UNLV | University Libraries
University of Nevada, Las Vegas

UNLV Theses, Dissertations, Professional Papers, and Capstones

December 2016

Occurrence and Proliferation of Antibiotics and Antibiotic Resistance in Wastewater Treatment Plants

Majid Neyestani
University of Nevada, Las Vegas, neyestan@unlv.nevada.edu

Follow this and additional works at: https://digitalscholarship.unlv.edu/thesesdissertations

Part of the Civil Engineering Commons, and the Environmental Engineering Commons

Repository Citation

Neyestani, Majid, "Occurrence and Proliferation of Antibiotics and Antibiotic Resistance in Wastewater Treatment Plants" (2016). *UNLV Theses, Dissertations, Professional Papers, and Capstones*. 2889.
https://digitalscholarship.unlv.edu/thesesdissertations/2889

This Dissertation is protected by copyright and/or related rights. It has been brought to you by Digital Scholarship@UNLV with permission from the rights-holder(s). You are free to use this Dissertation in any way that is permitted by the copyright and related rights legislation that applies to your use. For other uses you need to obtain permission from the rights-holder(s) directly, unless additional rights are indicated by a Creative Commons license in the record and/or on the work itself.

This Dissertation has been accepted for inclusion in UNLV Theses, Dissertations, Professional Papers, and Capstones by an authorized administrator of Digital Scholarship@UNLV. For more information, please contact digitalscholarship@unlv.edu.

المنارة للاستشارات

www.manaraa.com

OCCURRENCE AND PROLIFERATION OF ANTIBIOTICS AND ANTIBIOTIC RESISTANCE IN WASTEWATER TREATMENT PLANTS

By

Majid Neyestani

Bachelor of Science in Engineering – Civil Engineering
University of Qom
2010

Master of Science in Engineering – Civil and Environmental Engineering
Isfahan University of Technology
2012

A dissertation submitted in partial fulfillment
of the requirements for the

Doctor of Philosophy – Civil and Environmental Engineering

Department of Civil and Environmental Engineering and Construction
Howard R. Hughes College of Engineering
The Graduate College

University of Nevada, Las Vegas
December 2016

المنارة للاستشارات

www.manaraa.com

Copyright 2016 Majid Neyestani

All Rights Reserved

المنارة للاستشارات

www.manaraa.com

Dissertation Approval

The Graduate College
The University of Nevada, Las Vegas

November 23, 2016

This dissertation prepared by

Majid Neyestani

entitled

Occurrence and Proliferation of Antibiotics and Antibiotic Resistance in Wastewater Treatment Plans

is approved in partial fulfillment of the requirements for the degree of

Doctor of Philosophy – Civil and Environmental
Department of Civil and Environmental Engineering and Construction

Daniel Gerrity, Ph.D.
Examination Committee Chair

Dale Devitt, Ph.D.
Examination Committee Member

Donald Hayes, Ph.D.
Examination Committee Member

Jacimaria Batista, Ph.D.
Examination Committee Member

Eduardo Robleto, Ph.D.
Graduate College Faculty Representative

Kathryn Hausbeck Korgan, Ph.D.
Graduate College Interim Dean

المنارة للاستشارات
www.manaraa.com

Abstract

Solids retention time (SRT) is one of the most important factors in designing and operating activated sludge systems for biological wastewater treatment. Longer SRTs have been shown to alter the structure and function of microbial communities, thereby leading to improved treatment efficacy with respect to bulk and trace organics, nutrient removal, and membrane fouling. However, research has also shown that longer SRTs lead to increased prevalence of antibiotic resistant bacteria, perhaps due to increased exposure to antibiotics present in influent wastewater. The purpose of this study was to characterize changes in microbial community structure in a laboratory-scale activated sludge system as a function of SRT (2-20 days) and influent concentrations (1x-100x ambient concentrations) of five antibiotics: ampicillin, sulfamethoxazole, tetracycline, trimethoprim, and vancomycin. Also, this research aimed to characterize the role of SRT and elevated antibiotic concentrations on AR proliferation in biological treatment processes. Changes in microbial community structure were evaluated based on traditional plating methods and 16s rDNA sequencing, and microbial community function was evaluated based on changes in effluent water quality, including bulk organic matter characterization and antibiotic concentrations. Spread plate technique was used to determine the number of Gram positive *Staphylococcus/Streptococcus* strains. The extent of AR was also determined based on minimum inhibitory concentrations (MICs) of resistant isolates. The results indicated that SRT—but not antibiotic loading—had a significant impact on microbial community structure (e.g., reduction in relative prevalence of *Acinetobacter* and *Arcobacter*) and effluent water quality. Therefore, spikes in influent antibiotics (at sub-therapeutic concentrations) are not expected to adversely impact biological wastewater treatment. The results revealed that longer SRTs and higher antibiotic concentrations select for antibiotic resistant bacteria (ARBs).

المنارة للاستشارات
www.manaraa.com

The data obtained from this study suggests that longer SRTs may select for trimethoprim-resistant bacteria and/or result in false positives for trimethoprim resistance due to higher concentrations of free thymine or thymidine.

المنارة للاستشارات

www.manaraa.com

Acknowledgements

My most sincere thanks go to my advisor, Dr. Daniel Gerrity. I thank him for his guidance, encouragement and support. I would like to thank my committee members for their valuable advice and time. I would also like to thank the personnel at the study site, particularly Bruce Dacko, and Peter Faught at UNLV for their assistance in designing and setting up the experimental system.

Finally, I would like to express my eternal gratitude to my wife and my family for their everlasting love and support.

المنارة للاستشارات

www.manaraa.com

Dedication

I dedicate this dissertation to my wife, Elnaz, and to my parents, Abbas and Fatemeh, with my gratitude for their love, support, and inspiration throughout my life.

المنارة للاستشارات
www.manaraa.com

Table of Contents

1.0 Introduction ... 1
1.1 Environmental reservoirs of thymidine as a mechanism of trimethoprim resistance: ... 2
Background ... 2
Research question ... 3
Hypothesis ... 3
Approach ... 3
Outcome ... 3
1.2 Microbial community structure and function: ... 4
Background ... 4
Research question 1 ... 4
Hypothesis 1 ... 4
Research question 2: ... 5
Hypothesis 2 ... 5
Approach ... 5
Outcome ... 5
1.3 Relative abundance and extent of AR: ... 6
Background ... 6
Research question 1 ... 6
Hypothesis 1 ... 7
Research question 2 ... 7
Hypothesis 2 ... 7
Approach ... 7
Outcome ... 7
2.0 State of knowledge ... 8

المنارة للاستشارات
www.manaraa.com

2.1 Antibiotic Resistance as an Emerging Threat 8
2.2 Drivers of Antibiotic Resistance 15
I Conjugation 16
II Transduction 17
III Transformation 18
2.3 Influence of Metals, Quaternary Ammonium Compounds, and Antimicrobial Agents 19
2.4 Antibiotic Compounds and Resistance Mechanisms 20
2.4.1 Thymidine Interference with the Detection of Trimethoprim Resistant Bacteria 23
2.5 Methods for Quantifying Antibiotic Resistance 26
2.5.1 Culture Techniques 26
2.5.2 Molecular Techniques 27
2.6 Antibiotic Resistance in Wastewater Treatment Plants 30
2.6.1 Secondary Biological Treatment 31
2.6.2 Antibiotic Resistance in Biofilm 35
2.7 TOrC Removal by Biological Treatment 38
2.7.1 Occurrence of Indicator Trace Organic Compounds and Antibiotics 39
2.7.2 Modeling the Removal of Trace Organic Compounds in Biological Treatment Systems 41
3.0 An Evaluation of Bacterial Resistance to Trimethoprim and the Role of Wastewater as a Reservoir of Environmental Thymine and Thymidine 45
3.1 Abstract 45
3.2 Introduction 46
3.3 Materials and methods 50
3.3.1 Description of laboratory-scale sequencing batch reactors 50
3.3.2 Preparation of trimethoprim stock solution 53
3.4 Analytical methods 53

المنارة للاستشارات
www.manaraa.com

3.4.1 General water quality parameters 53

3.4.2 Trace organic compounds 54

3.5 Microbiological methods 56

3.5.1 Manual augmentation of thymidine with reagent-grade chemical 57

3.5.2 Manual augmentation of thymidine via cell lysing 58

3.5.3 Varying of SRT to evaluate the effects of cellular debris 59

3.5.4 Varying of SRT to evaluate multi-drug resistance 60

3.6 Statistical analysis 60

3.7 Results and discussion 61

3.7.1 General water quality 61

3.7.2 Trace organic compounds 64

3.7.3 Estimation of thymidine concentration 66

3.7.4 Manual augmentation of thymidine with reagent-grade chemical 67

3.7.5 Manual augmentation of thymidine via cell lysing 69

3.7.6 Varying of SRT to evaluate the effects of cellular debris 71

3.7.7 Varying of SRT to evaluate multidrug resistance among Gram positive bacteria 77

3.8 Conclusions 80

4.0 Impact of solids retention time and antibiotic loading in activated sludge systems on microbial community structure and bulk and trace organic matter 82

