

ABSTRACT

Title of Dissertation: ANTIBIOTIC CONCENTRATIONS AND THE COMPOSITION OF BACTERIAL COMMUNITIES IN MUNICIPAL WASTEWATER AND RECLAIMED WATER

Prachi Kulkarni, Doctor of Philosophy, 2016

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Before reclaimed water is used more widely within the current United States (U.S.) wastewater treatment infrastructure, it is important to examine the potential public health impacts of this emerging, alternative freshwater resource. My dissertation evaluated antibiotic concentrations and the composition of bacterial communities in conventionally treated municipal wastewater and resulting reclaimed water. I also evaluated the efficacy of a point-of-use filtration system in reducing antimicrobials present in reclaimed water. My objectives were to: 1) Assess the fate of antibiotics and; 2) Characterize the total bacterial community structure of differentially treated wastewater, and reclaimed water that has undergone on-site treatment and storage; and 3) Evaluate zero-valent iron (ZVI)-biosand filtration as a potential point-of use treatment technology for the reduction of antimicrobials from conventionally treated reclaimed water. I extracted nine antibiotics and total genomic deoxyribonucleic acid (DNA) from differentially treated wastewater and reclaimed

water samples from two Mid-Atlantic and two Midwest WWTPs, and one associated Mid-Atlantic spray irrigation site. I quantified the presence of antibiotics using high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS), and characterized total bacterial community structure using 16S rRNA gene sequencing. I also used HPLC-MS/MS to quantify the reduction of thirteen antimicrobials from conventionally treated reclaimed water after ZVI-biosand filtration. Statistical analyses included the Kruskal Wallis test, paired Wilcoxon signed-rank test, and differential abundance using normalization achieved by cumulative sum scaling. Activated sludge treatment used at all four WWTPs resulted in the reduction of some antibiotics and the increase of genera containing potentially pathogenic bacteria (*Mycobacterium* and *Legionella*). Treatment plant chlorination and spray irrigation site ultraviolet radiation (UV) treatment and open-air storage reduced the concentration of azithromycin and increased the relative abundance of *Mycobacterium*. ZVI-biosand filtration achieved significant reductions in azithromycin, ciprofloxacin, erythromycin, linezolid, oxolinic acid, pipemidic acid, penicillin and vancomycin. This research provided additional scientific evidence that activated sludge treatment and chlorination alone may not be sufficient for the removal of antimicrobials and potentially pathogenic bacteria from municipal wastewater and resulting reclaimed water. However, ZVI-biosand filtration may be an efficient reuse site technology for the reduction of antimicrobials from conventionally treated reclaimed water.

ANTIBIOTIC CONCENTRATIONS AND THE COMPOSITION OF BACTERIAL
COMMUNITIES IN MUNICIPAL WASTEWATER AND RECLAIMED WATER

by

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Dedication

For my husband Rob and mother Shaku.

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Table of Contents

Dedication	ii
Acknowledgements	iii
Table of Contents	iv
List of Tables	vii
List of Figures	viii
Chapter 1: Introduction	1
Chapter 2: Background	7
Municipal Wastewater Treatment in the United States	7
Reclaimed Water Use in the United States	9
Regulations Governing Reclaimed Water Use in the United States	10
Antibiotics in Wastewater and Reclaimed Water	12
Bacteria in Wastewater and Reclaimed Water	15
Exposure to Wastewater and Reclaimed Water	16
Agricultural and Landscape Irrigation with Reclaimed Water in the United States	18
Impact of Irrigation with Reclaimed Water	19
Chapter 3: The Application of Zero-valent Iron Technology for the Reduction of Antibiotic Residuals	24
Zero-valent Iron Technology	24
Laboratory Studies of Antibiotic Removal by Zero-valent Iron Technology	25
Chapter 4: Characterization of the Total Bacterial Community Structure of Environmental Samples	28
Introduction	28
Structure and Function of the 16S rRNA gene	29
Bacterial Identification Using the 16S rRNA gene	31
Bacterial Community Analysis using 16S rRNA gene Sequencing	32
Experimental Design and 16S rRNA gene Sequencing Data Analysis	34
Experimental Design	34
Sequence Filtering	35
Data Transformation	35
Taxonomic Summaries and Heatmaps	37
Diversity Analysis	37
Multivariate Exploratory Analyses	38
Multivariate Environmental Interpretation Analyses	38
Differences Between Groups	39
Longitudinal Analysis	39
Bacterial Community Analysis Using 16S rRNA gene Sequencing-Advantages ..	40
Bacterial Community Analysis Using 16S rRNA gene Sequencing-Limitations ..	40
16S rRNA gene Sequence	40
Multiple 16S rRNA Operon Copy Numbers	41
Genes and Species Definition	42
Assignment and Clustering Approches	43
PCR Bias	44

Database Limitations	44
Overcoming the Limitations of 16S rRNA gene Sequencing Analysis Methods...	45
Chapter 5: Antibiotic Concentrations Decrease During Wastewater Treatment but Persist at Low Levels in Reclaimed Water	46
Acknowledgements.....	46
Abbreviations.....	46
Abstract.....	48
Introduction.....	49
Materials and Methods.....	51
Study Sites	51
Sample Size and Description	51
Extraction and Analysis of Antibiotic Concentrations	52
Statistical Analysis.....	53
Results and Discussion	54
Antibiotic Concentrations in Influent Samples from all WWTPs	54
Antibiotic Concentrations in Effluent Samples from all WWTPs.....	55
Differences in Antibiotic Concentrations Between Same-Day Influent Versus Effluent Samples	56
Regional Differences Between Antibiotic Concentrations in Influent and Effluents	57
Differences in Antibiotic Concentrations Across Wastewater Treatment Processes	58
Differences in Antibiotic Concentrations From Mid-Atlantic WWTP1 to Mid-Atlantic SII	60
Limitations	61
Public Health Impacts and Future Research	62
References.....	64
Figures	71
Appendix A.....	76
Detailed description of all sampling sites included in the study.....	76
Supplementary Tables.....	79
Supplementary Figures	80
Chapter 6: Bacterial Community Structure of Conventionally Treated Wastewater and Reclaimed Water.....	82
Acknowledgements.....	82
Abreviations.....	82
Abstract.....	84
Introduction.....	85
Materials and Methods.....	87
Sampling Sites	87
Sample Collection.....	89
DNA Extraction	90
16S rRNA gene Amplification and Sequencing	91
Analysis Pipeline and Data Normalization	92
Statistical Analysis.....	93
Results.....	93

Sequencing	93
Influent Composition Across All WWTPs	94
Composition of Same-Day Influent-Effluent Pairs from All WWTPs	94
Community Changes Across Wastewater Treatment Processes	95
Changes in Community Structure from WWTP to Spray Irrigation Site	96
Discussion	96
Influent Composition Across All WWTPs	96
Composition of Same-Day Influent-Effluent Pairs from All WWTPs	97
Community Changes Across Wastewater Treatment Processes	98
Changes in Community Structure from WWTP to Spray Irrigation Site	98
Implications for Future Research	101
References	104
Figures	112
Appendix A	125
Supplementary Figures	125
Chapter 7: Zero-valent Iron-Biosand Filtration is Capable of Reducing Antimicrobial Concentrations in Unbuffered Conventionally-Treated Reclaimed Water	126
Acknowledgements	126
Abbreviations	126
Abstract	128
Introduction	130
Methods	132
Reclaimed Water Collection Site	132
ZVI-Biosand Filter	133
Sample Collection	134
Sample Processing	134
Environmental Parameters	136
Statistical Analysis	136
Results and Discussion	137
Collection of Chlorinated Effluent, Transport to Greenhouse and Storage in Rain Barrels	137
Filtration of Reclaimed Water Through the ZVI-Biosand Filter	139
Limitations and Implications for Future Research	143
References	145
Tables	151
Figures	155
Chapter 8: Conclusions, Public Health Significance and Future Research	159
Conclusions	159
Public Health Implications and Future Research	166
Bibliography	168

List of Tables

Chapter 5:

Appendix A:

Table S1 A list of the nine antibiotics analyzed with the corresponding mass-charge ratios (m/z) of their parent and daughter ions and their limit of detection (LOD) values (ng/mL) 79

Chapter 7

Table 1 List of tested antimicrobials and surrogate standards with corresponding limits of detection (LOD) and percent recoveries. 151

Table 2 Antimicrobials detected at concentrations below the limit of detection (LOD) by type of sample. 152

Table 3 Median concentrations (ng/ml) and interquartile ranges of antimicrobials in chlorinated effluent, reclaimed water, ZVI-biosand filtered reclaimed water, and tap water samples. Statistically significant reductions (p -value <0.01) in concentrations after ZVI-biosand filtration have been highlighted in bold. 153

Table 4 Median concentrations (ng/ml) and interquartile ranges of environmental parameters for reclaimed water, ZVI-biosand filtered reclaimed water and tap water samples. Statistically significant reductions (p -value <0.01) in concentrations after ZVI-biosand filtration have been highlighted in bold. 154

List of Figures

Chapter 5:

Figure 1: Concentrations (ng/mL) of antibiotics in influent samples collected from all four wastewater treatment plants (WWTPs) included in the study

AMP = Ampicillin; AZI = Azithromycin; CIP = Ciprofloxacin; LIN = Linezolid;
OXA = Oxacillin; OXO = Oxolinic Acid; PEN = Penicillin; PIP = Pipemidic Acid;
TET = Tetracycline 71

Figure 2: Concentrations (ng/mL) of antibiotics in effluent samples collected from all four wastewater treatment plants (WWTPs) included in the study

AMP = Ampicillin; AZI = Azithromycin; CIP = Ciprofloxacin; LIN = Linezolid;
OXA = Oxacillin; OXO = Oxolinic Acid; PEN = Penicillin; PIP = Pipemidic Acid;
TET = Tetracycline 72

Figure 3: Differences in antibiotic concentrations (ng/mL) between influent versus effluent samples collected on the same day from each of the four wastewater treatment plants (WWTPs) included in the study

AMP = Ampicillin; AZI = Azithromycin; CIP = Ciprofloxacin; LIN = Linezolid;
OXA = Oxacillin; OXO = Oxolinic Acid; PEN = Penicillin; PIP = Pipemidic Acid;
TET = Tetracycline 73

Figure 4: Differences in concentrations (ng/mL) of antibiotics across treatment processes used at all the wastewater treatment plants (WWTPs) included in the study

AMP = Ampicillin; AZI = Azithromycin; CIP = Ciprofloxacin; LIN = Linezolid;
OXA = Oxacillin; OXO = Oxolinic Acid; PEN = Penicillin; PIP = Pipemidic Acid;
TET = Tetracycline 74

Figure 5: Changes in antibiotic concentrations (ng/mL) as wastewater travels from the influent at Mid-Atlantic wastewater treatment plant 1 (Mid-Atlantic WWTP1), undergoes tertiary treatment and is then piped to Mid-Atlantic spray irrigation site 1 (Mid-Atlantic SII) for reuse. The sequential order of flow is as follows: 1) Raw influent; 2) Influent post screening; 3) Effluent; 4) Before UV treatment; 5) After UV treatment; 6) Inlet to storage pond; and 7) Inlet to pumphouse.

AMP = Ampicillin; AZI = Azithromycin; CIP = Ciprofloxacin; LIN = Linezolid;
OXA = Oxacillin; OXO = Oxolinic Acid; PEN = Penicillin; PIP = Pipemidic Acid;
TET = Tetracycline 75

Appendix A:

Figure S1: Differences in antibiotic concentrations (ng/mL) between influent samples collected from Mid-Atlantic versus Midwest wastewater treatment plants (WWTPs)
AMP = Ampicillin; AZI = Azithromycin; CIP = Ciprofloxacin; LIN = Linezolid;
OXA = Oxacillin; OXO = Oxolinic Acid; PEN = Penicillin; PIP = Pipemidic Acid;
TET = Tetracycline 80

Figure S2: Differences in antibiotic concentrations (ng/mL) between effluent samples collected from Mid-Atlantic versus Midwest wastewater treatment plants (WWTPs)
AMP = Ampicillin; AZI = Azithromycin; CIP = Ciprofloxacin; LIN = Linezolid;
OXA = Oxacillin; OXO = Oxolinic Acid; PEN = Penicillin; PIP = Pipemidic Acid;
TET = Tetracycline 81

Chapter 6:

Figure 1 Alpha diversity estimates and observed species number in influent samples from all four wastewater treatment plants (WWTPs). No statistically significant differences in alpha diversity estimates were found across influent samples from all four WWTPs. 112

Figure 2 Significantly differentially abundant (p -value <0.01) bacterial genera across influent samples from all four WWTPs. The most abundant bacteria belong to genera predominantly associated with the human microbiome and sewer infrastructure. 113

Figure 3 Alpha diversity estimates and observed species number in same-day influent-effluent pairs from all four WWTPs. Significant differences (p -value < 0.01) in observed species number were detected. 114

Figure 4 Significantly differentially abundant (p -value <0.01) bacterial genera across same-day influent-effluent pairs from all four WWTPs. The most abundant bacteria belong to genera predominantly associated with the human microbiome, sewer infrastructure and biological wastewater treatment processes. 115

Figure 5 PCoA plot using Bray-Curtis dissimilarity showing influent samples clustering apart from samples taken from downstream wastewater treatment processes. 116

Figure 6 Alpha diversity estimates and observed species number in treatment process samples from all four wastewater treatment plants (WWTPs). No statistically significant differences in alpha diversity estimates were found across treatment process samples from all four WWTPs. 117

Figure 7 Significantly differentially abundant (p -value <0.01) bacterial genera (top 10) across the various treatment processes performed at Mid-Atlantic WWTP1. 118

Figure 8 Significantly differentially abundant (p -value <0.01) bacterial genera (top 10) across the various treatment processes performed at Mid-Atlantic WWTP2. 119

Figure 9 Significantly differentially abundant (p -value <0.01) bacterial genera (top 10) across the various treatment processes performed at Midwest WWTP1. 120

Figure 10 Significantly differentially abundant (p -value <0.01) bacterial genera (top 10) across the various treatment processes performed at Midwest WWTP2. 121

Figure 11 Alpha diversity estimates and observed species number in samples from WWTP influent stage at Mid-Atlantic WWTP1 to spray irrigation site pumphouse stage at Mid-Atlantic SII. Significant differences in alpha diversity estimates were found for Shannon index ($F= 5.238$, p -value = 0.002) and observed species (OTU) number ($F= 8.945$, p -value = <0.01) estimates. 122

Figure 12 PCoA plot using Bray-Curtis dissimilarity showing pumphouse inlet samples clustering apart from samples after on-site treatment and storage (Before UV treatment, After UV treatment and Holding Pond inlet). 123

Figure 13 Significantly differentially abundant (p -value <0.01) bacterial genera (top 10) across the treatment at Mid-Atlantic WWTP1, transport to and treatment and storage, at Mid-Atlantic SII. 124

Appendix A:

Figure S1 Number of observed sequences compared to the estimated coverage with a histogram indicating the distribution of samples relative to the number of sequences per sample. Samples with fewer than 100 sequences were filtered. 125

Chapter 7:

Figure 1 Schematic illustrating experimental design. 155

Figure 2 Schematic illustrating treatment steps and the sampling location at the WWTP. 156

Figure 3 Antimicrobial concentration (ng/mL) reductions between reclaimed water and ZVI- biosand filtered reclaimed water samples collected on the same day. Statistically significant reductions (p -value <0.01) were observed for azithromycin, ciprofloxacin, erythromycin, linezolid, oxolinic acid, penicillin G, pipemidic acid and vancomycin.

AMP – Ampicillin, AZI – Azithromycin, CIP – Ciprofloxacin, ERY – Erythromycin, LIN – Linezolid, OXA – Oxacillin, OXO – Oxolinic Acid, PEN – Penicillin G, PIP – Pipemidic Acid, SUL – Sulfamethoxazole, TCC – Triclocarban, TET – Tetracycline, VAN – Vancomycin, RW – Reclaimed Water, ZVI – ZVI-biosand filtered reclaimed water. 157

Figure 4 Principal Component Analysis (PCA) plot illustrating the clustering of tap (TAP) and ZVI-biosand filtered reclaimed water (ZVI) samples with a distinct separation between the tap (TAP) and ZVI-biosand filtered reclaimed water (ZVI) groups from the reclaimed water (RW) group. The separation between the reclaimed water group (RW) and ZVI-biosand filtered reclaimed water group (ZVI) ($R^2 = 0.411$, p -value = 0.001) was much larger compared to that between the tap water group (TAP) and the ZVI-biosand filtered reclaimed water group (ZVI) ($R^2 = 0.227$, p -value = 0.003).

RW – Reclaimed water, TAP – Tap water, ZVI– ZVI-biosand filtered reclaimed water 158

Chapter 1: Introduction

As climate change influenced drought spreads across the United States (U.S.), states with historically low reclaimed water (treated wastewater effluent) use and less stringent wastewater and reclaimed water treatment regulations are turning to reclaimed water to address shortages of traditional freshwater sources for irrigation (Asano, 2007; EPA, 2012a). Landscape and agricultural irrigation are the primary applications for reclaimed water reuse in the U.S. (EPA, 2012a). Currently, California is the leading user of reclaimed water in the U.S. with agricultural irrigation being the largest user of the reclaimed water generated within the state (CA EPA, 2011). California allows the irrigation of raw-eaten food crops with reclaimed water, and requires extremely stringent treatment with regulations specifying not only quality, but also treatment parameters. Specifically, reclaimed water used for irrigation in California is required to undergo chlorination, dual-media filtration, coagulation, and flocculation under the Title 22 Code of Regulations related to Recycled Water (Asano, 2007; CA DPH, 2009). This type of treatment is not typical of conventional wastewater treatment in the U.S. and in the comparatively low use areas to which reclaimed water irrigation is rapidly spreading, it may not be possible to treat wastewater to the near potable quality required in California.

Currently, states have varying reclaimed water regulations or guidelines, due to the absence of legally binding federal regulations (EPA, 2012a). Most states, even ones with more rigid regulations, rely on indicator organism-based monitoring and do not monitor the presence of trace chemicals, such as pharmaceuticals and personal care products (EPA, 2012a). Almost all states require the monitoring and reporting of

treated wastewater effluent that leaves wastewater treatment plants but there is ample evidence of the deterioration of treated effluent quality within reclaimed water distribution systems (Asano, 2007; Jjemba, Weinrich, Cheng, Giraldo, & Lechevallier, 2010). Not all states call for reuse site water quality monitoring (Asano, 2007; EPA, 2012a). The primary objective of wastewater treatment in the U.S. is the degradation of organic matter and not the removal of pathogens and subsequently, various human pathogens, including antibiotic-resistant bacteria, have been found in wastewater treatment plants and at reclaimed water use sites (Carey et al., 2016; Maier, Pepper, & Gerba, 2009; Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014).

U.S. wastewater treatment plants are not designed to remove pharmaceuticals during treatment and pharmaceutically active compounds, including antibiotics, have been found in untreated as well as treated wastewater (Arvai, Klecka, Jasim, Melcer, & Laitta, 2014; Karthikeyan, 2006; Loganathan, Phillips, Mowery, & Jones-Lepp, 2009; Spongberg & Witter, 2008; Zhang & Li, 2011). Moreover, antibiotics are often present at sufficiently high concentrations to exert selective pressure to favor the proliferation of antibiotic resistance within these environments (Klaus Kümmerer, 2009). The ecotoxicological impact of pharmaceuticals in treated effluent has been examined, but the effect of chronic long-term exposure of humans to antibiotics in wastewater and reclaimed water is unknown (Kim & Aga, 2007). Research on irrigation with reclaimed water has also demonstrated the subsequent accumulation of antimicrobial substances in irrigated soil and plants (X. Wu, Dodgen, Conkle, & Gan, 2015).

Previous studies evaluating wastewater treatment and reclaimed water have relied on culture-based methods involving the isolation of specific pathogenic bacteria or indicator organisms (Crook, 2005; Sheikh, Cort, Kirkpatrick, Jaques, & Asano, 1990). Since pathogens normally exist as a part of complex microbial communities which are impacted by the environment in which they exist, studying pathogenic organisms in isolation may not provide all the information required to understand and optimize treatment processes and reuse site practices.

A report by the National Research Council (NRC) sponsored by several regulatory agencies including the U.S. Environmental Protection Agency (EPA) and the U.S. Centers for Disease Control (CDC) has called for further research on 1) residual contaminants in wastewater and their fate in the environment; 2) performance variability in pathogen removal during wastewater treatment; and 3) new technology for wastewater and reclaimed water treatment (NAS, 2012). The report highlights that filling in these data gaps would allow regulatory agencies to be able to determine the potential health impacts of chronic exposure to trace chemicals present in wastewater and to conduct improved health risk assessments of reuse projects (NAS, 2012). This information could also allow water resource managers to be able to optimize wastewater treatment processes (NAS, 2012). The report also states that increased reclaimed water use projections necessitate the exploration of new approaches and improvements in technology involved in wastewater and reclaimed water treatment (NAS, 2012). Therefore, in order to determine whether conventionally treated wastewater is safe for reclamation for irrigation applications we must evaluate 1) the capability of current treatment processes to reduce potentially

toxic bacterial and antibiotic constituents in treated effluent; and 2) the fate of these constituents as the reclaimed water reaches, and is applied to, its point of use.

The purpose of this research was to identify the impact of wastewater and reclaimed water treatment on antibiotic concentrations and total bacterial community structure within wastewater and reclaimed water with a goal towards examining the public health implications associated with reclaimed water use. My primary research objectives were as follows:

- 1) To quantify antibiotic concentrations in differentially treated wastewater from conventional WWTPs in distinct geographic locations and in reclaimed water undergoing on-site treatment and storage at a spray irrigation site
- 2) To characterize the total bacterial community structure of differentially treated wastewater from conventional WWTPs in distinct geographic locations and in reclaimed water undergoing on-site treatment and storage at a spray irrigation site
- 3) To evaluate antibiotic removals achieved through the use of a reuse site-based water treatment system

Each of the three research objectives is addressed in a separate manuscript included in this document. The dissertation document structure consists of eight chapters that are described below.

Chapter 2 provides background information on conventional U.S. wastewater treatment, reclaimed water use and regulations in the U.S., antibiotics and bacteria in

wastewater and reclaimed water, exposure to wastewater and reclaimed water, and agricultural and landscape irrigation and their impact on soil and plants.

Chapter 3 provides information on zero-valent iron technology and describes the findings of laboratory-based studies on antibiotic removal through zero-valent iron technology.

Chapter 4 provides background information, including data analysis techniques, on the use of culture-independent next-generation sequencing technology to perform the total bacterial community analysis of environmental samples.

Chapter 5 is a manuscript entitled “Antibiotic Concentrations Decrease During Wastewater Treatment But Persist At Low Levels in Reclaimed Water” that describes the variability in antibiotic reductions during various conventional wastewater treatment processes and the inefficiency of reuse site practices in achieving further antibiotic reductions.

Chapter 6 is a manuscript entitled “Characterization of the Bacterial Community Structure of Wastewater and Reclaimed Water” that describes the variability of bacterial community structure of differentially treated wastewater and reclaimed water. The findings from this study confirm that conventional wastewater treatment and current reuse treatment practices may not be sufficient at reducing potential pathogens from reclaimed water.

Chapter 7 is a manuscript entitled “Zero-valent Iron-biosand Filtration Is Capable of Reducing Antimicrobial Concentrations In Unbuffered Conventionally-Treated Reclaimed Water” that describes a greenhouse experiment conducted in order

to evaluate the efficacy of a zero-valent iron-biosand filtration in removing antimicrobial residues from conventionally treated wastewater.

Finally, Chapter 8 provides a conclusion and information on the public health significance of my findings as well as directions for future research.

Chapter 2: Background

Municipal Wastewater Treatment in the United States

In the United States (U.S.) wastewater treatment plants (WWTPs) treat municipal wastewater before discharging it to water bodies or distributing it for land application or for use in other reuse applications. Typical wastewater treatment in the U.S. consists of primary (large object removal), secondary (organic matter removal) and tertiary (filtration or disinfection beyond secondary treatment) treatment (EPA, 2004; Maier et al., 2009). Normally, most WWTPs conduct preliminary treatment prior to primary treatment, in order to remove large floating objects through screening or grinding, as well as sand and grit by settling, since these elements might damage operational equipment at the treatment plant (EPA, 2004; MDEQ, 2003). Primary treatment involves the partial removal of suspended solids through the use of sedimentation, chemical coagulation or filtration (Asano, 2007; EPA, 2004).

Fine and dissolved contaminants still remain in the wastewater after primary treatment (EPA, 2004). Up to 90% of organic matter in wastewater can be removed through the use of biological treatment processes which form the basis of secondary treatment, the basic principle being the use of microorganisms (bacteria, algae, fungi) and oxygen to degrade organic matter in wastewater (EPA, 2004). Biological treatment can be achieved by pumping wastewater and air through media containing microorganisms (trickling filters, biotowers, rotating biological contractors) or by suspending microorganisms in an activated water mixture (activated sludge, oxidation ditches, sequencing batch reactors) (EPA, 2004). Secondary clarifiers are used to

separate the activated biomass from the treated effluent which can then undergo further treatment or be discharged (EPA, 2004; MDEQ, 2003). Biodegradable organic matter and organic nitrogen containing matter can also be removed at this stage by converting ammoniacal nitrogen to nitrate and finally to nitrogen gas (EPA, 2004).

Municipal wastewater treatment in the U.S. is regulated under the Clean Water Act (CWA) which controls the release of contaminants into surface waters (EPA, 2004). Under the CWA, municipal wastewater treatment plant (WWTP) discharges must meet a minimum standard of secondary treatment (EPA, 2004). Tertiary or advanced treatment refers to any treatment processes used for contaminant removal beyond secondary treatment. It can include further removal of organic matter using filtration or sequential lagooning, nutrient removal through nitrification-denitrification and precipitation or pathogen removal via chlorination, ultraviolet (UV) treatment or ozonation (EPA, 2004). The primary objective of wastewater treatment in the U.S. is the degradation of organic matter and U.S. WWTPs are not designed to remove other contaminants like pharmaceuticals and personal care products (Arvai et al., 2014; EPA, 2012a; Spongberg & Witter, 2008).

As of 2014, there were 16,255 operational municipal WWTPs in the U.S., treating approximately 32 billion gallons of wastewater per day (EPA, 2014). In 2012, 94.5 million people in the U.S. were served by 7408 secondary, or less than secondary, WWTPs, which represent around 50% of all municipal WWTPs in the U.S. (EPA, 2012b). Approximately 34% of all municipal WWTPs in the U.S. performed treatment considered greater than secondary and around 15% of all municipal WWTPs in the U.S. produce no discharge at all (EPA, 2012b).

Reclaimed Water Use in the United States

The discharge of almost all of the treated wastewater effluent generated by WWTPs into surface water bodies was a fairly common practice in the U.S, but the escalating pressure on existing and readily available freshwater resources, combined with increasingly stringent regulations on effluent disposal, as well as government incentives to promote reuse have made the reuse of treated wastewater effluent an increasingly attractive alternative to surface water disposal (Asano, 2007; EPA, 2012a). Treated wastewater effluent, also known as “reclaimed water” is defined as “municipal wastewater that has been treated to meet specific water quality criteria with the intent of being used for beneficial purposes” (Crook, 2010). Typical uses of reclaimed water in the U.S. are landscape irrigation (golf courses and recreational fields), agricultural irrigation (food crops, non-food crops), livestock watering, impoundments (recreational and landscape), snowmaking, wetland or surface water augmentation, industrial reuse (cooling water, boiler water), toilet flushing, vehicle washing, groundwater recharge of non-potable aquifers and indirect potable reuse (augmentation of drinking water reservoirs followed by treatment at a drinking water treatment plant) (EPA, 2012a). Non-residential landscape irrigation and agricultural irrigation are the most common reuse applications of reclaimed water in the U.S. (Asano, 2007; EPA, 2012a).

The CWA requires WWTP effluent to undergo a minimum of secondary treatment before being discharged into surface waters (EPA, 2004). However, reclaimed water usually requires greater than secondary treatment since it has a

greater potential of direct contact with users compared to discharged effluent, with the extent of treatment dependent on the intended use of the reclaimed water (EPA, 2012a). After final treatment, reclaimed water can be delivered to the end-user directly from the WWTP, or indirectly, through a water reclamation facility, which may perform further treatment (EPA, 2012a; Rosenberg-Goldstein, 2010). Reclaimed water is transported via a reclaimed water distribution system that is separate from the potable water distribution system (EPA, 2012a). By the time the reclaimed water reaches its actual point of use it may have undergone further disinfection and monitoring within the distribution system, as well as post-treatment storage (EPA, 2012a; MRWPCA, 2013). The end-user may directly use the received reclaimed water, conduct further treatment before use, or store the reclaimed water (pre-or post-on-site treatment) until needed (Carey et al., 2016; EPA, 2012a; MRWPCA, 2013; Rosenberg-Goldstein, 2010). This is especially common in the case of agricultural or landscape irrigation where reclaimed water may be supplied, but not required, every day (Carey et al., 2016; EPA, 2012a; MRWPCA, 2013; Rosenberg-Goldstein, 2010).

Regulations Governing Reclaimed Water Use in the United States

The federal government has issued guidelines governing reclaimed water use in the U.S., but they are not legally binding, and therefore, currently there are no federal regulations governing reclaimed water use in the U.S. (EPA, 2012a). Reuse standards, where present, are established and applied by state and local regulatory agencies in the form of regulations or guidelines (EPA, 2012a). As a result, regulations and guidelines governing reclaimed water use display considerable

geographic variability (EPA, 2012a). As of 2012, 30 states had regulations and 15 states had guidelines governing reclaimed water use with no states having regulations that governed all possible uses of reclaimed water (Asano, 2007; EPA, 2012a).

State and local reuse regulations and guidelines vary from a primary focus on reuse to a primary focus on land disposal with incidental beneficial reuse (Asano, 2007; EPA, 2012a). Some states, with no official regulations or guidelines, allow reuse on a case-by-case basis (Asano, 2007; EPA, 2012a). Some states have very stringent regulations with standards based on water quality and minimum treatment requirements, while others prescribe water quality limits without specifying treatment requirements (Asano, 2007). State and local regulations and guidelines vary by the type of microbial quality testing indicator used, acceptable limits for water quality parameters, sampling requirements, and analytical methods (Asano, 2007).

In most regions where reclaimed water use is common, regulations are driven by the protection of public health, and reclaimed water treatment levels increase with increasing possibility of user contact (EPA, 2012a). Reuse applications differ by region, with most areas, except California and Florida, prohibiting the use of reclaimed water for the irrigation of raw-eaten food crops (EPA, 2012a). Parameters for reclaimed water quality assessment can range from basic measures using indicator organisms, biochemical oxygen demand and turbidity, to the inclusion of several additional water quality parameters listed in the Safe Drinking Water Act (SDWA) which includes both microbial as well as chemical contaminants (EPA, 2012a).

Several states require the monitoring of a chlorine residual, including parameters on concentration and contact time, within the reclaimed water distribution

system (Asano, 2007; EPA, 2012a). If UV radiation is used for wastewater disinfection then regulations vary from the absence of dosage or design or operation condition specifications to the requirement of compliance with the guidelines listed in the “UV Disinfection Guidelines for Drinking Water and Water Reuse” (Asano, 2007). Monitoring of reclaimed water quality, including that of the reclaimed water within the distribution systems, also varies by region in terms of frequency and manner, and states with extensive and historical reuse practices tend to have better developed and more comprehensive regulatory and monitoring practices (EPA, 2012a). Reclaimed water storage requirements specified by several state regulations usually do not differentiate between operational and seasonal storage, are focused mainly on the prevention of surface water discharge, and also differ from state to state (Asano, 2007). Guidelines and regulations governing reclaimed water quality at its actual point of use also vary from state to state (Asano, 2007; EPA, 2012a) .

Antibiotics in wastewater and reclaimed water

Antibiotics are widely used in human and veterinary medicine as well as for growth promotion in food-production animals (Levy, 1998). Most antibiotics are poorly absorbed by both humans and animals and are excreted, mostly unaltered, through feces and urine, and usually enter WWTPs through municipal influent and agriculturally influenced stormwater (Chee-Sanford et al.; Rosenberg Goldstein et al., 2012). Removal efficiencies of antibiotics from WWTPs are variable, and depend on initial influent concentrations, treatment processes and treatment plant operational parameters (Batt, Kim, & Aga, 2007). Furthermore, conventional municipal WWTPs

in the U.S. are not designed to remove pharmaceuticals from wastewater (EPA, 2010). Influent and effluent samples collected from WWTPs in the United States, have been found to contain antibiotics belonging to several classes (β -lactams, sulfonamides, quinolones, tetracyclines and macrolides) in the μg to ng/L range (Zhang & Li, 2011).

