et al., 2008; Aga et al., 2003; Campagnolo et al., 2002) and biosolids (Spongberg et al., 2008; Gbel et al., 2005; Lindberg et al., 2005). Indeed tetracycline concentrations in the range of several hundred µg/kg have been detected in soil samples even 10-12 months after manure application (Kummerer, 2004; Hamscher et al., 2002). Presence of tetracycline resistance genes have been previously found in manure and the soil environment using Polymerase Chain Reaction (PCR) (Srinavasan et al., 2008; Heuer and Smalla, 2007; Schmitt et al., 2006; Harris and Woodbine, 1967), however, the levels of the resistance genes in soil were not reported. Also, there is a lack of quantitative data on microbial resistance levels of sulfonamide in manure and in soils fertilized with manure (Heuer and Smalla, 2007; Schmitt et al., 2006; Snary et al., 2004). Brooks et al. (2007) reported the concentrations of only antibiotic resistance bacteria (ARB) in the background soil as 2.53×10^6 - 1.06×10^7 CFU/g that shows that these bacterial contaminants (i.e., ARB) reside in the soil media. It has been suggested that there is increasing occurrence of antibiotic-resistance genes in soil samples gathered in Netherlands between 1940 and 2008 (Knapp et al. 2010) so further studies need to be done in different parts of the world to better understand the observed trends.

The objectives of this study are (1) to quantify tetracycline and sulfonamide resistance gene levels in manure, biosolids and soil samples and (2) to evaluate the effects on antibiotic resistance genes after land application of manure and biosolids on the soil. This information will help to characterize biosolids and manure as alternatives for a nutrient amendment-material for land application based on their antibiotic resistance characteristics. In this study two tetracycline resistance genes (Tet-W and Tet-O) and a sulfonamide resistance gene (Sul-I) were quantified in soil, manure, and biosolid samples using Quantitative Polymerase Chain Reaction (Q-PCR) method. Findings of this study are important as it provides information about representative concentrations of Tet-W, Tet-O, and Sul-I genes in environmental soil media for the first time and also provides a comparison of biosolids and manure and their effect after application on soil.

Materials and Methods

Sample Collection:

Manure samples: Twenty four manure samples were collected from three different farms located across Michigan, USA (Table 3.S1, See Supplement Section). Sand-separated manure was collected from Dairy Farm A (Elsie, MI) which is one of the largest operating dairy farms in Michigan consisting of 9500 head of cattles. Dairy Farm B, (East Lansing, MI) consists of approximately 180 milking cows. Manure is collected in lagoons from all cows with no prior treatment. Manure applied on one of the site (referred ahead as Site A) was obtained from a nearby small Dairy Farm C, (Imlay, MI) consisting of 190 milking cows. The dairy farm does not treat manure.

Biosolids samples: Eighteen biosolids samples were collected from seven different wastewater treatment plants situated across Michigan (USA) with 2-4 sampling events from each of these plants for a period of about 10 months. The sludge treatment processes included dewatering, gravity thickening, anaerobic digestion, and lime stabilization methods described in Table 3.S2.

Soil samples: Background soil (defined as soil before the application of manure or biosolids, hereafter) and manure- or biosolids- amended soil samples (defined as soil fertilized with either manure or biosolids, hereafter) were collected from two different sites (Table 3.S3) in Michigan: Imlay city site (Site A) and the Kellogg Biological Station (KBS), Kalamazoo site (Michigan; Site B, hereafter), where manure and class B biosolids were applied, respectively.

Intermittent manure- and biosolids- amended soil sampling was done for a period of about four months in 2009. Site A (Imlay, MI) was an agricultural field containing a network of tile-drains. Class B biosolids (from Romeo WWTP) were applied on the field at three different time (23rd June, 1st and 3rd August) along with dairy manure, applied on some parts of the field (mostly in the buffer zone between the biosolids application boundary and the nearby ditch). Biosolids application events were conducted by Agronomics, Inc., Beulah, Michigan. During the first application event, biosolids were applied to the soil surface by spreading using a G-Force Front Pump System Nuhn Truck (Nuhn Industries Ltd.) with 8,500 gallons capacity tank (applied pressure: 15 pounds per square inch; moving velocity: 1.24 m/s) at an application rate of 10,947 gallons/acre (i.e., 218940 gallons or 10.2 liters/m2 with 5.7% solids). During subsequent events, biosolids were injected into the soil at the application rates of 6,375 gallons/acre (i.e., 127500 gallons with 5.2% solids) and 2,550 gallons/acre (i.e., 51,000 gallons with 7.8% solids), respectively. After land application, biosolids were allowed to sit on the soil before soil incorporation. Manure was applied twice (30th June and 29th August) in the field with surface application method. A total of four manure amended soil samples and seven biosolids amended soil were collected from this site at different intervals. In Site A, there was no application of biosolids in the previous year.