4.1 Abstract 82

4.2 Introduction 83

4.3 Materials and methods 87

4.3.1 Automation and operation of the laboratory-scale sequencing batch reactors 87

4.3.2 Preparation of antibiotic stock solutions 90

4.3.3 General water quality parameters 90

المنارة للاستشارات

www.manaraa.com

4.3.4 UV-Vis and fluorescence spectroscopy 91

4.3.5 Trace organic compounds 91

4.3.6 Molecular-based microbial community characterization of SBRs 92

4.4 Results and discussion 93

4.4.1 General water quality parameters 93

4.4.2 UV-Vis and fluorescence spectroscopy 97

4.4.3 Trace organic compounds 103

I. Phase 1 SRT testing 103

II. Phase 1 MLSS testing 106

III. Phase 2 antibiotic concentration testing 108

4.4.4 Molecular characterization of the microbial community 113

I. Phase 1 SRT testing 113

II. Phase 2 antibiotic concentration testing 125

4.4.5 Microbial community analysis with respect to the treatment aspects 125

4.5 Conclusions 126

5.0 Effect of solids retention time and elevated antibiotic concentrations on the fate of antibiotic resistance and micropollutants removal during biological wastewater treatment 128

5.1 Abstract 128

5.2 Introduction 128

5.3 Materials and methods 134

5.3.1 Description of laboratory-scale sequencing batch reactors 134

5.3.2 Preparation of antibiotic stock solutions 136

5.3.3 Analytical methods 136

I. General water quality parameters 136

II. Trace organic compounds 136

المنارة للاستشارات

www.manaraa.com

5.3.4 Microbiological methods ... 138

I. Detection of culturable antibiotic resistant bacteria and minimum inhibitory concentrations (MICs) ... 138

5.3.5 Statistical analysis ... 140

5.3.6 Limitations ... 140

5.4 Results and discussion ... 143

5.4.1 The effect of varying solids retention time on sequencing batch reactor performance ... 143

5.4.2 The effect of elevated antibiotic concentrations on the sequencing batch reactors performance ... 145

5.4.3 The effect of varying SRT on mitigation of micropollutants ... 147

5.4.4 The effect of elevated antibiotic concentrations on the SBRs performance with respect to trace organic compounds ... 151

5.4.5 The effect of varying SRT on culturable antibiotic resistant bacteria ... 154

5.4.6 The effect of elevated antibiotic concentrations on culturable antibiotic resistant bacteria 160

5.4.7 The effect of varying SRT on minimum inhibitory concentration among antibiotic resistant bacteria 167

5.4.8 The effect of elevated antibiotic concentrations on minimum inhibitory concentration among antibiotic resistant bacteria ... 174

5.5 Conclusions ... 179

6.0 Conclusions ... 180

7.0 Appendix A ... 182

8.0 Appendix B ... 188

9.0 Appendix C ... 190

10.0 References ... 199

المنارة للاستشارات

www.manaraa.com

Curriculum Vitae 220

المنارة للاستشارات
www.manaraa.com

List of Tables

Table 2-1. Target antibiotics and respective antibiotic resistance genes 23

Table 2-2- Classification of bacteria by electron donor, electron acceptor and source of carbon 32

Table 2-3. Threshold SRT for 80% TOrC degradation (Salveson et al., 2012).. 38

Table 3-1. Summary of methods for water quality parameters... 54

Table 3-2. Summary of experimental conditions 57

Table 3-3. Summary of calculation of Thymidine concentration in samples 67

Table 4-1. Summary of Spiked Antibiotic Concentrations in the SBRs.. 89

Table 4-2. Extent of nitrification and apparent denitrification 96

Table 4-3. Average peak fluorescence values during phase 1 SRT testing (5 sample events) 101

Table 4-4. Average peak fluorescence values during phase 2 antibiotic testing (3 sample events).......... 101

Table 4-5. Average regional fluorescence values during phase 1 SRT testing (5 sample events)........... 101

Table 4-6. Average regional fluorescence values during phase 2 antibiotic testing (3 events) 101

Table 4-7. Reductions in regional fluorescence and UV absorbance 103

Table 4-8. Summary of TOrC concentrations during phase 1 (variation of SRT)................................... 105

Table 4-9. Summary of TOrC concentrations during phase 1 (variation of MLSS concentration).......... 107

Table 4-10. Summary of TOrC concentrations during phase 2 (variation of antibiotic concentrations).. 112

Table 4-11. Comparison of target and observed concentrations in phase 2... 112

Table 4-12. Diversity and richness indices 115

Table 4-13. Relative abundance of microbial community structure (first sample set) 116

Table 4-14. Relative abundance of microbial community (second sample set)....................................... 117

Table 4-15. Comparison of the relative abundance of potential pathogens 125

Table 5-1. Summary of Spiked Antibiotic Concentrations 135

Table 5-2. Summary of minimum inhibitory concentrations (MICs) for each antibiotic 139

المنارة للاستشارات
www.manaraa.com

Table 5-3. Summary of TOrC concentrations in the PE and effluent from the SBRs as a function of SRT 150

Table 5-4. Summary of TOrC concentrations in the PE and effluent from the SBRs with elevated antibiotic concentrations 152

Table 5-5. Comparison of Target and Observed Antibiotic Concentrations in Primary Effluent............. 153

Table 5-6. The effect of varying SRT on culturable Staph/Strep resistant bacteria (Sample Event 1)..... 158

Table 5-7. The effect of varying SRT on culturable Staph/Strep resistant bacteria (Sample Event 2)..... 158

Table 5-8. The effect of varying SRT on culturable Staph/Strep resistant bacteria (Sample Event 3)..... 158

Table 5-9. The effect of elevated antibiotic concentrations on culturable Staph/Strep resistant bacteria (Sample Event 1).. 165

Table 5-10. The effect of elevated antibiotic concentrations on culturable Staph/Strep resistant bacteria (Sample Event 2).. 165

Table 5-11. The effect of elevated antibiotic concentrations on culturable Staph/Strep resistant bacteria (Sample Event 3).. 165

Table 5-12. Comparison of target antibiotic concentrations used in the well trays for the MIC assay 168

Table 5-13. Summary of Task 1 MIC Data for Primary Effluent 171

Table 5-14. Summary of Task 1 MIC Data for 2-Day SRT (Varying SRT)... 171

Table 5-15. Summary of Task 1 MIC Data for 7-Day A SRT (Varying SRT)....................................... 172

Table 5-16. Summary of Task 1 MIC Data for 7-Day B SRT (Varying SRT) 172

Table 5-17. Summary of Task 1 MIC Data for 20-Day SRT (Varying SRT)... 173

Table 5-18. Summary of Task 2 MIC Data for Primary Effluent 176

Table 5-19. Summary of Task 2 MIC Data for 1X Antibiotic Concentrations....................................... 176

Table 5-20. Summary of Task 2 MIC Data for 10X (A) Antibiotic Concentrations 177

Table 5-21. Summary of Task 2 MIC Data for 10X (B) Antibiotic Concentrations 177

Table 5-22. Summary of Task 2 MIC Data for 100X Antibiotic Concentrations.................................... 178

المنارة للاستشارات

www.manaraa.com

List of Figures

Figure 2-1. Timeline of Antibiotic Resistance 9

Figure 2-2. Conjugation mechanism 17

Figure 2-3. Transduction mechanism 18

Figure 2-4. Transformation mechanism 19

Figure 2-5. Relationship between cell debris and SRT 25

Figure 2-6. Relationship between total fluorescence and SRT (different full-scale facilities). 35

Figure 2-7 Surviving cell fractions of P. aeruginosa 37

Figure 3-1. Pathways describing the disruption of DNA synthesis 49

Figure 3-2. Sequencing batch reactors (varying SRT) 51

Figure 3-3. Sequencing batch reactors (varying antibiotic concentrations) 52

Figure 3-4. The effect of thymidine on total bacterial growth 58

Figure 3-5. Average concentrations of sCOD, MLSS and MLVSS in the SBRs 62

Figure 3-6. Average concentrations of ammonia, nitrate, and nitrite in the SBRs 62

Figure 3-7. Effect of temperature on sCOD, ammonia, and nitrate 63

Figure 3-8. Concentration of trimethoprim, sulfamethoxazole, and atenolol 65

Figure 3-9. Bacterial growth in the presence of different thymidine concentrations 68

Figure 3-10. Apparent TMP resistance in the presence of thymidine 68

Figure 3-11. Bacterial growth in the presence of post-sonication filtrate 70

Figure 3-12. Apparent TMP resistance in the presence of PSF 70

Figure 3-13. Relationship between theoretical concentration of cellular debris 72

Figure 3-14. Apparent TMP resistance in the presence of filtered MLSS 73

Figure 3-15. Bacterial growth in the presence of different concentrations of MLSS filtrate (2-day SRT). 74

Figure 3-16. Apparent TMP resistance in the presence of MLSS filtrate (2-day SRT) 74

Figure 3-17. Bacterial growth in the presence of different concentrations of MLSS filtrate (7-day SRT). 75

Figure 3-18. Apparent TMP resistance in the presence of MLSS filtrate (7-day SRT) 75

المنارة للاستشارات

www.manaraa.com

Figure 3-19. Bacterial growth in the presence of different concentrations of MLSS filtrate (20-day SRT) 76
Figure 3-20. Apparent TMP resistance in the presence of MLSS filtrate (20-day SRT) 76
Figure 3-21. Ambient air temperature and apparent SMX/TMP resistance 79
Figure 4-1. Average concentrations of (A) sCOD and (B) ammonia 94
Figure 4-2. Average concentrations of (A) nitrate and (B) nitrite 95
Figure 4-3. Temporal variability in the sCOD, ammonia, nitrate, and nitrire 97
Figure 4-4. Representative EEMs as a function of SRT 99
Figure 4-5. Representative EEMs as a function of influent antibiotic concentration 100
Figure 4-6. Average reductions in peak fluorescence 101
Figure 4-7. Average reductions in regional fluorescence during (A) phase 1 and (B) phase 2 102
Figure 4-8. Goods coverage plot for phase 1 115
Figure 4-9. Microbial community structure (first sample set) 119
Figure 4-10. Microbial community structure (second sample set) 120
Figure 4-11. Results of principal component analyses for phase 1 and phase 2 124
Figure 5-1. Schematic of spread plate analysis 141
Figure 5-2. Schematic of minimum inhibitory concentration analysis 142
Figure 5-3. Average concentrations of sCOD, MLSS and MLVSS 144
Figure 5-4. Average concentrations of ammonia, nitrate, and nitrite (varying SRT) 145
Figure 5-5. Effect of temperature on sCOD, ammonia, and nitrate (varying SRT) 145
Figure 5-6. Average concentrations of sCOD, MLSS and MLVSS (elevated antibiotic concentrations) 147
Figure 5-7. Average concentrations of ammonia, nitrate (elevated antibiotic concentrations) 147
Figure 5-8. Summary of TOrC mitigation as a function of SRT 149
Figure 5-9. Percentage of Resistant Staph/Strep in the SBRs with varying SRT (Sample Event 1) 155
Figure 5-10. Percentage of Resistant Staph/Strep in the SBRs with varying SRT (Sample Event 2) 155
Figure 5-11. Percentage of Resistant Staph/Strep in the SBRs with varying SRT (Sample Event 3) 156