Trimethoprim and sulfamethoxazole were found to occur in WWTP effluents at concentrations greater than those found in the influent stream (Bendz, Paxeus, Ginn, & Loge, 2005). Antibiotic removal mechanisms in conventional WWTPs, which mostly depend on biological processes for organic matter degradation, include hydrolysis, adsorption and biodegradation (Zhang & Li, 2011). However, antibiotic removal through conventional wastewater treatment was variable (Batt et al., 2007; EPA, 2010). A study comparing antibiotic removal among eight WWTPs in China determined that the removal efficiencies of fluoroquinolones, sulfonamides and macrolides ranged from 39% to 72% (L. Gao et al., 2012). Activated sludge was not found to be effective in the removal of trimethoprim (Paxeus, 2004). An extended sludge treatment process was able to reduce the concentrations of sulfamethoxazole, sulfadimethoxine, sulfamethazine and trimethoprim by 64 to 93% as long as their corresponding concentrations in the influent stream were between 1 and $5\mu\text{g/L}$ (Yu, Lin, Lateef, Lin, & Yang, 2009). The use of disinfection, through chlorination and ultraviolet radiation, has resulted in some antibiotic removal (Kim & Aga, 2007). Advanced treatment processes such as ozonation and membrane filtration were more efficient at further elimination of antibiotics (Zhang & Li, 2011). However, disinfection is not always used by all WWTPs, some of which only disinfect

seasonally (Kim & Aga, 2007). Advanced treatment processes are even less frequently used during conventional wastewater treatment. Therefore, antibiotics continue to persist in treated wastewater effluent.

Several studies have found antibiotics, in the $\mu\text{g/L}$ to ng/L range, (ciprofloxacin, sulfamethoxazole, and clindamycin: 0.043 to 0.076 $\mu\text{g/L}$, sulfamethoxazole: 300 ng/L , and erythromycin, sulfamethoxazole, ofloxacin, ciprofloxacin, norfloxacin and vancomycin: 4.2 to 1435 ng/L) in surface water into which treated effluent was discharged (Batt, Bruce, & Aga, 2006; Brown, Kulis, Thomson, Chapman, & Mawhinney, 2006; Tuc Dinh et al., 2011). This finding is significant since treated effluent that might usually be discharged, may also be transported from WWTPs to reclaimed water use sites for applications such as spray irrigation. Monitoring of antibiotic concentrations is currently not part of U.S. state regulations, or guidelines governing reclaimed water meant for reuse (EPA, 2012a). Transport to, and storage at, the reuse sites may also impact the final concentrations of antibiotics in reclaimed water that actually comes in contact with soil, plants and people at the reuse sites. Treated effluent from an urban WWTP supplying treated effluent to a reuse site for landscape irrigation was found to contain, over a period of five months, trimethoprim (1.96 ng/L to 42 ng/L), sulfamethoxazole (2.61 to 59.2 ng/L) and erythromycin (154 to 611 ng/L) (Kinney, Furlong, Werner, & Cahill, 2006).

Bacteria in Wastewater and Reclaimed Water

Pathogenic, as well as non-pathogenic species of bacteria, present on the skin, in the gastrointestinal tract, urogenital tract and respiratory tract enter WWTPs, as part of raw influent, through the sewage system (Cai, Ju, & Zhang, 2014b). Since wastewater treatment usually consists of biological treatment to break down organic matter, and remove nutrients, non-pathogenic bacteria (saprophytic, nitrifying, denitrifying, floc-forming etc.) are also added to wastewater specifically for the purpose of wastewater treatment (Gerardi, 2006). However, since the primary purpose of wastewater treatment in the U.S. is the breakdown of organic matter, and not the removal of pathogens, (Maier et al., 2009) WWTPs contain both non-pathogenic as well as pathogenic bacteria, including opportunistic pathogens. Furthermore, antibiotics present in WWTPs occur at concentrations high enough to exert selective pressures for allowing for the transfer and development of antibiotic resistance (Kummerer, 2001). Naturally stress-tolerant strains of *Escherichia coli* (*E. coli*), methicillin resistant *Staphylococcus aureus* (MRSA), and vancomycin-resistant enterococci (VRE) have been isolated from WWTPs (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014; Zhi et al., 2016). Bacterial pathogens including *Salmonella* spp., *Legionella* spp., *Clostridium* spores, MRSA, *E. coli*, enterococci and VRE have been isolated from treated wastewater effluent (Brissaud, Blin, Hemous, & Garrelly, 2008; Garcia et al., 2007; Koivunen, Siitonen, & Heinonen-Tanski, 2003; Levantesi et al., 2010; Rosenberg Goldstein et al., 2012).

Aeromonas spp., *Legionella* spp., *Mycobacterium* spp., found to be non-detectable or in low quantities in treated wastewater effluent, have been known to regrow in chlorinated reclaimed water distribution systems (Jjemba et al., 2010). Biofilm formation as well as bacterial encapsulation have been shown to aid *Klebsiella pneumoniae* in resisting chlorination (LeChevallier, Cawthon, & Lee, 1988). VRE has been isolated at U.S. landscape spray irrigation sites using reclaimed water (Carey et al., 2016). Wastewater treatment efficiency, treated wastewater quality and reclaimed water quality are all currently monitored through the measurement of indicator bacteria using culture based methods (Asano, 2007; EPA, 2012a).

Exposure to Wastewater and Reclaimed Water

In the U.S., and other developed countries, exposure to municipal wastewater is most likely to occur within WWTPs, with both acute as well as chronic exposure being possible. Exposure could occur through inhalation of aerosols, through dermal contact and through ingestion (Hansen, Hilden, Klausen, & Rosdahl, 2003). Occupational exposure studies have found that workers in WWTPs may be exposed to bacterial pathogens including *Klebsiella* spp., *E. coli*, *Clostridium perfringens*, fecal streptococci, *Leptospira* spp. as well pharmaceuticals present in wastewater (Hansen et al., 2003). Both MRSA and VRE have been found within U.S. WWTPs (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014).

Exposure to pathogens and pharmaceuticals in reclaimed water may occur through aerosolization during spray irrigation, or direct contact with, or ingestion of, reclaimed water or soil, turf or crops irrigated with reclaimed water (Asano, 2007). Elevated air densities of fecal coliforms, fecal streptococci and mycobacteria were detected, above background levels, at least 200 m downwind from areas that had undergone spray irrigation with secondary treated wastewater (Camann, 1988). A reclaimed water spray irrigation site was found to have bioaerosols containing total coliforms (18-1,076 CFU/m³) (Teltsch, Kedmi, Bonnet, Borenzstajn-Rotem, & Katzenelson, 1980). Another study of spray irrigation with reclaimed water detected aerosolized coliforms at a concentration greater than 10³ coliforms per milliliter of reclaimed water (Teltsch & Katzenelson, 1978).

Exposure to reclaimed water during irrigation depends on several factors, including the quality of the reclaimed water, type of irrigation (drip versus spray), setback distances and timing of irrigation (Asano, 2007). Thus far, there have been no documented U.S. reports of adverse health events due to exposure to pathogens through the ingestion of reclaimed water irrigated food crops, or exposure to reclaimed water through spray irrigation, but there might be sporadic cases which may be difficult to link conclusively to reclaimed water exposure (Crook, 2005). Currently limited information is available on the health effects of exposure to pharmaceuticals present in reclaimed water (Kim & Aga, 2007). Exposure to the large number of pharmaceuticals present in low concentrations in reclaimed water is of concern with respect to chronic exposure to reclaimed water or soil or plants irrigated with reclaimed water (Kim & Aga, 2007).

Epidemiological studies specifically examining health risks associated with exposure to reclaimed water are scarce, and have produced conflicting results (Crook, 2005; Shuval, 1991). Contact with partially treated wastewater (retention in either one or two reservoirs) resulted in an increased rate of diarrheal disease in children, compared to controls, but not in adults, as seen by a study in Mexico (Peasey, Blumenthal, Mara, & Ruiz-Palacios, 2000). An increased risk of diarrheal disease was also observed, compared to controls, among individuals who consumed onions and green tomatoes irrigated with partially treated wastewater (Peasey et al., 2000). However, an examination of gastrointestinal effects associated with exposure to reclaimed water (having undergone sand-anthracite filtration and chlorination up to 4-6 mg/L) used for landscape irrigation, resulted in the observation that contact with wet grass combined with elevated concentrations of indicator bacteria, and not exposure to the reclaimed water itself, was associated with an increased incidence of gastrointestinal illness (Durand & Schwebach, 1989).

Agricultural and Landscape Irrigation with Reclaimed Water in the United States

California uses 37% of the reclaimed water generated within the state for the irrigation of food crops, including raw-eaten food crops (CA EPA, 2011). Other states that are major users of reclaimed water for food crop irrigation are Florida, Arizona, Hawaii, Nevada, Texas and Washington (EPA, 2012a). As of 2012, 27 U.S. states had either guidelines or regulations governing the planned or incidental use of reclaimed water for the irrigation of food crops (EPA, 2012a). Reclaimed water used for agricultural irrigation of food crops in California is required to have undergone

oxidation, coagulation, filtration and disinfection, making it closer in quality to potable water (CA DPH, 2009). Other states that are leading users of reclaimed water either follow California regulations or have their own public health based regulations (Asano 2007). Some localities within California have regulations that also include the monitoring of constituents included in the Safe Drinking Water Act (SDWA) (EPA, 2012a).

Landscape irrigation in restricted and unrestricted areas is also a common application of reclaimed water in the U.S., with golf course irrigation being a major use of reclaimed water (EPA, 2012a). Florida and California are leading users of reclaimed water for landscape irrigation with Arizona, Colorado, Hawaii, Nevada, New Mexico, Texas and Utah following close behind (EPA, 2012a). Florida and California also allow landscape irrigation of residential areas with reclaimed water (EPA, 2012a). As of 2012, 36 U.S. states had either guidelines or regulations governing the planned or incidental use of reclaimed water for landscape irrigation (EPA, 2012a). Reclaimed water used for landscape irrigation in California is required to have undergone oxidation, coagulation, filtration and disinfection (Asano, 2007). Treatment requirements for reclaimed water used for landscape irrigation are lower than those required for agricultural irrigation but other protective measures such as setback distances and timing restrictions are usually required (EPA, 2012a).

Impact of Irrigation with Reclaimed Water

Soil irrigated, for a period of five months, with reclaimed water, known to contain the antibiotics trimethoprim, sulfamethoxazole and erythromycin, was found

to retain erythromycin in amounts much greater than the concentrations found in the reclaimed water used for irrigation (Kinney et al., 2006). Soil irrigated with reclaimed water containing trimethoprim and sulfamethoxazole showed exceedingly large sorption of trimethoprim and limited sorption of sulfamethoxazole indicating that trimethoprim may remain in the top layer of soil and affect the soil microbiome or may be absorbed by plants growing in the soil while sulfamethoxazole may have a potential to contaminate groundwater (Chefetz, Mualem, & Ben-Ari, 2008; Lin & Gan, 2011; Thiele-Bruhn, 2003). Soil from parks irrigated with reclaimed water for one month was found to contain up to 145.2 mg/kg of tetracyclines and 79.2 mg/kg of quinolones (F.-H. Wang et al., 2014). Leachate collected after irrigation of mature turfgrass, for six months, with tertiary treated effluent containing trimethoprim and sulfamethoxazole was found to contain trimethoprim (mean concentration - 10.2 ng/L) and sulfamethoxazole (mean concentration - 12.4 ng/L) (Bondarenko et al., 2012). Groundwater samples from land irrigated with treated wastewater effluent containing between 90 and 150 ng/L sulfamethoxazole were found to contain 20 ng/L sulfamethoxazole (Avisar, Lester, & Ronen, 2009). 53 ng/L and 298 ng/L of triclosan was detected in soil from a golf course irrigated with reclaimed water at 75cm and 105 cm depths respectively (Snyder et al., 2004).

Several studies have been conducted to determine the uptake of pharmaceuticals, including antibiotics, by plants, but most of them have been under hydroponic conditions or in field conditions using treated effluent that has been spiked with additional antibiotics. Hydroponic studies have detected sulfamethoxazole, sulfamonomethoxine, sulfadimethoxine, trimethoprim, triclosan,

triclocarban enrofloxacin, chlortetracycline, monensin and amoxicillin, added to nutrient solutions, in leaves, stems and roots of cabbage, pea, lettuce, spinach, cucumber, pepper and red cabbage subsequently irrigated with the amended nutrient solutions. (Chowdhury, Langenkamper, & Grote, 2016; Herklotz, Gurung, Heuvel, & Kinney, 2010; Tanoue et al., 2012; X. Wu, Ernst, Conkle, & Gan, 2013). The concentrations of antibiotics analyzed in these hydroponic studies were often present at much higher concentrations in the nutrient solutions used as growth mediums than would normally be present in reclaimed water.

Field or greenhouse studies using treated wastewater effluent that has been spiked with pharmaceuticals, including antibiotics, or freshwater containing pharmaceuticals, including antibiotics, at concentrations similar to those found in treated wastewater effluent, have demonstrated the uptake of antibiotics such as sulfamethoxazole in sweet potatoes and carrots; roxithromycin and clindamycin in carrot roots and Bermuda grass roots; sulfamethoxazole in tomato leaves; sulfapyridine in cucumber leaves; lincomycin and ofloxacin in arugula leaves; lincomycin in corn grains; and triclocarban and triclosan in soybean roots and beans (Goldstein, Shenker, & Chefetz, 2014; Tammy L Jones-Lepp, Sanchez, Moy, & Kazemi, 2010; Malchi, Maor, Tadmor, Shenker, & Chefetz, 2014; Marsoni et al., 2014; C. Wu, Spongberg, Witter, Fang, & Czajkowski, 2010). Limited studies of irrigation with unfortified reclaimed water known to contain antibacterial agents, namely azithromycin, sulfamethoxazole, roxithromycin, trimethoprim, triclosan and triclocarban, above the limit of detection, have mostly found no antimicrobial uptake

by plants except in the case of triclosan in carrots (Tammy L Jones-Lepp et al., 2010; X. Wu, Conkle, Ernst, & Gan, 2014).

Effluent from waste stabilization ponds used for the irrigation of 29 food crops in Peru resulted in the detection of *Salmonella*, enterotoxigenic *E.coli* and enteropathogenic *E.coli* with the most contaminated crop being lettuce followed by parsley, spinach and carrot. Waiting for eight days to harvest after irrigation resulted in the reduction of *E. coli* and elimination of *Salmonella* on the crop samples analyzed (Peasey et al., 2000). A Portuguese study examining the effect of spray irrigation of lettuce with trickling filter effluent found that the indicator bacteria detected in lettuce were similar to those detected in the water immediately after irrigation. Five days after irrigation, zero *Salmonella* organisms were detected on the lettuce and after seven days the fecal coliform levels observed were similar to those seen in lettuce irrigated with fresh water (Vaz da Costa Vargas, Bastos, & Mara, 1996). Drip and furrow irrigation of lettuce and radish crops with effluent derived from treatment by an aerated waste stabilization pond followed by a facultative pond in Portugal resulted in the detection of 10^3 to 10^4 *E. coli* organisms per 100 mg of radish and lettuce respectively with no detection of *Salmonella* under dry conditions, but under rainy conditions both *E. coli* and *Salmonella* counts increased, possible due to transfer from soil (Bastos & Mara, 1995). Greenhouse based experiments conducted in the United Kingdom on the furrow irrigation of lettuces with trickling filter effluent resulted in the lettuce being *E. coli* free three days after irrigation (Bastos & Mara, 1995). Studies from Israel have shown that fecal coliform transfer from reclaimed water to vegetable and salad crops was highly dependent on the

concentrations of coliforms present in the reclaimed water used for irrigation (Armon, Dosoretz, Azov, & Shelef, 1994). Lettuce harvested after 60 days of irrigation with trickling filter effluent in Spain, showed zero presence of *Salmonella* spp. but significantly higher levels of total and fecal coliforms, fecal streptococci and *Clostridium* sr compared to controls (Mañas, Castro, & de las Heras, 2009).

The most extensive research into the long-term use of reclaimed water for agricultural irrigation in the U.S. has been conducted in California. Reclaimed water irrigation of food crops was first implemented in California in the 1980s and continues today with reclaimed water being used to irrigate crops often eaten raw, such as lettuce and strawberries (MRWPCA, 2013). Title 22 quality reclaimed water was determined to be safe for use in agricultural irrigation following a 10-year study, the Monterey Wastewater Reclamation Study for Agriculture (MWRSA), which examined the presence of bacteria (coliforms, *Salmonella* and *Shigella*), viruses (naturally occurring animal viruses), parasites (*Ascaris lumbricoides*, *Entamoeba histolytica*) and heavy metals in reclaimed water and reclaimed water irrigated soil and crops (Sheikh et al., 1990).

Chapter 3: The application of zero-valent iron technology for the reduction of antibiotic residuals

Zero-valent Iron Technology

Zero-valent iron (ZVI) has been used for groundwater remediation as part of subsurface permeable reactive barriers (PRB) for more than twenty years (Chiu, 2013; Ingram et al., 2012; You, Han, Chiu, & Jin, 2005). Remediation through the use of ZVI is achieved by reduction followed by precipitation or co-precipitation, or immobilization through adsorption resulting in non-toxic filtration products (EPA, 2015). ZVI can also be used to remove organic and inorganic compounds through oxidation through hydroxyl radicals, ferryl ions, and superoxide radicals (Stieber, Putschew, & Jekel, 2011). ZVI technology was initially developed in order to remove chlorinated organic compounds in groundwater but, since then, the use of ZVI has expanded to the elimination of several other organic and inorganic contaminants including heavy metals, energetic compounds, Freons, pesticides and nutrients (EPA, 2015; Gillham, Vogan, Gui, Duchene, & Son, 2010; You et al., 2005). In recent years, research on ZVI treatment technology has also expanded to include the removal of contaminants of relevance to drinking water quality, such as chlorine and natural organic matter along with associated disinfection by-products as well as bacteria and viruses (Chiu, 2013).

During remediation applications, ZVI is sometimes stabilized by mixing with porous inert materials like sand in order to avoid any cementation that may occur due to the formation of corrosion products if ZVI is used alone (Gottinger, McMartin, Wild, & Moldovan, 2013). This ZVI-sand mixture also allows for contamination

removal by screening which is improved with time as the ZVI corrosion process causes the corrosion products to fill pore spaces between the sand and iron mixture (Noubactep & Caré, 2010). However, a ZVI-sand ratio balance must be attained since there is a limit to the screening potential achieved, after which the corrosion product expansion into pore spaces can result in loss of filter permeability (Gottinger et al., 2013).

Laboratory Studies of Antibiotic Removal by Zero-valent Iron Technology

ZVI technology has been analyzed, in laboratory studies, at granular, micro- and nano-scale, for the removal of antibiotics from aqueous solutions. Ciprofloxacin (fluoroquinolone class) degradation of 80 to 92% was achieved with 120 minutes of contact with granular ZVI under oxic conditions with degradation found to be due to a combination of hydroxylation of quinolone and benzene rings and partial defluorination of ciprofloxacin (Perini, Silva, & Nogueira, 2014). Stieber et al. (2011) achieved 99% ciprofloxacin elimination through reductive as well as oxidative processes, following eight hours of granular ZVI contact in the presence of oxygen with elimination dependent on time, amount of iron used and pH (Stieber et al., 2011). 99% of tetracycline (tetracycline family) removal was obtained through the use of nanoscale ZVI modified with starch, with 69% of the elimination being attributed to flocculation and the rest to adsorption and degradation (Fu et al., 2015). This study was conducted in order to analyze the long term effect of nanoscale ZVI contact with tetracycline and elimination was found to occur in two stages, the first being rapid adsorption and degradation, which occurred in one to four hours and the

second being slow flocculation which took four to 30 days (Fu et al., 2015).

Tetracycline and oxytetracycline (tetracycline class) removal by microscale ZVI indicated pH to be the most important factor for removal efficacy (optimal pH=3), which was enhanced by increasing temperature and iron dose (Hanay, Yıldız, Aslan, & Hasar, 2014) . At pH=3 tetracycline as well as oxytetracycline removal was approximately 100% (Hanay et al., 2014). 4-epi-tetracycline, the main transformation product of tetracycline was adsorbed onto micro-scale ZVI within 15 minutes (Hanay et al., 2014). Oxytetracycline transformation product concentrations were found to be much lower than 4-epi-tetracycline (Hanay et al., 2014). Tetracycline and oxytetracycline, as well as their respective transformation products, were all found to be adsorbed by micro-scale ZVI within 15 to 240 minutes (Fu et al., 2015; Hanay et al., 2014). Tetracycline and oxytetracycline removal mechanisms were attributed more to adsorption compared to degradation in this study (Hanay et al., 2014).

Amoxicillin and ampicillin (β -Lactam class) removal by contact with micro- and nano-scale ZVI was achieved by reduction via the rupture of the β -Lactam ring, by adsorption onto iron corrosion products and by sequestration within the matrix of iron hydroxides co-precipitating iron hydroxides (Ghauch, Tuqan, & Assi, 2009). Initial concentrations of 20mg/L of ampicillin and amoxicillin had half-lives, after ZVI contact, of approximately 60.3 ± 3.1 and 43.5 ± 2.1 minutes respectively under oxic conditions, and 11.5 ± 0.6 and 11.2 ± 0.6 minutes respectively, under anoxic conditions (Ghauch et al., 2009). 100% metronidazole (nitrometronidazole class) elimination was achieved within 5 minutes of contact with nanoscale ZVI, and removal depended on initial ZVI pH, dose and metronidazole concentration (Fang et

al., 2011). The elimination mechanism was attributed to a combination of degradation and adsorption (Fang et al., 2011).

Chapter 4: Characterization of the total bacterial community structure of environmental samples

Introduction

Bacterial identification has progressed from the sole use of morphologic and phenotypic descriptions of known bacterial strains to the use of the bacterial deoxyribonucleic acid (DNA), specifically, the 16S ribosomal ribonucleic acid (rRNA) gene, which has become the most commonly used molecular marker in microbial community analysis due to its essential function, ubiquity and evolutionary properties (Böttger, 1989; Case et al., 2007; Garrity & Holt, 2001; Harmsen, 2004; Kolbert, 1999; Palys, 1997; Tortoli, 2003; C. R. Woese, 1987). Advances in sequencing technology have now made it possible to study complex mixtures of organisms that commonly occur in environmental samples (Shendure, Mitra, Varma, & Church, 2004). This approach provides several advantages compared to traditional culture based methods. First, sequencing technologies enable the ability to access and analyze organisms that may be viable but non-culturable and may not be able to survive outside their environmental niches (Tringe & Rubin, 2005). Second, since genomic DNA is extracted directly from the bacterial constituents of an environmental sample, information about community dynamics and the influence of the host environment can be determined (Tringe & Rubin, 2005).

Structure and Function of the 16S rRNA gene

Bacterial ribosomes are cytoplasmic nucleoprotein particles, composed of proteins and rRNA molecules and are responsible for messenger RNA (mRNA) translation and protein synthesis (Han, 2006; Hong, 2006; H. F. Noller et al., 1987). These proteins and rRNA molecules are arranged into two distinct sections of the ribosome known as the large subunit (LSU) and small subunit (SSU). rRNAs participate directly in the protein translation process and ribosomes have been hypothesized to have evolved from functional rRNA molecules (Crick, 1968; H. F. Noller et al., 1987; H. Noller & Woese, 1981; C. Woese, 1980). Ribosomes within prokaryotic cells consist of a small (30S) subunit composed of 16S rRNA and 21 proteins, and a large (50S) subunit composed of the 5S rRNA, 23S rRNA and 31 proteins (H. F. Noller et al., 1987). The 16S rRNA gene is approximately 1500 base pairs (bp) long, highly conserved at both ends, and contains nine hypervariable regions resembling hairpins (Mongodin, 2015; Stiegler, Carbon, Zuker, Ebel, & Ehresmann, 1981). 16S rRNA plays an important role in transfer RNA (tRNA)-ribosomal binding and tRNA translocation because of the bases and tRNA binding sites contained within it (Carter et al., 2000; H. F. Noller et al., 1987; Shi, Chiu, Ghosh, & Joseph, 2009). The 3'-terminus of the 16S rRNA is involved in the initiation of protein synthesis and, along with ribosomal proteins, 16S rRNA plays a structural role within the 30S subunit (Burma, Nag, & Tewari, 1983; H. F. Noller et al., 1987; Shine & Dalgarno, 1974; Wimberly et al., 2000). Antibiotics interact with specific features within 16S rRNA leading to interference during protein synthesis

and mutations in 16S rRNA can affect translational accuracy (H. F. Noller et al., 1987; Vallabhaneni, 2009).

Genes encoding 5S, 16S, 23S rRNAs are typically structured into operons within bacterial genomes with one to 15 operon copy numbers per bacterial genome (Klappenbach, Dunbar, & Schmidt, 2000; Rainey, Ward-Rainey, Janssen, Hippe, & Stackebrandt, 1996). Multiple copies of rRNA operons multiply translation in order to achieve high growth rates in response to environmental change and are indicative of an evolutionary development within bacteria for the acquisition of a competitive advantage (Case et al., 2007; Klappenbach et al., 2000). Functional differentiation between rRNA operons leads to their differential expression in response to environmental change (Case et al., 2007). Studies of *E. coli* have showed that one rRNA operon copy is insufficient, with eight operon copies being maintained in order to synthesize the number of ribosomes required to achieve maximum growth rates (Bremer, 1975; Condon, Liveris, Squires, Schwartz, & Squires, 1995). Furthermore, in *E. coli*, the higher the number of inactivated rRNA operons, the longer the time required for growth increase in response to added resources (Condon et al., 1995). The number of rRNA operons present in a bacterial genome may regulate the speed at which organisms synthesize ribosomes and respond to favorable growth conditions due to the high demand for rRNA transcription and the integral role of rRNAs in the regulation of ribosome generation (Condon et al., 1995; Stevenson & Schmidt, 1998). However, the capacity to respond rapidly to fluctuating conditions comes at a metabolic expense due to the overproduction of ribosomes (Klappenbach et al., 2000).

Bacterial Identification using the 16S rRNA gene

rRNAs are present in all known living cells and the 16S rRNA gene is universal in bacteria (C. R. Woese, 1987; C. R. Woese, Stackebrandt, Macke, & Fox, 1985). The 16S rRNA gene sequence seemingly behaves like a “molecular chronometer”, its degree of conservation attributed to its importance as an ancient and critical component of cell function and rRNA-protein interaction (Clarridge, 2004; Doolittle, 1999; C. R. Woese, 1987). The 16S rRNA gene sequence consists of both conserved and variable regions whose sequences have diverged over evolutionary time (Clarridge, 2004; Han, 2006; Stiegler et al., 1981). Each bacterial species generally has a unique 16S rRNA sequence (with occasional exceptions) which is conserved enough within, and sufficiently variable between, most bacterial species (Clarridge, 2004; Fox et al., 1980; Hong, 2006). This allows for the use of the 16S rRNA gene as a target for species identification (Clarridge, 2004; Hong, 2006). Universal primers complementary to the conserved regions are usually used to determine the phylogenetic relationship between distant organisms while primers complementary to the variable regions are used for family- and genus- level differentiation between closely related organisms (Case et al., 2007; Greisen, Loeffelholz, Purohit, & Leong, 1994; Han, Pham, Tarrand, Sood, & Luthra, 2002; Hong, 2006). The 16S rRNA sequences of several bacteria have been determined and are available for comparison in accessible databases such as SILVA (Pruesse et al., 2007), Greengenes (DeSantis et al., 2006), Ribosomal Database Project (RDP) (Maidak, 1996), GenBank (Benson, Karsch-Mizrachi, Lipman, Ostell, & Wheeler,

2007) etc. Comparison with the 16S rRNA gene sequence allows for the differentiation between organisms at the genus and species level across all major bacterial phyla, with sequence lengths of at least 200 bp commonly used to obtain meaningful results, and the entire 1500 bp (approximate) length used in order to describe new species (Clarridge, 2004; Han, 2006; Sacchi, 2002a, 2002b).

Bacterial Community Analysis using 16S rRNA gene sequencing

Bacterial genomic DNA is extracted from whole cells, either directly from environmental or clinical samples, or from a pure culture followed by polymerase chain reaction (PCR) amplification of the 16S rRNA gene sequence using universal primers. After purification the PCR products undergo cycle sequencing to obtain the 16S rRNA nucleotide sequences within the samples. This dataset of DNA sequences is used to describe the qualitative and quantitative distribution of organisms within and between samples, and to determine the correlation between taxonomic changes and environmental, chemical or biological parameters associated with the samples (Thomas, Gilbert, & Meyer, 2012). The general outline of sequence data processing involves assembly, clustering and annotation followed by statistical analysis. The dataset of “raw” sequences undergoes quality assessment including primer and barcode trimming followed by either reference-based or *de novo* sequence assembly or a hybrid of the two approaches (Mongodin, 2015; Thomas et al., 2012). The assembled sequences are then assigned to their samples of origin (if originally multiplexed) and either each DNA sequence is compared to a reference database to obtain a classification, or the DNA sequences are clustered into groups of taxon

independent operational taxonomic units (OTUs) that represent individual genomes, or genomes from closely related organisms, through the use of a sequence similarity threshold (Huse et al., 2008; Huse, Welch, Morrison, & Sogin, 2010; Zongzhi Liu, DeSantis, Andersen, & Knight, 2008; Mongodin, 2015; Navas-Molina, 2013; Schloss et al., 2009; Schloss & Handelsman, 2005; Thomas et al., 2012; Q. Wang, Garrity, Tiedje, & Cole, 2007).

Typically, OTUs are considered analogous to the traditional taxonomic grouping of organisms into candidate taxa based on phenotypic similarity and are formed based on sequence identity using a user-defined identity threshold (Navas-Molina, 2013). Usually 97% sequence similarity is used since it is conventionally assumed to represent bacterial species (Drancourt et al., 2000). Clustering can be performed using a *de novo* approach (grouping based on sequence identity), a closed reference approach or an open-reference approach (Navas-Molina, 2013). The last two approaches are based on matching sequences to a reference sequence database with sequences failing to match the database discarded when using the closed reference approach and forming new clusters, as well as being added as new references to the reference database, when using the open-reference approach (Navas-Molina, 2013). Finally, chimeras are detected and removed and the clustered sequences are annotated i.e. provided with taxonomic assignments (Mongodin, 2015; Navas-Molina, 2013; Thomas et al., 2012). Large 16S rRNA gene databases and alignments provide the reference framework for comparing the fragmented sequences which represent the many microbial taxa present in the sampled community (Eren et al., 2013). These annotated sequences, along with a mapping file of sample details

(metadata including environmental, chemical, biological parameters) can be used to conduct diversity and statistical analyses allowing for the investigation of relationships between microbial community structures and their host or ecosystem.

Experimental Design and 16S rRNA gene Sequencing Data Analysis

Experimental design

Biological and technical variation should be accounted for when planning a microbial community analysis using 16S rRNA gene sequencing (Thomas et al., 2012). Furthermore, the effect of temporality should also be considered during analysis if multiple samples are collected over a period of time. This is because microbial systems are highly dynamic and the timing of sample collection can have a major impact on microbial community within the samples being analyzed (Thomas et al., 2012). Pilot tests should be performed in order to determine sample size, replicate number and sequencing depth (Prosser, 2010). Strategies for the sampling and analysis of replicates should be carefully considered prior to collection since splitting up samples may only provide technical, but not biological, replicates and pooling multiple samples may lead to the loss of information on variability (Thomas et al., 2012). In order to be able to obtain information on the relationship between sample parameters and sample microbial community, precise and detailed metadata should be recorded and used during data analysis (Thomas et al., 2012).

Sequence filtering

Statistical analysis of the annotated sequences is based on sample parameters, experimental design and hypotheses using quantitative ecology techniques and conventional statistical tools to describe correlations and statistically significant patterns (Thomas et al., 2012). 16S rRNA sequence datasets are usually assembled in a matrix (OTU table) of rows of sample names and columns of OTUs and their corresponding taxonomic identifiers (Kuczynski et al., 2011). The number of sequences assigned to each biological sample for each OTU is listed in the matrix and can be used to calculate the relative abundance of each OTU. Several steps need to be performed in order to prepare the dataset for downstream analyses. To reduce spurious OTUs, abundance-based quality filtering should be performed (ex. removal of OTUs with number of sequences less than 0.005% of the total number of sequences) (Navas-Molina, 2013). Community composition data may contain zero abundance values which may require the dataset to be transformed in order to perform distance based ordination analyses (Legendre & Gallagher, 2001; Ramette, 2007).

Data transformation

Transformations commonly used include Hellinger distance and chord distance, and are important since they reduce the weight given to rare species in the dataset which contribute more distance measures than common species (Legendre & Gallagher, 2001; Ramette, 2007). Specific unrelated OTUs (ex. chloroplast sequences or host DNA sequences) should be removed from the table (Navas-Molina, 2013).

Samples with low OTU counts may indicate low quality reads and may have to be removed in order to obtain good quality data for downstream analysis (Navas-Molina, 2013). Since samples with similar number of sequences tend to appear to be similar to one another during diversity analyses an optimal sampling depth value (random subset of sequences selected for per sample) should be determined and all samples that are below the optimal subsampling depth should be removed (Navas-Molina, 2013). The optimal sampling depth is usually dependent on the data obtained and should fit all OTU clustering approaches used (if multiple clustering approaches are used) (Navas-Molina, 2013). Usually a depth of over 1000 sequences per sample is recommended (Navas-Molina, 2013). However, since different types of samples will have different levels of community diversity 1000 sequences per sample may be sufficient for some sample types while it may be more than necessary for others. Since the number of species often exceeds the number of samples, appropriate corrections (ex. Bonferroni correction for t-test based analyses) need to be performed (Thomas et al., 2012). The dataset can then be used to perform exploratory analyses (ex. Principal Component Analysis (PCA) or Principal Coordinate Analysis (PCoA)) based on OTU abundance and environmental interpretation analyses (ex. Redundancy analysis (RDA)) which account for sample parameters as well (Ramette, 2007). A combination of the two is ideal since exploratory analyses are limited to the detection of patterns such as similarity and dissimilarity between groups and the depth provided by environmental interpretation analyses is needed in order to determine the factors driving those patterns (Ramette, 2007).