The Site B field is used for research purposes and multiple experimental lysimeters (1.5 m wide and 2.1 m deep) have been installed in the site. The biosolids application rate for this site was approximately 5 gallons per lysimeter. After land application, biosolids were allowed to sit on the soil for ~12 hours followed by simulated

rainfall on each of the lysimeters using portable rainfall simulators (rainfall rate: 2.5"/hr) on a semi-continuous basis. A total of twelve biosolid-amended soil samples were collected from the lysimeter area site where Class B biosolids (from St. Clair WWTP) were applied in year 2009. The Site B field has been reported to be previously (>10 years before) fertilized with manure or compost manure (Basso and Ritchie, 2005). Soil samples were analyzed for nutrient contents by the biosolids application company at A & L Great Lakes Laboratories, Inc. (Fort Wayne, Indiana, U.S.A.). The nutrient composition of the soil from both the sites are given in Table 3.S3.

Sample Processing:

All manure, biosolids, and soil samples were transported to the Water Quality Laboratory at Michigan State University (East Lansing, MI) in coolers within 4 hours of collection. It was aliquoted into smaller vials within 24-36 hrs and stored at -80° C for DNA extraction. Solid contents of these samples were also determined along with other molecular analysis.

DNA Extraction:

DNA was extracted manually from the concentrated samples using QIAamp^(b) DNA stool extraction kit (Qiagen) for manure and biosolids samples and UltraClean^(b) Soil DNA Isolation kit (Mo Bio laboratories) for the soil samples. The extracts were then stored in a freezer at -20 C for further quantification.

Quantification:

Real-time Polymerase Chain reaction was used for quantification antiobiotic resistance genes including tetracycline ARGs [Tet-W and Tet-O] and one sulfonamide ARG [Sul-I]. SybrGreen analysis approach was used for the reactions. The primers and the probes for the tetracycline and sulfonamide resistance genes were referred from Aminov et al. (2001) and Pei at al. (2006), respectively. For the quantification of the Eubacterial 16sRNA gene, TaqMan approach was followed previously described by Suzuki et al. (2001). The primer and probe sequences of the antibiotic resistance genes and 16sRNA are summarised in Table 3.S4. Roche Light Cycler 1.5 was used for all the analysis which was done in a set of duplicate or triplicate for better results.

Standard Curves:

Standards for the positive control were constructed by transforming gene bearing plasmids into the *E. coli* using TOPO Cloning kit (InvitrogenTM). Initially the biosolids samples were collected from wastewater treatment plant (East Lansing, MI) and the antibiotic resistance genes were detected using Polymerase Chain Reaction (PCR) and Gel Electrophoresis. Fresh PCR product from the samples showing the target antibiotic resistance gene was mixed with the cloning solution containing the vector. This mixture was then transformed into the competent *E.coli* cells followed by growth of these cells on media. Plamid was extracted according to the QlAprep[®] Spin Miniprep Kit from the culture suspension of transformed colonies. Plasmid extract was purified and DNA

concentration was checked using a NanoDrop Spectrophotometer (NanoDrop[®] ND-1000, Wilmington, DE). Standards with different range of copies per mL were prepared by serial dilutions of purified plasmid extract. Plasmid extracts were initially diluted based on amplicon size, plasmid concentration ($ng/\mu L$) and copies per reaction desired and further 10× fold serial dilutions were prepared. Absolute quantification was done using Q-PCR assay and the CP (Crossing Point) value calculated in the quantification graphs for each respective concentration was used to generate the standard curve.