المنارة للاستشارات
www.manaraa.com

Figure 5-12. Temporal trend in antibiotic resistant percentage (Varying SRT) 159

Figure 5-13. Percentage of Resistant Staph/Strep in the SBRs with elevated antibiotic concentrations (Sample Event 1)... 162

Figure 5-14. Percentage of Resistant Staph/Strep in the SBRs with elevated antibiotic concentrations (Sample Event 2)... 163

Figure 5-15. Percentage of Resistant Staph/Strep in the SBRs with elevated antibiotic concentrations (Sample Event 3)... 163

Figure 5-16. Temporal trend in antibiotic resistant percentage (Elevated antibiotic concentrations)....... 164

Figure 5-17. Linear correlation between predicted values and experimental observations of target antibiotic resistant bacteria 167

www.manaraa.com

1.0 INTRODUCTION

The discovery of antibiotics is considered a turning point in human health history because antibiotics have been responsible for saving millions of people each year. The term "antibiotic" is defined as any class of organic molecule that kills or inhibits microbes by specific interactions with bacterial targets (Davis and Davis, 2010). Unfortunately, the intensive use of antibiotics for therapeutic and non-therapeutic purposes has significantly decreased the effectiveness of antibiotics over the past 60 years. Although there are no regulated statistics available on the quantity of antibiotics used in the United States, it is estimated that over 20 million pounds of antibiotics are used in agriculture and veterinary medicine, which is about 80 percent of the total antibiotics sold in the U.S. each year.

In addition to general concerns related to antibiotic occurrence and exposure, recent studies suggest a link between wastewater treatment and the occurrence of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) (Auerbach et al., 2007; Zhang et al., 2015). In fact, wastewater treatment plants (WWTPs), which are critical for protecting human and environmental health from pollution in wastewater, are now considered significant reservoirs of antibiotic resistance (AR) (Novo and Manaia, 2010). The presence of antibiotics in wastewater matrices can form a selective pressure that increases the concentration of ARBs by inhibiting antibiotic-susceptible bacteria and increasing the probability of mutation and horizontal gene transfer (Schwartz et al., 2003; Martinez, 2008; Wang et al., 2011). As such, ARB and ARGs are now considered wastewater-derived contaminants of emerging concern (CECs) that pose a threat to public health (Pruden et al., 2006). However, the role of WWTPs in the dissemination and proliferation of ARB and ARGs is still unclear, and there is still a lack of comprehensive studies assessing the effects of operational conditions in biological processes on the prevalence of

المنارة للاستشارات
www.manaraa.com

antibiotic resistance in treated wastewater. For example, solids retention time (SRT), or the average amount of time bacteria are recycled within an activated sludge system, has been shown to impact microbial community structure (e.g., with respect to nitrification) so SRT may also have an impact on the prevalence of antibiotic resistance and the ability of the microbial community to biodegrade CECs commonly found in wastewater, including antibiotics.

A general goal of this research is to provide a better understanding of the effects of several operational and water quality variables on the occurrence, proliferation, and mitigation of antibiotics and antibiotic resistance in wastewater. This research was divided into three tasks, each of which focused on specific research questions:

1.1 Environmental reservoirs of thymidine as a mechanism of trimethoprim resistance:

Background: Trimethoprim disrupts the conversion of dihydrofolate to tetrahydrofolate—a process involved in the synthesis of nucleotides. Although trimethoprim blocks the tetrahydrofolate pathway, bacteria might still be able to obtain thymidine or thymine from growth media or from their environment, thereby artificially elevating the observed level of AR. Bacterial cells lyse when they enter the death phase, which releases their cellular contents (e.g., thymine and thymidine) into their surrounding environment. In theory, more cell debris may be indicative of higher concentrations of free thymine and thymidine in a biological reactor. Because there is a higher bacterial death rate at longer SRTs, such systems may be characterized by greater trimethoprim resistance due to water quality differences (i.e., higher free thymine and/or thymidine) rather than—or in addition to—changes in the microbial community. With some culture-based techniques, like membrane filtration, bacteria are separated from their aqueous environment, but with other techniques, such as the spread plate method, an aliquot of

المنارة للاستشارات
www.manaraa.com

the aqueous sample is transferred to the growth media. Therefore, the number of trimethoprim resistant bacteria might be influenced by the method used. In this task, culture-based methods will be used to quantify the level of trimethoprim resistance as a function of media used (i.e., media with low levels of thymine/thymidine vs. media with higher levels of thymine/thymidine) and as a function of the quantity of cellular debris.

Research question: How does thymidine in environmental samples interfere with the detection of trimethoprim resistant bacteria and how does SRT impact environmental reservoirs of thymidine-like compounds?

Hypothesis: Because thymine/thymidine can be found in some nutrient media or the intracellular components of lysed bacteria from biological treatment systems, bacteria can access these environmental reservoirs, thereby bypassing traditional mechanisms of thymine/thymidine production, and grow in the presence of clinical concentrations of trimethoprim. Longer SRTs also yield greater quantities of cellular debris, thereby yielding greater numbers of bacteria with apparent trimethoprim resistance.

Approach: The SBRs will be operated with similar conditions to those described above. The microbiological components of the study will be divided into four sets of experiments: (1) manual augmentation of thymidine with reagent-grade chemical, (2) manual augmentation of thymidine via cell lysing, (3) varying of SRT in the SBRs to evaluate the effects of cellular debris, and (4) varying of SRT in the SBRs to evaluate single- and multi-drug resistance.

Outcome: Results from this research will show that some trimethoprim sensitive bacteria can be reported as resistant bacteria, which may erroneously overestimate the role of biological treatment systems in proliferating AR.

المنارة للاستشارات
www.manaraa.com

1.2 Microbial community structure and function:

Background: Longer SRTs may alter microbial community structure and function, which can lead to reductions in TOrC concentrations, improved nutrient removal, and greater transformation of bulk organic matter. Despite efforts to understand the microbial communities in WWTPs, specifically in biological treatment systems, there are still many uncertainties regarding microbial community structure and function. Contradictory outcomes may arise when studies focus on different influent wastewater qualities, different treatment technologies and/or operational conditions, and even different methodologies for assessment of microbial community structure. Therefore, additional studies and analyses are needed to assess the role of wastewater treatment processes and their operational conditions (e.g., SRT) on microbial community structure. Furthermore, with current rates of antibiotic production and consumption, it is quite possible to expect higher concentrations of antibiotics in raw wastewaters. Moreover, accidental releases of untreated industrial wastewater (e.g., pharmaceutical manufacturing) may increase the risk of biological treatment failure if bacteria are exposed to unusually high concentrations of antibiotics. This may adversely impact microbial community structure and function, reduce the efficacy of wastewater treatment by inhibiting critical subpopulations, and possibly lead to the failure of biological treatment. Therefore, a more comprehensive understanding of the effects of operational conditions, specifically SRT, and varying influent antibiotic concentrations on wastewater treatment is needed.

Research question 1: What is the effect of varying solids retention time (SRT) on *(i)* microbial community structure and *(ii)* trace organic compound (TOrC) concentrations?

Hypothesis 1: Biological treatment systems with longer SRTs may select for slowly growing bacteria capable of degrading a wider variety of TOrCs and achieving lower effluent TOrC

المنارة للاستشارات
www.manaraa.com

concentrations. Longer SRTs will promote a shift in microbial community structure, as determined by 16s rDNA sequencing.

Research question 2: What is the effect of varying influent antibiotic concentrations on *(i)* microbial community structure and *(ii)* TOrC concentrations?

Hypothesis 2: Higher antibiotic concentrations in biological reactors may inhibit the growth and metabolic activity of some microorganisms, thereby promoting a shift in microbial community structure and hindering TOrC degradation.

Approach: In Task 1, laboratory-scale sequencing batch reactors (SBRs) were designed to mimic an activated sludge process operating with SRTs of 2 days, 7 days (in duplicate), and 20 days to understand the role of varying SRT on microbial community structure. In Task 2, the SBRs will be operated at a constant SRT of 7 days, but the reactor influent will be spiked with target antibiotics at concentrations of 1x (ambient primary effluent concentrations), 10x (in duplicate), and 100x. Ambient concentrations of the target antibiotics (ampicillin, sulfamethoxazole, tetracycline, trimethoprim, and vancomycin) will be determined during Task 1. Despite the elevated spiking levels, the concentrations are still likely to be sub-inhibitory when compared against clinical standards. Samples will be collected from the SBRs for 16s rDNA sequencing. After extraction and purification, the DNA will be shipped to Research and Testing Laboratory (Lubbock, TX) for amplification with universal primers for Bacteria and analysis with a MiSeq sequencer (Illumina, San Diego, CA). After preliminary data processing, statistical analyses will be performed on the top 10 most abundant genera by principal component analysis (PCA).