Taxonomic summaries and heatmaps

The most basic manner in which to identify patterns and differences between samples involves the visualization of the relative abundance of various taxa present in the sample at multiple taxonomic levels (Navas-Molina, 2013). Any observed differences can be then analyzed for statistical significance (Navas-Molina, 2013). OTU heatmaps can also be used to visualize relationships between OTUs and samples by using gradations in color intensity corresponding to relative abundances of OTUs in each sample (Navas-Molina, 2013).

Diversity analysis

Species diversity describes species richness (number of species) and species evenness (equality of species abundance) (Hill, 1973; Tuomisto, 2010a, 2010b). The two most commonly used categories of diversity measures are α - and β -diversity (R. Whittaker, 1972; R. H. Whittaker, 1960), corresponding to diversity within a sample, and differences in diversity between samples, respectively (Ramette, 2007). α -diversity is commonly measured using Shannon's index and Simpson's index with a high α -diversity indicating a higher number of species with similar abundances (Magurran, 2011; Shannon & Weaver, 1948; Simpson, 1949). β -diversity is commonly measured using the Bray-Curtis dissimilarity with a high β -diversity indicating greater dissimilarity between two groups (Bray & Curtis, 1957; Magurran, 2011).

Multivariate exploratory analyses

PCA and PCoA are two popular multivariate exploratory analysis methods. Multidimensional distance or dissimilarity matrices of β -diversities are transformed into a new set of orthogonal axes using methods such as PCA or PCoA in order to account for as much variation of the original data as possible (Gower, 1966; Hotelling, 1933; Mardia, Kent, & Bibby, 1979; Navas-Molina, 2013). Taxonomic information superimposed onto PCoA plots can identify the taxa that are driving the differences observed between the microbial communities (Navas-Molina, 2013).

Multivariate environmental interpretation analyses

In order to explain which sample parameters most significantly explain the variation in microbial community composition regression methods such as RDA are used, with species data being considered the “dependent variable” and sample parameters the “independent variables” (Ramette, 2007; Rao, 1964). RDA explains patterns of species variation corresponding to measured sample parameters and provides correlation coefficients between each species and each measured sample parameter (Ramette, 2007). When used with statistical tools, RDA can provide total variation in species composition as explained by the sample parameters as well as the overall statistical significance of the relationships between the species and the sample parameters (Ramette, 2007).

Differences between groups

Statistically significant differences in OTU abundance between groups can be determined using datasets standardized to a pre-determined number of sequences using non-parametric t-tests, non-parametric multivariate analysis of variance (NPMANOVA) or non-parametric Kruskal-Wallis tests along with Wilcoxon rank-sum tests (Anderson, 2001; Paulson, Pop, & Bravo, 2016; Segata et al., 2011). Non-parametric tests are often used for these analyses since ecological data may not always satisfy the assumptions (ex. normal distribution) required by conventional multivariate statistical methods (Anderson, 2001).

Longitudinal analysis

In order to analyze microbial community stability of samples collected from the same location or host over multiple time points, the normalized Jensen-Shannon divergence index is used to evaluate dissimilarities between community states (J. Lin, 1991; Romero, 2014). A community state refers to the relative abundance of all phylotypes at a particular time point within a sample (Romero, 2014). Lower Jensen-Shannon divergence scores indicate higher similarity between two community states while higher scores indicate higher dissimilarity between two community states (Romero, 2014). When using read count data obtained from longitudinal experiments in order to compare differential features between groups, generalized estimation equations or linear mixed-effects models are used to model the data while assuming a Poisson or negative binomial data distribution pattern (Romero, 2014).

Bacterial Community Analysis using 16S rRNA Gene Sequencing – Advantages

16S rRNA gene sequencing has several advantages compared to traditional phenotypic bacterial identification methods. Namely, it allows for the discovery and description of novel bacterial taxa, and for the precise identification of poorly described, rarely isolated, phenotypically aberrant strains of bacteria, mycobacteria and other fastidious organisms, uncultivated, or viable but non-culturable, bacteria, and adherent, diverse and unknown bacteria in mats (ex. biofilms) (Clarridge, 2004; Han, 2006; Hugenholtz, Goebel, & Pace, 1998; Relman, 1999). The use of 16S rRNA gene sequencing analysis has led to organisms previously defined (through phenotypic methods) as the same species, or part of the same genus, or even part of different genera, as actually being genotypically too dissimilar, or too similar, to be part of the same species, or genera, or being part of completely different genera (Clarridge, 2004).

Bacterial Community Analysis using 16S rRNA Gene Sequencing – Limitations

16S rRNA gene sequence

The 16S rRNA gene is very sensitive - a single nucleotide difference at the 16S rRNA gene level can predict significant genomic variation (Thompson et al., 2005; Ward, Ferris, Nold, & Bateson, 1998). However, it is not very specific – two organisms taxonomically distant from one another may have identical 16S rRNA gene sequences, due to horizontal gene transfer (Eren et al., 2013). Furthermore, although the 16S rRNA gene has broad applicability across all taxonomic groups,

making it ideal for identifying unknown organisms, its sequence does not have enough variation or encode virulence factors, making it far from ideal when comparing bacterial species for epidemiological purposes, differentiating between all species within a certain genus, or detecting virulent species of bacteria (Clarridge, 2004).

Multiple 16S rRNA operon copy numbers

Multiple heterogeneous copies of the 16S rRNA gene within bacterial genomes introduce bias into microbial community analysis (Crosby & Criddle, 2003; Dahllof, Baillie, & Kjelleberg, 2000) since the copies can have different sequences leading to the incorrect identification of multiple unique organisms instead of one organism (Case et al., 2007). Copy numbers have been observed to be mostly taxon-specific, but variations among strains of the same species have also been observed (Acinas et al., 2004). 16S rRNA gene copy numbers are variable even at the family and genus level and bacterial genomes with more 16S rRNA copies tend to contain more diverse variants of the gene (Větrovský & Baldrian, 2013). The existence of multiple, variable copies of 16S rRNA genes when constructing OTU clusters could lead to unreliable estimates of relative abundance and diversity of microbial communities in complex samples (Větrovský & Baldrian, 2013). Diversity estimates obtained by OTU clustering tend to be inflated and abundance estimates tend to underestimate the abundance of taxa with low 16S rRNA gene copy numbers and overestimate the abundance of taxa with high 16S rRNA gene copy numbers (Větrovský & Baldrian, 2013). Moreover, the *de facto* threshold of 97% leads to

species and even genomes to be clustered as different OTUs, or species of different genera to be clustered together, because it is not low enough to capture these intra-genomic and intra-species differences (Case et al., 2007; Větrovský & Baldrian, 2013).

Genus and species definition

The ambiguity in the definition of bacterial genus or species found in conventional culture-based microbiology also extends to the use of 16S rRNA gene sequence comparisons and algorithms used to generate and analyze the sequencing data (Clarridge, 2004). This is especially true when determining the exact extent of genetic difference that captures species differentiation, intra-species variability (ex. all strains within a species do not always have identical 16S rRNA gene sequences) (Clarridge, 2004). Furthermore, the use of different algorithms and different databases often leads to varying results (Clarridge, 2004). Therefore, the sole reliance on genotype for definition may not be the best approach. Due to the similarity or likeness of sequences between species within or between genera, closely related but distinct species may not be accurately differentiated (Clarridge, 2004; Han, 2006). This issue is compounded if a portion of the gene, rather than the whole gene is amplified prior to sequencing (Han, 2006). Identical sequences are more common at the subspecies level and organisms with similar or same genotype but different phenotype may be designated as different species, using 16S rRNA gene sequencing analysis, when they are actually different strains of the same species (Clarridge, 2004; V. Hall, 2003).

Assignment and clustering approaches

The current approaches used to partition 16S rRNA datasets have some limitations. Classifying reads of organisms from high diversity environments using reference databases may lead to poor resolution of diversity measures (Eren et al., 2013). This is because databases, which often use reference classifications based on isolated organisms, are unable to capture the entire microbial diversity often found in environmental samples, especially since they lack a large portion of 16S rRNA gene sequences, mostly from uncultured samples (Eren et al., 2013; Huse et al., 2010; Pace, 1997; Quast et al., 2013; Sogin et al., 2006). On the other hand, clustering approaches based on sequence similarity often result in a large number of OTU groupings (Eren et al., 2013). However, in order to minimize OTU number inflation due to random sequencing errors, researchers have to use the relatively low *de facto* sequence similarity threshold of 97% making it very difficult to identify community organisms that differ by a very small number of nucleotides (Eren et al., 2013; Kunin, Engelbrekton, Ochman, & Hugenholtz, 2010; McLellan, Huse, Mueller-Spitz, Andreishcheva, & Sogin, 2010). Environmental samples often contain distinct organisms which are closely related but have small differences in gene sequences and both clustering and database comparison methods do not always provide the resolution required to classify these closely related organisms into distinct units (Eren et al., 2013).

PCR bias

Since the use of 16S rRNA sequencing relies on PCR amplification of the 16S rRNA gene prior to sequencing, several PCR associated drawbacks associated can negatively affect downstream processing: 1) primer mismatch due to the use of universal primers 2) background bacterial contamination (ubiquity of bacteria, bacterial origin of Taq polymerase, contamination during sampling or extraction) can be amplified due to the use of universal primers, 3) co-extraction of PCR inhibitors can inhibit enzymes, 4) different genome sizes and 16S rRNA copy numbers can lead to differential PCR amplification and 5) mixtures of 16S rRNA genes can lead to the formation of chimeras (Sipos et al., 2007; Wintzingerode, Göbel, & Stackebrandt, 2006).

Database limitations

Sequence comparison can be affected by the length of sequence being analyzed, the alignment tool and quality of the reference database being used. Databases may be unverified, not peer reviewed, may not contain all possible reference sequences and may be unable to capture intra-species variability due to the lack of all possible strains within a species (Clarridge, 2004). Species may be designated incorrectly in reference databases (ex. strains with minor genetic variability (less than 1%) being designated as separate species and species associated with several genera (ex. *Enterobacter*) listed under one genus) (Clarridge, 2004). 16S rRNA species designation variability tends to be seen more in the case of less well known or less pathogenic species and curated databases are continuously being

improved with the sequencing of more bacterial species using improved sequencing technology (Clarridge, 2004).

Overcoming the limitations of current 16S rRNA gene sequencing analysis methods

Sequences can be re-compared to several curated databases as they are updated. The use of databases which contain bacterial genomes relevant to the sample being analyzed will ensure accurate bacterial identification. If possible, biochemical tests can be used in conjunction with sequencing to provide a more definitive identification (L. Hall, Doerr, Wohlfiel, & Roberts, 2003). The submission of metagenomics data and associated metadata to curated databases can improve databases and future analyses. To obtain finer resolution at species level in complex environmental samples, oligotyping can be used in addition to clustering and reference database comparison (Eren et al., 2013). Unlike comparing all positions in sequence reads, which forms the basis of database comparison and clustering, oligotyping focuses only on the variable sites of the 16S rRNA gene sequence to define taxonomic units (Eren et al., 2013). Any OTU inflation caused by multiple 16S rRNA gene copies must be addressed during data analysis. The use of shotgun sequencing could help overcome the limitations of analysis based on the PCR amplification of the 16S rRNA gene; however, genome size variation associated with the use of shotgun sequencing for the examination of community composition should be addressed during analysis (Větrovský & Baldrian, 2013).

Chapter 5: Antibiotic Concentrations Decrease During Wastewater Treatment But Persist At Low Levels in Reclaimed Water

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Abbreviations

AMP	Ampicillin
AZI	Azithromycin
CIP	Ciprofloxacin
HLB	Hydrophilic-Liphophilic Balance
HPLC-MS/MS	High Performance Liquid Chromatography-Tandem Mass Spectrometry
LIN	Linezolid
LOD	Limit of Detection
MRSA	Methicillin Resistant <i>Staphylococcus aureus</i>

OXA	Oxacillin
OXO	Oxolinic Acid
PEN	Penicillin G
PIP	Pipemidic Acid
SI	Spray Irrigation
SRT	Solids Retention Time
TET	Tetracycline
U.S.	United States
UV	Ultraviolet
VRE	Vancomycin Resistant enterococci
WWTP	Wastewater Treatment Plant

Abstract

Reclaimed water has emerged as a potential irrigation solution to freshwater shortages. However, limited data exist on the persistence of antibiotics in reclaimed water used for irrigation. Therefore, we examined the fate of nine commonly-used antibiotics (ampicillin, azithromycin, ciprofloxacin, linezolid, oxacillin, oxolinic acid, penicillin G, pipemidic acid and tetracycline) in differentially treated wastewater and reclaimed water from two U.S. regions. We collected 72 samples from two Mid-Atlantic and two Midwest treatment plants, and one Mid-Atlantic spray irrigation site. Antibiotic concentrations were measured using liquid-chromatography-tandem mass spectrometry. Data were analyzed using Mann-Whitney-Wilcoxon tests and Kruskal Wallis tests. Overall, antibiotic concentrations in effluent samples were lower than that of influent samples. Mid-Atlantic plants had similar influent but lower effluent antibiotic concentrations compared to Midwest plants. Azithromycin was detected at the highest concentrations (of all antibiotics) in influent and effluent samples from both regions. For most antibiotics, transport from the treatment plant to the irrigation site resulted in no changes in antibiotic concentrations, and UV treatment at the irrigation site had no effect on antibiotic concentrations in reclaimed water. Our findings show that low-level antibiotic concentrations persist in reclaimed water used for irrigation; however, the public health implications are unclear at this time.

Introduction

The use of reclaimed water (treated municipal wastewater) for landscape and agricultural irrigation is projected to rise in the United States (U.S.) (EPA, 2012a). However, research conducted on the safety of irrigating with reclaimed water has focused predominantly on the presence of microbial pathogens (EPA, 2012a; Sheikh et al., 1990), heavy metals (EPA, 2012a; Sheikh et al., 1990) and organics (EPA, 2012a; Sheikh et al., 1990), with limited data available on the occurrence of pharmaceuticals, including antibiotics, in reclaimed water (Kinney et al., 2006; X. Wu et al., 2014, 2015). Antibiotics are extensively used in the U.S. for therapeutic use among humans, and therapeutic, prophylactic, and non-therapeutic use among food-production animals (Center for Veterinary Medicine, 2015; Kim & Aga, 2007). Consequently, most antibiotic residues enter wastewater due to incomplete metabolism or incorrect disposal (Kummerer, 2001). Conventional wastewater treatment plants (WWTPs) in the U.S. are not designed to remove or monitor pharmaceuticals (Pruden et al., 2013), resulting in the frequent detection of multiple antibiotics in municipal wastewater, and treatment plant effluents (USGS, 2016; Zhang & Li, 2011).

Although the concentrations of antibiotics in wastewater effluent are relatively low (EPA, 2012a), the combination of antibiotics, nutrients and bacteria in reclaimed water, and in soil and plants subsequently irrigated with reclaimed water, could potentially result in the selection of antibiotic resistance among bacterial populations present in these environments (Fahrenfeld, Ma, O'Brien, & Pruden, 2013; Negreanu, Pasternak, Jurkevitch, & Cytryn, 2012). Methicillin-

resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) have been detected in influent, activated sludge, secondary clarifier, post aeration and effluent samples from U.S. WWTPs (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014). In addition, VRE have been detected at a U.S. reclaimed water spray irrigation site (Carey et al., 2016).

Antibiotics also have the potential to accumulate in soil and plants irrigated with wastewater and reclaimed water (Kinney et al., 2006; Pan, Wong, & Chu, 2014; Ternes, Bonerz, Herrmann, Teiser, & Andersen, 2007; X. Wu et al., 2015). Erythromycin was found to accumulate over five months in soil irrigated with reclaimed water (Kinney et al., 2006), and six tetracyclines, 4-epianhydrotetracycline, doxycycline and six quinolones (F.-H. Wang et al., 2014) accumulated in soil during a one-month period of reclaimed water irrigation. However, there are few studies that have compared different wastewater treatment technologies with regard to their impacts on antibiotic concentrations in reclaimed water. In addition, to our knowledge, there are little data regarding the impact of reclaimed water transport and additional reclamation site treatments on levels of antibiotics in reclaimed water.

Therefore, the goal of this study was to characterize antibiotic concentrations in differentially treated wastewater and reclaimed water from a spray irrigation site in order to evaluate the impact of treatment process variation and reuse site practices on the fate of antibiotic residues in reclaimed water intended for reuse. To our knowledge, this is the first study to analyze antibiotic concentrations throughout the treatment train from wastewater influent to reclaimed water utilized at an associated

reuse site for spray irrigation. Our findings inform the further exploration of treatment plant and reuse site practices, as well as future regulations, that may reduce the occurrence of antibiotics in reclaimed water.

Materials and Methods

Study sites

Wastewater samples collected from four U.S. wastewater treatment plants that supply treated effluent to reuse sites were included in this study: two WWTPs in the Mid-Atlantic region, previously described as Mid-Atlantic WWTP1 (Rosenberg Goldstein et al., 2012) and Mid-Atlantic WWTP2 (Rosenberg Goldstein et al., 2012); and two WWTPs in the Midwest region, previously described as Midwest WWTP1 (Rosenberg Goldstein et al., 2012) and Midwest WWTP2 (Rosenberg Goldstein et al., 2012). Reclaimed water samples from one spray irrigation site in the Mid-Atlantic region, previously described as Mid-Atlantic SI1 (Carey et al., 2016) (that receives treated effluent from Mid-Atlantic WWTP1 for landscape irrigation), were also tested in the study. All sites were chosen based on the willingness of the site operator to participate. A detailed description of each of the sites is included in Supplementary Information.

Sample size and description

Grab samples were collected, throughout the treatment process, from May 2009 to October 2010, with sampling timing dependent on the availability of the WWTP operators and spray irrigation site managers. Schematics of our sampling

locations have been previously described in Rosenberg et al (2012) (Rosenberg Goldstein et al., 2012) and Carey et al (2016) (Carey et al., 2016). All samples were collected in 1L sterile polyethylene Nalgene® Wide Mouth Environmental Sampling Bottles (Nalgene, Lima, OH), transported to the laboratory at 4 °C and stored at -80 °C until antibiotic residues were isolated and quantified in 2011. A total of 72 samples were included in this analysis: 45 wastewater samples (16 from Mid-Atlantic WWTP1, 7 from Mid-Atlantic WWTP2, 11 from Midwest WWTP1, and 11 from Midwest WWTP2) and 27 reclaimed water samples from Mid-Atlantic SI1. In total, 15 influent, 4 activated sludge, 3 post aeration, 6 secondary clarifier, 4 (lagoon) cell B and 13 effluent samples were collected from all WWTPs. From the Mid-Atlantic SI1 site, 6 samples were collected before UV treatment, 7 after UV treatment, 6 at the open-air storage pond inlet, and 8 at the pumphouse inlet.

Extraction and analysis of antibiotic concentrations

Nine antibiotics commonly used in the U.S. (U.S. National Library of Medicine. National Institutes of Health., 2015), and previously detected in wastewater samples (Zhang & Li, 2011), were analyzed: β lactams - ampicillin (AMP), oxacillin (OXA) and penicillin G (PEN); a macrolide - azithromycin (AZI); an oxazolidinone - linezolid (LIN); quinolones - ciprofloxacin (CIP), oxolinic acid (OXO) and piperimidic acid (PIP); and a tetracycline - tetracycline (TET). Antibiotic concentrations in all samples were quantified using a previously published method (Sapkota, Heidler, & Halden, 2007), with modifications. A 10 μ L aliquot of a methanol stock solution containing 10 μ g/ml of surrogate standard (Linezolid-d3, Toronto Research Chemical

Inc., Toronto Canada, Cat # L466502) was added to a 200 mL aliquot of each sample, followed by thorough mixing and equilibration. All samples were then extracted using Oasis HLB (60 mg) cartridges (Waters Corp; Milford MA), conditioned with 3 mL methanol followed by a 3 mL water rinse. The samples were loaded under minimal vacuum using Visiprep 12-port Vacuum Manifolds (Sigma-Aldrich, St. Louis, MO). Cartridges were then washed with 1 mL of water containing 5% methanol by volume and analytes were eluted with 6 mL of acetonitrile with 0.2% formic acid followed by 3 mL of methanol:acetone mix (50:50; vol:vol) under minimal vacuum. Each extract was dried under nitrogen at 40°C and reconstituted in 1 mL of acetonitrile:0.1 % formic acid mix (50:50; vol:vol) followed by the addition of a 10 µL aliquot of 10 µg/mL internal standard (OxolinicAcid-d5, Toronto Research Chemical Inc., Toronto, Canada). High performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) was used to detect and quantify antibiotics using an Applied Biosystem ABI3000 tandem mass spectrometer with positive electrospray ionization and chromatographic separation was achieved by an Xterra MS C18 2.5 µm, 2.1x50 mm column (Waters Corporation, Milford, MA) with a pre-column filter (Phenomenex, Torrance CA). The list of antibiotics included in the analysis and their corresponding limits of detection (LOD) is provided in Supplementary Table S1.

Statistical analysis

All statistical analyses were performed using R (version 3.2.4 2016 The R Foundation for Statistical Computing). Due to several samples with antibiotic concentrations below the LOD, certain antibiotics with very high concentrations

(reflective of prescription patterns and thus, considered representative of true sample concentrations), and small sample size at some WWTPs, a conservative, but robust, non-parametric rank-based approach was used for analysis (Helsel, 2012).

Differences between groups were determined using non-parametric Mann-Whitney-Wilcoxon test, or Kruskal Wallis test, based on the number of groups being compared (Helsel, 2012). The Bonferroni correction was used to adjust p -values when conducting multiple comparisons. In all cases, p -values ≤ 0.05 were defined as statistically significant, except when Bonferroni corrections were employed.

Results and Discussion

Antibiotic concentrations in influent samples from all WWTPs

Figure 1 summarizes the antibiotic concentrations detected in influent samples across all WWTPs. Antibiotic detection ranges in ng/mL were as follows – ampicillin (< LOD to 49.7), oxacillin (1.39 to 18), penicillin (< LOD to 23.8), azithromycin (22.2 to 336), ciprofloxacin (3.28 to 69.5), oxolinic acid (5.35 to 9.43), piperidic acid (5.23 to 55.1), linezolid (3.05 to 61.5) and tetracycline (< LOD to 188).

Azithromycin was detected at the highest concentrations compared to all antibiotics in influent samples recovered from all WWTPs, with the highest concentration occurring in influent samples collected from Midwest WWTP1. Concentrations of azithromycin in both the Midwest WWTP1 and the Mid-Atlantic WWTP1 influents were, on average, an order of magnitude higher than those detected at the other WWTPs. Azithromycin concentrations were also the highest of all antibiotics analyzed, in influent, activated sludge as well as effluent samples, at

another U.S. wastewater treatment plant located in Kentucky (Loganathan et al., 2009). Azithromycin, which is the most commonly prescribed human-use antibiotic in the U.S. (Centers for Disease Control and Prevention (CDC), 2015; Hicks et al., 2015) and has been found at fairly high concentrations in biosolids (Walters, McClellan, & Halden, 2010) with a relatively long half-life in biosolid-amended soil (Walters et al., 2010), may have entered Mid-Atlantic WWTP1 through domestic and hospital wastewater (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014) and Midwest WWTP1 through domestic and agriculturally-influenced stormwater (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014).

β -lactams were found at the lowest concentrations (compared to other antibiotics) in influent samples from all WWTPs, with 20% of influent samples containing ampicillin below the LOD and 33% of influent samples containing penicillin G below LOD. Despite being one of the most highly used classes of antibiotics in the U.S. (Centers for Disease Control and Prevention (CDC), 2015), β -lactams are not usually found in high concentrations in influent samples (Zhang & Li, 2011) due to chemical hydrolysis in the influent stream, or cleavage of the unstable β -lactam ring by β -lactamases (Zhang & Li, 2011).

Antibiotic concentrations in effluent samples from all WWTPs

The antibiotic concentrations detected in effluent samples from all WWTPs are displayed in Figure 2. Antibiotic detection ranges in ng/mL were as follows – ampicillin (2.31 to 42.2), oxacillin (< LOD to 10.1), penicillin (< LOD to 20.3),

azithromycin (0.82 to 183), ciprofloxacin (2.71 to 16.4), oxolinic acid (< LOD to 7.94), pipemidic acid (3.76 to 26), linezolid (< LOD to 22.1) and tetracycline (< LOD to 23.6). Oxacillin, penicillin G, tetracycline and pipemidic acid occurred at concentrations below the LOD in 54%, 46%, 23% and 8% of all effluent samples, from all WWTPs, respectively. The β -lactams would have undergone further cleavage and hydrolysis during wastewater treatment (Zhang & Li, 2011), while tetracycline, due to its extremely high sludge-wastewater partition coefficient (Batt et al., 2007), may have been adsorbed into activated sludge.

Differences in antibiotic concentrations between same-day influent versus effluent samples

Antibiotic concentration differences between influent and effluent samples collected on the same day from each of the WWTPs are illustrated in Figure 3. In general, concentrations of most antibiotics were lower in the effluent samples compared to influent samples, with differences, at marginal significance, between influent and effluent concentrations observed only for oxacillin ($W = 54$, p -value = 0.004) and pipemidic acid ($W = 53$, p -value = 0.006). To account for multiple comparisons, p -values at or below 0.005 were considered to be statistically significant. Statistically significant differences for just two of the nine antibiotics analyzed may have been due to the cross sectional nature of the grab samples and our irregular access to some WWTPs (which was dictated by plant operators).

Regional differences between antibiotic concentrations in influents and effluents

Antibiotic concentration differences between Mid-Atlantic and Midwest WWTP influents can be seen in Supplementary Figure S1. Generally, most influent antibiotic concentrations were similar between the two regions, except for azithromycin concentrations which were higher, though not statistically significantly, in the Midwest WWTP influents, compared to the Mid-Atlantic treatment plant influents. Azithromycin levels may have been higher in the raw influent of Midwest WWTPs (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014) compared to Mid-Atlantic plants, because Midwest influents were comprised of both domestic wastewater and agriculturally-influenced stormwater. Since the Midwest plants are located in rural areas where biosolids are applied to agricultural land (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014), runoff from this land during rain events could have increased levels of azithromycin in the waste stream.

Antibiotic concentration differences between effluents from the Midwest and Mid-Atlantic regions are shown in Supplementary Figure S2. In spite of most antibiotics being at similar concentrations in all influent samples, ampicillin, oxacillin, oxolinic acid, penicillin G and tetracycline were found at higher concentrations in the effluents from Midwest WWTPs while azithromycin and linezolid were found at higher concentrations in the effluents from Mid-Atlantic WWTPs. None of these differences, however, were statistically significant.

The observed variability in antibiotic removal could be attributed to treatment process variations, namely treatment plant capacity, nature of influent and type of tertiary treatment. Other differences could have been due to WWTP reactor type and solid-retention time (SRT) both of which impact microbial population characteristics of activated sludge (Batt et al., 2007; Jelic, Gros, Petrovic, Ginebreda, & Barcelo, 2012). Pharmaceutical degradation is achieved by nitrifying bacteria (through the production of monooxygenase (including ammonia monooxygenase and dioxygenase enzymes (Dorival-García et al., 2013)) which increase with longer SRT (Batt, Kim, & Aga, 2006) and occur at higher concentrations in activated sludge from a nitrification reactor compared to a conventional activated sludge reactor (Kim & Aga, 2007). Variability could have been due to activated sludge reactor type (Popple, Williams, May, Mills, & Oliver, 2016; Walters et al., 2010) and although all four plants in our study contained an activated sludge process, the types varied from a conventional continuous activated sludge reactor (Mid-Atlantic WWTP1), aeration tanks (Mid-Atlantic WWTP2), a sequencing batch reactor (Midwest WWTP2) or activated sludge lagoons (Midwest WWTP2). SRT variability also could have influenced the observed differences between plants; however, this information was not obtained during the study.

Differences in antibiotic concentrations across wastewater treatment processes

Antibiotic concentration differences across all treatment processes utilized at all WWTPs are described in Figure 4. In general, most antibiotics partitioned into samples from various treatment processes based on the chemical and physical

properties of the class to which they belong. Statistically significant differences were found only for oxacillin, between influent and effluent samples ($W = 28$, p -value = 0.0002), and activated sludge and effluent samples ($W = 89$, p -value = 0.0005). To account for multiple comparisons, p -values at or below 0.0005 were considered to be statistically significant.

Ciprofloxacin and piperidic acid were relatively abundant in activated sludge samples due to their non-volatility (Batt et al., 2007) and fairly high sludge-wastewater partition coefficient (Batt et al., 2007). These antibiotics are also resistant to microbial degradation (Jelic et al., 2012; T L Jones-Lepp & Stevens, 2007) but susceptible to photochemical degradation (Jelic et al., 2012; T L Jones-Lepp & Stevens, 2007). However, the large amounts of organic matter in activated sludge may have blocked light and resulted in reduced photochemical degradation.

Azithromycin, despite having a relatively low sludge-wastewater partition coefficient (Zhang & Li, 2011), and oxacillin and penicillin G, despite being more prone to hydrolysis (Zhang & Li, 2011), were also found at high concentrations in activated sludge. Azithromycin may have continued to persist in activated sludge due to its high influent concentrations. Activated sludge samples from another U.S. treatment plant in Kentucky also contained high azithromycin concentrations (Loganathan et al., 2009). Higher than expected antibiotic concentrations of other antibiotics, including β -lactams, may have also occurred due to interactions with proteins, nucleic acids, and polysaccharide cell-wall components of activated sludge bacteria (Jelic et al., 2012), and bonding and complexation with lipids, fats and other particulate matter in activated sludge, allowing compounds with low octanol-water

and sludge-wastewater coefficients to easily adsorb into activated sludge (Jelic et al., 2012). Tetracycline, a non-volatile compound (Batt et al., 2007) with a high sludge-wastewater partition coefficient (Batt et al., 2007), and the ability to undergo polarization or complexation with solid particles (Golet, Strehler, Alder, & Giger, 2002; Jelic et al., 2012), was found at unexpectedly low concentrations in activated sludge samples, possibly due to the relatively low therapeutic use of tetracycline among humans (Zhang & Li, 2011).

Differences in antibiotic concentrations from Mid-Atlantic WWTP1 to Mid-Atlantic SII

Figure 5 illustrates the changes in antibiotic concentrations in samples obtained sequentially from the influent at Mid-Atlantic WWTP1 through the Mid-Atlantic SII pumphouse sprinkler. For all antibiotics, transport from the WWTP to the spray irrigation site resulted in virtually unchanged median concentrations. The only observed decrease in median concentration was for azithromycin (56.6 ng/mL to 38.6 ng/mL). Similarly, the median concentrations of almost all of the antibiotics remained unchanged after UV treatment at the spray irrigation site. Open-air storage at the spray irrigation site resulted in a decrease in the median concentration of azithromycin (44.85 ng/mL to 8.79 ng/mL), but almost all other antibiotics remained at virtually unchanged levels before and after storage.

Ampicillin concentrations were statistically significantly different between “after UV treatment” samples and “pumphouse inlet” samples ($W = 14$, p -value = 0.0006). Azithromycin concentrations were statistically significantly different

between “holding pond inlet” samples and “pumphouse inlet” samples ($W = 112$, p -value = 0.0001), “after UV treatment” samples and “pumphouse inlet samples” ($W = 154$, p -value < 0.0001), “before UV treatment” samples and “pumphouse inlet samples” ($W = 140$, p -value < 0.0001) and between Mid-Atlantic WWTP1 influent samples and the “pumphouse inlet” samples ($W = 112$, p -value = 0.0001). To account for multiple comparisons p -values at or below 0.0006 were considered statistically significant.

Distribution system characteristics, such as residual chlorine, pH, temperature, biofilm community structure and dissolved organic matter (parameters we were unable to assess) could have influenced antibiotic concentrations during transport; however, our data showed that the effects were negligible. On-site UV radiation treatment was performed at a wavelength (254 nm) that has previously been found to be ineffective at reducing antibiotic concentrations (Batt et al., 2007). Azithromycin may have undergone photodegradation in the storage pond influenced by direct photolysis due to direct excitation from solar radiation, or indirect photolysis due to interaction with reactive intermediates generated by humic acids (Tong, Eichhorn, Pérez, Wang, & Barceló, 2011).

Limitations

The main limitations of this study were the collection of grab samples and unequal sample sizes resulting from limited access to some collection sites. Furthermore, since we could only include one spray irrigation site in our study, our findings may not be applicable to all U.S. spray irrigation sites. However, by studying

four conventional WWTPs across two regions, our observations could be representative of multiple types of conventional wastewater treatment processes commonly employed in different regions of the U.S.

Public health impacts and future research

Antibiotics have the potential to exert selective pressures on existing bacterial communities within WWTPs (Kim & Aga, 2007) and in reclaimed water (Fahrenfeld et al., 2013), potentially contributing to increased levels of antibiotic resistance within these environments (Rosenberg Goldstein et al., 2012). Both MRSA and VRE have been detected in the same WWTP effluents that were tested in this study and sent to reuse applications (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014), and VRE was detected in the reclaimed water that we tested from the Mid-Atlantic spray irrigation site (Carey et al., 2016). Thus, it is possible that the trace levels of antibiotics that we observed in the wastewater and reclaimed water samples could have contributed to the selection of bacteria that are resistant to those specific antibiotics. In addition, the variable impact of different treatment technologies on antibiotic degradation is also a potential concern since some antibiotics (ciprofloxacin, ofloxacin) have been shown to be genotoxic (K Kümmerer, Al-Ahmad, & Mersch-Sundermann, 2000). Our data show that antibiotics remain at low levels in reclaimed water, but the effect of chronic human exposures to complex mixtures of antibiotics, and other pharmaceuticals in reclaimed water is unclear and deserves further study (Malchi, Maor, & Chefetz, 2015).