Statistical Analysis:

Gene concentrations of samples were statistically analysed using t-test. A t-test is a statistical hypothesis test and is commonly applied when the test statistic would follow a normal distribution. Pairs of samples showing significant difference in their gene concentration levels have p-values less than 0.05. Ninety percent confidence interval of gene concentration levels were calculated by subtracting 5th percentile values from 95th percentile values (Kammen et al. 1999).

Results

Gene concentrations of manure, biosolids and soil samples:

Relative antibiotic resistant gene concentrations in manure samples obtained from different farms are shown in Fig. 3.1-a. Manure from the Dairy Farm A showed significantly higher (p<0.05) concentration for Tet-W and Sul-I genes compared to Dairy Farm C. The concentration of antibiotic resistance genes (Sul-I genes) in the manure from the Dairy Farm C was significantly lower (p<0.05) than that from Dairy Farm B.

Lime stabilized biosolids showed lower concentration of the antibiotic resistance genes compared to that from other treatment methods, suggesting that the advanced sludge treatment is more effective (Fig. 3.1-b). Tet-O gene concentration was found to be significantly lower (p<0.05) in anaerobic digested biosolids compared to dewatered biosolids. However, no significant difference (p>0.05) was observed in concentrations of the antibiotic resistance genes among biosolids produced by other sludge treatment methods.

No significant difference was observed in Sul-I gene levels between soil samples from different sites (Site A and Site B) (p>0.05) (Fig. 3.1-c). Tet-O gene was not detected in background soil sample from the Site A (Imlay site). Tet-W gene concentration was observed to be a magnitude lower in the Site A soil compared to the Site B soil, which might correspond to difference in environmental conditions, and soil type suggesting the possibility of regional differences in diversity of antibiotic-resistance genes.

Comparison of gene concentrations of antibiotic resistance genes in manure, biosolids, soil samples:

This study detected levels of antibiotic resistance genes (Tet-W, Tet-O, and Sul-I) in different manure and biosolids, along with untreated soil, and manure- and biosolidsamended soil samples from field sites. High concentrations of antibiotic resistance genes were observed both in manure and biosolids samples (Fig. 3.2). Table 3.1 summarizes the detected concentration of antibiotic resistance genes (Tet-W, Tet-O and Sul-I) in manure, biosolids and background soil samples. Gene concentrations of Tet-W and Tet-O in manure samples were observed to be significantly higher (p<0.05) than that in biosolids samples (~1-2 magnitude higher). However, Sul-I gene and 16srRNA gene were not observed to differ significantly (p>0.05) in manure and biosolids samples.

The background soil samples also showed presence of tetracycline- and sulfonamide-resistance genes but at lower concentrations compared to manure and biosolids samples (Fig. 3.2). Significant difference in concentration of tetracycline- and sulfonamide-resistance genes was observed between background soil and manure samples (p<0.05) and also between background soil and biosolids samples (p<0.05).

Significant increases (p<0.05) in concentrations of all the antibiotic resistance genes (Tet-W, Tet-O and Sul-I) were observed in the manure- amended soil samples at Site A after the land application of manure (from Imlay City Dairy Farm C, Table 3.S1) compared to that in background soil (Fig. 3.3-a). This observation signifies the introduction of additional antibiotic resistance genes from manure into the soil environment after its land application. Similar observations of significant increase (p<0.05) in gene concentrations of antibiotic resistance genes in manure- amended soil samples were also seen for the biosolids land application at the Site A (Imlay site) (biosolids obtained from Romeo WWTP, Table 3.S2) except for Tet-W gene (p>0.05) (Fig. 3.3-b). In comparison of observations between manure application and biosolids application, it seems that biosolids application has a lesser impact on gene concentrations in soil, which may be due to the presence of lower concentration levels of antibiotic resistance genes in the biosolids samples compared to that in manure samples (assuming similar application and degradation rates).

In contrast, at Site B no significant increase (p>0.05) in gene concentrations of antibiotic resistance genes was observed after biosolids application on soil (biosolids obtained from St. Clair WWTP, Table 3.S2) (Fig. 3.3-c). The concentration range of antibiotic resistance genes was observed to be in similar levels before and after biosolids application. The percentage changes in gene concentration of the antibiotic resistance genes after manure and biosolids application are summarized in Table 3.2.