Outcome: Microbial community structure in biological wastewater treatment processes has been previously analyzed, but the analyses have generally been performed on large systems with

www.manaraa.com

limited experimental control. The results from this study will clarify the roles of SRT and influent antibiotic concentrations on microbial community structure and function with respect to TOrC degradation. Also, SBR performance and treatment efficiency at ambient and elevated concentrations of antibiotics will help predict the risk of treatment process failure during transient antibiotic loading conditions.

1.3 Relative abundance and extent of AR:

Background: Despite the efforts to elucidate the role of WWTPs in relation to antibiotic resistance, there is still no clear evidence that WWTPs, specifically the biological treatment processes, are contributing to the proliferation of antibiotic resistance. Some studies suggest that WWTPs achieve a significant reduction in the number of ARB (Guo et al., 2015; Huang et al., 2012), while other research indicates that WWTPs serve as major contributors of ARB and ARGs (Kim et al., 2010). It is important to remember that comparing the results from different WWTPs with different influent wastewater quality, treatment trains and technologies, and operational conditions may not yield meaningful relationships between antibiotic resistance and biological treatment. In fact, the contradictions reported in the existing literature might be attributable to such differences. Therefore, in order to truly understand the role of biological treatment systems in relation to AR, more controlled experiments are needed to reduce the number of uncertainties. By using laboratory-scale SBRs fed with primary effluent from a single WWTP, it is possible to isolate the effect of SRT and elevated antibiotic concentrations on the target AR metrics.

Research question 1: What is the effect of varying SRT on relative abundance and extent of antibiotic resistance in biological treatment systems?

المنارة للاستشارات
www.manaraa.com

Hypothesis 1: Prolonged exposure of bacteria (i.e., longer SRTs) to sub-inhibitory concentrations of antibiotics, heavy metals, and other antimicrobial agents (e.g., triclosan) will lead to *(i)* higher rates of antibiotic resistance, as measured by relative abundance of ARBs and *(ii)* greater extent of antibiotic resistance, as measured by minimum inhibitory concentrations (MICs) of antibiotic resistant isolates.

Research question 2: What is the effect of varying influent antibiotic concentrations on relative abundance and extent of antibiotic resistance in biological treatment systems?

Hypothesis 2: Prolonged exposure of bacteria to elevated, yet still sub-clinical, concentrations of antibiotics in primary wastewater effluent will lead to *(i)* higher rates of antibiotic resistance, as measured by relative abundance of ARBs and *(ii)* greater extent of antibiotic resistance, as measured by MICs of antibiotic resistant isolates.

Approach: The SBRs will be operated with similar conditions to those described above. In order to detect culturable ARB, the spread plate technique will be used. Colony counts on Mueller-Hinton (MH) agar containing Staph/Strep Supplement will serve as the 'total' culturable count, and the colony counts on the same media supplemented with the target antibiotics at standard clinical concentrations will serve as the AR counts. The AR percentage will be reported to account for variations in total bacteria as a function of SRT. A total of eight random AR isolates will also be harvested for each antibiotic, and pure cultures of each isolate will be assayed with the MIC method.

Outcome: The results from this study will provide a better understanding of the role of SRT and influent antibiotic concentrations on relative AR abundance and extent of AR in biologically treated wastewater effluents.

المنارة للاستشارات
www.manaraa.com

This dissertation has a general literature review on antibiotic resistance followed by three chapters, which are presented as standalone papers. Each paper answers one of the main research questions and contains 5 sections including: (1) abstract, (2) critical literature review, (3) research methods, (4) results and discussions, and (5) conclusions.

2.0 STATE OF KNOWLEDGE

2.1 Antibiotic Resistance as an Emerging Threat

In 2011, the World Health Organization (WHO) identified antibiotic resistance (AR) as "one of the three greatest threats to human health" (WHO, 2011). Recent scientific studies have also established a link between recycled water and environmental occurrence of AR (Fahrenfeld et al., 2013).

The discovery of antibiotics is considered a turning point in human health history. The first class of sulfonamide antibiotics was introduced in the mid-20th century. Due to the initial efficacy of the sulfonamides, these antibiotics became more prevalent, which presumably led to the rise of sulfonamide resistance (Costa et al., 2006). After the discovery of sulfonamides, other classes of antibiotics, such as penicillin and streptomycin, were also discovered and administered, which led to further antibiotic resistance. Similar cycles of drug development, widespread use, and increased resistance have since been observed.

In recent decades, many pathogenic bacteria have evolved into multi-drug resistant (MDR) bacteria. Concern is growing about MDR bacteria because of their resistance to a wide range of antibiotics. In fact, previous studies confirmed that patients who visited hospitals more frequently were more susceptible to MDR pathogens. (McAdam et al., 2010). Unfortunately, the rate of development and production of new antibiotics has significantly declined over the past 30

المنارة للاستشارات
www.manaraa.com

years. Therefore, the emergence of single- and multi-drug resistance coupled with the decreasing number of effective antibiotics necessitates a coordinated global strategy to slow the spread of AR. Figure 2-1 shows the antibiotic resistance evolution over the last 80 years and suggests that society may be returning to the pre-antibiotic era.

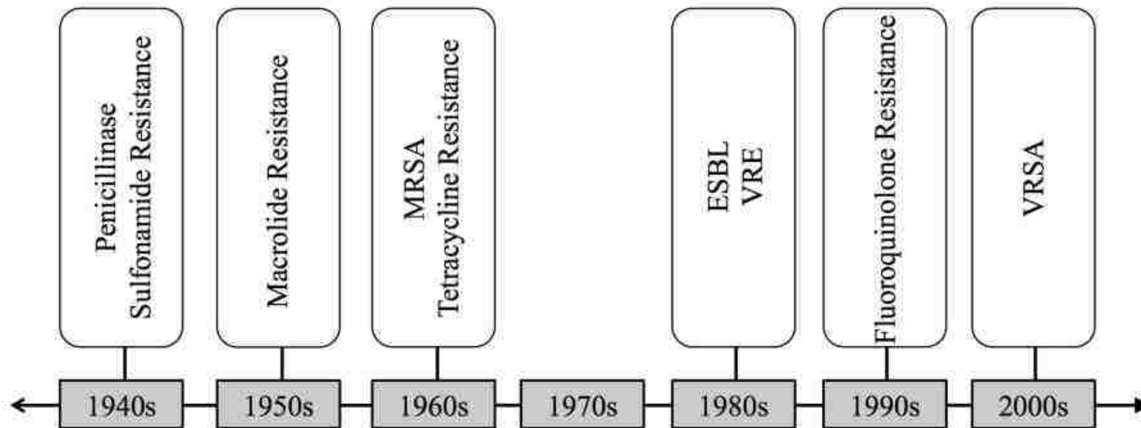


Figure 2-1. Timeline of Antibiotic Resistance

*VRSA = vancomycin-resistant *Staphylococcus aureus*, ESBL = extended spectrum beta-lactamases, VRE = vancomycin-resistance *Enterococcus*, MRSA = methicillin-resistant *Staphylococcus aureus*.

Wastewater treatment plants play an important role in protecting human and environmental health from pollution in wastewater, but they are also considered significant reservoirs for AR. Previous studies investigated the role of wastewater treatment plants in the proliferation or mitigation of antibiotic resistant bacteria (ARBs) and antibiotic resistance genes (ARGs) (Zhang et al., 2015; Guo et al., 2015.; Su et al., 2014). These studies highlighted the selection pressure exerted on bacteria in wastewater matrices (Schwartz et al., 2003). Specifically, the presence of antibiotics can form a selective pressure that increases the concentration of ARBs by inhibiting antibiotic-susceptible bacteria. This medium also increases the chance of mutation and horizontal gene transfer (HGT) (Wang et al., 2011; Martinez, 2008).

المنارة للاستشارات
www.manaraa.com

Data reported in previous publications are sometimes inconsistent and contradictory. For example, Aminov et al. (2001) and Auerbach et al. (2007) showed that due to the continuous exposure of bacteria to sub-inhibitory concentrations of antibiotics, wastewater treatment plants provide an environment that is potentially suitable for proliferation of ARGs and ARBs. However, Suller et al. (2000) showed that continuous exposure of a triclosan-sensitive *Staphylococcus aureus* strain to sub-inhibitory concentrations of triclosan did not promote any changes in triclosan susceptibility or to other targeted antibiotics.

Despite the efforts to elucidate the role of wastewater treatment plans (WWTPs) in relation to antibiotic resistance, there is still no clear evidence that WWTPs, especially the biological treatment processes, are contributing to the proliferation of antibiotic resistance. Some studies suggest that WWTPs achieve a significant reduction in the number of ARBs (Guo et al., 2015; Huang et al., 2012), while other research indicates that WWTPs serve as major contributors of ARBs and ARGs (Kim et al., 2010). These uncertainties may arise from research evaluating different treatment technologies, operational conditions, influent wastewater quality or wastewater constituents, and different methodologies for the detection of ARBs and ARGs. Therefore, additional studies and analyses are needed to assess the role of wastewater treatment processes on proliferation and mitigation of antibiotic resistance.