We confirmed that conventional continuous activated sludge processes alone may not effectively remove antibiotics from municipal wastewater. We also observed the persistence of antibiotics in reclaimed water at a spray irrigation site, in spite of on-site UV treatment. If conventionally-treated municipal wastewater is increasingly used for downstream purposes such as irrigation, additional cost-effective, onsite technologies may need to be developed to reduce the occurrence of persisting contaminants, including antibiotics, in the reclaimed water and prevent the dissemination of these contaminants into the environment and human populations.

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Figures

Figure 1: Concentrations (ng/mL) of antibiotics in influent samples collected from all four wastewater treatment plants (WWTPs) included in the study

AMP = Ampicillin; AZI = Azithromycin; CIP = Ciprofloxacin; LIN = Linezolid;
OXA = Oxacillin; OXO = Oxolinic Acid; PEN = Penicillin; PIP = Pipemidic Acid;
TET = Tetracycline

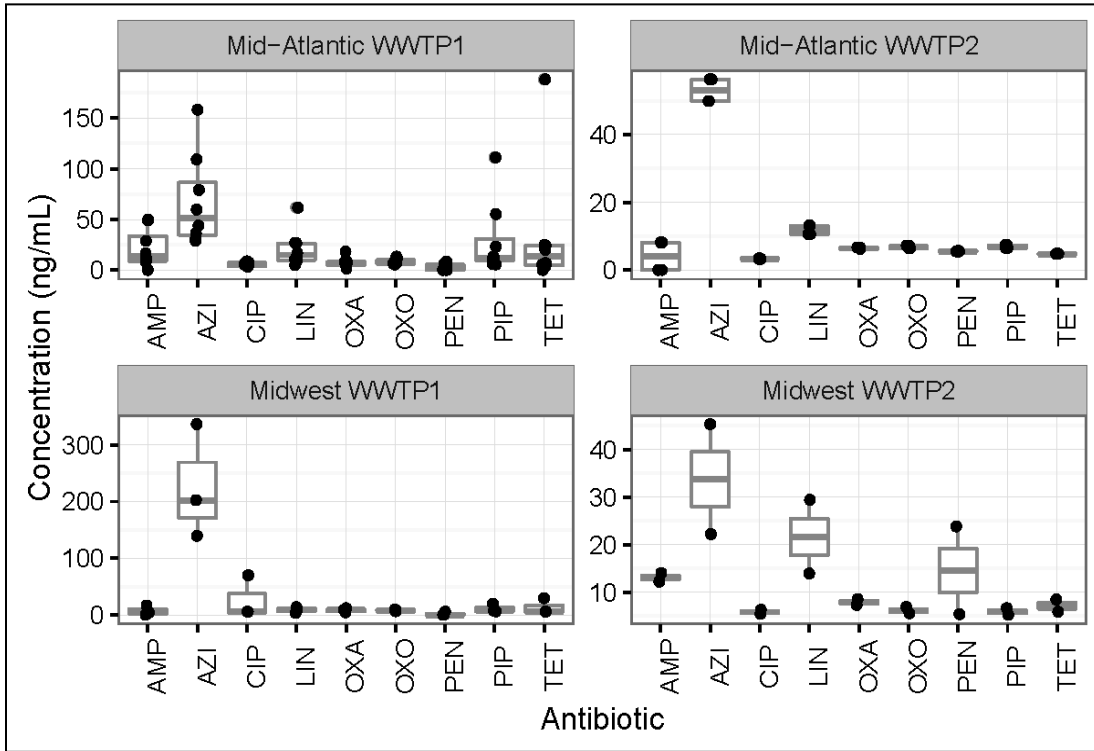


Figure 2: Concentrations (ng/mL) of antibiotics in effluent samples collected from all four wastewater treatment plants (WWTPs) included in the study

AMP = Ampicillin; AZI = Azithromycin; CIP = Ciprofloxacin; LIN = Linezolid;
 OXA = Oxacillin; OXO = Oxolinic Acid; PEN = Penicillin; PIP = Pipemidic Acid;
 TET = Tetracycline

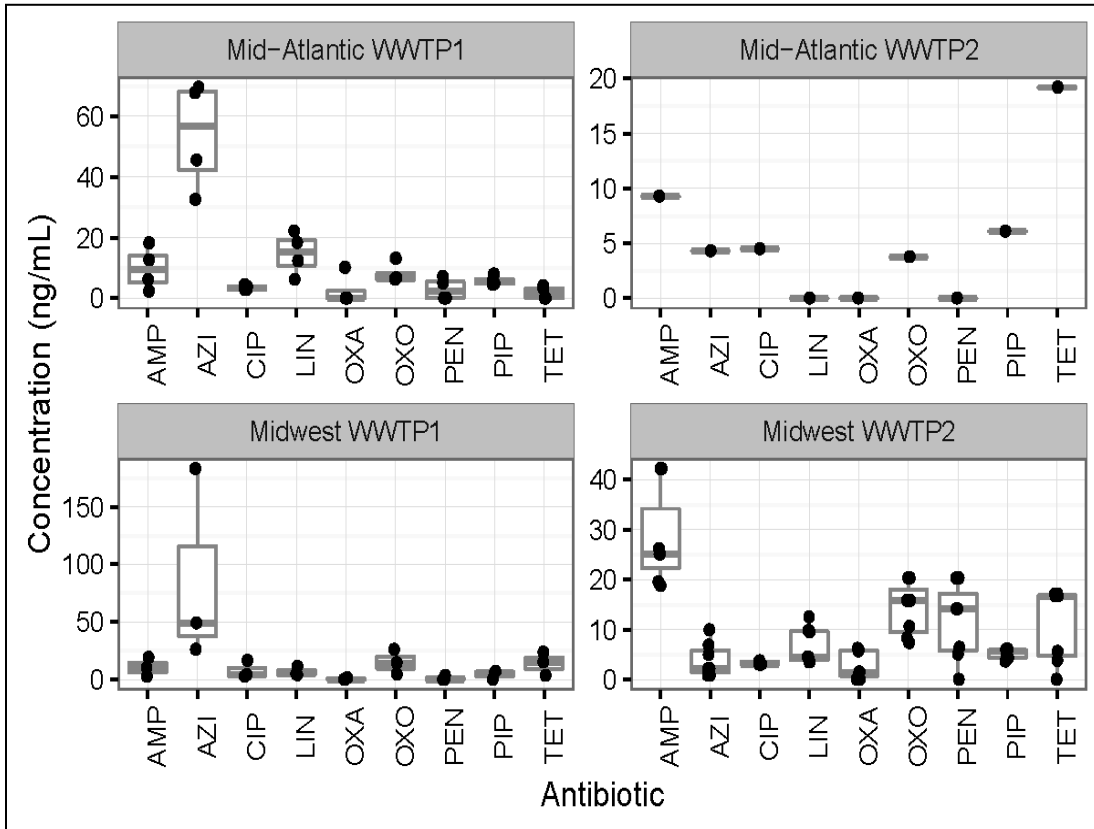


Figure 3: Differences in antibiotic concentrations (ng/mL) between influent versus effluent samples collected on the same day from each of the four wastewater treatment plants (WWTPs) included in the study

AMP = Ampicillin; AZI = Azithromycin; CIP = Ciprofloxacin; LIN = Linezolid;
 OXA = Oxacillin; OXO = Oxolinic Acid; PEN = Penicillin; PIP = Pipemidic Acid;
 TET = Tetracycline

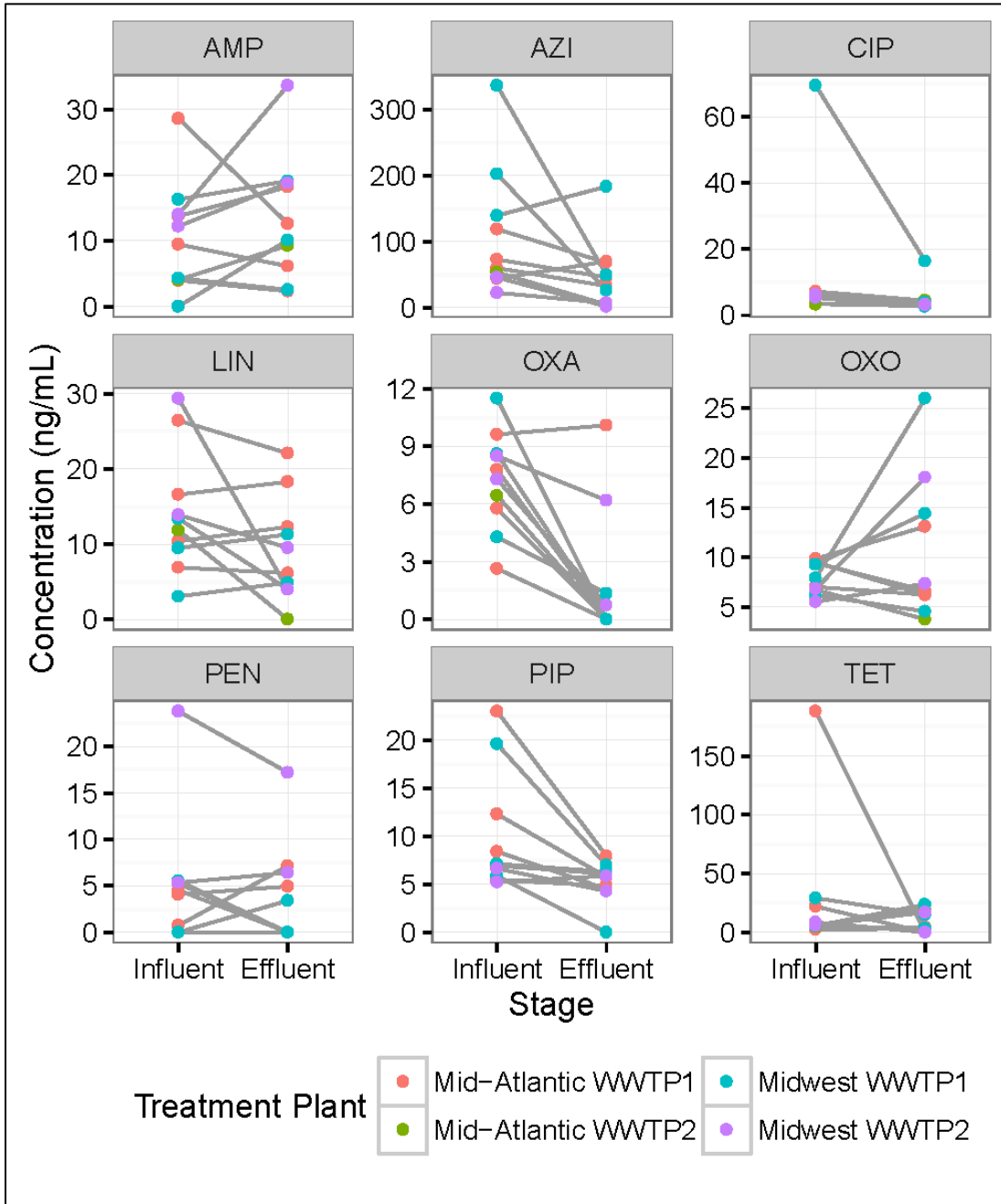


Figure 4: Differences in concentrations (ng/mL) of antibiotics across treatment processes used at all the wastewater treatment plants (WWTPs) included in the study
 AMP = Ampicillin; AZI = Azithromycin; CIP = Ciprofloxacin; LIN = Linezolid;
 OXA = Oxacillin; OXO = Oxolinic Acid; PEN = Penicillin; PIP = Pipemidic Acid;
 TET = Tetracycline

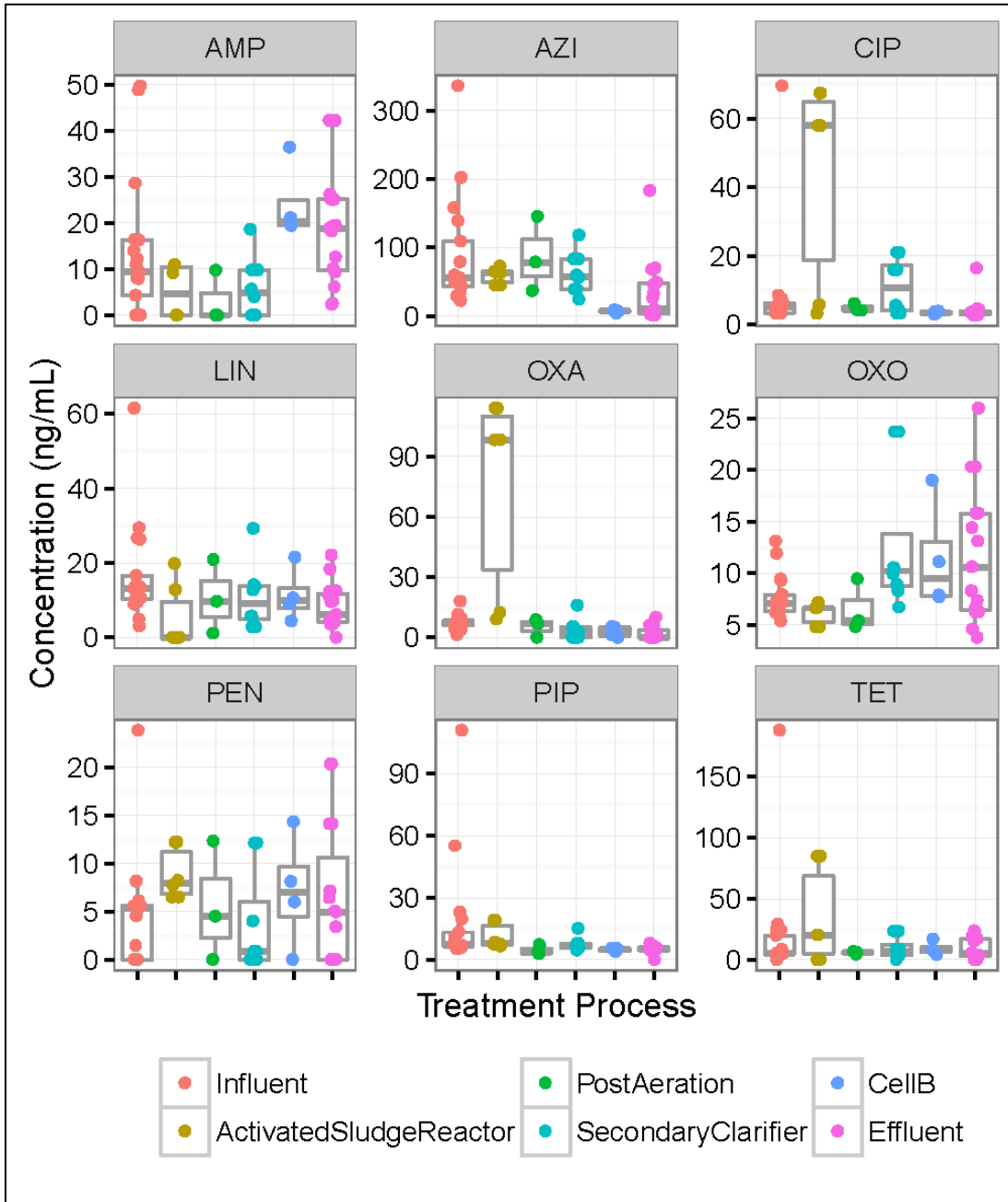
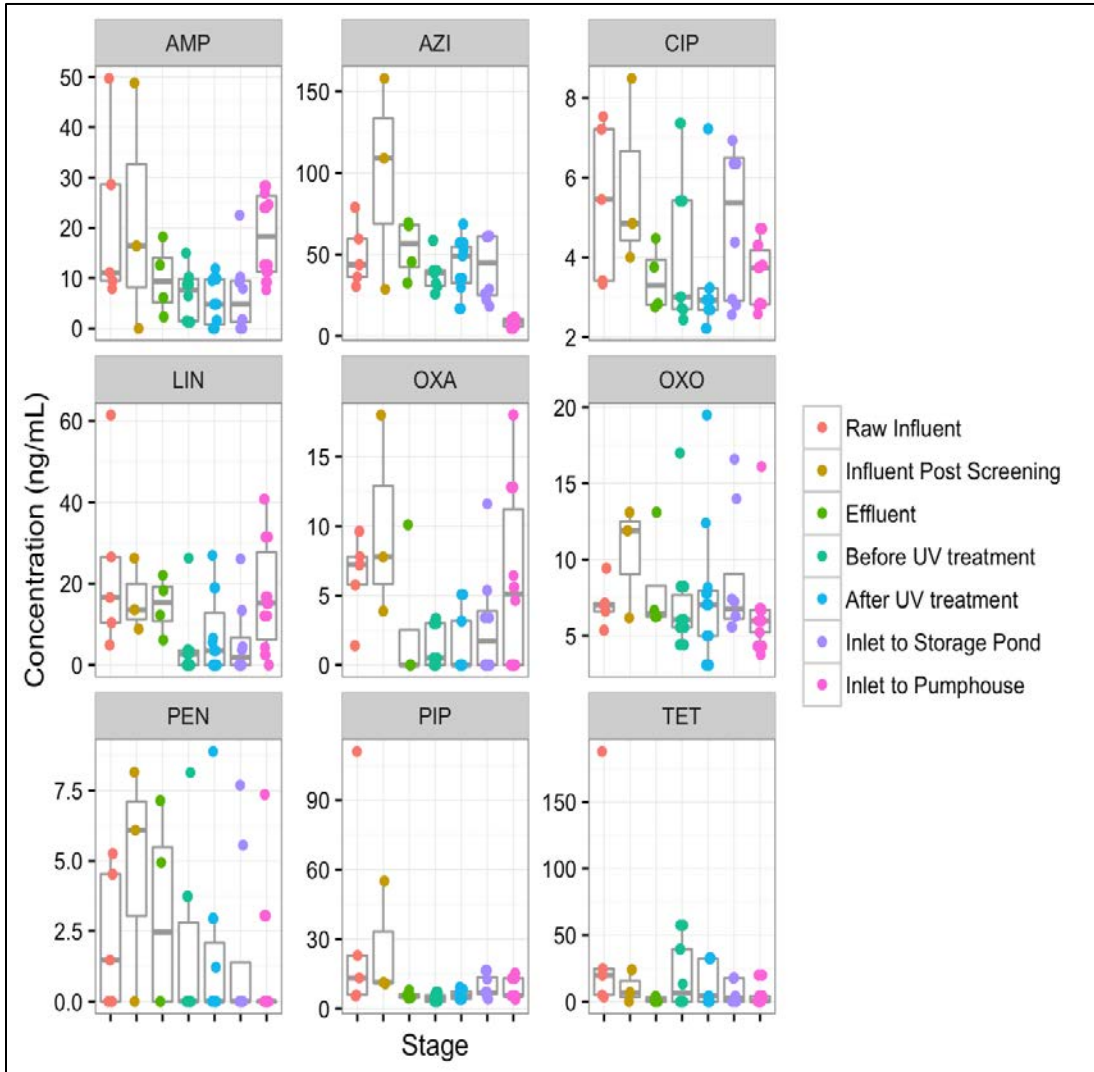


Figure 5: Changes in antibiotic concentrations (ng/mL) as wastewater travels from the influent at Mid-Atlantic wastewater treatment plant 1 (Mid-Atlantic WWTP1), undergoes tertiary treatment and is then piped to Mid-Atlantic spray irrigation site 1 (Mid-Atlantic SII) for reuse. The sequential order of flow is as follows: 1) Raw influent; 2) Influent post screening; 3) Effluent; 4) Before UV treatment; 5) After UV treatment; 6) Inlet to storage pond; and 7) Inlet to pumphouse.

AMP = Ampicillin; AZI = Azithromycin; CIP = Ciprofloxacin; LIN = Linezolid;
 OXA = Oxacillin; OXO = Oxolinic Acid; PEN = Penicillin; PIP = Pipemidic Acid;
 TET = Tetracycline



Appendix A

Detailed description of all sampling sites included in the study

All sites were chosen based on the willingness of the site operator to participate. Mid-Atlantic WWTP1 is an urban tertiary wastewater treatment plant processing 681,390 m³ of wastewater per day with a peak capacity of 1.51 x 10⁶ m³/d (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014). The influent includes domestic and hospital wastewater (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014). Treatment steps at this plant are screens, primary clarifier, activated sludge reactors, secondary clarifier, sand filters, chlorination (dose of 2 mg/L to 3 mg/L), dechlorination (with sodium bisulfite) and effluent discharge (chlorine residual of <0.1 mg/L) (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014). Effluent from this plant is piped to a landscaping site (Mid-Atlantic SII) for reuse in spray irrigation (Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014; Rosenberg Goldstein, Micallef, Gibbs, He, et al., 2014). Mid-Atlantic SII performs on-site treatment and storage prior to spray irrigation (Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014; Rosenberg Goldstein, Micallef, Gibbs, He, et al., 2014). On-site treatment includes screening (double-walled aluminum screen) and ultraviolet (UV) disinfection (minimum of 30,000 $\mu\text{W}/\text{cm}^2$ with 254 nm wavelength UV bulbs) (Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014; Rosenberg Goldstein, Micallef, Gibbs, He, et al., 2014). The UV treated reclaimed water is then pumped to an open air storage pond (peak capacity 15,142 m³) at a rate of 3.29 m³/d (Rosenberg Goldstein, Micallef, Gibbs, George, et

al., 2014; Rosenberg Goldstein, Micallef, Gibbs, He, et al., 2014). Water from the storage pond is then pumped to spray heads based on irrigation needs (Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014; Rosenberg Goldstein, Micallef, Gibbs, He, et al., 2014). Site employees use backpack sprayers to apply reclaimed water to locations not reached by spray heads (Rosenberg Goldstein, Micallef, Gibbs, He, et al., 2014).

Mid-Atlantic WWTP2 is a suburban tertiary treatment plant processing 7,570 m³ of wastewater per day with a peak capacity of 45,425 m³/d (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014). The influent includes domestic and hospital wastewater (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014). Treatment steps at this plant are screens, primary clarifier, primary aeration tank, secondary aeration tank, secondary clarifier, multimedia filter, chlorination (dose of 2 mg/L to 3 mg/L), de-chlorination (with sodium bisulfite) and effluent discharge (chlorine residual of <0.1 mg/L) (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014). Effluent from this plant is transported to a landscaping site for reuse via spray irrigation (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014).

Midwest WWTP1 is a rural tertiary treatment plant processing 1,363 m³ of wastewater per day with a peak capacity of 10,978 m³/d (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014). The influent at this plant includes domestic wastewater and agriculturally influenced stormwater (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et

al., 2014). Treatment steps at this plant are screens, activated sludge lagoons, clarifiers, seasonal chlorination (in June, July and August; dose of 4 mg/L) and dechlorination, and effluent discharge (chlorine residual of 0 mg/L) (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014). Effluent from this plant is transported to a landscaping site for reuse via spray irrigation (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014).

Midwest WWTP2 is a rural tertiary treatment plant processing 1,439 m³ of wastewater per day with a peak capacity of 7,571 m³/d (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014). The influent includes domestic, food production and agriculturally influenced wastewater (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014). Treatment steps at this plant are screens, sequencing batch reactor, lagoon cell A, lagoon cell B, lagoon cell C, lagoon cell D, lagoon cell E and effluent discharge (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014). There is no on-site disinfection and unchlorinated effluent from this plant is transported to an agricultural site for irrigation of animal feed crops (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014).

Supplementary Tables

Table S1: A list of the nine antibiotics analyzed with the corresponding mass-charge ratios (m/z) of their parent and daughter ions and their limit of detection (LOD) values (ng/mL)

Antibiotic	Parent Ion (m/z)^a	Daughter Ion (m/z)^a	LOD (ng/mL)
Ampicillin (AMP)	366.7	206.9	0.0242
Azithromycin (AZI)	375.0	113.1	0.0092
Ciprofloxacin (CIP)	331.5	287.4	0.0131
Linezolid (LIN)	337.5	295.4	0.0217
Oxacillin (OXA)	402.0	158.2	0.0201
Oxolinic Acid (OXO)	261.1	243.0	0.0213
Penicillin G (PEN)	334.6	158.2	0.0308
Pipemidic Acid (PIP)	303.4	215.9	0.0279
Tetracycline (TET)	445.0	409.9	0.0107

^amass-charge ratio

Supplementary Figures

Figure S1: Differences in antibiotic concentrations (ng/mL) between influent samples collected from Mid-Atlantic versus Midwest wastewater treatment plants (WWTPs)
AMP = Ampicillin; AZI = Azithromycin; CIP = Ciprofloxacin; LIN = Linezolid;
OXA = Oxacillin; OXO = Oxolinic Acid; PEN = Penicillin; PIP = Pipemidic Acid;
TET = Tetracycline

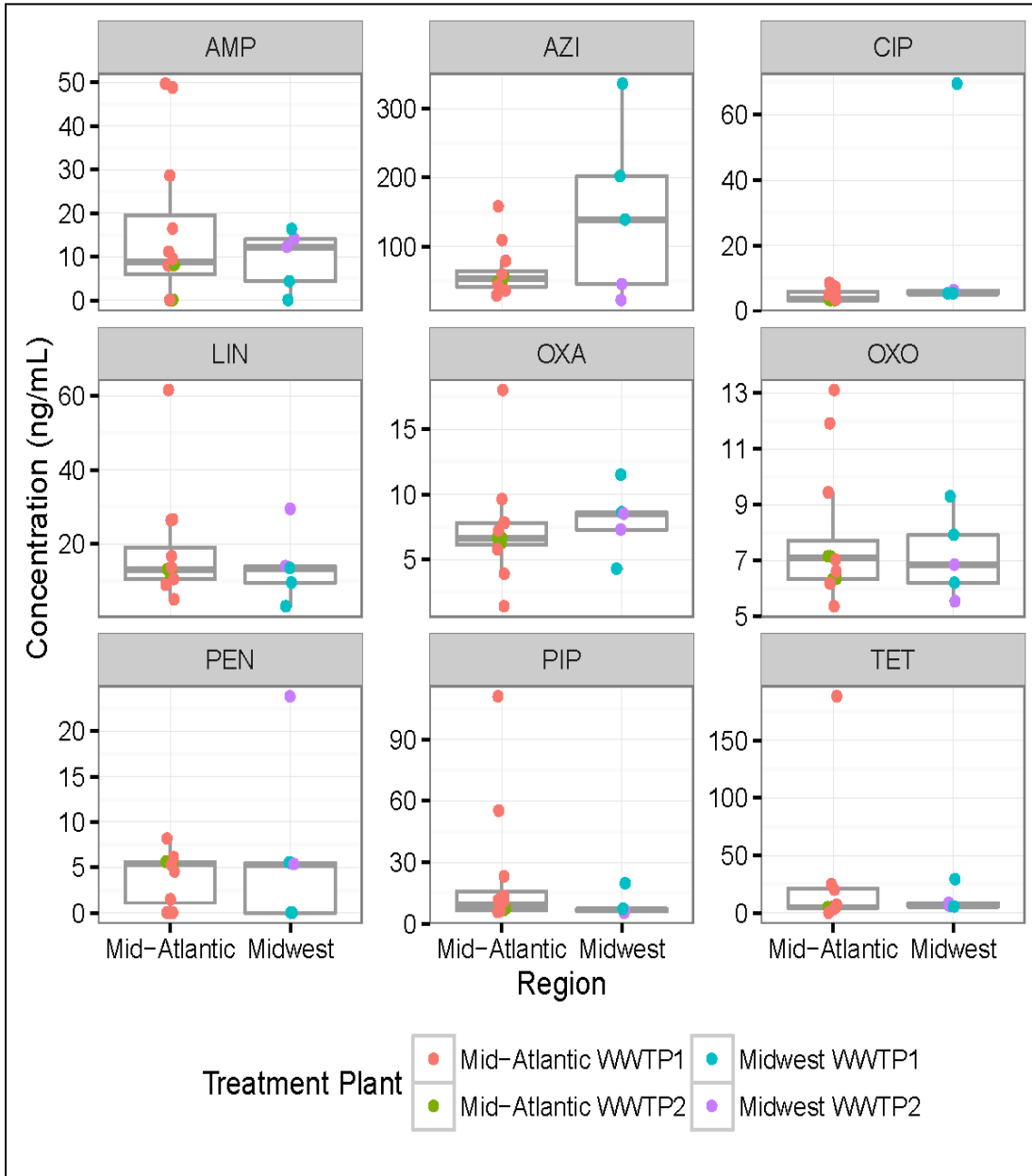
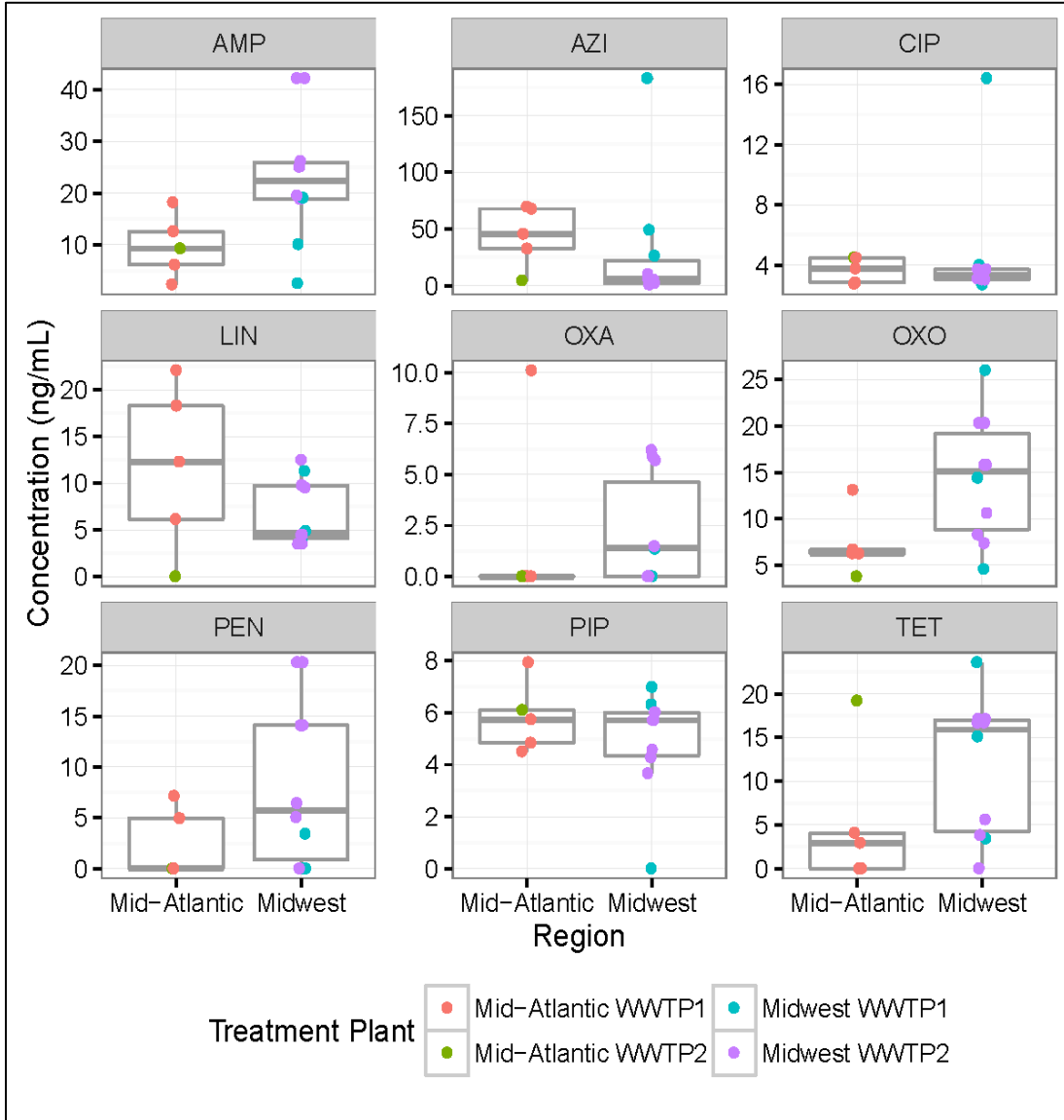


Figure S2: Differences in antibiotic concentrations (ng/mL) between effluent samples collected from Mid-Atlantic versus Midwest wastewater treatment plants (WWTPs)
 AMP = Ampicillin; AZI = Azithromycin; CIP = Ciprofloxacin; LIN = Linezolid;
 OXA = Oxacillin; OXO = Oxolinic Acid; PEN = Penicillin; PIP = Pipemidic Acid;
 TET = Tetracycline



Chapter 6: Bacterial Community Structure of Conventionally Treated Wastewater and Reclaimed Water

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Abbreviations

AOC	Assimilable Organic Carbon
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
BSA	Bovine Serum Albumin
DNA	Deoxyribonucleic Acid
OUT	Operational Taxonomic Unit
PANDAseq	Paired-End Assembler for DNA sequences
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PES	Polyethersulfone
PCCP	Pharmaceuticals and Personal Care Products
RDP	Ribosomal Database Project

RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
SDS	Sodium Dodecyl Sulfate
SI	Spray Irrigation
U.S.	United States
UV	Ultraviolet
WWTP	Wastewater Treatment Plant

Abstract

As the use of reclaimed water spreads across the United States (U.S.), from areas that have access to reclaimed water that has undergone advanced potable level treatment, to areas that may only have performed conventional wastewater treatment it has become necessary to examine the public health impacts of conventionally-treated reclaimed water. Currently reclaimed water regulations and treatment practices vary geographically within the U.S. Many regulations are based on culture-based research, and the use of indicator organisms to determine treatment quality. However, pathogens exist as members of complex microbial communities which may be impacted by wastewater treatment processes, treatment plant parameters and wastewater constituents and indicator organisms may not always correlate with the pathogen presence. Therefore, we use 16S rRNA gene sequencing to characterize total bacterial communities present in differentially treated wastewater and reclaimed water (n=67) from four wastewater treatment plants (WWTPs) and one associated spray irrigation site conducting on-site treatment and storage. Final effluent structure was influenced by influent constituents, sewer infrastructure and treatment processes. *Legionella* and *Mycobacteria* genera were abundant in samples collected from the WWTP effluent and the inlet to the pumphouse supplying the sprinkler system at the spray irrigation site, most likely due to resistance to disinfection and open air storage. As reclaimed water use is projected to increase even further, results from this study could be used to design more comprehensive water quality guidelines and regulations that are protective of human health.