Discussion

It has been suggested that manure, in addition to introducing antibiotic resistance genes into the soil, may enhance horizontal gene transfer to soil bacteria as it provides nutrients for activation of transfer as well as helps in mobilizing genetic elements (Smalla et al., 2000; Gotz and Smalla, 1997). Contradictorily, it has been reported that manure or biosolids application have not shown much impact on levels of antibiotic resistance culturable bacteria above background soil levels (Brooks et al.,2007; D'Costa et al, 2006). The findings suggest there must be some dilution effect in the soil which did not result in a significant change in gene concentrations in soil samples even after application events. In our study, the background soil samples have been observed to contain antibiotic resistance genes even before manure or biosolids application, and an increase in concentration is seen after manure application.

Antibiotic resistance bacteria have been found in high numbers in a residential garden which was fertilized with manure obtained from a nearby dairy farm (Esiobu et al., 2002). In a study conducted in Netherland, only temporary influence in the levels of tetracycline resistance in the soil has been reported after addition of pig manure slurry (Knapp et al., 2010). The amount of manure slurry spread on the soil was observed to

have an effect on the occurrence of tetracycline resistance levels (Sengelov et al., 2003). It is suggested that the method of manure storage and manure treatment can have considerable impacts on the occurrence and the spread of tetracycline-resistance genes in the agricultural soil environment (Dolliver et al., 2008; Yu et al., 2005). Also, it is reported that composted dairy manure is an environmentally friendly soil amendment material (Edrington et al., 2009). It is therefore proposed that some manure treatment must also be done before land application to lower the level of antibiotics and antibiotic resistance bacteria and their genes in the environment.

To better understand the source of high antibiotic resistance in manure samples, Dairy Farm B was surveyed in terms of their antibiotic usage and manure treatment. As surveyed, dry cows were observed to be treated with penicillin dihydro-streptomycin while the sick cows were observed to be given oxytetracycline treatment or sulfadimethoxine (rarely), hereby, providing justifications of observing high antibioticresistance levels in the dairy's manure samples. Also the milking equipment washes contain antibiotics which may flow into the pits directly and get mixed with the manure. The Dairy farm A is a large facility which uses antibiotics on a regular basis and therefore shows similar levels of antibiotic resistance in the manure. The manure applied on the Imlay site was obtained from the small dairy farm (Dairy Farm C) which has almost negligible antibiotic usage and disposes the manure periodically, thus accounting for the lower levels of antibiotic resistance gene (Fig. 3.1-a).

Biosolids are also commonly applied in agricultural land. In our study, the observations at two different sites after application of biosolids are found to be contradictory. Previous studies have also reported such opposing observations (Brooks et

al., 2007; D'Costa et al., 2006; Gotz and Smalla, 1997). The genetic diversity and natural characteristics of soil play important roles in minimizing the effect of introduction of genes in soil environment by biosolids application events. Based on the observed occurrence of antibiotic resistance genes in the soils at background levels and the different effects of manure and biosolids application, it is clear that there are regional differences in diversity of antibiotic-resistance genes pools which might be responsible for these variations.

The presence of the antibiotic-producing bacteria and their resistance genes in nutrient-enriched environment could be one of the reasons for occurrence of antibiotic resistance genes in the soil environment at background levels. The antibiotics produced can help soil bacterial community to acquire resistance genes under natural selection process (Seveno et al., 2002). It has been implied that the soil has been loaded with genes encoding antibiotics and their related resistance almost certainly for many millions of years (Clewell, 2008; Thiele-Bruhn, 2003; Esiobu et al., 2002; Arai, 1991). Further long-term research is needed to help determine the representative correlations between occurrence of antibiotic resistance microbes and antibiotic resistance genes in multiple soil environments.

Conclusions

Significantly high concentrations of antibiotic resistance genes were observed in different manure and biosolids samples. Significant differences (p<0.05) were observed in concentrations of antibiotic resistance genes (Tet-W and Tet-O) between manure and biosolids samples. Antibiotic resistance genes were also found in the background soil samples from different sites, implying the presence of an indigenous antibiotic resistance

gene pool in the soil environment. Additionally, significantly higher gene concentration levels in manure- or biosolids- amended soil samples were observed after the land application of manure or biosolids in one of the sites. More work is required to understand long-term persistence of antibiotic resistance genes in the soil environment after manure or biosolids application.