Human and animal gastrointestinal systems are likely significant contributors to antibiotic resistance in the environment. As demonstrated in the recent literature, one of the potential pathways for the release of human-derived AR into the environment is through the discharge of treated wastewater effluent (Kim et al., 2010). Wastewater-impacted surface waters have been shown to contain many tetracycline residues (Kim et al., 2010) at concentration as high as 4 μg/L (Kolpin et al., 2002). Raw sewage entering the plant contains a wide variety of inorganic and

المنارة للاستشارات
www.manaraa.com

organic contaminants, including heavy metals, antibiotics, and detergents that have been linked to AR development. The raw sewage also contains a baseline level of AR in the form of ARBs and ARGs (Zhang et al., 2009). Once these compounds and AR building blocks reach the secondary biological treatment process, they enter an ideal environment for bacterial activity, growth, horizontal gene transfer (Kim et al., 2010), and co- or cross-resistance to antibiotics (Silver and Phung, 1996; Alonso et al., 2001; Baker-Austin et al., 2006). However, as mentioned earlier, it is not yet clear whether the secondary biological treatment process actually contributes to the proliferation of AR or whether it actually provides some level of mitigation due to AR bacteria being outcompeted.

Kim et al. (2010) performed a mass balance to clarify the role of the activated sludge process in proliferating or attenuating tetracycline resistant bacteria (TRBs) and tetracycline resistance genes (TRGs) in different WWTPs. They found that WWTPs neither amplified nor attenuated the TRBs and TRGs. The results also indicated that among 20 different TRGs tested, *tet*(O) and *tet*(W) genes were the most abundant genes throughout the treatment train. Zhang et al. (2015) investigated the fate of antibiotic-resistant phenotypes of cultivable heterotrophic bacteria and ARGs in three WWTPs in China. The research team monitored thirteen ARGs in activated sludge from anaerobic, anoxic, and aerobic zones. These ARGs were from tetracycline, sulfonamide, streptomycin, and β-lactam resistance classes. The result from this study indicated that WWTPs displayed considerable reduction in the total cultivable heterotrophic bacteria containing resistance elements. They also showed that ARGs are more frequent in influent than effluent suggesting that the wastewater treatment facilities contribute to a decrease in the prevalence of antibiotic resistance. However, the results confirmed that during activated sludge

المنارة للاستشارات
www.manaraa.com

process, ARGs abundance increased, thereby suggesting that ARGs accumulate in sludge rather than remaining in the aqueous phase.

Wang et al. (2015) monitored the fate of 10 subtypes of ARGs for sulfonamide, tetracycline, β-lactam class, and macrolide resistance and the class 1 integrase gene (*intI*1) across each stage of 5 full-scale pharmaceutical WWTPs in China. The results showed that the WWTPs can reduce the number of ARGs by 0.5-2.5 orders of magnitude in the aqueous phase, but a significant amount of ARGs are discharged in dewatered sludge. The total load of ARGs in dewatered sludge was 7-fold to 308-fold higher than raw influent and 16-fold to 638-fold higher than final effluent. The results also showed the proliferation of ARGs in the biological treatment processes.

Shi et al. (2013) evaluated the effect of chlorination on microbial antibiotic resistance in drinking water treatment plants. 16S rRNA gene cloning indicated that Proteobacteria are the main ARB in drinking water. The results also showed that after chlorination, resistance to chloramphenicol, trimethoprim, and cephalothin was higher among surviving bacteria, possibly indicating a link between resistance to disinfection and resistance to antibiotics.

Pruden et al. (2012) investigated the occurrence of ARGs in different environments, including river sediments, dairy lagoons, irrigation ditches, and wastewater and drinking water treatment plants. The polymerase chain reaction (PCR) technique was used to detect several tetracycline and sulfonamide ARGs. Quantitative PCR (qPCR) assays were performed to further quantify two tetracycline ARGs *tet*(W) and *tet*(O) and two sulfonamide ARGs *sul*(I) and *sul*(II). The results confirmed that ARG concentrations in environments impacted by human/agricultural activity are higher than pristine environments.

Kristiansson et al. (2011) investigated the microbial communities in river sediments receiving wastewater from pharmaceutical companies. A culture-independent shotgun metagenomic

المنارة للاستشارات
www.manaraa.com

technique was applied to determine the microbial communities. In order to characterize the resistome, the research team searched the metagenomes for signatures of known antibiotic resistance genes. The results showed that significant differences were found among resistance genes associated with different classes of antibiotics, including sulfonamides, fluoroquinolones, and aminoglycosides in downstream locations compared to upstream locations. The same pattern was observed for integrons, transposons, and plasmids. The relative abundance of class 1 integrases, transposase (associated with insertion sequence common regions (ISCRs)), and two plasmids (RSF1010 and pMTSm3) were considerably higher in downstream locations compared to upstream locations, suggesting the higher mobility of resistance genes in downstream. These genes could eventually be transferred from environmental microbes to human pathogens.

In 2009, a strain of *Klebsiella pneumonia* with a broad range of antibiotic resistance was identified from a Swedish patient previously hospitalized in India (Yong et al., 2009). The antibiotic resistance determinant was recognized as a novel metallo-β-lactamase (MBL) and designated the New Delhi metallo-*β*-lactamase (NDM-1), which is an enzyme that confers resistance to a broad range of antibiotics. Bacteria with this type of multidrug resistance pose a great risk to global health (Luo et al., 2014). Luo et al. (2014) investigated the proliferation of multidrug resistant New Delhi metallo-*β*-lactamase genes in different processes in WWTPs in China. The results indicated that NDM-1 gene prevailed throughout several treatment units, including the discharged effluent, and that NDM-1 genes were found in higher concentrations in dewatered sludge.

Chen et al. (2013) conducted research to evaluate the removal rate of ARGs in WWTPs in China. Three WWTPs with different advanced treatment systems (biological aerated filter, constructed wetland, and UV disinfection) were selected to quantify the concentration of ARGs. In this

المنارة للاستشارات
www.manaraa.com

study, the concentrations of 16S rRNA genes, *tet*(M), *tet*(O), *tet*(Q), *sul*(I), *sul*(II) and *intI*1 were measured in wastewater and biosolids. The results revealed that ARGs concentration decreased by 1.3-2.1 orders of magnitude in the constructed wetland and by 1.0-1.2 orders of magnitude in the biological aerated filter. However, only small changes were observed for the targeted ARGs between influent and effluent of the UV disinfection system. The same observation was made by McKinney and Pruden (2012) regarding the limited potential of UV disinfection to damage ARGs in wastewater effluents.

Although many of the recent AR studies focus on molecular methods, some studies are assessing AR occurrence and fate through culture-based methods. Zhang et al. (2015) studied AR among heterotrophic bacteria using traditional spread plating and streaking techniques. The bacterial isolates were tested for susceptibility to 12 different antibiotics based on the standard concentrations identified by the CLSI. One of the major findings from the study was that wastewater treatment plants typically reduced the extent of multi-drug resistance in the treated effluent. In other words, bacteria present in the effluent were resistant to fewer antibiotics than bacteria present earlier in the treatment train. Through sequencing, they also discovered that Gram negative bacteria dominated the wastewater influent, while Gram positive bacteria dominated the effluent.

Amador et al. (2015) evaluated the role of hospitals and wastewater treatment plants as contributors of AR in Portugal. The ampicillin-resistant *Enterobacteriaceae* were enumerated and isolated and were tested for antimicrobial susceptibility using the disk diffusion method. The study measured the resistance to the β-lactam group of antibiotics, including cefoxitin and the combination of amoxicillin and calvulanic acid, and the non-β-lactam group, including tetracycline and the combination of trimethoprim and sulfamethoxazole. The results showed that

المنارة للاستشارات
www.manaraa.com

wastewater treatment plant effluent contained a higher rate of multidrug resistance compared with the untreated influent. A similar study was performed by Nagulapally et al. (2009) to examine the occurrence of ciprofloxacin, trimethoprim/sulfamethoxazole, and vancomycin resistant bacteria in a wastewater treatment plant. The results revealed that a significant number of fecal coliforms, *E. coli*, and enterococci exhibited resistance to the target antibiotics in municipal treatment plants.

2.2 Drivers of Antibiotic Resistance

The rapid appearance and widespread proliferation of multidrug resistant phenotypes was a relatively unexpected phenomenon. It was originally believed that mutation had a key role for spreading antibiotic resistance, but the emergence of bacteria with multidrug resistance led to the discovery of transferable genetic materials like conjugative R-plasmids and transposons (Row-Magnus and Mazel, 2002). Integrase is a type of site-specific recombinase that promotes recombination between two defined sequences in DNA. One typical example is integration of phage DNA into a bacterial chromosome. An integron is an integrase with a specific site for integration of gene cassettes, which might include ARGs. Integrons are thought to be one of the important actors in the dissemination of resistance genes among diverse Gram-negative isolates (Hall and Stokes, 1993; Bennett, 1999). Great attention is given to the Gram-negative bacteria of the *Enterobacteriaceae* since they are responsible for common food-borne diseases. However, Gram-positive bacteria are also a major reservoir of class 1 antibiotic resistance integrons (Nandi et al., 2004).

Plasmids are known as extrachromosomal genetic material carrying multiple genes, including ARGs. Plasmids are circular molecules of double-stranded or linear DNA and may vary in size

المنارة للاستشارات
www.manaraa.com

from a few thousand to hundreds of thousands of base pairs. Bacterial cells carry plasmids because they provide gene products that benefit the bacteria in special circumstances. However, bacteria may lose their plasmids when they are no longer in need of those benefits (i.e., when encountering reduced or eliminated selective pressure). Bacteria can 'justify the costs' of harboring resistance genes when the risks of exposure to antibiotics or heavy metals are increased (Baquero and Coque, 2014). A study by Gullberg et al. (2014) showed that continuous exposure of bacteria to sublethal concentrations of antibiotics and heavy metals is sufficient to justify the maintenance cost of harboring resistance elements. Plasmids are of interest due to their ability to code for multidrug resistance and also their ability to spread genes via bacterial conjugation, which is one form of horizontal gene transfer (HGT). HGT can be accomplished by three main mechanisms: conjugation, transduction, and transformation.