Introduction

Reclaimed water use is rapidly expanding in the United States (U.S.) (Asano, 2007; EPA, 2012a), from historically high-use areas like California, where reclaimed water users have access to treated wastewater that has undergone chlorination, dual-media filtration, coagulation and flocculation (CA DPH, 2009) , to areas which may only have conventionally treated wastewater available for reuse applications. Furthermore, since the U.S. currently has no legally binding federal regulations governing reclaimed water use , regulations vary from state to state (EPA, 2012a). Not all states specify the exact type of process required in order to obtain the level of treatment mandated within their particular regional guidelines or regulations, and even though most state regulations focus on the quality of wastewater treatment plant (WWTP) effluent (EPA, 2012a), not all states require reuse site monitoring and reporting (Asano, 2007).

Most regulations and guidelines regarding bacterial pathogens in wastewater and reclaimed water are based on research utilizing culture-based methods analyzing single strains of bacteria in nutrient rich environments (Marcus, Wilder, Quazi, & Walker, 2013; Sheikh et al., 1990), and the use of indicator bacteria (EPA, 2012a). Therefore, these methods may not provide a comprehensive analysis of water quality (Marcus et al., 2013) since pathogens exist as a part of complex microbial communities (Marcus et al., 2013) and indicator pathogens have been found to have poor correlation with the actual presence of pathogens (Harwood et al., 2005; Jjemba et al., 2010). The complex microbial community within wastewater and reclaimed water may be impacted by wastewater treatment processes (Cydzik-Kwiatkowska &

Zielińska, 2016), operational parameters (Cyzdik-Kwiatkowska & Zielińska, 2016), wastewater constituents like heavy metals, xenobiotics and pharmaceuticals and personal care products (PPCPs), as well as reuse-site practices.

Although most state regulations require the use of chlorine residuals in reclaimed water distribution systems, the decline in the microbiological quality of reclaimed water by the time it reaches the reuse site has been previously documented (Jjemba et al., 2010). Opportunistic pathogens (*Aeromonas* spp., *Mycobacterium* spp. and *Legionella* spp.) have been observed to regrow in disinfected reclaimed water distribution systems due to biofilm development (Lehtola et al., 2007) and disinfectant dissipation (Jjemba et al., 2010), and have also been detected more often than routinely used indicator bacteria (Jjemba et al., 2010).

Therefore, in order to provide a comprehensive analysis of the public health impacts associated with reclaimed water use, it is worthwhile to characterize total bacterial communities from conventionally treated wastewater and reclaimed water. In this study, we used 16S rRNA (ribosomal RNA) gene sequencing to explore the total bacterial community structure of differentially treated wastewater from four WWTPs, that provide treated effluent for reuse, in two distinct geographic regions. We also analyzed samples from a spray irrigation site that receives treated effluent from one of the four WWTPs and performs on-site ultraviolet (UV) treatment and open-air storage before use. Our findings can advance current knowledge of the impact of conventional wastewater treatment processes, operational parameters as well as reclaimed water distribution and reuse site practices on the bacterial community structure of wastewater and reclaimed water.

Materials and Methods

Sampling Sites

Samples were collected from four WWTPs previously described as Mid-Atlantic WWTP1 (Rosenberg Goldstein et al., 2012), Mid-Atlantic WWTP2 (Rosenberg Goldstein et al., 2012), Midwest WWTP1 (Rosenberg Goldstein et al., 2012) and Midwest WWTP2 (Rosenberg Goldstein et al., 2012) and a landscape spray irrigation site, previously described as Mid-Atlantic SII (Carey et al., 2016), receiving treated effluent from Mid-Atlantic WWTP1. All sites were chosen based on the willingness of the site operator to participate.

Treatment processes at Mid-Atlantic WWTP1, an urban tertiary wastewater treatment plant processing 681,390 m³ of wastewater (including domestic and hospital wastewater) per day (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014), are screens, primary clarifier, activated sludge reactors, secondary clarifier, sand filters, chlorination (2-3 mg/L), de-chlorination and effluent discharge (<0.1 mg/L chlorine residual) (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014). A portion of the treated effluent from this plant is transported, through an enclosed pipe, to Mid-Atlantic SII for reuse in spray irrigation activities (Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014; Rosenberg Goldstein, Micallef, Gibbs, He, et al., 2014) where it undergoes screening and ultraviolet (UV) disinfection ($>30,000 \mu\text{W}/\text{cm}^2$) followed by storage in an open-air pond (peak capacity 15141.65 m³) (Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014; Rosenberg Goldstein, Micallef, Gibbs, He, et al., 2014). Water from the storage pond is then pumped to spray heads based on

irrigation needs (Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014; Rosenberg Goldstein, Micallef, Gibbs, He, et al., 2014). Spray irrigators also use backpack sprayers to apply reclaimed water to locations that are not reached by spray heads (Rosenberg Goldstein, Micallef, Gibbs, He, et al., 2014).

Treatment processes at Mid-Atlantic WWTP2, a suburban tertiary wastewater treatment plant processing 7,570 m³ of wastewater (including domestic and hospital wastewater) per day (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014), are screens, primary clarifier, primary aeration tank, secondary aeration tank, secondary clarifier, multimedia filter, chlorination (2-3 mg/L), de-chlorination and effluent discharge (< 0.1 mg/L chlorine residual) with a portion of the effluent being transported to a landscaping site for re-use via spray irrigation (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014).

Midwest WWTP1 is a rural tertiary treatment plant processing 1,363 m³ of wastewater (including domestic wastewater and agriculturally influenced storm-water) per day, with treatment processes being screens, activated sludge lagoons, clarifiers, seasonal chlorination and de-chlorination (4 mg/L in June, July and August) and effluent discharge (chlorine residual of 0 mg/L) (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014). A portion of effluent from this plant is transported to a landscaping site for re-use via spray irrigation (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014).

Midwest WWTP2 is a rural tertiary treatment plant processing 1,439 m³ of wastewater (domestic, food production and agriculturally influenced wastewater) per day with treatment processes being screens, sequencing batch reactor, lagoon cell A, lagoon cell B, lagoon cell C, lagoon cell D, lagoon cell E, and effluent discharge (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014). There is no on-site disinfection and unchlorinated effluent is transported to an agricultural site for irrigation of animal feed-crops (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014).

Sample Collection

Grab samples were collected throughout the treatment process at all WWTPs and the Mid-Atlantic SII site between May 2009 and October 2010 with sampling event timing dependent on WWTP and SI site manager availability and schedule. Sampling locations schematics have been previously described in Rosenberg et al (2012) (Rosenberg Goldstein et al., 2012) and Carey et al (2016) (Carey et al., 2016). Sterile one-liter polyethylene Nalgene® Wide Mouth Environmental Sampling Bottles (Nalgene, Lima, OH) were used to collect samples which were transported to the laboratory at 4 °C and stored at -80 °C until filtration and DNA extraction in 2013. A total of 67 samples were included in this analysis: 11 from Mid-Atlantic WWTP1, 7 from Mid-Atlantic WWTP2, 10 from Midwest WWTP1, 9 from Midwest WWTP2 and 30 from Mid-Atlantic SII. In total, 11 influent, 4 activated sludge, 2 post aeration, 6 secondary clarifier, 4 cell B, 10 effluent, 7 pre-UV treatment, 8 post-

ultraviolet (UV) treatment, 7 holding-pond-inlet and 8 pumphouse-inlet samples were included in this analysis.

DNA Extraction

Samples were thawed completely and 500 ml of each sample was vacuum filtered through a 0.2 μm , 47mm hydrophilic polyethersulfone (PES) filter (Pall Corporation, Port Washington, NY). Molecular biology grade water (MoBio Laboratories, Carlsbad, CA) was similarly filtered to serve as a negative control. Total genomic DNA was extracted from the filters by adapting previously published procedures (Jackson et al., 2014; Zupancic et al., 2012) utilizing both enzymatic as well as mechanical lysis. Briefly, each filter was aseptically placed in a sample lysis tube (Lysing Matrix B) (MP Biomedicals, Solon, OH) followed by the addition of ice-cold molecular biology grade 1X Phosphate Buffered Saline (PBS) (Gibco-Life Technologies, Grand Island, NY), lysozyme from chicken egg white (10mg/ml, Sigma-Aldrich, St. Louis, MO), lysostaphin from *Staphylococcus staphylolyticus* (5mg/ml Sigma-Aldrich, St. Louis, MO) and mutanolysin from *Streptomyces globisporus* ATCC 21553 (1mg/ml Sigma-Aldrich, St. Louis, MO) and incubated at 37 °C for 30 minutes. A second enzymatic lysis step followed, with the addition of Proteinase K (20mg/ml, Invitrogen-Life Technologies, Grand Island, NY) and 10% (w/v) sodium dodecyl sulfate (SDS) (BioRad, Hercules, CA) and incubation at 55 °C for 45 minutes. The samples were then mechanically lysed at 6.0 m/s for 40 seconds using the FastPrep®-24 benchtop homogenizer (MP Biomedicals, Irvine, CA). DNA purification was achieved by using the QIAmp DSP DNA mini kit 50, v2 (Qiagen,

Valencia, CA), according to the manufacturer's protocol, followed by additional purification using sodium acetate. DNA quality was assessed using a NanoDrop® spectrophotometer (NanoDrop Technologies, Wilmington, DE) and gel electrophoresis.

16S rRNA gene amplification and sequencing

Polymerase chain reaction (PCR) amplification of the V3-V4 hypervariable region of the 16S rRNA gene was achieved, using previously published procedures (Caporaso et al., 2012; Fadrosch et al., 2014; Sellitto et al., 2012), through the use of the 16S rRNA universal primers 319F/806R. Unique 12 base pair (bp) sequence tags were included with the 806R primer, to barcode each sample, to allow for multiplexing several samples in a single Illumina MiSeq (Illumina, San Diego, CA) run (Fadrosch et al., 2014). PCR amplification was performed using Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, Waltham, MA) and additional bovine serum albumin (BSA) (20 mg/ml Sigma-Aldrich St. Louis, MO) (to overcome PCR inhibition) in a DNA Engine Tetrad 2 thermal cycler (Bio-Rad, Hercules, CA). The cycling parameters were as follows: 30 seconds at 98°C, followed by 30 cycles of 10 seconds at 98°C, 15 seconds at 66°C and 15 seconds at 72°C and a final step of 5 minutes at 72°C. Negative controls excluding templates were also processed per primer pair. Amplicon presence was confirmed using gel electrophoresis and quantified using a KAPA library quantification kit (KAPA Biosystems, Wilmington, MA). Equimolar (25 ng) PCR amplicons, from each sample, were mixed in a single tube and amplification primers and reaction buffers were removed using the AMPure kit (Agencourt Biosciences, Beverly, MA). Amplicons were pooled and sequenced

according to the manufacturer's protocol using the Illumina MiSeq (Illumina, San Diego, CA).

Analysis pipeline and data normalization

The analysis pipeline used was similar to a previously published method (Pop et al., 2016). The high-throughput multiplexed 16S rRNA reads were screened for low quality base calls and insufficient raw read lengths. Paired-end sequences were assembled using Paired-End Assembler for DNA sequences (PANDAseq) (Masella, Bartram, Truszkowski, Brown, & Neufeld, 2012) and resulting high-quality consensus sequences were de-multiplexed, trimmed of artificial barcodes and 5' and 3' primer regions followed by *de novo* clustering into operational taxonomic units (OTUs) using DNAClust (Ghodsi, Liu, & Pop, 2011) to 99% identity. Taxonomic annotation was performed using the Ribosomal Database Project (RDP) (Cole et al., 2014) (rdp.cme.msu.edu, release 10.4) database. OTUs without a match to the RDP database and with > 97% identity by the Basic Local Alignment Search Tool (BLAST)(Madden, 2003), were assigned an OTU identifier. Chimeras were identified and filtered using Perseus/UCHIME (Edgar, Haas, Clemente, Quince, & Knight, 2011).

The number of observed sequences compared to the estimated coverage can be seen in Figure S1. Sufficient sequencing depth was obtained and samples containing fewer than 100 sequences were excluded from downstream analysis (Figure S1). Data were normalized with cumulative sum scaling using metagenomeSeq (Paulson et al., 2016).

Statistical analysis

Observed number of OTUs and measures of evenness, were estimated using the normalized data and the Shannon Index (Shannon & Weaver, 1948) and Simpson's Diversity Index (Simpson, 1949) using R statistical software version 3.3.0 packages phyloseq (McMurdie & Holmes, 2013) version 1.16.2, vegan (Dixon, 2003) version 2.3.5. ggplot2 (Hadley. Wickham, 2009) version 2.1.0 was used for the visualization of results. The Kruskal-Wallis test was used to determine statistically significant differences in alpha diversity estimates across groups. Paired t-test was used to determine statistically significant differences in alpha diversity estimates across same-day influent-effluent sample pairs. Beta diversity was estimated using Bray-Curtis dissimilarity (Bray & Curtis, 1957) and compared using Analysis of similarities (ANOSIM) on the normalized data with 999 permutations. Pairwise differences were calculated using betadisper with significance assessed using Tukey's test at $p < 0.0$ using the R packages: biom, vegan, ggplot2, phyloseq. Differential abundance across samples was estimated using metagenomeSeq (Paulson et al., 2016) version 1.14.2, and visualized using ggplot2 (Hadley. Wickham, 2009) version 2.1.0.. Differences were considered statistically significant at $p < 0.05$.

Results

Sequencing

After quality control, a total of 6.1×10^6 sequences were obtained for a total of 67 samples. A total of 1494 unique assigned-species OTUs were identified and 339 unique unassigned-species OTUs were identified in total.

Influent composition across all WWTPs

Influent samples from all four WWTPs had similar α -diversity (Figure 1), and no statistically significant differences were detected in the observed number of OTUs, Simpson's index and Shannon index estimates across influent samples from all four WWTPs. Significant differences (p -value <0.01) were seen at the genus level for *Bifidobacterium*, *Blautia*, *Clostridium*, *Collinsella*, *Dorea*, *Eubacterium*, *Faecalibacterium*, *Lactococcus*, *Rhodobacter* and *Streptococcus* genera (Figure 2).

Composition of same-day influent-effluent pairs from all WWTPs

Observed number of OTUs were significantly higher in influent samples compared to effluent samples ($F = 3.38$, p -value = 0.01), however no significant differences were seen between influent and effluent samples with respect to Shannon index as well as Simpson's index estimates (Figure 3). Significant differences in relative abundance across same-day influent-effluent sample pairs from all four WWTPs were seen at the genus level for *Bifidobacterium*, *Brooklawnia*, *Faecalibacterium*, *Lactococcus*, *Mycobacterium*, , *Propionibacterium*, *Pseudomonas*, *Streptococcus* and *Trichococcus* and an unclassified OTU (OTU_489593) (Figure 4). The relative abundance of bacteria belonging to the *Mycobacterium* genus was higher in the effluent samples compared to the influent samples (Figure 4).

Community Changes Across Wastewater Treatment Processes

Figure 5 shows that influent samples were distinct from other samples collected from downstream treatments. No significant differences were detected in the observed number of OTUs, Simpson's index and Shannon index estimates within wastewater samples collected at different stages of treatment (Figure 6). Figures 7 through 10 illustrate the differentially abundant genera detected in treatment process samples from Mid-Atlantic WWTP1 (Figure 7), Mid-Atlantic WWTP2 (Figure 8), Midwest WWTP1 (Figure 9) and Midwest WWTP2 (Figure 10). At Mid-Atlantic WWTP1 the relative abundance of *Mycobacterium* was higher in effluent samples compared to influent samples and at Midwest WWTP1 both *Mycobacterium* and *Legionella* were higher in effluent samples compared to influent samples. The relative abundance of *Mycobacterium* at Mid-Atlantic WWTP1 decreased during biological treatment but increased after filtration and chlorination. However the relative abundance of *Mycobacterium* was lower in the effluent compared to the influent. At the Midwest WWTP1 the relative abundance of both *Mycobacterium* & *Legionella* increased during biological treatment and seasonal chlorination with both having higher relative abundance in effluent samples compared to influent samples. The relative abundance of *Mycobacterium* decreased during biological treatment at Midwest WWTP2, but remained stable after lagooning, with the relative abundance in the effluent being lower than the influent.

Changes in community structure from WWTP to spray irrigation site

Figure 11 shows the differences in observed number of OTUs and the Shannon index and Simpson's index estimates within samples across stages from influent through to the inlet to the pumphouse from Mid-Atlantic WWTP1 to Mid-Atlantic SI1. Significant differences were seen between influent, effluent and spray irrigation samples for Shannon index ($F= 5.238$, $p\text{-value} = 0.002$) and observed OTU number ($F= 8.945$, $p\text{-value} = <0.01$) estimates. Figure 12 illustrates that the samples taken from the pumphouse inlet, after treated effluent had undergone UV treatment and storage at the spray irrigation site clustered separately from all on-site treatment (pre- and post) and storage samples. Figure 13 illustrates the differentially abundant genera across influent and effluent samples (from Mid-Atlantic WWTP1) and spray irrigation site samples (from Mid-Atlantic SI1) before and after on-site treatment and storage. The relative abundance of *Mycobacterium* was similar, and the *Legionella* was lower, in pumphouse inlet samples compared to effluent samples.

Discussion

Influent composition across all WWTPs

Nine of the ten genera with the highest relative abundance found in influent samples were *Bifidobacterium*, *Blautia*, *Clostridium*, *Collinsella*, *Dorea*, *Eubacterium*, *Faecalibacterium*, *Lactococcus* and *Streptococcus*. Bacteria belonging to these genera are typical components of the human microbiome (dermal, intestinal, urogenital, oral and lung) (Erb-Downward et al., 2011; Marchesi, 2014). *Lactococcus*, though found at relatively low abundance in human fecal samples was observed to be

enriched in sewer environments (Vandewalle et al., 2012). The tenth, *Rhodobacter*, however, is more commonly found further downstream in WWTPs (Cyzdik-Kwiatkowska & Zielińska, 2016). *Rhodobacter* spp. are photosynthetic denitrifying bacteria which are usually isolated from freshwater or marine environments (LPSN, 2016) and animal manure lagoons (Weeks, 2012) and may have entered the influent streams at these WWTPs through surface run-off or proliferated in sewer environments with access to sunlight.

Composition of same-day influent-effluent pairs from all WWTPs

Final effluent structure could have been influenced by WWTP operational parameters, xenobiotics, metals, PPCPs, as well as microbial interactions within the WWTP. *Bifidobacterium* spp., *Faecalibacterium* spp. and *Streptococcus* spp. are common constituents of the fecal microbiome (Marchesi, 2014) and may have also played a role in fermentation during anaerobic processes within activated sludge treatment (Mara & Horan, 2008). Therefore, they were either enriched during activated sludge treatment or carried over from influent and remained abundant throughout treatment. *Pseudomonas* and *Trichococcus*, are some of the most abundantly detected genera in sewer systems and WWTPs (Gerardi, 2006; Y. Liu, Dong, & Shi, 2015; McLellan et al., 2010; Saunders, Albertsen, Vollertsen, & Nielsen, 2016; Vandewalle et al., 2012). The *Pseudomonas* genus was found to be dominant in aerobic sections, specifically manholes (Y. Liu et al., 2015), and the *Trichococcus* genus was detected in anaerobic sections of sewage systems, specifically sewage pipes (Y. Liu et al., 2015). Some of the most common nitrite

oxidizing species of bacteria isolated from activated sludge systems belong to the genus *Pseudomonas* (Rodriguez-Sanchez et al., 2014) and since *Pseudomonas* spp. are able to break down a large number of substrates (Gerardi, 2006) they are abundant in WWTPs . Bacteria belonging to the *Trichococcus* genus are also commonly isolated from activated sludge (Saunders et al., 2016).

Community Changes Across Wastewater Treatment Processes

All four WWTPs included in our study conducted biological treatment of wastewater through the use of activated sludge. However only Mid-Atlantic WWTP1 and Mid-Atlantic WWTP2 conducted chlorination throughout the year, but Midwest WWTP1 only chlorinated in the summer and Midwest WWTP2 did not chlorinate.

Mycobacterium spp. are ubiquitous in aquatic environments (Kumar, 2003) but are also used for phosphate removal in activated sludge treatment (Gerardi, 2006).

Legionella are also ubiquitous in aquatic environments (United States Environmental Protection Agency, 2001) and within WWTPs, *Legionella* spp. are known to grow in *Acanthamoeba*, *Hartmannella* and *Naegleria* present in activated sludge systems and in aerated ponds in the presence of oxygen (Caicedo, Beutel, Scheper, Rosenwinkel, & Nogueira, 2016).

Changes in community structure from WWTP to spray irrigation site

The relative abundance of *Legionella* was lower higher in effluent samples compared to influent samples and from samples collected after UV compared to those collected before UV. However, the relative abundance of *Legionella* was slightly

lower in samples collected from the pumphouse inlet compared to those collected from the inlet to the holding pond. *Mycobacterium* relative abundance remained relatively stable in all stages from influent through to the pumphouse inlet. The *Mycobacterium* and *Legionella* genera both contain potentially pathogenic species and both these genera contain species that are ubiquitous in aquatic environments (Kumar, 2003; United States Environmental Protection Agency, 2001). Bacteria belonging to the *Mycobacterium* genus are commonly detected in WWTP effluent, especially in WWTPs using biological treatment (Cai, Ju, & Zhang, 2014a; Cai & Zhang, 2013; Kaevska, Videnska, & Vasickova, 2016). Mycobacteria are hydrophobic, attach to surfaces or water-air interfaces and are resistant to chemical disinfectants (Brennan & Nikaido, 1995). Mycobacteria are also known to aggregate in water, and aggregates of *Mycobacterium* spp. larger than 41 µm in wastewater were shown to be resistant to UV and chlorine disinfection (Bohrerova & Linden, 2006). Parasitic *Legionella* spp., within amoebic hosts, are also known to be resistant to disinfection (Caicedo et al., 2016).

Mycobacterium spp. have also been shown to be correlated with assimilable organic carbon (AOC) (Jjemba et al., 2010) which is present in very high concentrations in reclaimed water storage and distribution systems (Jjemba et al., 2010) and both *Mycobacterium* spp. and *Legionella* spp. are known to survive in biofilms (Jjemba et al., 2010) which are often present in reclaimed water distribution systems (Narasimhan et al., 2005). Both *Mycobacterium* spp. and *Legionella* spp. are known to be associated with amoebae and ciliates and their occurrence is known to be correlated (Jjemba et al., 2010). The symbiotic relationship with protozoa may also

protect bacteria belonging to the *Mycobacteria* and *Legionella* genera against disinfection (Jjemba et al., 2010).

Jjemba et al (2010) have also demonstrated the regrowth of *Mycobacterium* spp. and *Legionella* spp. in effluent reservoirs and reclaimed water distribution systems due to the loss of chlorine residual (Jjemba et al., 2010). *Mycobacterium* spp. and *Legionella* spp. were often detected more frequently (at least 10-fold higher concentrations) compared to indicator bacteria (enterococci, coliforms, and *Escherichia. coli*) (Jjemba et al., 2010). Furthermore, the increases compared to indicator bacteria were found to be significantly higher at conventional WWTPs, and these opportunistic pathogens were detected numerous times within reclaimed water distribution systems in the absence of indicator bacteria (Jjemba et al., 2010). Both *Legionella* spp. and *Mycobacterium* spp. have been known to resist UV treatment at the wavelength used by Mid-Atlantic S11 (Bohrerova & Linden, 2006; Linden & Sobsey, 2005; Zeming Liu et al., 1995). The study by Bohrerova et al (2006) showed that *Mycobacterium* inactivation may be hindered by aggregation and the study by Liu et al (1995) demonstrated that scale accumulation on UV lamps could hinder *Legionella* inactivation.

A study of *Mycobacterium* behavior in French WWTPs demonstrated that primary treatment with physical-chemical decantation using lamellar settlers along with a ferric chloride coagulant and an anionic polymeric flocculant and secondary treatment with biofiltration (aerated and anoxic biofilters) was successful at the removal of approximately 98.6 % of mycobacteria during wastewater treatment (Radomski et al., 2011). None of the WWTPs in our study performed this type of

treatment. Only two of the four WWTPs (Mid-Atlantic WWTP1 and Mid-Atlantic WWTP2) performed primary settling, but with conventional settling tanks. All four WWTPs used conventional activated sludge processes rather than biofilters and Mid-Atlantic WWTP1 used sand filtration, while Mid-Atlantic WWTP2 used multimedia filtration, prior to chlorination. Midwest WWTP1 and Midwest WWTP2 did not perform any kind of filtration with Midwest WWTP1 only conducting chlorination in the summer and Midwest WWTP2 conducting no chlorination at all. Mid-Atlantic SII conducted UV treatment and open-air storage before spraying.

The study by Jjemba et al (2010) determined that several treatment configurations including trickling filters with tertiary treatment with sand filtration, activated sludge with secondary filtration, membrane bioreactor processes were able to reduce indicator bacteria but opportunistic pathogens like *Mycobacterium* spp. and *Legionella* spp. were able to regrow within the reclaimed water distribution system to concentrations higher than those of indicator bacteria (Jjemba et al., 2010).

Implications for future research

The patterns observed in this exploratory analysis provide only a cross-sectional view of the bacterial communities present in conventionally treated wastewater and reclaimed water due to the fact that we were only able to take grab samples and had limited access to treatment plants, which also resulted in an unbalanced sampling pattern. Furthermore, all effluent samples had fewer reads compared to influent samples which may have led to bias while estimating the number of observed OTUs. This difference in sequencing depth may have contributed

towards some of the difference observed between influent and effluent samples. The observed species number may have also been inflated due to spurious OTU artifacts. The observed differences in the same-day pairs may have been heavily influenced by the Mid-Atlantic WWTP1 and Midwest WWTP2 samples, the WWTPs with the most complete pairs of same-day samples available for analysis. Furthermore, since not all samples compared were collected on the same day some of the structural differences could be attributed to this temporal difference. The treatment performed at these two WWTPs, however, could be considered representative of their particular geographic regions. We also included one spray irrigation site in our analysis so the effects seen there may not be wholly generalizable but this spray irrigation site could also be considered typical of landscape irrigation sites in this region.

16S rRNA gene sequencing does not allow us to determine whether the abundant genera found in our samples were metabolically active or provide us with any information on their functional roles. 16S rRNA gene sequencing does not always have the discriminatory power to provide species level and strain level information, so we cannot be absolutely certain of the detection of genera that contain potentially pathogenic species. However, numerous studies have found similar patterns in wastewater and reclaimed water using both culture-based and culture-independent techniques. The findings from this study could also be used to develop long term studies of wastewater and reclaimed water using composite sampling, which may provide us with a more comprehensive evaluation of community structure. Future analysis can build on the findings of this study by utilizing more advanced techniques such as metatranscriptomics. Results from this

study could be compared to the analysis of samples from more advanced wastewater and reclaimed water treatment processes like those used in areas of the U.S. that permit the use of reclaimed water for the irrigation of food crops. This would allow us to determine the community differences between conventionally treated wastewater and wastewater that has undergone more advanced treatment. Finally results from such studies can be used to develop water quality parameters and regulations that are more protective of public health and to also optimize treatment processes and operational parameters and develop effective reuse-site treatment technologies.

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Figures

Figure 1 Alpha diversity estimates and observed species number in influent samples from all four wastewater treatment plants (WWTPs). No statistically significant differences in alpha diversity estimates were found across influent samples from all four WWTPs.

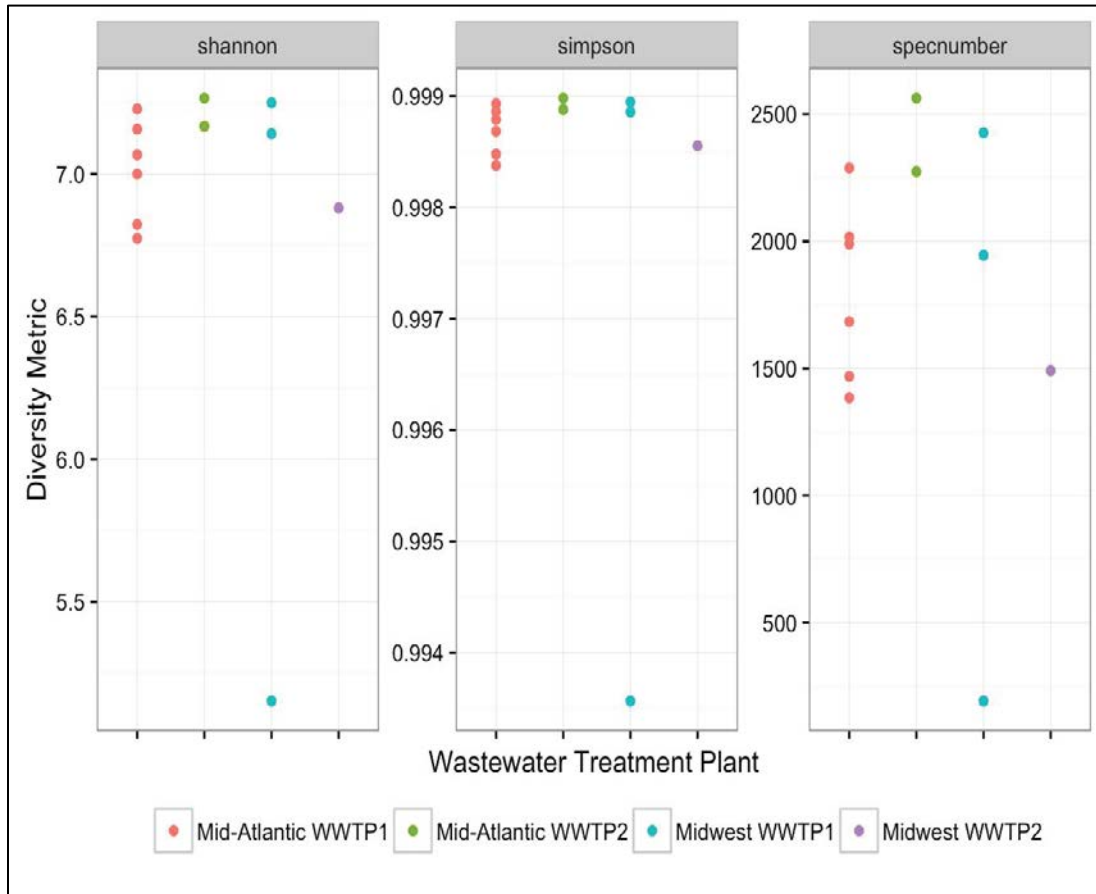


Figure 2 Significantly differentially abundant (p -value <0.01) bacterial genera across influent samples from all four WWTPs. The most abundant bacteria belong to genera predominantly associated with the human microbiome and sewer infrastructure.