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Appendix A: Supplemental Material

Information regarding manure, biosolids and soil characteristics has been included in the supplemental section of the manuscript in Table 3.S1, 3.S2 and 3.S3. Details on the primers and probes used in the assays have also been mentioned in Table 3.S4.

Figures and Tables



(a) Different Manure samples.

Figure 3.1(a-c): Individual sample type-characterization of gene (Tet-W, Tet-O, Sul-I and 16srRNA) levels in bar graphs. Note: 'n' indicate no. of samples and X-axis labels indicate the sampling site.Error bars indicate standard deviation around mean value; KBS-The Kelloggs Biological Station



(b)Different Biosolids samples

Figure 3.1(a-c) (cont'd)



(c) Background soil samples from two different sites.

Figure 3.1(a-c) (Cont'd)





(a)

Figure 3.2(a-d): Concentration ranges of antibiotic resistance genes (Tet-W, Tet-O and Sul-I) and 16s rRNA genes in manure, biosolids, soil, manure amended soil and biosolids amended soil samples. Note: 'n' indicate no. of samples, X-axis labels indicate the type of samples, Rectangular boxes indicate the interquartile range of the data, Median value is indicated by the horizontal line inside the box, Small circles represent the mean values. Asterisks(*) represents the outlier data in the ranges.





Figure 3.2(a-d) (Cont'd)



Figure 3.2(a-d) (Cont'd)

16sRNA



Figure 3.2(a-d) (Cont'd)



⁽a) Site A: Imlay, MI

Figure 3.3(a-c): Effect of land application of manure and biosolids on gene (Tet-W, Tet-O, Sul-I and 16srRNA) levels in soils. Note: Manure and biosolids amended soil sampling was done for a period of about four months. KBS-The Kellogg Biological Station.



(b) Site A: Imlay, MI

Figure 3.3(a-c) (Cont'd)



(c) Site B: KBS, MI

.

Figure 3.3(a-c) (Cont'd)

Table 3.1: Concentration of antibiotic resistance genes (Tet-W, Tet-O and Sul-I) detected in manure, biosolids and background soil samples.

	MEAN CONCENTRATION (Copies/g)			90% CONFIDENCE INTERVAL RANGE (Copies/g)			
Target Gene	Manure	Biosolids	Soil	Manure	Biosolids	Soil	
Tet-W	5.03 ×10 ⁹	9.53×10^{8}	3.61 ×10 ⁴	$3.44 \times 10^8 - 1.15 \times 10^{10}$	2.09×10^7 - 3.66×10^9	$2.79 \times 10^3 - 6.09 \times 10^4$	
Tet-O	1.54×10^{10}	3.15×10^{8}	1.05×10^{6}	$3.66 \times 10^8 - 7.78 \times 10^{10}$	1.20×10^{7} - 9.42×10^{8}	$1.26 \times 10^{5} - 1.73 \times 10^{6}$	
Sul-I	1.51 ×10 ⁸	6.04 ×10 ⁸	4.19×10 ⁴	$8.89 \times 10^{6} - 2.39 \times 10^{8}$	$8.10 \times 10^{6} - 2.81 \times 10^{9}$	2.25×10^4 -7.57×10 ⁴	

Table	3.2:	Percentage	change	in	gene	concentrations	in	soil	after	manure	or
biosoli	ds ap	plication									

	Tet-W	Tet-O	Sul-1
Manure Application		1	I
Site A: Imlay site	+76.8%	+100% †	+35.7%
Biosolids Application			
Site A: Imlay site	+22.7%	+100% 🕇	+13.9%
Site B: KBS site	-9.6%	+6.0%	+2.1%

Note: All calculations are done using log values.

'+' and '-' signs mean an increase and decrease in concentration levels after application.

'†' means since Tet-O gene was not present in background soil, so 100% increase in manure- and biosolids- amended soil.