I Conjugation

Bacterial conjugation occurs when genetic material is transferred between two cells by direct cell-to-cell contact. In this system, the donor cell provides the transferable genetic elements, such as a plasmid or transposon, via pilus attachment (**Error! Reference source not found.**). A transposon is a DNA sequence that can use transposase (a specialized recombinase) to freely move along the DNA from one place to another. The mechanism of conjugation has been highlighted in systems with high concentrations of both bacteria and antibiotics (Shoemaker et al., 2001; Davies and Davies, 2010; Dodd et al., 2012), such as human or animal gastrointestinal tracts, but some studies have also demonstrated the potential for conjugative gene transfer to occur in wastewater and the environment (Alcaide and Garay, 1984; Dodd et al., 2012).

المنارة للاستشارات
www.manaraa.com

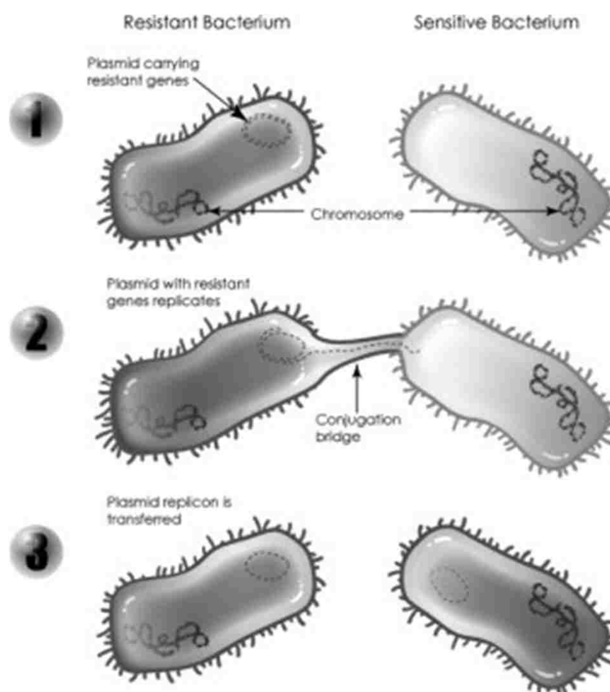


Figure 2-2. Conjugation mechanism

Reprinted from Antibiotic Resistance: A Guide for Effective Prescribing in Women's Health, Vol 53, Valerie A. Roe, Copyright 2008, with permission from Elsevier

II Transduction

In transduction, bacterial genetic elements, such as a piece of DNA, is transferred from one bacterium to another by a bacteriophage, which is a virus that infects bacteria. In transduction, direct contact between two cells is not required because the transfer occurs by a vector (i.e., the phage). Assuming the transferred DNA codes for antibiotic resistance, the ARG can potentially be transferred to the new host (Dodd et al., 2012). This mechanism has recently been observed in wastewater (Muniesa et al., 2004; Parsley et al., 2010; Colomer-Lluch et al., 2011; Dodd et al., 2012). **Error! Reference source not found.** illustrates the bacterial transduction mechanism. Although the bacteriophages are thought to have a role in proliferation of antibiotic resistant bacteria, recent studies were focused on applying lytic phages to kill antibiotic resistant bacteria (Sulakvelidze, 2005). Lytic bacteriophages are effective in killing bacteria via mechanisms that

المنارة للاستشارات
www.manaraa.com

are different from those of antibiotics (Sulakvelidze, 2005). Thus, phage therapy may be a promising option for treating pathogenic antibiotic resistant bacteria (Sulakvelidze, 2005).

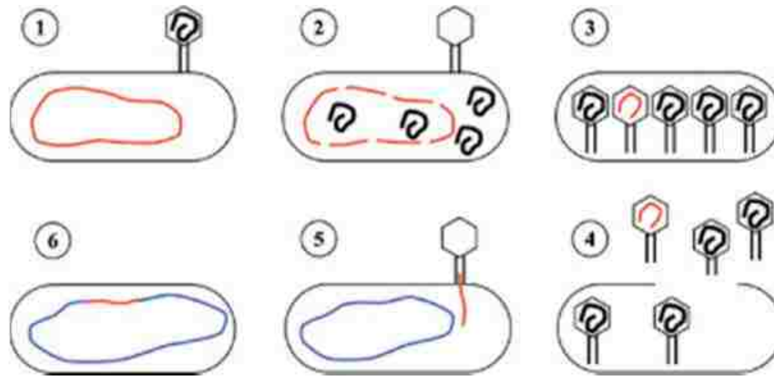


Figure 2-3. Transduction mechanism

Reprinted from Escherichia coli as a model active colloid: A practical introduction, Vol 137, Schwarz-Linek et al, Copyright 2016, with permission from Elsevier

Bacteria which are infected by phages can either participate in a lysogenic cycle or a lytic cycle. In a lysogenic cycle, the cell continues to live and reproduce, but in a lytic cycle, the host cell is lysed after infection.

III Transformation

During transformation, bacteria will uptake foreign, free-floating genetic elements from the surrounding environment. The genetic elements could be naked DNA or a plasmid. For transformation to occur, a bacterium must be in a state of competence which can be promoted by changing environmental conditions. It appears that most bacteria are not able to take up DNA in an efficient way, but some chemicals may make them more permeable. On the other hand, there are bacteria that are able to take up DNA from their environment without any chemical treatment, which are called naturally transformable. Even naturally transformable bacteria cannot always take up DNA, and they must be at certain stage in their life cycle. Competence is referred

المنارة للاستشارات
www.manaraa.com

to the state that bacteria have in their life cycle in which they are able to take up naked DNA from their environment (Molecular Genetics of Bacteria, 2013). This mechanism of gene transfer has been specifically linked to streptococci and meningococci (Davies and Davies, 2010; Dodd et al., 2012), but it is not exclusive to these genera (Dodd et al., 2012). Figure 2-4 illustrates bacterial transformation.

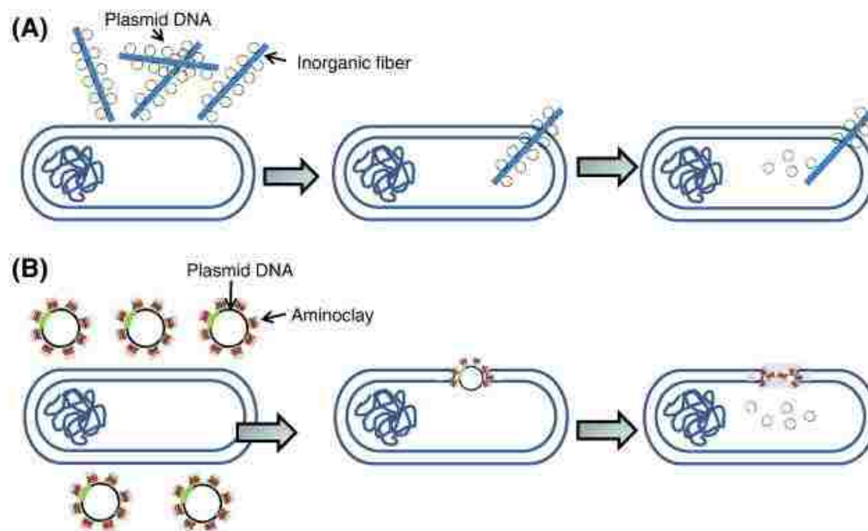


Figure 2-4. Transformation mechanism

Reprinted from A simple bacterial transformation method using magnesium- and calcium-aminoclays, Vol 95, Choi et al. Copyright 2013, with permission from Elsevier

2.3 Influence of Metals, Quaternary Ammonium Compounds, and Antimicrobial Agents

The presence of quaternary ammonium compounds (QACs), heavy metals, and antimicrobial agents is another driving force for the development of antibiotic resistance. For metabolism, maintenance, and growth, bacteria require trace concentrations of some metals like zinc, nickel, chromium, and copper. Theses metals are necessary for bacterial metabolic activity and enzyme production, but they can also be toxic at higher concentrations (Seiler and Berendonk., 2012). Becerra-Castro et al. (2015) showed that copper or zinc at concentrations >60 mg/L may select

المنارة للاستشارات
www.manaraa.com

for antibiotic resistant phenotypes. Other heavy metals including silver, mercury, and lead are toxic to bacteria even at low concentrations (Seiler and Berendonk, 2012). In fact, the application of heavy metals, particularly silver, in industry and agriculture is common due to their bactericidal potential.

A study by Salyers and Amabile-Cuevas (1997) suggested that decreases in antibiotic usage do not necessarily limit the spread and maintenance of AR because agents other than antibiotics can promote cross-resistance (Baker-Austin et al., 2006). For example, bacteria have evolved mechanisms of metal tolerance, including (1) complexation or sequestration of metals, (2) intercellular ion reduction via proteins (e.g., reduction of Hg^{2+} to Hg^0 by MerA protein), and (3) efflux systems (Seiler and Berendonk, 2012). These mechanisms are relevant to antibiotic resistance because the bacterial response to antibiotics and heavy metals is similar (Koditschek and Guyre, 1974). The resistance can be described as "cross-resistance," in which the same mechanism (e.g., efflux pumps) is responsible for protection against metals and antibiotics, or "co-resistance," in which resistance to metals and antibiotics is genetically coded in close proximity on a plasmid (Seiler and Berendonk, 2012). Both Gram negative and Gram positive bacteria are susceptible to metal resistance, but for some heavy metals like cadmium, Gram positive bacteria are more sensitive (Babich and Stotzky, 1977). Gram negative bacteria also have an outer cell membrane which may limit the penetration of metals.