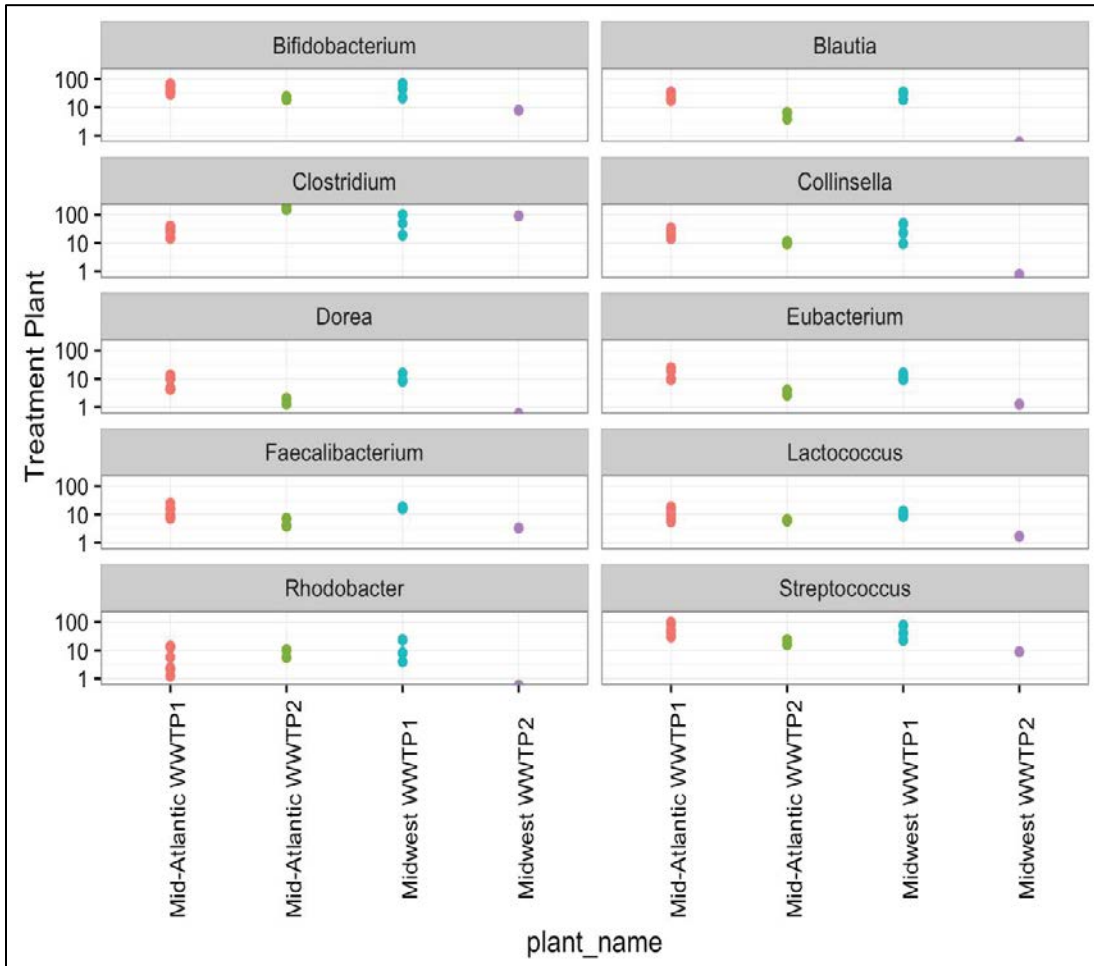


Figure 3 Alpha diversity estimates and observed species number in same-day influent-effluent pairs from all four WWTPs. Significant differences (p -value < 0.01) in observed species number were detected.

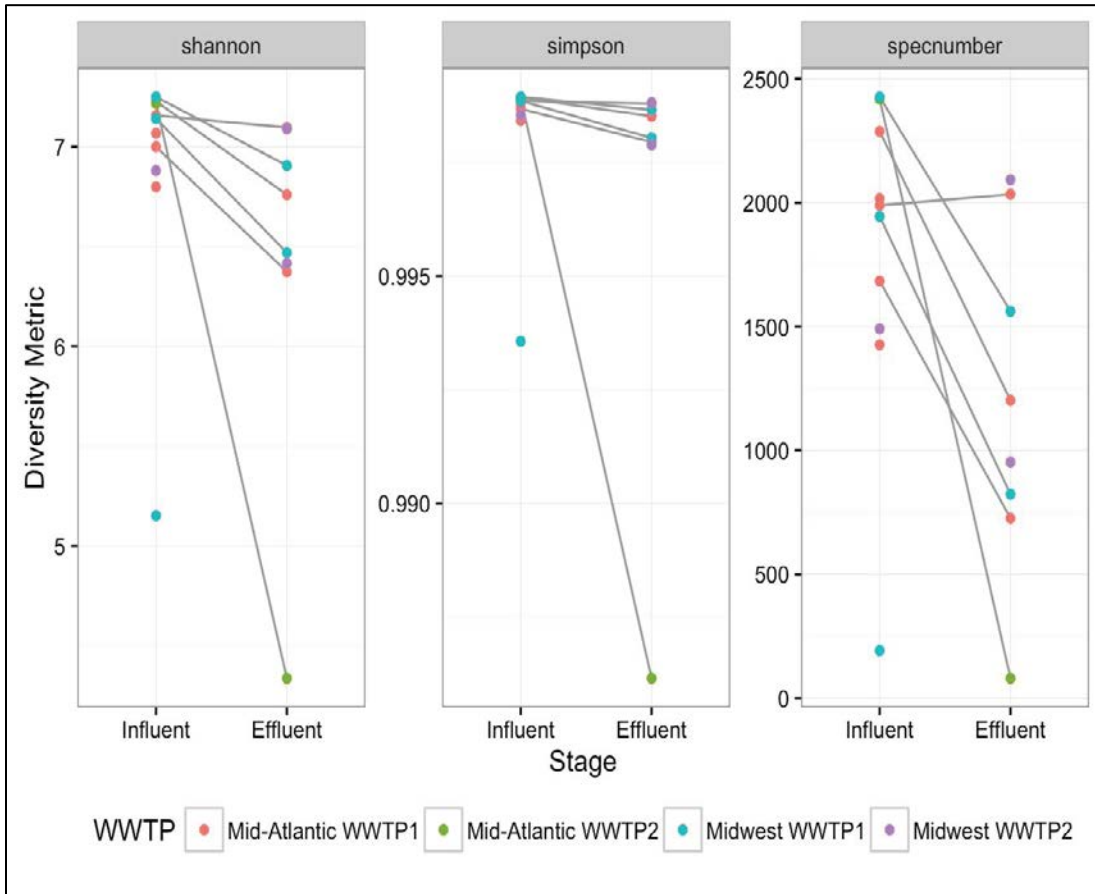


Figure 4 Significantly differentially abundant (p -value <0.01) bacterial genera across same-day influent-effluent pairs from all four WWTPs. The most abundant bacteria belong to genera predominantly associated with the human microbiome, sewer infrastructure and biological wastewater treatment processes.

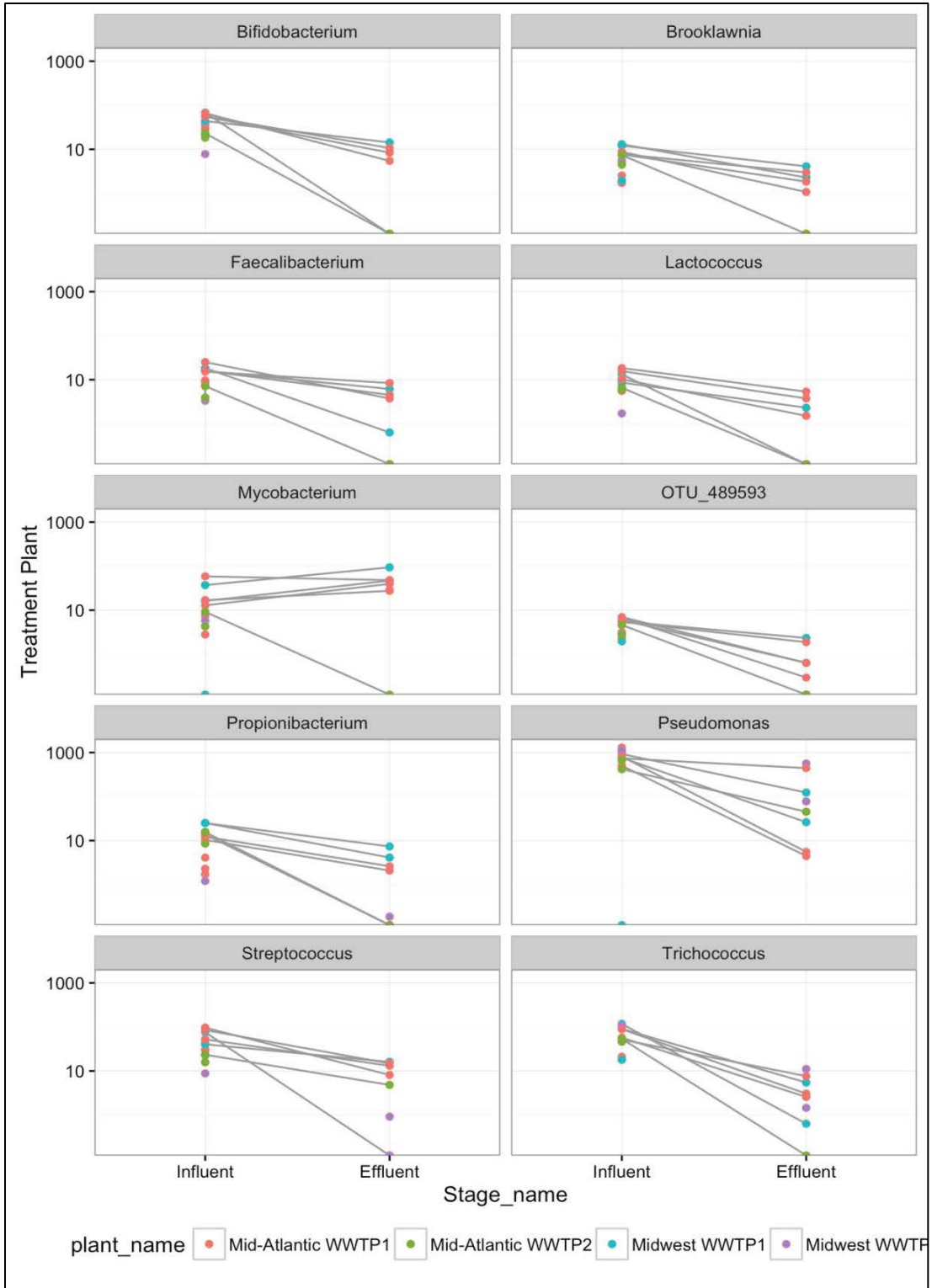


Figure 5 PCoA plot using Bray-Curtis dissimilarity showing influent samples clustering apart from samples taken from downstream wastewater treatment processes.

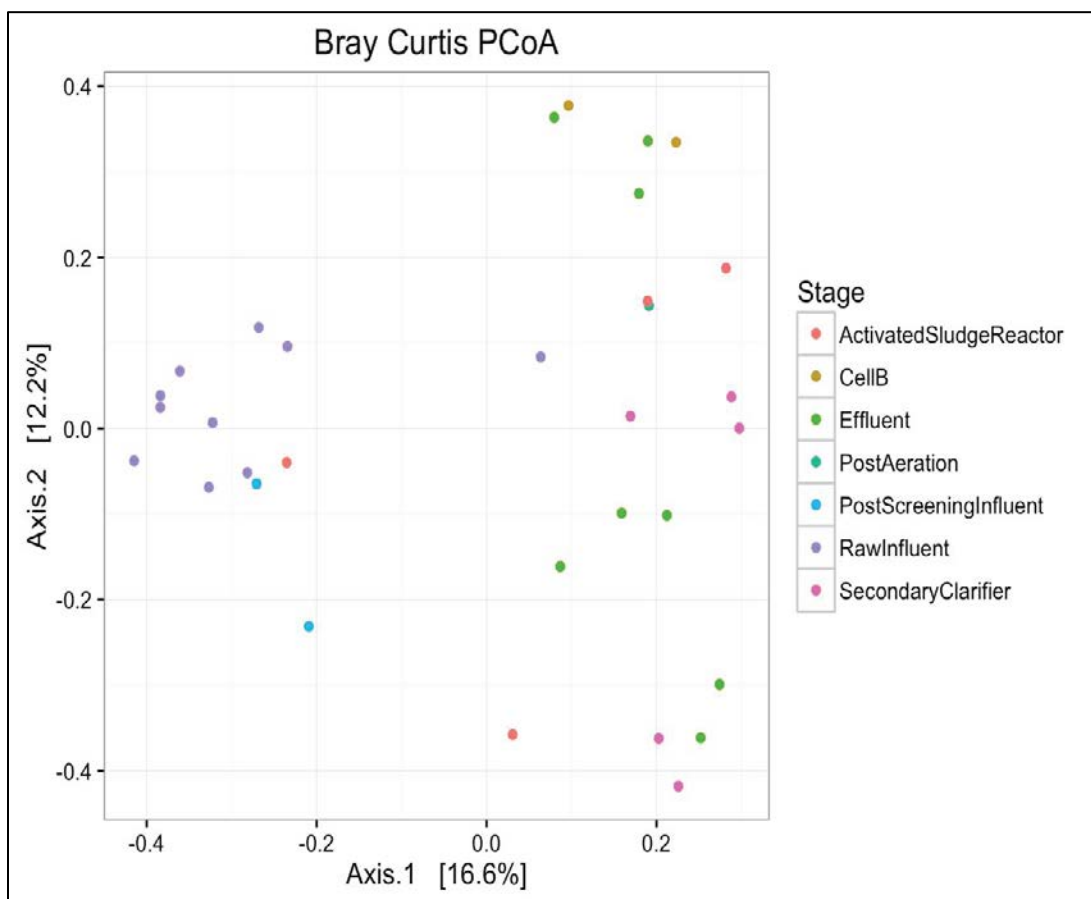


Figure 6 Alpha diversity estimates and observed species number in treatment process samples from all four wastewater treatment plants (WWTPs). No statistically significant differences in alpha diversity estimates were found across treatment process samples from all four WWTPs.

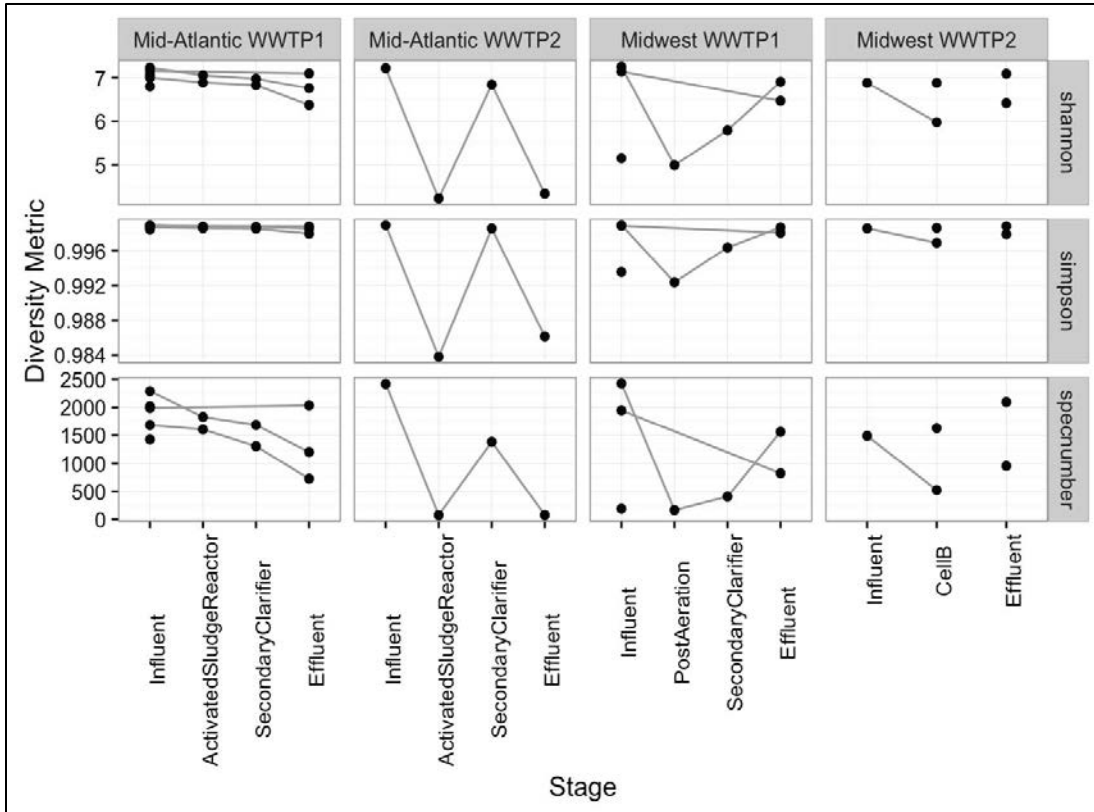


Figure 7 Significantly differentially abundant (p -value <0.01) bacterial genera (top 10) across the various treatment processes performed at Mid-Atlantic WWTP1.

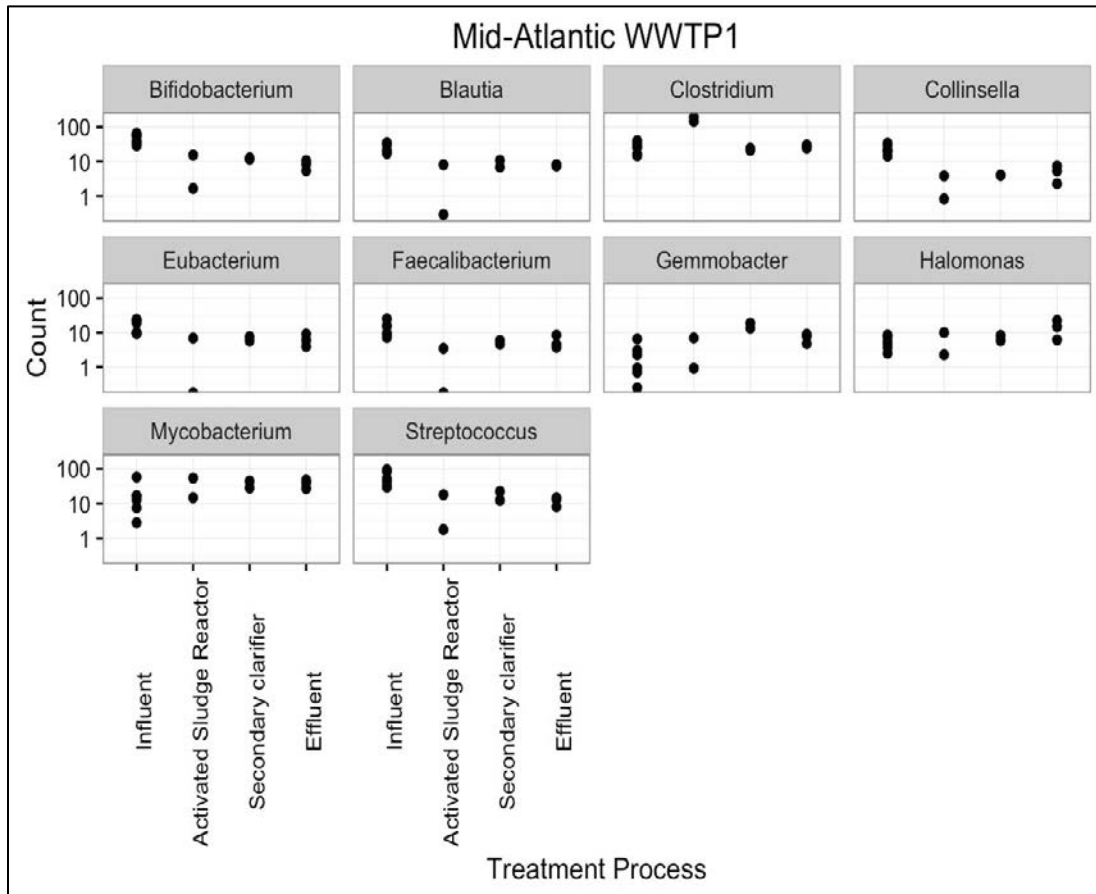


Figure 8 Significantly differentially abundant (p -value <0.01) bacterial genera (top 10) across the various treatment processes performed at Mid-Atlantic WWTP2.

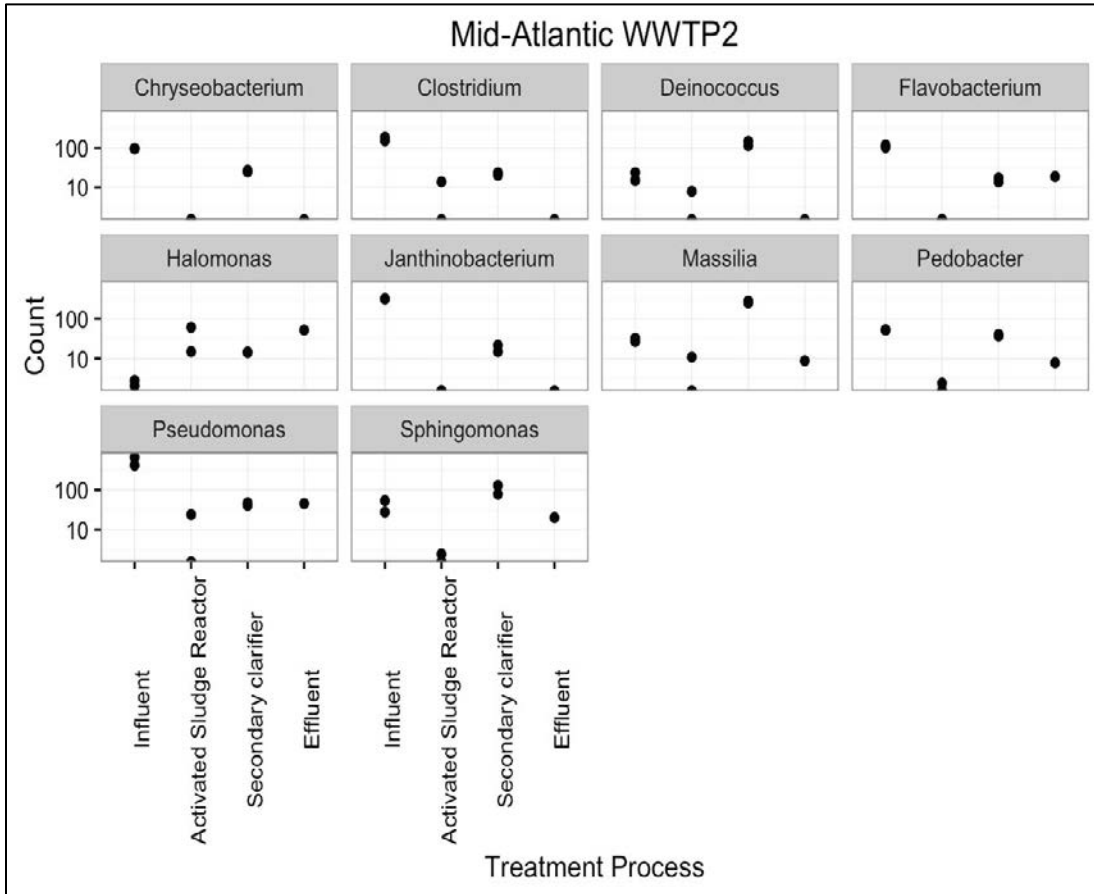


Figure 9 Significantly differentially abundant (p -value <0.01) bacterial genera (top 10) across the various treatment processes performed at Midwest WWTP1.

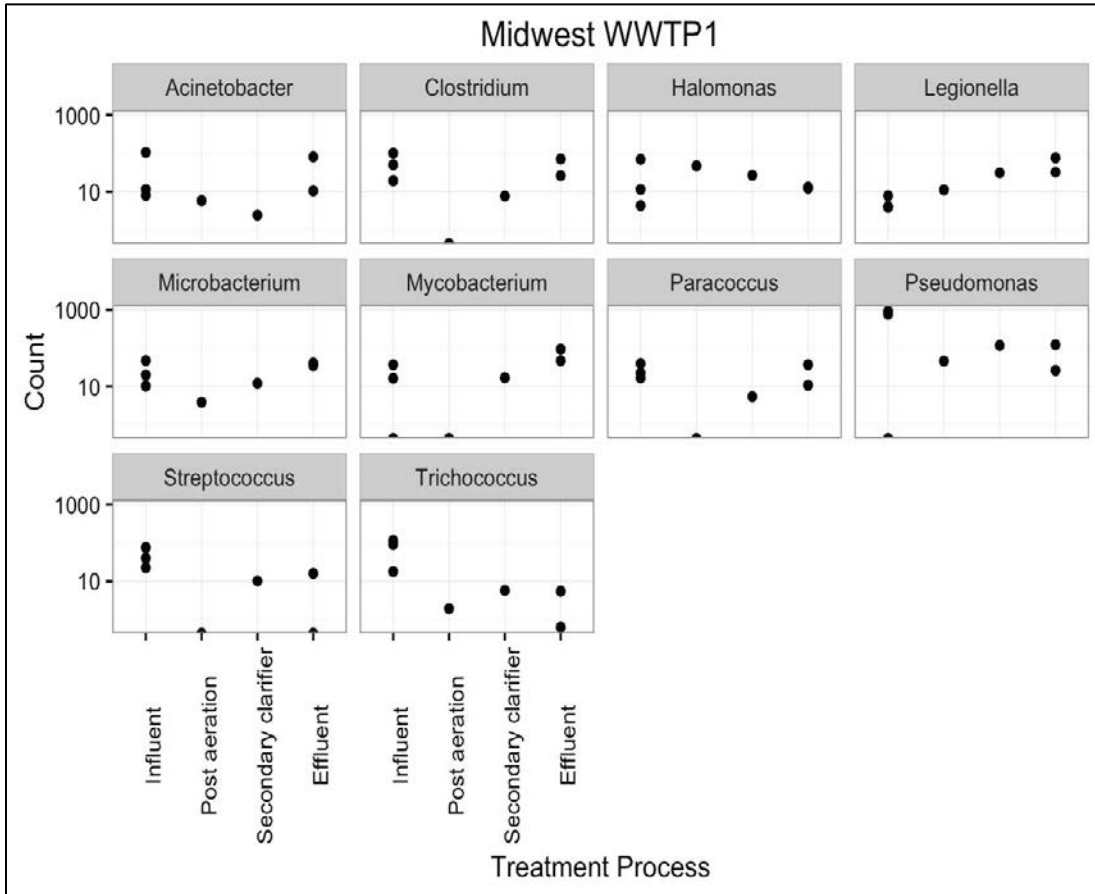


Figure 10 Significantly differentially abundant (p -value <0.01) bacterial genera (top 10) across the various treatment processes performed at Midwest WWTP2.

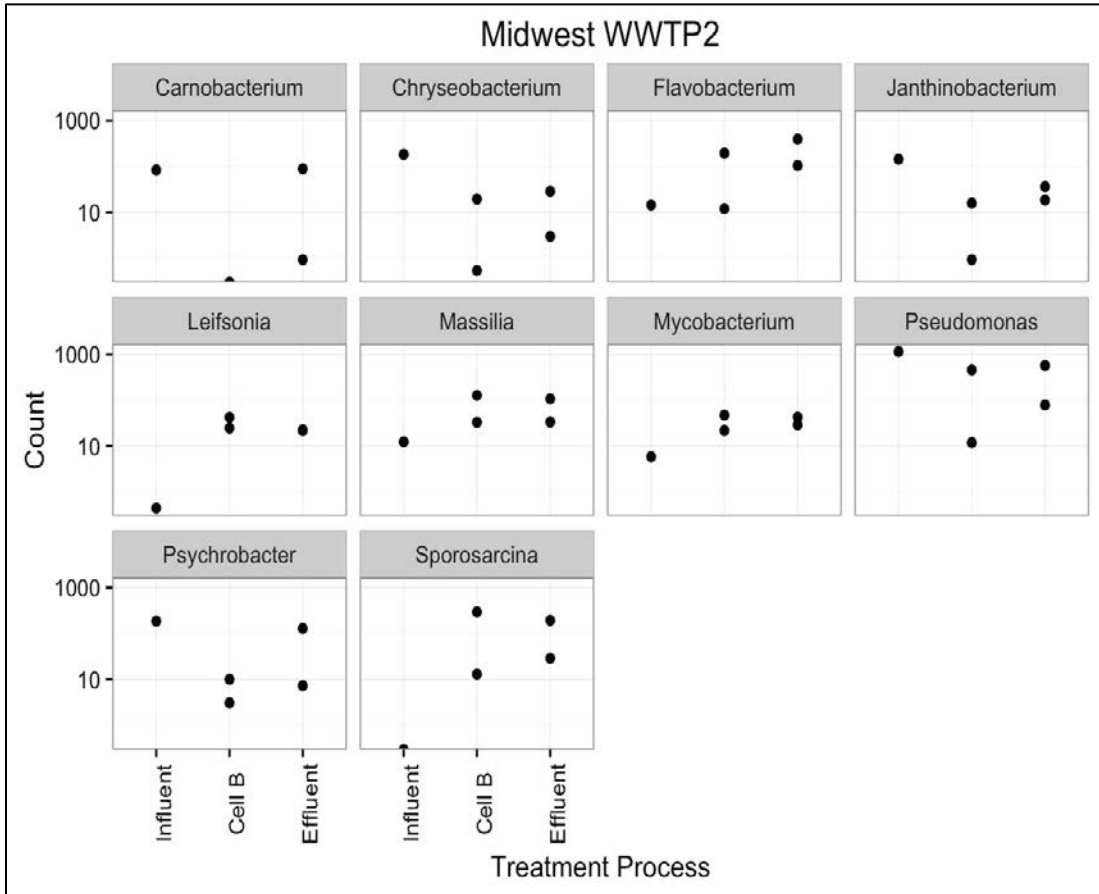


Figure 11 Alpha diversity estimates and observed species number in samples from WWTP influent stage at Mid-Atlantic WWTP1 to spray irrigation site pumphouse stage at Mid-Atlantic SI1. Significant differences in alpha diversity estimates were found for Shannon index ($F= 5.238$, p -value = 0.002) and observed species (OTU) number ($F= 8.945$, p -value = <0.01) estimates.

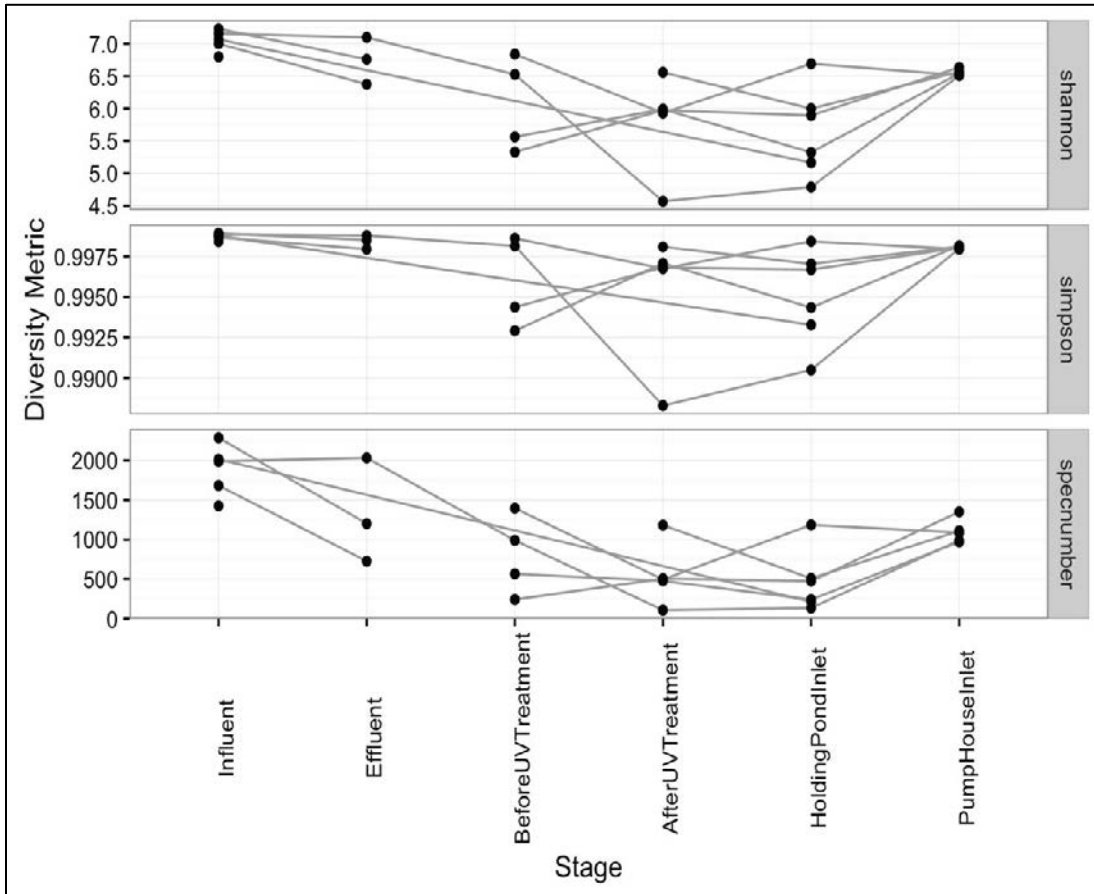


Figure 12 PCoA plot using Bray-Curtis dissimilarity showing pumphouse inlet samples clustering apart from samples after on-site treatment and storage (Before UV treatment, After UV treatment and Holding Pond inlet).