Supplementary Material

Table 3.S1:	Manure	Characterstics
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	Dairy Farm A	Dairy Farm B	Dairy Farm C
	Elsie,MI	East Lansing, MI	Imlay, MI
Manure treatment	Sand Seperated	No treatment	No treatment
Average % solid	3.4%	1.7%	26.9%

Table 3.S2: Biosolid Treatment Characteristics

	EAST LANSING	IMLAY	St. CLAIR	PLAINWELL	ROMEO	TRAVERSE CITY	LANSING
Sludge treatment	Dewatering (No Anaerobic Digestion)	Gravity Thickening (No Anaerobic Digestion)	Anaerobic Digestion	Anaerobic Digestion	Anaerobic Digestion	Anaerobic Digestion	Lime stabilization
Disposal of sludge	Landfill	Agricultural land	Agricultural land	Agricultural land	Agricultural land	Agricultural land	Agricultural land
% solid	18.1%	1.5%	5.8%	4.0%	8.0%	4.9%	9.2%

Table 3.S3: Soil Characterstics

	Site A	Site B
	Imlay City, MI	Kelloggs Biological Station,
		Kalamazoo, MI
Soil Classification	Loam	Sandy Loam
% Organic Matter	6.9%	2.1%
Total Phosphorus (ppm)	32	60
Total Potassium (ppm)	66	114

Target	Primers	Sequences (5'-3')	Annealing temperature (°C)	Amplicon Size (bp)	References
Tet-W	tet(W)-FV	GAGAGCCTGCTATATGCCAGC	64	168	Aminov et al. 2001
	tet(W)-RV	GGGCGTATCCACAATGTTAAC			
Tet-O	tet(O)-FW	ACGGARAGTTTATTGTATACC	60	171	Aminov et al. 2001
	tet(O)-RV	TGGCGTATCTATAATGTTGAC			
Sul-I	sul(I)-FW	CGCACCGGAAACATCGCTGCAC	55.9	163	Pei et al. 2006
	sul(I)-RW	TGAAGTTCCGCCGCAAGGCTCG			
Bacteria	BACT1369F	CGGTGAATACGTTCYCGG	56	143	Suzuki et al. 2001
16srRNA	PROK1492R	GGWTACCTTGTTACGACTT			
	TM1389F	CTTGTACACACCGCCCGTC			
	(Probe)				

 Table 3.S4: Primers and probes used in this study

CHAPTER 4

Conclusions: Engineering significance

CONCLUSIONS

Occurrence of antibiotic resistance bacteria (ARB) and genes (ARGs) in environmental systems pose human health threats. Identification of environmental reservoirs that harbor ARB and ARGs is important. Until now conventional techniques such as modified heterotrophic plate counts have been successful for the enumeration of antibiotic resistance bacteria in the environmental samples. With the advancement in molecular genetic techniques, antibiotic resistance genes associated with those bacteria or free ARGs in the environment can be detected and also quantified using regular PCR (Polymerase Chain Reaction) and Q-PCR (Quantitative polymerase chain reaction) techniques respectively.

The aim of this study was to analyze the antibiotic resistance patterns in microorganisms in samples collected from raw influent, secondary clarifier (SC) effluent and disinfected effluent wastewater and quantify the occurence and release of antibiotic resistant genes (ARGs) and antibiotic resistant bacteria (ARB) into the environment through the effluent and biosolids of different wastewater treatment. Samples were monitored for tetracycline resistant genes (*tetW* and *tetO*) and sulfonamide resistant gene (*sul-1*) by utilizing molecular techniques. The findings of the study suggest that there is significant reduction in concentration of ARB and ARGs in the final effluent in comparison to raw influent. A comparison between different wastewater treatment and biosolids treatment methods was performed to conclude that both advanced wastewater treatment process (Membrane Biological Reactor) and advanced biosolids treatment

processes (anaerobic digestion and lime stabilization) provide higher removal efficiency for antibiotic resistant bacteria and antibiotic resistant genes. Also disinfection methods did not prove to have a significant contribution in the removal of ARB and ARGs within the wastewater plants.

Tetracycline and sulfonamide resistance gene was also quantified in manure, biosolids and soil samples to evaluate the effects on antibiotic resistance gene levels after land application of manure and biosolids on the soil. This information will help to characterize biosolids and manure as alternatives for a nutrient amendment-material for land application based on their antibiotic resistance characteristics. Findings of this study show that concentrations of ARGs were significantly higher in manure samples than in biosolids samples and on land application, their contribution to the soil depends upon the background soil concentration and the soil characteristics. The practice of biosolids or manure land application on soils should be dealt with more caution with respect to the spread of the resistant determinants in the environment. REFERENCES

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