2.4 Antibiotic Compounds and Resistance Mechanisms

Antibiotics have established themselves as some of the most powerful tools against pathogenic diseases in humans and animals. As a result, many research projects have been conducted to discover new antibiotics and to understand their structure and mechanisms of action. A subset of

www.manaraa.com

clinically significant antibiotics is presented in this section. These antibiotics will serve as the target compounds for the research described later.

The sulfonamide antibiotic class includes sulfamethoxazole (SMX), which is often administered in conjunction with the dihydrofolate reductase inhibitor trimethoprim (Bushby and Hitchings, 1968). The combination of these two antibiotics is effective against a variety of Gram positive and Gram negative bacteria. Sulfamethoxazole is an antimicrobial substance with the formula of $C_{10}H_{11}N_3O_3S$. SMX is a member of the sulfonamide antibiotic class, which was the first antibiotic to be used for clinical practice on a large scale (Zhang et al., 2009). Sulfonamide antibiotics disrupt folate synthesis by inhibiting dihydropteroate synthetase (DHPS), which is responsible for transformation of para-aminobenzoic acid to dihydrofolate. Dihydrofolate has a pivotal role in DNA precursor synthesis in that DNA synthesis cannot be performed in the absence of dihydrofolate.

Trimethoprim (TMP) also targets folic acid synthesis and was first used in 1962 to treat human infections (Huovinen et al., 2001). TMP disrupts the conversion of dihydrofolate to tetrahydrofolate—a process involved in the synthesis of nucleotides. TMP kills the cell by depleting it of dihydrofolate reductase, which catalyzes the transformation of dihydrofolate to tetrahydrofolate.

The β-lactam antibiotic class includes ampicillin (AMP). β-lactam antibiotics interfere with the cross-linking of peptidoglycan in bacterial cell walls, which prevents cell division and ultimately results in bacterial cell lysis (Struthers et al., 2003). Ampicillin is effective against Gram positive and some Gram negative bacteria. Enterobacterial *ampC* is a chromosomal antibiotic resistance gene (ARG) for the synthesis of β-lactamase, which hydrolyzes ampicillin at the β-lactam ring.

المنارة للاستشارات
www.manaraa.com

The *ampC* ARG has also been described as an indicator of fecal contamination in wastewater, surface water, and drinking water (Schwartz et al., 2003; Volkmann et al., 2004).

Tetracycline is another class of antibiotics used against a broad range of Gram-negative and Gram-positive bacteria (Auerbach et al., 2007; Struthers et al., 2003). Tetracycline interrupts protein synthesis in the bacterial cell. Tetracycline resistant bacteria are able to eject the antibiotic from its cytoplasm through efflux pump mechanisms. Resistant bacteria are also able to eliminate tetracycline from their ribosome via ribosomal protection proteins (RPPs) (Roberts, 2005). The *tetW* ARG, which has been detected in wastewater treatment plants, is responsible for RPP. Usually, conjugative plasmids or transposons carry *tetR* genes, which make them easier to be transferred via horizontal gene transfer mechanisms (Auerbach et al., 2007). With the introduction of tetracycline, at least 38 different tetracycline resistance genes (*tet*) have been detected in a variety of bacterial genera (Auerbach et al., 2007; Dancer et al., 1997; Roberts, 2005). Among all *tet* genes, 22 genes have been identified in water environments.

Vancomycin (VA) is an antibiotic of last resort for the treatment of bacterial infections when other antibiotics therapies have been failed. Vancomycin is a glycopeptide antibiotic prescribed for serious Gram-positive bacterial infections. VA is not able to pass across the cell membrane of Gram-negative bacteria due to the large glycopeptide molecule. Therefore, most Gram negative bacteria (e.g., *E. coli*) are intrinsically resistant to VA. Vancomycin-resistant *Enterococcus* (VRE) is a severe bacterial infection associated with outbreaks of hospital-acquired infections around the world (Schwartz et al, 2003). Six different types of vancomycin ARGs have been discovered, but the *van*A ARG is the most abundant in surface water and wastewater (Messi et al., 2006; Volkmann et al., 2004). The VA ARGs typically result in morphological changes to

المنارة للاستشارات
www.manaraa.com

the bacterial cell wall that hinder VA attachment. Table 2-1 summarizes these antibiotics and their related genes.

Table 2-1. Target antibiotics and respective antibiotic resistance genes

Antibiotic Class	Target Antibiotic	Antibiotic Mode of Action	Target AR Gene	Antibiotic Resistance Mode of Action
β-lactam	Ampicillin	Interferes with the cross-linking of peptidoglycan in bacterial cell walls; bactericidal	*ampC* (Gram negative bacteria)	Hydrolysis of antibiotic at β-lactam ring
Sulfonamide	Sulfamethoxazole	Inhibits folate synthesis; bacteriostatic	*sulI* *sulII*	Modification to membrane permeability, efflux pumps, and target enzymes
Dihydrofolate Reductase Inhibitor	Trimethoprim	Inhibits conversion of dihydrofolate to tetrahydrofolate; bacteriostatic	*dfrA*	Modification to membrane permeability, efflux pumps, and target enzymes
Tetracycline	Tetracycline	Protein synthesis inhibition; bacteriostatic	*tetW*	Efflux pumps or elimination from ribosome
Glycopeptide	Vancomycin	Interferes with the cross-linking of peptidoglycan in bacterial cell walls; bactericidal	*vanA* (Gram positive bacteria)	Extrinsic: morphological changes that inhibit attachment; Intrinsic: impermeable membrane of Gram negative bacteria

2.4.1 Thymidine Interference with the Detection of Trimethoprim Resistant Bacteria

A study by Amyes and Smith (1972) revealed that the presence of thymidine or thymine reduces the antibacterial efficacy of trimethoprim. As mentioned earlier, trimethoprim interferes with the

المنارة للاستشارات
www.manaraa.com

ability of bacteria to generate thymidine/thymine for DNA replication. However, if bacteria are able to utilize secondary reservoirs of thymine/thymidine, trimethoprim is rendered ineffective. This was observed in Amyes and Smith (1972), which used different types of media with different ambient concentrations of thymidine/thymine for testing trimethoprim sensitivity. For this reason, some manufacturers are producing nutrient media with limited or reduced thymine/thymidine content to reduce potential interference when testing trimethoprim sensitivity. However, free thymine/thymidine in environmental samples may still cause overestimation of AR prevalence unless bacteria are separated from their matrix before assay (e.g., with membrane filtration).

Metcalf and Eddy (2014) presented an equation to obtain the volatile suspended solids (VSS) production rate in terms of substrate removed, influent nonbiodegradable VSS, and kinetic coefficient as follows:

$$P_{X,VSS} = \frac{QY(S_0 - S)}{1 + (k_d)SRT} + \frac{(f_d)(k_d)YQ(S_0 - S)SRT}{1 + (k_d)SRT} + QX_{o,i} \quad \text{(Eq. 2-1)}$$

$P_{X,VSS}$ = net waste activated sludge produced each day, kg VSS/d
S_0 = influent substrate concentration, mg/L
S = effluent substrate concentration, mg/L
Q = influent flowrate, m3/d
f_d = fraction of cell mass remaining as cell debris, g/g
Y = synthesis yield coefficient for heterotrophic bacteria, g VSS/g COD
$X_{o,i}$ = nbVSS concentration in influent, g/m3
k_d = specific endogenous decay coefficient, g VSS/g VSS.d

or

$$P_{X,VSS} = A + B + C \quad \text{(Eq. 2-2)}$$

المنارة للاستشارات
www.manaraa.com

The first part of Eq. 2-1 (Part A in Eq. 2-2) represents the amount of heterotrophic biomass production. Biomass production by heterotrophic bacteria is a function of flow rate, bacterial growth yield, amount of substrate consumed in the process, endogenous decay coefficient, and SRT. The second part represents the amount of cell debris produced in the process. In this part, SRT is a very important parameter since there is a direct correlation between SRT and the amount of cell debris. According to the Eq. 2-1, a longer SRT results in higher cell debris present in the biological reactor (Figure 2-5).

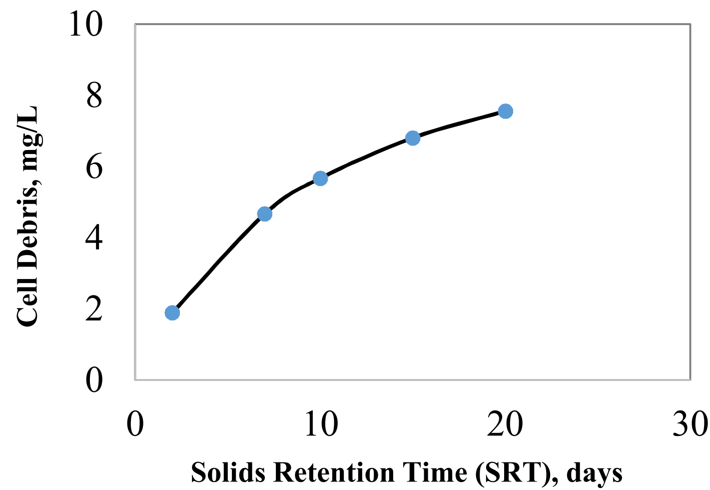


Figure 2-5. Relationship between cell debris and SRT

Bacterial cells begin to lyse when they enter the death phase, which releases their cellular contents (e.g., thymine and thymidine) into their surrounding environment. In theory, more cell debris may result in higher concentrations of free thymine and thymidine in a biological reactor. Therefore, systems with longer SRTs may be characterized by greater trimethoprim resistance due to water quality rather than changes in the microbial community.