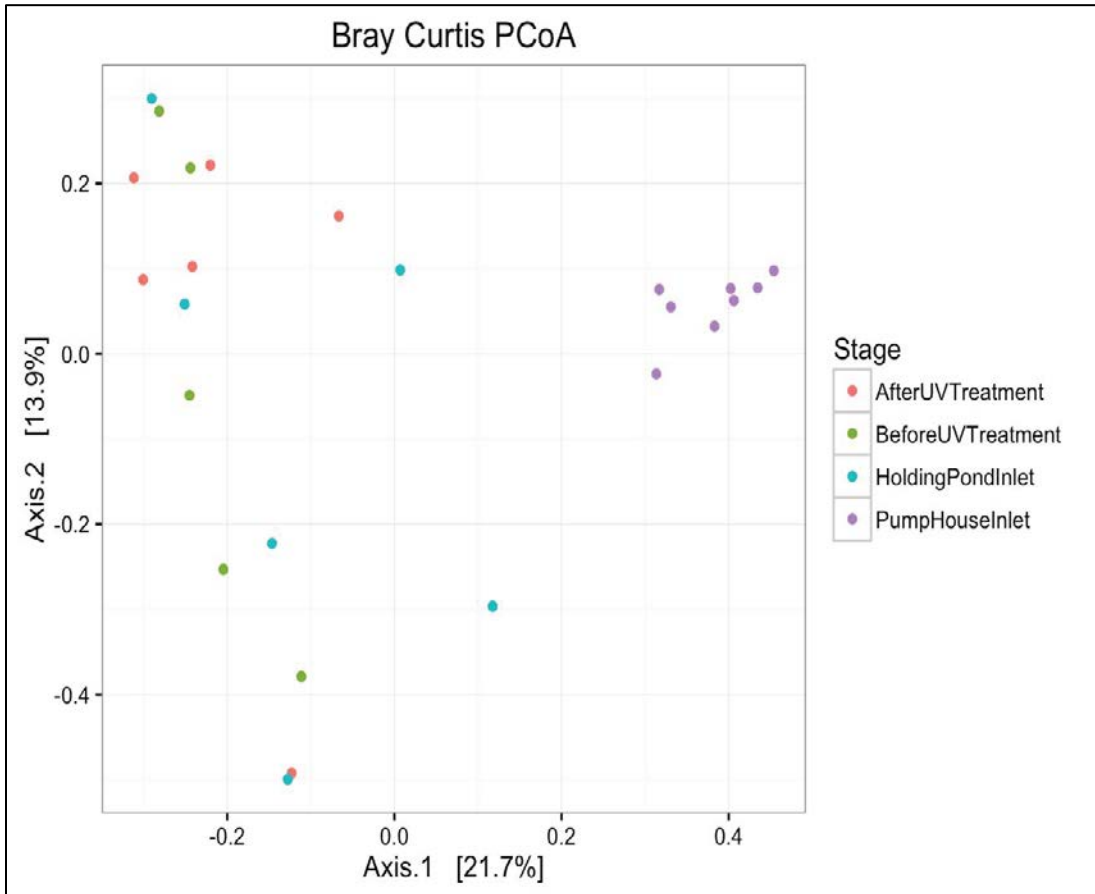
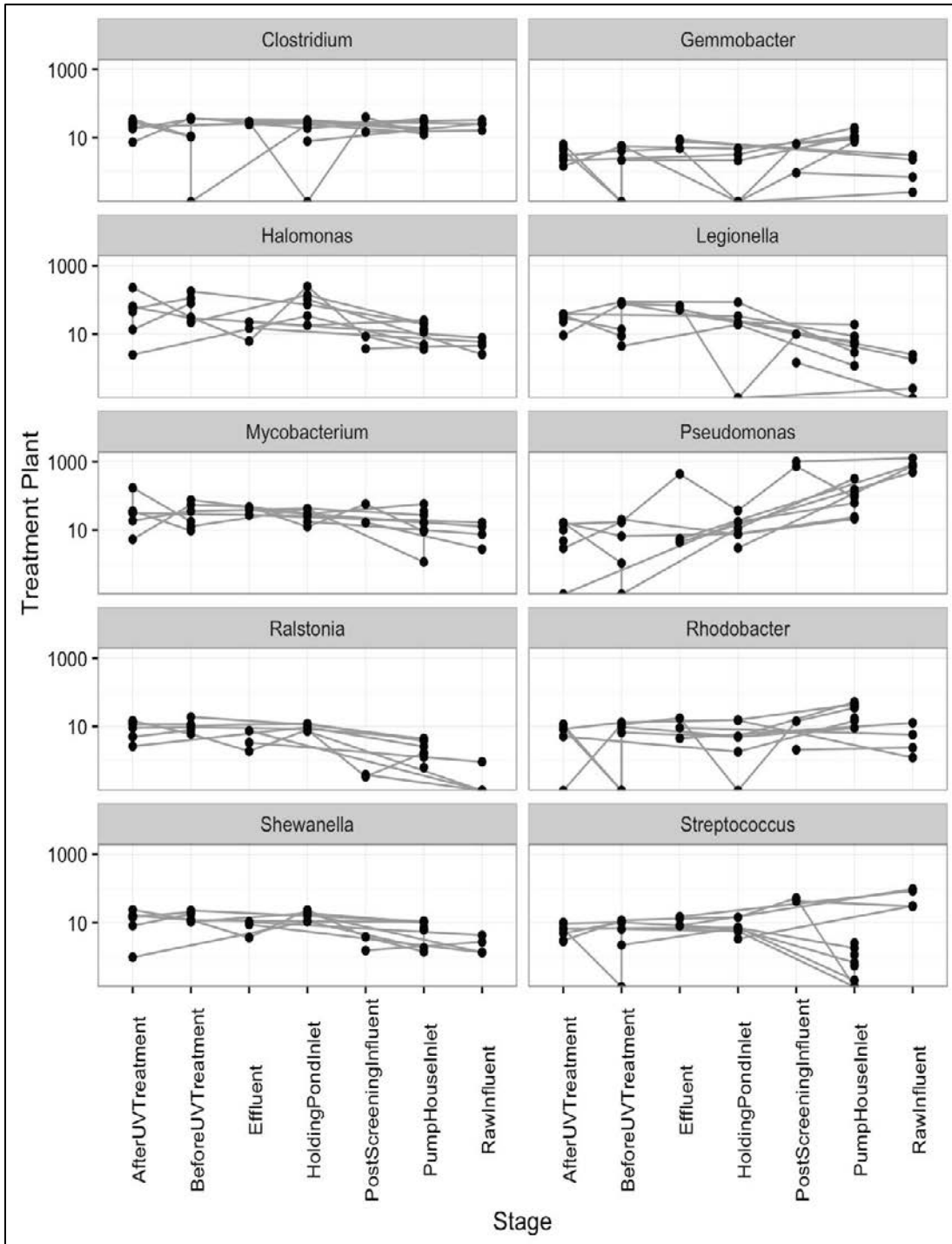


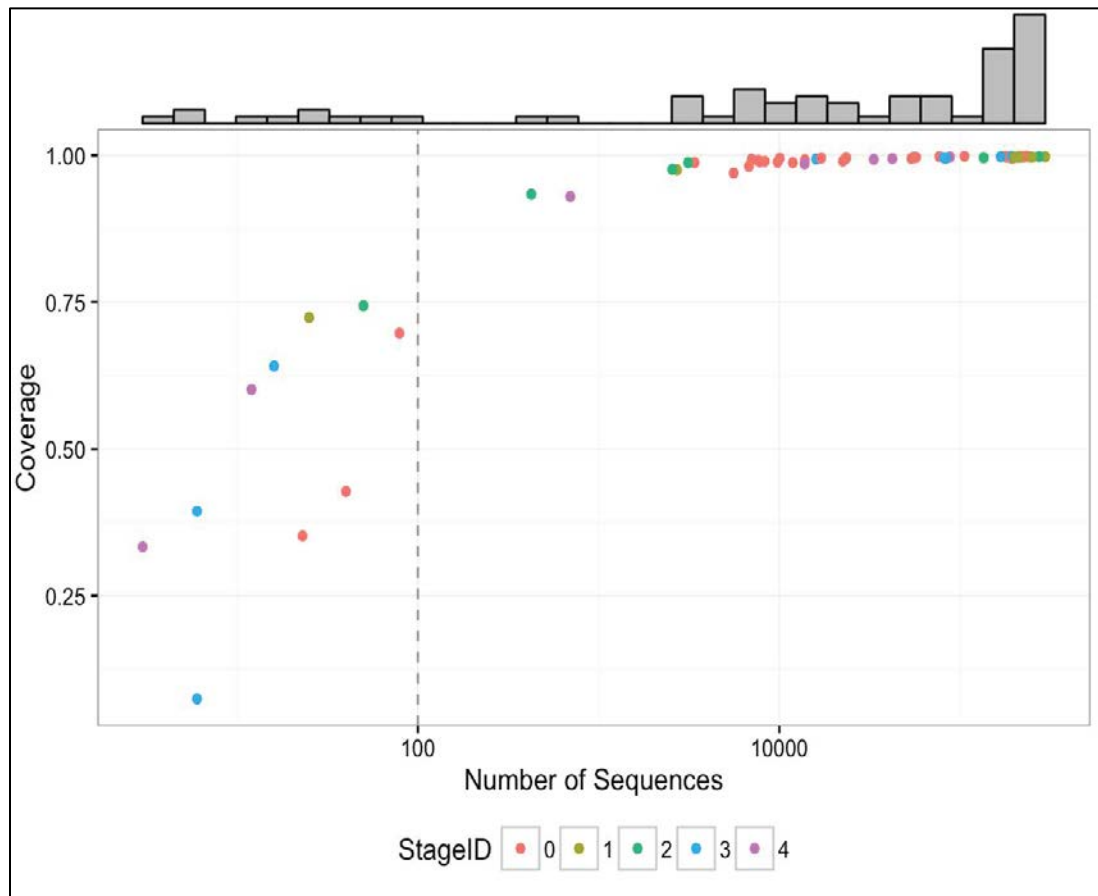
Figure 13 Significantly differentially abundant (p -value <0.01) bacterial genera (top 10) across the treatment at Mid-Atlantic WWTP1, transport to and treatment and storage, at Mid-Atlantic SII.



Appendix A

Supplementary Figures

Figure S1 Number of observed sequences compared to the estimated coverage with a histogram indicating the distribution of samples relative to the number of sequences per sample. Samples with fewer than 100 sequences were filtered.



- 0 – Spray Irrigation Site
- 1 – Influent, Post-Screening Influent
- 2 – Activated Sludge, Post Aeration
- 3 – Secondary Clarifier, Cell B
- 4 – Effluent

Chapter 7: Zero-valent iron-biosand filtration is capable of reducing antimicrobial concentrations in unbuffered conventionally-treated reclaimed water

(Prachi Kulkarni, Greg A Raspanti, Anthony Q Bui, Rhodel N Bradshaw, Manan Sharma, Amir Sapkota, Amy R Sapkota)

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Abbreviations

AMP	Ampicillin
AZI	Azithromycin
CIP	Ciprofloxacin
ERY	Erythromycin
FC	Free Chlorine
FDR	False Discovery Rate
HLB	Hydrophilic-Liphophilic Balance

HPLC-MS/MS	High Performance Liquid Chromatography-Tandem Mass Spectrometry
LIN	Linezolid
LOD	Limit of Detection
OXA	Oxacillin
OXO	Oxolinic Acid
PCA	Principal Component Analysis
PEN	Penicillin G
PERMANOVA	Permutational Multivariate Analysis of Variance
PIP	Pipemidic Acid
PPCP	Pharmaceuticals and Personal Care Products
SUL	Sulfamethoxazole
TC	Total Chlorine
TCC	Triclocarban
TDS	Total Dissolved Solids
TET	Tetracycline
U.S.	United States
VAN	Vancomycin
WWTP	Wastewater Treatment Plant
ZVI	Zero-valent Iron

Abstract

The use of reclaimed water may be a necessary element of water management and irrigation programs as freshwater resources continue to dwindle. However, if reclaimed water is going to be adopted as an alternative freshwater resource within the existing conventional wastewater treatment infrastructure typically found across the U.S., it is necessary to investigate reuse site-based treatment solutions that can further reduce contaminants that persist in reclaimed water. We explored the efficacy of a zero-valent iron (ZVI)-biosand filter in removing residual antimicrobials present in conventionally treated reclaimed water. 13 antimicrobials commonly found in reclaimed water were quantified using high performance-liquid chromatography-tandem mass spectrometry in unbuffered chlorinated effluent from a tertiary treatment plant before and after filtration through a 50:50;v:v macro-scale ZVI-biosand filter over a two-month period using a greenhouse-based experiment designed to simulate reuse site conditions. Several classes of antimicrobials were included in the study – β -lactam (ampicillin, oxacillin, penicillin G), quinolone (ciprofloxacin, oxolinic acid, and pipemidic acid), macrolide (azithromycin, erythromycin), glycopeptide (vancomycin), oxazolidinone (linezolid), sulfonamide (sulfamethoxazole), tetracycline (tetracycline) and an antimicrobial agent (triclocarban). Significant (p -value <0.01) reductions in concentrations were observed for all quinolones (ciprofloxacin, oxolinic acid, pipemidic acid) and macrolides (azithromycin, erythromycin), one β -lactam (penicillin), linezolid and vancomycin. 100% reduction was achieved for erythromycin. The median concentration of ciprofloxacin, the most predominant antimicrobial detected, was reduced from 233.5 ng/mL, in reclaimed

water, to 29.1 ng/mL in ZVI-biosand-filtered reclaimed water. Long-term analysis including the impact of ZVI-biosand filtration on other pharmaceutical and personal care products (PPCPs), bacterial, viral and parasitic pathogens, and salinity is required in order to determine the efficacy of ZVI-biosand filtration as a comprehensive point-of-use technology for reclaimed water used in irrigation applications.

Introduction

Several areas of the United States (U.S.) are adopting the use of nontraditional water sources, such as reclaimed water, due to freshwater resource stresses resulting from climate, demographic, and land-use changes (Asano, 2007; EPA, 2012a; U.S.Global Change Research Program, 2015). Historically drought-prone areas like California have extensive reclaimed water use patterns and reuse regulations that are protective of public health (EPA, 2012a). For instance, California state regulations, under Title-22 of the California Department of Public Health Regulations Related to Recycled Water, require reclaimed water used for agricultural, as well as landscape, irrigation to be oxidized, coagulated, filtered and disinfected (Asano, 2007; CA DPH, 2009). This type of extensive treatment is not common in many areas in which reclaimed water use is now emerging (EPA, 2012a). These previously low-use areas, including Maryland, New Jersey and Delaware, are conducting proactive water resource management but existing practices, reclaimed water use patterns and infrastructure limitations in these regions may present challenges to the development of sustainable reclaimed water use solutions that are protective of public health (Asano, 2007; EPA, 2012a; U.S.Global Change Research Program, 2015).

Additional challenges are posed by the limitations of current reclaimed water regulations in the U.S. Specifically, the absence of legally binding federal regulations and the resulting geographical variation in regulations and treatment requirements, the lack of monitoring of trace constituents such as pharmaceuticals and personal care products (PPCPs), including antimicrobials, and the variability of reuse site monitoring requirements (EPA, 2012a). Although present at relatively low

concentrations in reclaimed water, multiple antimicrobial classes have been detected and the human health impacts of chronic exposure to antimicrobials present in reclaimed water is unknown (Kim & Aga, 2007). Furthermore, the combination of antimicrobials, nutrients and bacteria in reclaimed water could potentially result in the selection of antibiotic resistance among bacterial populations present in this water type (Fahrenfeld et al., 2013; Negreanu et al., 2012).

If reclaimed water is going to continue to be explored and adopted as an alternative freshwater resource within the existing conventional wastewater treatment infrastructure typically found across the U.S., it is necessary to investigate reuse site-based treatment solutions that can further reduce contaminants that persist in reclaimed water. A potential candidate for reuse site-based treatment technology is zero-valent iron (ZVI)-biosand filtration. Initially developed for the remediation of groundwater contaminated with chlorinated compounds, ZVI is now also used for the elimination of several other contaminants (heavy metals, pesticides, nutrients etc.) (EPA, 2015; Gillham et al., 2010; You et al., 2005). ZVI-based remediation is achieved by chemical reduction followed by precipitation or co-precipitation, or immobilization through adsorption (EPA, 2015). Research is also being conducted on the ability of ZVI treatment to remove drinking water contaminants including disinfection by-products and pathogenic bacteria and viruses (Chiu, 2013; Ingram et al., 2012).

Laboratory-scale studies of filtration systems using macro- and nano-scale ZVI have demonstrated the achievement of concentration reductions ranging from 80 to 99% of ciprofloxacin (Perini et al., 2014; Stieber et al., 2011), tetracycline and

oxytetracycline (Fu et al., 2015; Hanay et al., 2014), amoxicillin and ampicillin (Ghauch et al., 2009), and metronidazole (Fang et al., 2011) among others. Many of these studies analyzed pH-buffered solutions of single antibiotics at concentrations that may not always be relevant to conventionally-treated reclaimed water.

Maintenance of an artificially controlled pH is not feasible if ZVI-biosand filtration is to be used as a point-of-use treatment for agricultural or landscape irrigation, and the unknown health effects of nanoparticles (Gwinn & Vallyathan, 2006) makes the use of nano-scale ZVI undesirable for this type of application. Therefore, our goal was to explore the efficacy of macro-scale ZVI-biosand filtration in reducing concentrations of a mixture of antimicrobial residues present in unbuffered reclaimed water that had undergone conventional wastewater treatment.

Methods

Reclaimed Water Collection Site

Reclaimed water was collected from a tertiary wastewater treatment plant (WWTP) located in the Mid-Atlantic United States. A schematic of the WWTP, including the specific sampling locations at the plant, is illustrated in Figure 1. The WWTP is located in a rural town with a population of 4,808 at the time of the 2010 census (U.S. Census Bureau, 2016) with land use including suburban developments and farmland (Maryland Department of Commerce, 2016). The WWTP treats between 1135.62 and 1419.53 m³ of wastewater per day and has a maximum daily capacity of 1892.70 m³. Incoming raw wastewater undergoes grinding for large debris removal followed by grit removal and then activated sludge treatment followed by

secondary clarification. From December 1st to February 28th the secondary clarified wastewater undergoes ultraviolet radiation treatment followed by surface water discharge. From March 1st to November 30th the secondary clarified wastewater is piped to an open-air lagoon for consistent volume maintenance and chlorinated before land application by spray irrigation onto fields of reed canarygrass (*Phalaris arundinacea* L.) to achieve further nutrient removal and ultimate groundwater recharge. Permission to collect reclaimed water for the purposes of this study was granted by the Town Administrator.

ZVI-Biosand Filter

A commercially available biosand filter (HydrAid® BioSand Water Filter, NativeEnergy, Burlington, VT) was adapted for this experiment. The filter is made of opaque plastic with a height of 0.77 m and a diameter of 0.42 m. Sand (provided with the filter) and ZVI (Peerless Metal Powders and Abrasives Company, Detroit, MI) were sieved to achieve a particle size range of 400 µm to 625 µm. Equal parts by volume of sand and ZVI were thoroughly mixed and added to the filter. Once every week, from March 31 to June 21, 2016, the filter was flushed with 20 L of ultrapure water. ZVI filtration of reclaimed water began on June 21, 2016. The approximate flow rate of the ZVI-biosand filter was 5.6 L/min and since reclaimed water was manually filtered, a mark was made on the ZVI filter to maintain an approximately consistent flow rate throughout the experiment.

Sample Collection

A schematic describing the experimental design, including sample collection, is illustrated in Figure 2. The experiment was designed to simulate reuse site conditions. Chlorinated effluent was collected from the WWTP and delivered to the reuse site (University of Maryland (UMD) Research Greenhouse Complex), where it was stored in 189 L rain barrels (Cat # 81313 Algreen Products Inc., Ontario, Canada) (Rain barrel composites) until needed. Every five days, water from the rain barrels (Reclaimed Water) was filtered (ZVI-biosand filtered reclaimed water) at the point of use. Tap water, supplied to the greenhouse from a drinking water treatment plant, was included in the analysis in order to determine the concentration of antimicrobials in potable quality water. All samples were collected in June, July and August of 2016, brought to the laboratory on ice after collection and stored at -80 °C until extraction. All samples were collected in 500 mL sterile polyethylene Nalgene® Wide Mouth Environmental Sampling Bottles (Nalgene, Lima, OH).

Sample Processing

All samples were analyzed in September 2016. 13 antimicrobials, commonly used in the U.S. (Sapkota et al., 2007; U.S. National Library of Medicine. National Institutes of Health., 2015), and previously detected in wastewater samples (Sapkota et al., 2007; Zhang & Li, 2011), were analyzed: antibacterial agent – triclocarban (3,4,4'-trichlorocarbanilide; TCC); β lactams - ampicillin (AMP), oxacillin (OXA) and penicillin G (PEN); a glycopeptide – vancomycin (VAN); macrolides - azithromycin (AZI) and erythromycin; an oxazolidinone - linezolid (LIN); quinolones

- ciprofloxacin (CIP), oxolinic acid (OXO) and pipemidic acid (PIP); a sulfonamide – sulfamethoxazole (SUL) and a tetracycline - tetracycline (TET). Caffeine, an indicator of human fecal contamination (Potera, 2012), was also analyzed.

Antimicrobial concentrations in all samples were quantified using a previously published method (Sapkota et al., 2007), with modifications. Samples were thawed at room temperature 24 hours prior to extraction and a 200 mL aliquot was used for extraction. A 10 μ L aliquot of a methanol stock solution containing 10 μ g/mL each of surrogate standards (Linezolid-d3, Oxolinic Acid d5, Triclocarban-13C6, Toronto Research Chemicals Inc., Cat #s L466502, O857502 and T774202 respectively and Caffeine-13C3, Sigma-Aldrich Co. LLC, Cat # C0582) was added to each 200 mL aliquot of each sample, followed by thorough mixing. All samples were then extracted using Oasis HLB (60 mg) cartridges (Waters Corp; Milford MA), conditioned with 3 mL methanol followed by a 3 mL water rinse. The samples were loaded under minimal vacuum using Visiprep 12-port Vacuum Manifolds (Sigma-Aldrich, St. Louis, MO). Cartridges were then washed with 1 mL of water containing 5% methanol by volume and analytes eluted with 6 mL of acetonitrile with 0.2% formic acid followed by 3 mL of methanol:acetone mix (50:50; vol:vol) under minimal vacuum. Each extract was dried under nitrogen at 40°C and reconstituted in 1 mL of acetonitrile:0.1 % formic acid mix (50:50; vol:vol). High performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) was used to detect and quantify antimicrobials using an Agilent 1290 Infinity II LC System tandem mass spectrometer (Agilent Technologies Inc., Santa Clara, CA). Chromatographic separation was achieved by an Agilent Zorbax Eclipse Plus C18 Rapid Resolution

HD 2.1x50mm, 1.8 μm column (Agilent Technologies Inc., Santa Clara, CA) with a pre-column filter (Phenomenex, Torrance CA). Raw concentration readings were adjusted for recovery. The complete list of antimicrobials, surrogate standards, their corresponding limits of detection (LOD) and percent recoveries are listed in Table 1.

Environmental Parameters

The concentrations of environmental parameters free chlorine (FC), total chlorine (TC), nitrate (NO_3) and nitrite (NO_2) were measured, for all samples, using a DR900 Colorimeter (Hach Company, Loveland, CO) using the reagents DPD Free Chlorine Reagent Powder Pillow, DPD Total Chlorine Reagent Powder Pillow, NitraVer 5 Nitrate Reagent Powder Pillow, NitriVer 2 Nitrite Reagent Powder Pillow (Hach Company, Loveland, CO, Cat #s 2105669, 2106169, 2105569, 2107569 respectively). pH was measured for all samples using Fisher Scientific™ accumet™ AB15+ Basic pH meter (Fisher Scientific, Hampton, NH) and total dissolved solids (TDS) using an Etekcity Digital Handheld TDS Meter (Etekcity, Anaheim, CA). The average daytime and nighttime temperatures in the greenhouse were 29.9 °C and 25.4 °C, respectively.

Statistical Analysis

All statistical analyses were performed using R (version 3.2.3) (R Foundation for Statistical Computing, 2016). A conservative non-parametric rank-based approach was used for analysis due to the relatively small sample size, certain antimicrobials occurring at high concentrations (reflective of prescription patterns) and certain

antimicrobials having a large number of non-detects (reflective of its particular class) (Helsel, 2012). Differences between reclaimed water and ZVI-biosand-filtered reclaimed water groups were determined using the Wilcoxon signed-rank test for paired samples and the Kruskal-Wallis test was used to compare differences between all independent samples using the stats package version 3.4.0 (R Foundation for Statistical Computing, 2016). Pairwise testing was conducted using the Dunn's test with a false discovery rate (FDR) correction. Principal component analysis (PCA) was used to explore which groups were closely associated and permutational multivariate analysis of variance (PERMANOVA) was used to quantify the dissimilarity between the groups using the package vegan version 2.4-1 (Oksanen et al., 2016) using Euclidean distance and 999 permutations. Visualization was performed using the package ggplot2 version 2.1.0 (H Wickham, 2009). In all cases, p -values ≤ 0.05 were defined as statistically significant and the FDR used was 5%.

Results and Discussion

Collection of chlorinated effluent, transport to greenhouse and storage in rain barrels

Caffeine was detected at levels above the LOD in all samples from all groups, including the chlorinated effluent and reclaimed water groups, indicating that the antimicrobials present in the samples had a human fecal origin (Potera, 2012). Chlorinated effluent and reclaimed water groups contained samples in which ampicillin, oxacillin and tetracycline were found at below the LOD concentrations (Table 2). The chlorinated effluent group also contained samples in which pipemidic

acid was detected at concentrations below the LOD (Table 2). Oxacillin was found at concentrations above the LOD only in the reclaimed water group and in only three of all nine samples belonging to this group (Table 2). Ciprofloxacin, followed by sulfamethoxazole and triclocarban, were detected at the highest concentrations, and tetracycline and oxacillin at the lowest concentrations, in chlorinated effluent samples (Table 3). Ciprofloxacin and sulfamethoxazole are among the most commonly prescribed antibiotics in the U.S., and triclocarban is a ubiquitous ingredient of personal care products (Centers for Disease Control and Prevention (CDC), 2015; Sapkota et al., 2007). All three have also been detected at high concentrations in WWTP effluents or downstream from WWTP discharge locations (Sapkota et al., 2007; Zhang & Li, 2011). Tetracycline is often found at very low concentrations in WWTP effluent due to its low therapeutic use, and oxacillin belongs to the β -lactam class which undergoes rapid reduction in WWTPs due to beta- lactamase action and hydrolysis (Zhang & Li, 2011). Ampicillin, also a β -lactam, was found below the LOD in chlorinated effluent samples. Penicillin G, another β -lactam, and belonging to a very commonly prescribed group (penicillins), however, was found above the LOD in all chlorinated effluent samples (Hicks et al., 2015). No significant differences in concentrations of antimicrobials and environmental parameters were observed between chlorinated effluent samples collected on different days.

Median antimicrobial concentrations were not statistically significantly different between chlorinated effluent and reclaimed water samples (Table 3), suggesting that storage of chlorinated effluent in the rain barrels prior to use may have had minimal impact. The opacity of the covered rain barrels and the consistency

in ambient conditions within the greenhouse may have also influenced these findings. However, free and total chlorine concentrations were statistically significantly lower ($\chi^2 = 7.7201$, p -value = 0.005 and $\chi^2 = 8.6908$, p -value = 0.003198 respectively) and NO_2 concentrations were statistically significantly higher ($\chi^2 = 8.2617$, p -value = 0.004049) in reclaimed water compared to chlorinated effluent samples (Table 4), possibly due to chlorine dissipation and algae accumulation with subsequent additions of chlorinated effluent to the rain barrels. No significant differences in concentrations of antimicrobials and environmental parameters were observed for reclaimed water samples collected on different days throughout the duration of the experiment.

Filtration of reclaimed water through the ZVI-biosand filter

The four antimicrobials with the highest concentrations in chlorinated effluent (ciprofloxacin, sulfamethoxazole, triclocarban and penicillin G) were also found to be the most predominant in reclaimed water, and subsequently, in ZVI-biosand-filtered reclaimed water samples (Table 3). ZVI-biosand filtration resulted in statistically significant decreases in the concentrations of azithromycin ($V = 45$, p -value = 0.004), ciprofloxacin ($V = 45$, p -value = 0.004), erythromycin ($V = 45$, p -value = 0.004), linezolid ($V = 44$, p -value = 0.008), oxolinic acid ($V = 45$, p -value = 0.004), penicillin G ($V = 44$, p -value = 0.008), pipemidic acid ($V = 45$, p -value = 0.004) and vancomycin ($V = 45$, p -value = 0.004), with concentration decreases observed for all antimicrobials on all collection days (Figure 3). Erythromycin was reduced to below LOD levels in all nine ZVI-biosand-filtered reclaimed water samples and the median concentration of ciprofloxacin was reduced from 233.5 ng/mL, in reclaimed water

samples, to 29.1 ng/mL after ZVI filtration (Table 3). Oxacillin, which was detected at above the LOD in three of all nine reclaimed water samples, was also reduced to below LOD levels after ZVI filtration (Table 3, Figure 3).

Only NO₂ was found to be statistically significantly lower in ZVI-biosand-filtered reclaimed water samples compared to reclaimed water samples ($V = 36$, p -value = 0.01) (Table 4). The median pH of reclaimed water samples was 7.72 while that of ZVI-biosand-filtered reclaimed water was 8.97 (Table 4). The median concentrations of TDS in reclaimed water (325), ZVI-biosand-filtered reclaimed water (317.5) and tap water (116) (Table 4) were all below the maximum contaminant level (500 mg/L) for TDS under the National Primary Drinking Water Regulations established by the Environmental Protection Agency (EPA) (U.S Environmental Protection Agency., 2016). TDS is a secondary standard that is used for aesthetic considerations, such as taste, color, and odor and no significant decrease was found in TDS after ZVI filtration.

ZVI-biosand-filtered reclaimed water contained antimicrobials at concentrations similar to those detected in tap water as illustrated by the clustering patterns seen in Figure 4. This is significant since the tap water was of potable quality and supplied to the greenhouse from a drinking water treatment plant. ZVI-biosand-filtered reclaimed water samples were seen to cluster very closely with tap water samples, with distinct separation from reclaimed water samples. Statistically significant separation was observed for reclaimed water and ZVI-biosand-filtered reclaimed water ($R^2 = 0.411$, p -value = 0.001), and although significant separation

was also seen between the ZVI-biosand-filtered reclaimed water and tap water samples ($R^2 = 0.227$, p -value = 0.003), the effect size was much smaller.

ZVI-biosand-filtered reclaimed water and tap water groups both contained samples with below LOD concentrations of ampicillin, erythromycin, oxacillin, pipemidic acid and tetracycline (Table 1). In addition, the ZVI-biosand-filtered reclaimed water group also contained samples with below LOD concentrations of azithromycin, while the tap water group also contained samples with below LOD concentrations of ciprofloxacin (Table 1). The five antimicrobials with the highest median concentrations in tap water were penicillin G, sulfamethoxazole, linezolid, oxolinic acid and triclocarban (Table 2).

Near perfect reductions have been demonstrated for several antibiotics by artificially controlling pH conditions through buffering and by increasing contact surface area by using nano-scale ZVI in lab-scale studies of single antibiotics in solution (Ghauch et al., 2009; Hanay et al., 2014; Perini et al., 2014). Still, despite the use of macro-scale ZVI, on unbuffered conventionally treated reclaimed water, we were able to achieve 100% reduction for erythromycin and significant reductions for eight antimicrobials including one occurring at the higher concentrations compared to all others (Table 3). We were not able to achieve significant reductions for ampicillin, sulfamethoxazole, triclocarban and tetracycline, however, median concentrations of all these antimicrobials were lower in ZVI-biosand-filtered reclaimed water compared to reclaimed water (Table 3). Moreover, sulfamethoxazole and triclocarban were also among the antimicrobials found at the highest concentrations in tap water (Table 3).

Though not artificially maintained, the pH of reclaimed water and ZVI-biosand-filtered reclaimed water both remained close to the median levels of 7.72 and 8.97 throughout the duration of the experiment. It has been proposed that maintaining a pH of 8 or 9 might prolong the life of the ZVI-biosand filter and prevent ZVI particles from dissolving (Bae & Hanna, 2015). The presence of antimicrobials as mixtures in reclaimed water and the use of commercially available ZVI and sand, make it difficult to determine the exact reclaimed water and filter characteristics influencing reduction as well as the specific reduction mechanisms involved for individual antimicrobials. Furthermore, the precise reduction mechanisms may be dependent on reclaimed water and filter characteristics. The impact of pH, agitation (which increases dissolved oxygen), temperature, and iron dose are among the many factors that influence antimicrobial reduction by ZVI (Noubactep, 2008). Reclaimed water characteristics (pH, NO₃, NO₂, antimicrobial concentrations) and ambient temperature remained fairly constant throughout the duration of the experiment but changes in filter characteristics like iron dose reduction, corrosion and permeability changes were unknown.

Only some of the antimicrobials included our study have been studied with respect to ZVI removal efficiency and many of these analyses are based on nano-scale ZVI. Ampicillin (at an initial pH of 6.6) reduction was shown to occur due to β -lactam ring rupture, adsorption on to, and co-precipitation with, iron corrosion products with the addition of halide salts (NaCl) having a positive impact on reduction (Ghauch et al., 2009). Tetracycline was found to have almost 100% removal efficiency at pH 3.0 and 6.5 but only 53.5% and 43.1% at pH 8.0 and pH

10.0 respectively (Chen et al., 2011). Fu et al. (2015) also observed this decreased efficiency at pH 8 and pH 10 with a pH of 6 resulting in 99% tetracycline reduction (Fu et al., 2015). However, Hanay et al. (2014) found a pH of 3.0 to be optimal for tetracycline and oxytetracycline (Hanay et al., 2014). Interestingly, tetracycline is known to undergo sorption with iron oxides resulting in the dissolution of the iron oxides (Gu & Karthikeyan, 2005). These complexes can be dissociated under low pH conditions (Fu et al., 2015). Stieber et al. (2011) examined the efficacy of zero-valent iron treatment (in the presence of oxygen) for the reduction of antibiotics, cytostatic drugs and diagnostic agents and demonstrated that antibiotic (piperaciline, cefuroxime, ciprofloxacin) removal efficiency was higher under acidic conditions (Stieber et al., 2011). Finally, the reduction efficiency of ciprofloxacin has been demonstrated to have a linear relationship with iron dose and pH (Perini et al., 2014).

Limitations and implications for future research

Further examination of these reduction trends is necessary, using a longer sampling duration, with increased sampling frequency, in order to determine the effect of seasonal prescription trends, weather related effects, as well as daily WWTP operational variations on antibiotic reductions. The persistence of these effects should also be examined when performing continuous filtration, which would be the case during irrigation. Since effluent from only one WWTP was included in this study these findings may not be generalizable. However, the treatment performed at this WWTP is typical of conventional wastewater treatment across the U.S. and this area is an ideal example of a mixed-use irrigation pattern location.

Another important factor to be examined in future studies is long-term efficiency, which may be impacted by seasonal variations in antibiotic prescription patterns, organic matter deposition due to prolonged filtration, and reduction in contact time due to ZVI corrosion, resulting in preferential flow. The potential for the development of antibiotic resistance and opportunistic pathogens within the biosand biofilm community must be examined. Sand is used for the stabilization of ZVI particles in order to maintain permeability as the development of corrosion products over time can lead to cementation and reduced flow (W. Gao et al., 2015; Gottinger et al., 2013). Different ZVI to sand ratios must be examined to determine impact on antimicrobial reduction. The contributions of the ZVI itself towards reductions in water quality must also be examined along with the generation of transformation products and metabolites. These initial results on antimicrobial reduction are promising, however, in order to determine whether this filter can serve as a comprehensive reuse site-based treatment technology the reduction of other PPCPs, bacterial, viral and parasitic pathogens as well as salinity must also be investigated.

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Tables

Table 1 List of tested antimicrobials and surrogate standards with corresponding limits of detection (LOD) and percent recoveries.

Antimicrobial	Parent Ion (<i>m/z</i>) ^a	Product Ion (<i>m/z</i>) ^a	LOD (ng/mL)	% Recovery
Ampicillin	350	160.4	4.55	7.3
Azithromycin	749.5	591.4	2.38	84.2
Caffeine	195	138.2	1.16	93.5
Caffeine-13C3	198	140	-	-
Ciprofloxacin	332.1	314.1	3.68	280.3
Erythromycin	734.5	158.2	0.55	13.5
Linezolid	338.2	195	0.85	110.0
Linezolid-d3	341.2	297.2	-	-
Oxacillin	402	144	4.74	95.0
Oxolinic Acid	262	244	0.89	140.0
Oxolinic Acid-d5	267	249.1	-	-
Penicillin G	335	159.9	1.01	17.0
Pipemidic Acid	304	217.4	2.19	78.1
Sulfamethoxazole	254	108	1.64	24.1
Tetracycline	445	154.2	1.22	21.2
Triclocarban	313	160	0.49	116.6
Triclocarban-13C6	319	160	-	-
Vancomycin	725	144	5.21	48.0

^amass-charge ratio

Table 2 Antimicrobials detected at concentrations below the limit of detection (LOD) by type of sample.

Antimicrobial	Sample Type and % below LOD			
	Chlorinated WWTP Effluent	Reclaimed Water	ZVI-biosand filtered reclaimed water	Tap Water
Ampicillin	40	11.1	22.2	37.5
Azithromycin	-	-	33.3	-
Ciprofloxacin	-	-	-	62.5
Erythromycin	-	-	100	100
Oxacillin	100	66.7	100	100
Pipemidic Acid	20	-	44.4	75
Tetracycline	80	11.1	22.2	50

Table 3 Median concentrations (ng/ml) and interquartile ranges of antimicrobials in chlorinated effluent, reclaimed water, ZVI-biosand filtered reclaimed water, and tap water samples. Statistically significant reductions (p -value <0.01) in concentrations after ZVI-biosand filtration have been highlighted in bold.

Antimicrobial	Median concentration (ng/mL) (interquartile range)			
	Chlorinated WWTP Effluent	Reclaimed water	ZVI-biosand filtered reclaimed water	Tap water
Ampicillin	0.20 (0.00 - 0.90)	9.26 (4.55 - 15.27)	2.09 (1.23 - 3.29)	0.09 (0.00 - 0.58)
Azithromycin	16.89 (11.23 - 17.86)	13.26 (12.13 - 29.79)	0.23 (0.00 - 0.38)	0.33 (0.28 - 0.36)
Ciprofloxacin	103.54 (88.37 - 121.71)	233.49 (156.20 - 248.61)	29.15 (16.27 - 62.85)	0.00 (0.00 - 0.44)
Erythromycin	4.89 (3.96 - 9.05)	13.20 (12.82 - 17.05)	0.00 (0.00 - 0.00)	0.00 (0.00 - 0.00)
Linezolid	7.72 (5.72 - 8.43)	10.26 (9.57 - 12.27)	8.14 (7.32 - 8.78)	6.91 (6.33 - 7.20)
Oxacillin	0.00 (0.00 - 0.00)	0.00 (0.00 - 2.48)	0.00 (0.00 - 0.00)	0.00 (0.00 - 0.00)
Oxolinic Acid	9.94 (9.34 - 10.75)	16.21 (13.68 - 20.11)	9.34 (7.00 - 9.88)	4.72 (4.44 - 5.04)
Penicillin G	30.65 (30.61 - 30.65)	31.40 (30.85 - 32.72)	30.56 (30.53 - 30.67)	30.74 (30.62-30.95)
Pipemidic Acid	1.73 (0.91 - 2.71)	6.07 (3.78 - 7.61)	0.12 (0.00 - 0.39)	0.00 (0.00 - 0.01)
Sulfamethoxazole	48.74 (36.12 - 55.34)	35.88 (28.99 - 71.64)	27.33 (26.74 - 32.04)	23.50 (22.82 - 24.28)
Tetracycline	0.00 (0.00 - 0.00)	7.12 (6.04 - 14.93)	1.89 (0.40 - 3.61)	0.07 (0.00 - 3.51)
Triclocarban	41.23 (39.96 - 47.99)	26.94 (21.06 - 54.27)	12.38 (8.48 - 15.51)	2.30 (1.26 - 5.64)
Vancomycin	6.39 (1.81 - 9.12)	8.33 (5.19 - 12.25)	1.00 (0.83 - 1.13)	0.96 (0.87 - 1.52)

Table 4 Median concentrations (ng/ml) and interquartile ranges of environmental parameters for reclaimed water, ZVI-biosand filtered reclaimed water and tap water samples. Statistically significant reductions (p -value <0.01) in concentrations after ZVI-biosand filtration have been highlighted in bold.

Parameter	Median concentration (ng/mL) and (interquartile range)			
	Chlorinated WWTP effluent	Reclaimed water	ZVI-biosand filtered reclaimed water	Tap water
Free chlorine (mg/L)	0.10 (0.09 - 0.14)	0.00 (0.00 - 0.01)	0.00 (0.00 - 0.00)	0.01 (0.00 - 0.02)
Total chlorine (mg/L)	1.63 (1.62 - 1.79)	0.03 (0.02 - 0.03)	0.00 (0.00 - 0.00)	0.08 (0.03 - 0.12)
NO ₃ (mg/L)	5.30 (3.20 - 5.70)	12.88 (8.13 - 19.00)	7.50 (6.08 - 7.73)	1.08 (0.60 - 1.15)
NO ₂ (mg/L)	6.00 (4.00 - 6.00)	13.50 (10.25 - 17.00)	7.00 (6.75 - 8.00)	5.00 (5.00 - 6.00)
pH	7.34 (7.34 - 7.51)	7.72 (7.71 - 7.85)	8.97 (8.84 - 9.11)	7.43 (7.27 - 7.65)
Total dissolved solids (mg/L)	352.00 (334.00-369.00)	325.00 (316.50-341.50)	317.50 (304.00-319.00)	116.00 (101.00-131.00)

Figures

Figure 1 Schematic illustrating experimental design.

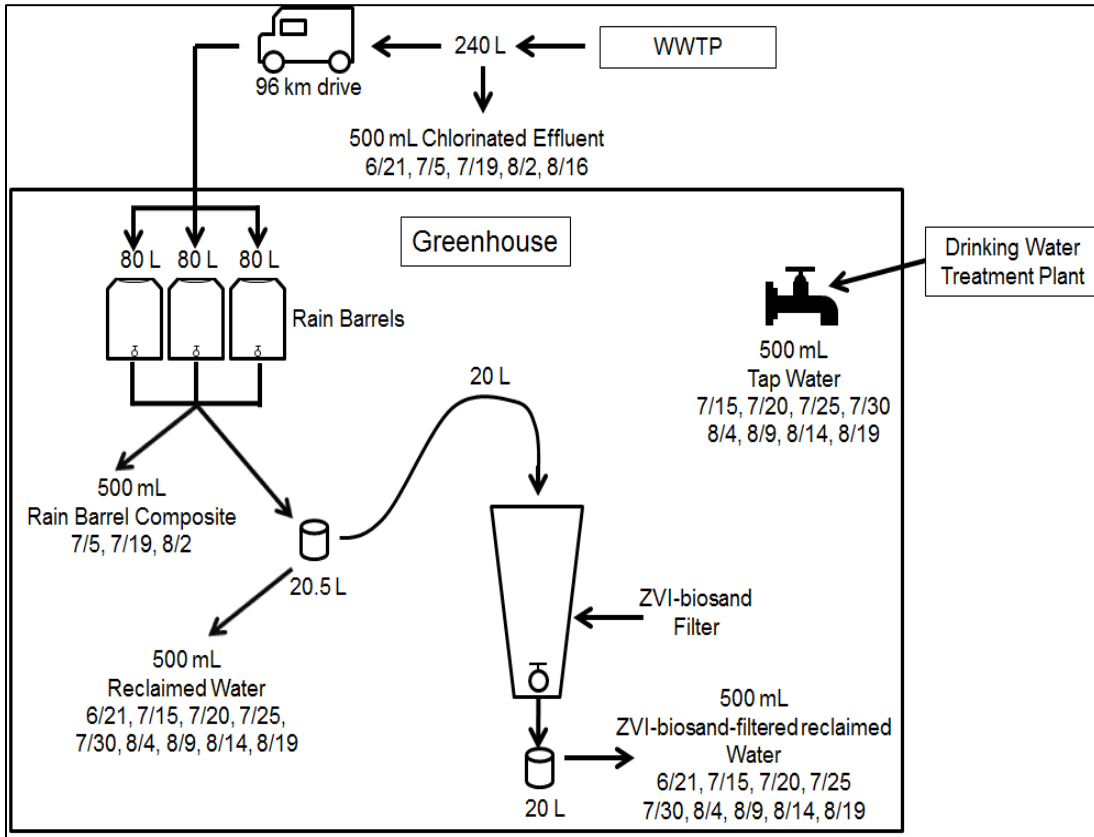


Figure 2 Schematic illustrating treatment steps and the sampling location at the WWTP.

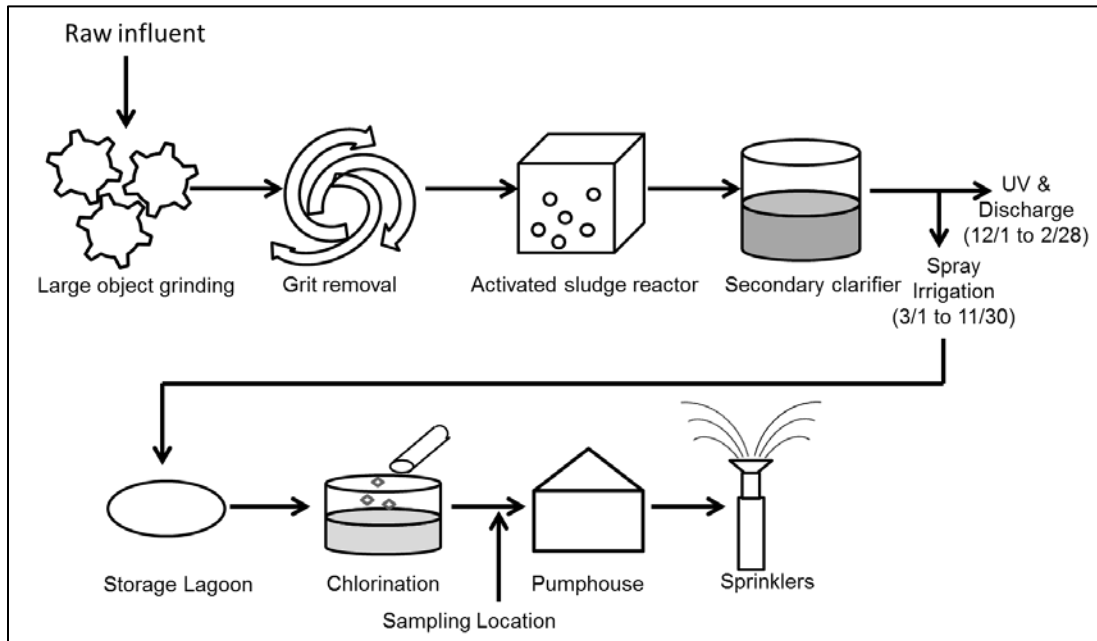


Figure 3 Antimicrobial concentration (ng/mL) reductions between reclaimed water and ZVI- biosand filtered reclaimed water samples collected on the same day. Statistically significant reductions (p -value <0.01) were observed for azithromycin, ciprofloxacin, erythromycin, linezolid, oxolinic acid, penicillin G, pipemidic acid and vancomycin.

AMP – Ampicillin, AZI – Azithromycin, CIP – Ciprofloxacin, ERY – Erythromycin, LIN – Linezolid, OXA – Oxacillin, OXO – Oxolinic Acid, PEN – Penicillin G, PIP – Pipemidic Acid, SUL – Sulfamethoxazole, TCC – Triclocarban, TET – Tetracycline, VAN – Vancomycin, RW – Reclaimed Water, ZVI – ZVI-biosand filtered reclaimed water

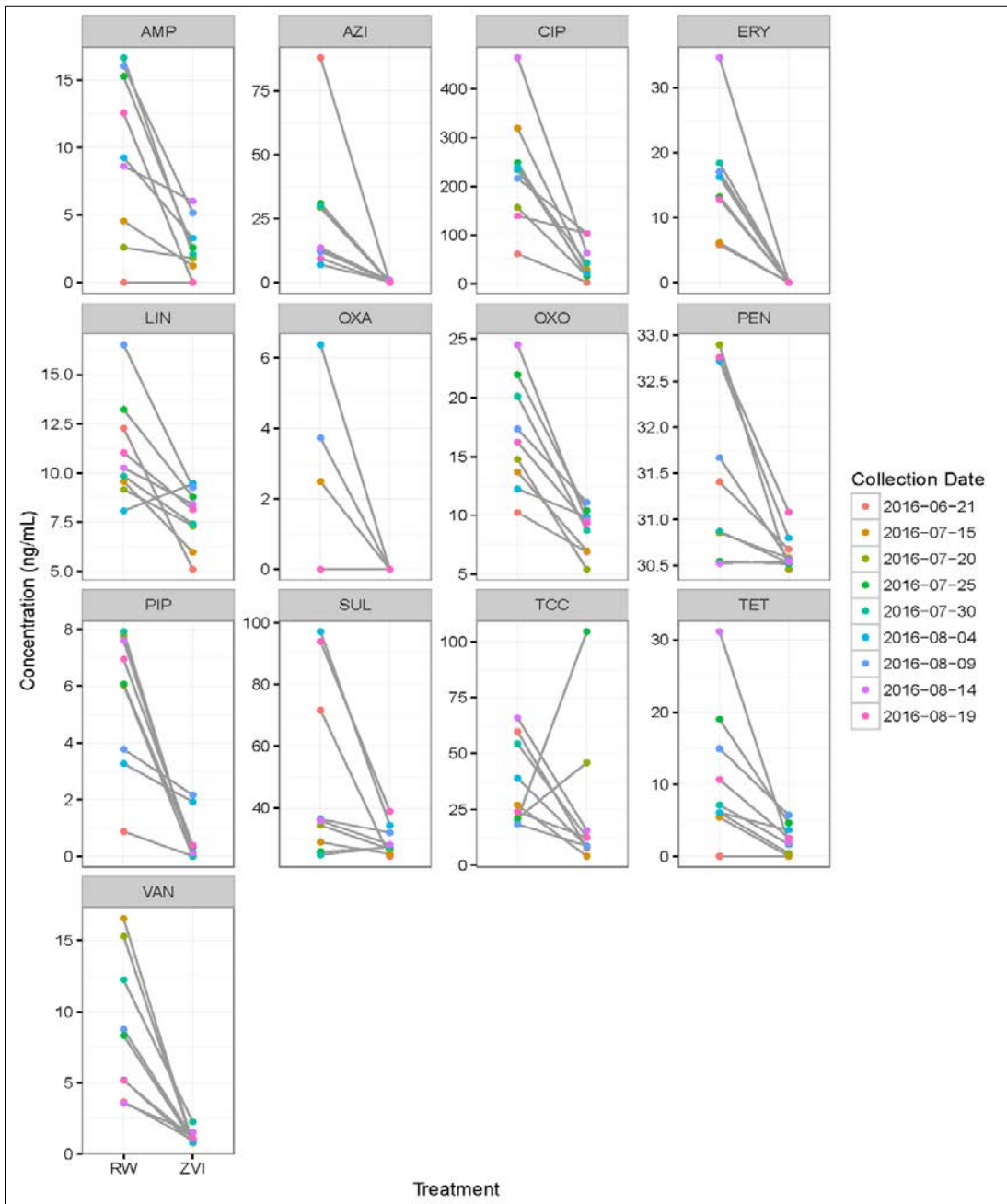
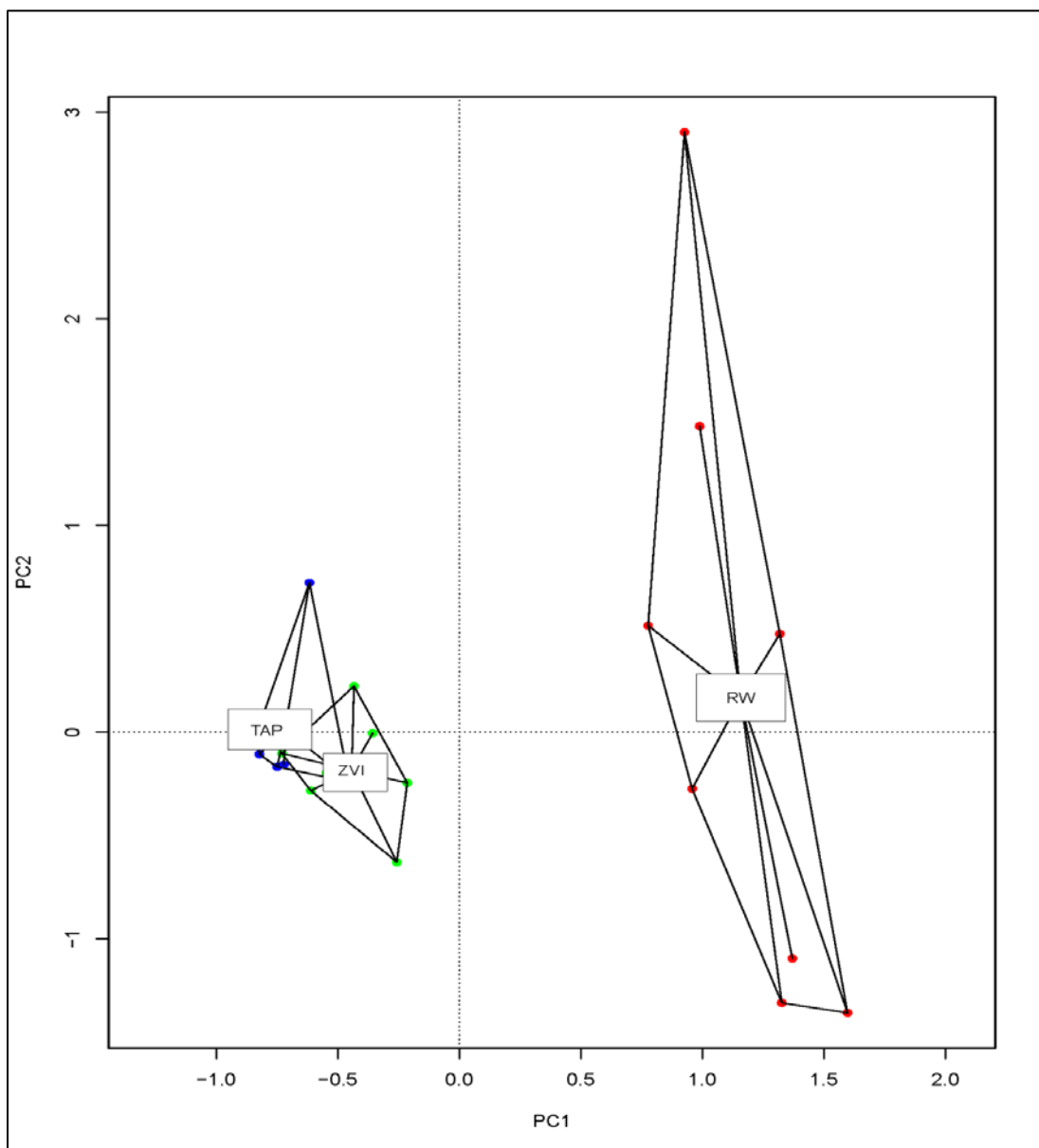


Figure 4 Principal Component Analysis (PCA) plot illustrating the clustering of tap (TAP) and ZVI-biosand filtered reclaimed water (ZVI) samples with a distinct separation between the tap (TAP) and ZVI-biosand filtered reclaimed water (ZVI) groups from the reclaimed water (RW) group. The separation between the reclaimed water group (RW) and ZVI-biosand filtered reclaimed water group (ZVI) ($R^2 = 0.411$, p -value = 0.001) was much larger compared to that between the tap water group (TAP) and the ZVI-biosand filtered reclaimed water group (ZVI) ($R^2 = 0.227$, p -value = 0.003).

RW – Reclaimed water, TAP – Tap water, ZVI– ZVI-biosand filtered reclaimed water



Chapter 8: Conclusions, Public Health Significance and Future Research

Conclusions

Climate, demographic, and land-use changes are putting increasing pressure on the availability of water for irrigation in the U.S., and many areas of the U.S. are responding with adaptive changes in water resource management (Asano, 2007; EPA, 2012a; U.S.Global Change Research Program, 2015). Historically drought-prone areas in the U.S. have taken the lead in embracing nontraditional water sources, such as reclaimed water, conducting research, and developing protective regulations governing its use (Asano, 2007). However, climate change is expected to intensify long-term drought conditions, and compromise groundwater and freshwater quality, in areas of the U.S. previously considered as being water-rich, such as the Mid-Atlantic and the Southeast (Asano, 2007; EPA, 2012a; U.S.Global Change Research Program, 2015). These areas are also experiencing added water stress due to population growth (National Research Council, 2012). Proactive water resource management in these areas is increasing but existing practices in these areas may present challenges in developing sustainable solutions that are also protective of public health (Asano, 2007; EPA, 2012a; U.S.Global Change Research Program, 2015).

The planned use of reclaimed water is considered a promising solution to address the decline in freshwater sources, and as of 2011, between 5-6% of municipal wastewater effluent, approximately 2.22 billion gallons per day, was being reclaimed and reused in the United States (Miller, 2011). If 100% of the approximately 12 billion gallons of treated municipal wastewater discharged every day is reused, it

could provide an equivalent of 6% of the total amount of water used in the U.S. (National Research Council, 2012). Additionally, reclaimed water has the added benefit of being generated every day, making it an extremely dependable source of water (EPA, 2012a). Currently, agricultural and landscape irrigation are the largest applications of reclaimed water use in the U.S. accounting for 29% and 18% of total reclaimed water use respectively (EPA, 2012a).

California, is a leading user of reclaimed water in the U.S. with 37% and 24% of reclaimed water generated within the state being used for agricultural and landscape irrigation respectively (California EPA, 2012). California state regulations require reclaimed water to be used for agricultural irrigation of food crops and for reclaimed water to be used for landscape irrigation of both restricted as well as unrestricted areas to be oxidized, coagulated, filtered and disinfected (Asano, 2007; CA DPH, 2009). This level of treatment is not common in the previously low-use areas to which reclaimed water use is now spreading. These areas may not have the infrastructure to treat wastewater to the almost potable quality required in California and may also have more of a mixed-use pattern of irrigation (Asano, 2007). Furthermore, the absence of legally binding federal regulations has resulted in geographically variable regulations governing reclaimed water use (EPA, 2012a). Trace constituents of reclaimed water, like antibiotics, that may have a potentially negative health impact due to chronic exposure are not regulated and most regulations are based on research that relies on culture-based analysis which may not capture all possible information on harmful microbial constituents in reclaimed water (Asano, 2007; EPA, 2012a). Most state regulations specify treatment requirements but not all

states stipulate the exact treatment processes necessary to achieve those requirements (Asano, 2007). Although, almost all state regulations focus on treated wastewater leaving wastewater treatment plants, not all states have reuse site monitoring or reporting requirements (Asano, 2007).

Therefore, in order to facilitate safe adoption of reclaimed water, it is necessary to examine the quality of conventionally treated wastewater and work within the existing infrastructure to investigate point-of-use treatment solutions since centralized high level treatment may not always be feasible. Moreover, in order to improve existing practices as well as increase safe adoption it is also necessary to address current research and regulatory gaps associated with reclaimed water.

Advances in technology have now made it possible to address previous research gaps in order to optimize treatment processes, improve reuse practices and update future regulations. Therefore, my goal was to conduct an exploratory analysis of the impact of conventional wastewater treatment and reuse site practices on antibiotic residues and total bacterial community structure in wastewater and reclaimed water and examine the efficacy of zero-valent iron as a potential point-of-use filter for the reduction of antibiotic residues from conventionally treated reclaimed water.

I investigated the presence of nine commonly used antibiotics in conventionally treated wastewater, at various stages of treatment, from four tertiary level wastewater treatment plants from two distinct geographic locations in the U.S. Two plants were from urban and suburban locations in the Mid-Atlantic and two from rural locations in the Midwest. All four WWTPs use suspended growth biological treatment in the form of activated sludge and provide treated effluent for spray irrigation, either for

landscape irrigation or for the irrigation of animal-food crops. Two of the plants performed chlorination, one performed seasonal chlorination, and one did not chlorinate before discharge. I also analyzed samples from one of the four spray irrigation sites associated with these WWTPs. This site, in the Mid-Atlantic, received tertiary treated wastewater from one of the two Mid-Atlantic WWTPs. This WWTP performed activated sludge treatment, sand filtration and chlorination. At the spray irrigation site, the treated effluent was screened and underwent ultraviolet (UV) disinfection before being stored in an open-air storage pond before being pumped through a pumphouse to sprinklers for landscape irrigation.

The antibiotics analyzed were ampicillin, azithromycin, ciprofloxacin, linezolid, oxacillin, oxolinic acid, penicillin G, pipemidic acid and tetracycline. Overall, antibiotic concentrations in effluent samples were lower compared to influent samples. Mid-Atlantic plants and Midwestern plants had similar influent antibiotic concentrations but effluent antibiotic concentrations were lower in the Mid-Atlantic plants compared to the Midwestern plants. At the spray irrigation site azithromycin was the only antibiotic which showed a statistically significant decrease.

Azithromycin concentration was lower at the irrigation site before on-site UV treatment, compared to its concentration in treated effluent. Azithromycin was also lower after on-site UV treatment and after on-site open-air storage before spraying.

There may have been several potential factors driving the observed variability in antibiotic removal. Namely differences in influent concentrations and inter-plant operational variability such as differences in treatment process, treatment plant capacity and type of tertiary treatment. The variability in antibiotic removal was

especially significant since samples collected from all treatment processes at these wastewater treatment plants have been found to be positive for MRSA and VRE isolates (Rosenberg Goldstein et al., 2012; Rosenberg Goldstein, Micallef, Gibbs, George, et al., 2014). Variations in distribution system characteristics, including chlorine residual, dissolved organic matter and biofilm community structure may have influenced antibiotic concentration variability. The inability of on-site UV radiation treatment and on-site storage to reduce the concentration of only one antibiotic was also significant since non-*Enterococcus faecalis* isolates recovered from this spray irrigation site were found to be resistant to several antibiotics, including quinupristin/dalfopristin, vancomycin, tetracycline, penicillin and ciprofloxacin (Carey et al., 2016). The main limitations of this study were its cross sectional nature, unbalanced sampling scheme and the inclusion of only one spray irrigation site.

Pathogens present in wastewater and reclaimed water are a part of a complex microbial community which is impacted by wastewater treatment processes, treatment plant operational parameters and wastewater constituents including xenobiotics and pharmaceuticals and personal care products (PPCPs). Regulations governing reclaimed water use are often based on research that relies on culture based methods utilized to look for specific pathogens in these environments. However, it may not always be possible to culture certain potentially pathogenic bacteria, for instance, bacteria that form spores, or are viable-but-non-culturable (VBNC). Therefore, culture based research may not provide adequate information on the impact of wastewater and reclaimed water treatment on pathogens present in these

environments. This may be illustrated by the fact that several state regulations rely on the use of indicator bacteria to determine treatment efficacy, but potentially pathogenic *Mycobacterium* spp. and *Legionella* spp. have been isolated from treated wastewater effluent and reclaimed water in the absence of indicator bacteria (Jjemba et al., 2010). Therefore, it may be useful to investigate the presence of other potentially pathogenic bacteria, and the structure of the bacterial communities in which they reside, throughout conventional wastewater treatment processes and reclaimed water treatment and use.

To achieve this goal, I performed an exploratory analysis of 72 samples of differentially treated wastewater from the four conventional WWTPs and one associated spray irrigation site, described above, by extracting total genomic deoxyribonucleic acid (DNA) directly from these samples and conducting 16S rRNA gene sequencing analysis to determine the total bacterial community structure of samples taken from various wastewater treatment processes, and from the on-site treatment processes and open-air storage pond at a spray irrigation site.

I discovered that influent samples from all four WWTPs were similar in structure while final effluent structure was influenced by influent constituents, sewer infrastructure and treatment processes and the bacterial genera found to be abundant across treatment process across all plants showed functional similarity. Effluent structure and open-air storage had the most impact on the communities at the inlet to the pumphouse supplying the sprinklers. *Legionella* and *Mycobacteria* genera were abundant in WWTP effluent and at the inlet to the pumphouse, possibly due to their

presence in activated sludge, resistance to disinfection, chlorine dissipation, resistance to UV disinfection and open-air storage.

The main limitations of this study were the grab sample and unbalanced nature of sampling and since more samples were collected from Mid-Atlantic WWTP1 and Midwest WWP2, the observations from these samples may have influenced the overall findings. 16S rRNA gene sequencing does not allow us to determine whether the communities found are metabolically active, therefore future work using advanced techniques such as metatranscriptomics can be used to fill in this gap.

I demonstrated that conventionally reclaimed water contained antimicrobial residues as well as bacteria belonging to genera that contain potential opportunistic pathogens. If we want to be able to safely use this reclaimed water for irrigation it is necessary to examine potential on-site treatment technologies that can be applied to conventionally treated reclaimed water. As an initial step, I examined the efficacy of one such treatment technology – zero-valent iron (ZVI)-biosand filtration for the reduction of thirteen antimicrobials widely used in the U.S. and commonly present in reclaimed water – ampicillin, azithromycin, ciprofloxacin, erythromycin, linezolid, oxacillin, oxolinic acid, penicillin G, pipemidic acid, sulfamethoxazole, tetracycline, triclocarban and vancomycin. The ZVI-biosand filter consisted of equal volumes of commercially available macro-scale sand and ZVI. Chlorinated, unbuffered effluent from a tertiary treatment plant was filtered through this filter. Tap water from a drinking water treatment plant was used as a comparison. After filtration, significant (p -value <0.01) reductions in concentrations were observed for ciprofloxacin, oxolinic acid, pipemidic acid, azithromycin, erythromycin, penicillin, linezolid and

vancomycin with 100% reduction achieved in the case of erythromycin. The antibiotic concentrations in ZVI-biosand-filtered reclaimed water were found to be similar to those found in tap water. The limitations of this study were its short duration, batch filtration, and the inability to separate the effects of ZVI from biosand. I also collected effluent from only one WWTP. However, treatment performed at this WWTP is typical of conventional wastewater treatment and the WWTP is in an area with a mixed-use irrigation pattern.

Public Health Implications and Future Research

I was able to confirm that conventional activated sludge process-based wastewater treatment and chlorination, ultraviolet radiation treatment and open-air storage of reclaimed water may not effectively reduce antibiotics, and bacterial genera that could contain potentially pathogenic bacteria, namely *Mycobacterium* and *Legionella*, from wastewater. However, zero-valent-iron-biosand filtration can reduce concentrations of antimicrobials present in reclaimed water to levels close to those present in potable water.

This research can provide the foundation for future long-term studies using a composite sampling scheme that can be conducted on the fate of bacterial, viral, and parasitic pathogens; xenobiotics; and pharmaceuticals and personal care products (PPCPs) present in conventionally treated reclaimed water and their eventual impacts on the development of antibiotic resistance and transfer to, and accumulation in, soils and plants irrigated with conventionally treated reclaimed water. Since areas that allow the use of reclaimed water for food crop irrigation often perform advanced

biological treatment as well as tertiary treatment, a comparative long-term analysis of conventionally treated wastewater and reclaimed water and wastewater that has undergone advanced treatment may provide further information towards developing on-site solutions for treatment when using conventionally treated wastewater. The move towards increased reclaimed water use is intriguing; however, widespread use should not occur unless treatment technologies and regulatory frameworks can be further developed to ensure that public health is protected. My research can be applied to help develop future reclaimed water regulations and practices that are protective of public health.

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