المنارة للاستشارات
www.manaraa.com

2.5 Methods for Quantifying Antibiotic Resistance

Antibiotic resistance can be quantified by two main approaches: culture methods and molecular methods. These two techniques are used in this study to evaluate the level of antibiotic resistance in wastewater. It is important to note that the combined use of culture and molecular methods is novel in that studies generally rely on one approach or the other, which might not provide a complete understanding of the AR issue.

2.5.1 Culture Techniques

Culture methods such as spread plates or membrane filtration are typically used to enumerate and isolate specific groups of bacteria. The use of selective culture media is very common when a specific group of bacteria are of interest. With respect to AR, disc diffusion and micro-dilution methods are commonly used to differentiate between resistant and susceptible strains. Spread plates with selective culture media supplemented with antibiotics can be used to determine the ratio between the number of AR bacteria and the 'total' number of cultivable bacteria in a sample. For example, the Staph/Strep selective supplement, which contains a mixture of nalidixic acid and colistin sulfate, can be used to select for Gram positive cocci, including Staphylococcus aureus, streptococci, and enterococci, while inhibiting the growth of Pseudomonas, Klebsiella, Proteus, Enterobacter, Clostridium, and Escherichia. Nalidixic acid is a bacteriostatic antibiotic, and colistin is a bactericidal antibiotic with a similar mode of action to that of the QACs (i.e., solubilizing bacterial membranes to release intracellular components). Just as with other antibiotics, resistance to nalidixic acid and colistin is possible. However, colistin, in particular, is considered a 'last resort' antibiotic for multidrug-resistant Gram negative bacteria (Blair et al., 2015), and resistance to this particular antibiotic is uncommon in wastewater (Zhang

المنارة للاستشارات
www.manaraa.com

et al., 2009). Nalidixic acid is also used to a much lesser extent than other antibiotics (Watkinson et al., 2009), and one study indicated that the median concentration of nalidixic acid in Australian wastewaters was below the detection limit (Watkinson et al., 2009), which suggests that there is less selective pressure to develop and maintain resistance to this antibiotic. The colonies that grow in the presence of the Staph/Strep selective supplement without the target antibiotics can be assumed to represent the total cultivable Staph/Strep in the sample, while the colonies that grow in the presence of the Staph/Strep selective supplement in addition to the target antibiotic(s) can be assumed to represent the antibiotic resistant, cultivable, Staph/Strep.

In order to determine the extent to which bacteria are resistant to antibiotics, the minimum inhibitory concentration (MIC) assay can be used. Briefly, antibiotic concentrations are serially diluted, and the minimum concentration at which the growth of pure culture isolates is inhibited is described as the MIC. The Clinical and Laboratory Standards Institute (CLSI) publishes the currently accepted MICs for a variety of antibiotics, although these numbers typically increase over time to account for greater levels of antibiotic resistance.

2.5.2 Molecular Techniques

A study by Amann et al. (1995) showed that only a small portion of aquatic bacterial communities can be cultured by standard methods. Therefore, any type of culture-based method may only provide information on a small portion of bacterial communities. Instead, molecular methods can be used to detect microorganisms for which culture-based methods are ineffective or those that grow too slowly relative to the larger microbial community (Oliver, 2005, 2010; Trevors, 2010). Molecular methods, such as real-time polymerase chain reaction (qPCR), provide a highly sensitive and specific alternative without the need for cultivation. After sample

المنارة للاستشارات
www.manaraa.com

collection and DNA extraction and purification, qPCR employs target-specific primers and fluorescent probes to quantify the original number of copies of the target sequence in the sample. The process involves repeated denaturing of the DNA, annealing of the primers and probes, and enzymatic extension of the primers and initiation of the fluorescent probes. Once the fluorescence reaches an established threshold, the cycle number is noted and compared to a corresponding standard curve. Despite the utility of qPCR, the method's basic reliance on the presence of DNA means that it cannot always distinguish between extracellular vs. intracellular DNA, damaged vs. intact DNA, or non-viable vs. viable microorganisms.

Genetic characterization of antibiotic resistant mutants is of interest since it provides further information about responsible mutations. For years, genetic mapping was the only way to locate a mutation in the genome and the gene responsible for that mutation. Now the genome of the bacterium can be sequenced easily, and many genes can be identified by annotation. In order to locate the mutations in the genome sequence, marker rescue or complementation techniques can be used to clone the region containing mutations. The clones can then be sequenced and located in the annotated sequence of the genome. When the mutation is located and identified, the appropriate primers can be designed. In order to design the forward and reverse primers in PCR and qPCR, it is important to pay attention to the length of primers, the annealing and melting temperature, and the GC content, since these factors can affect the efficiency of amplification (Molecular Genetics of Bacteria, 2013).

Target-specific methods such as qPCR are now being supplemented with non-specific metagenomics approaches, such as pyrosequencing, which allows one to simultaneously identify a large number of microorganisms or non-specific gene sequences present in a sample. Pyrosequencing involves the stepwise addition of nucleotides to a complementary strand of

المنارة للاستشارات
www.manaraa.com

DNA. Nucleotide availability is controlled by the system (i.e., only one of four possible nucleotides is available at any given time), and each time a nucleotide is added to the strand of DNA, pyrophosphate is released and converted to adenosine triphosphate (ATP), which then combines with luciferin to emit light. Increasing light intensity indicates that the same nucleotide has been added repeatedly. Once the signal stabilizes, the remaining nucleotides and ATP are destroyed, and the system moves on to the next of the four possible nucleotides. The resulting sequences are then assembled into genomes. Kristiansson et al. (2011) employed multiplexed massively parallel pyrosequencing to characterize the microbial communities upstream and downstream of sites impacted by pharmaceutical wastewater discharge and a control site impacted by municipal wastewater discharge. The sites were also tested for antibiotic accumulation in river sediment. The pharmaceutical sites showed relatively consistent detection of fluoroquinolones in the sediments, but the upstream samples were orders of magnitude lower in concentration. The upstream and downstream Swedish sites registered below the method detection limits for all antibiotics. With respect to the pyrosequencing data, the researchers were able to simultaneously identify a range of microorganisms and detect an assortment of antibiotic resistance genes, integrons, plasmids, and transposons. The authors found that several antibiotic resistance genes, including those encoding resistance to sulfamethoxazole (sulII) and streptomycin (strA and strB), were detected at significantly higher concentrations (22-62 times higher) downstream of the pharmaceutical wastewater discharge points. The ARGs were supplemented with an abundance of integrons, transposons, and plasmids, which are critical in facilitating horizontal gene transfer. On the other hand, quinolone resistance genes were detected at lower concentrations downstream of the pharmaceutical wastewater discharge sites. Presumably due to the lower concentrations of antibiotics in the Swedish wastewater effluent, the

www.manaraa.com

ARGs were much less abundant at those locations. Despite the utility of pyrosequencing for characterizing a wide variety of genetic elements related to AR, it is a relatively costly approach and is not well suited for the objectives of the current study.

2.6 Antibiotic Resistance in Wastewater Treatment Plants

Wastewater is a source of constituents of concern, including pathogenic bacteria, nutrients, heavy metals, and trace organic contaminants (TOrCs), including antibiotics. Therefore, wastewater treatment plants have the potential to continuously expose bacteria to sub-inhibitory concentrations of a wide range of antimicrobial compounds (Aminov et al., 2001; Auerbach et al., 2007).

A typical WWTP usually has three major treatment steps: (1) preliminary/primary, (2) secondary treatment, and (3) tertiary/advanced treatment. During primary treatment, large solids and grit are physically removed by screening and sedimentation. In secondary treatment, a major portion of the biodegradable organic matter, or biochemical oxygen demand (BOD), is removed via cellular respiration by native biomass. In addition to BOD removal via aeration, the biological process can be engineered to achieve nitrification (aerobic), denitrification (anoxic), and phosphorus removal (sequential anerobic and aerobic). The secondary process also involves physical removal of the biomass by sedimentation in secondary clarifiers or by membranes in membrane bioreactors. In many WWTPs, secondary effluent is then subjected to tertiary treatment involving filtration and disinfection.

In particular, biological treatment processes in WWTPs provide an ideal environment for the proliferation of AR. Bacteria in these systems are exposed to sub-inhibitory concentrations of a suite of antibiotics and other AR inducing elements and compounds (Aminov et al., 2001;

المنارة للاستشارات
www.manaraa.com

Auerbach et al., 2007). Depending on the operational conditions, bacteria remain in the bioreactors for varying amounts of time depending on the treatment target (i.e., BOD removal or BOD/nutrient removal). Solids retention time (SRT) is one of the key operational parameters in a suspended growth bioreactor and refers to the average amount of time the bacteria stay in the system before being ‘wasted.’ With longer SRTs, bacteria may have a greater chance of obtaining antibiotic resistance elements through horizontal gene transfer mechanisms. Those elements can then be propagated via vertical gene transfer, or bacterial replication. Despite the potential for AR transfer, it is not yet clear whether the biological treatment process actually contributes to the proliferation of AR or whether it actually provides some level of mitigation due to AR bacteria being outcompeted.

2.6.1 Secondary Biological Treatment

Biological treatment processes are designed to transform dissolved and particulate biodegradable components of wastewater. Before the 1980s, the main goal of the biological treatment process was to remove BOD and total suspended solids (TSS). Since then, with more stringent discharge limits, several modifications and configurations have evolved to remove nutrients, specifically nitrogen and phosphorous, from wastewater biologically (Metcalf and Eddy, 2014). Typical secondary biological treatment processes now incorporate multiple tanks operated in anaerobic, anoxic, and/or aerobic conditions to meet specific treatment goals.

Multiple technologies are available for biological treatment but are typically applied in one of three forms: (1) trickling filters, which rely on attached biomass growth (i.e., biofilms) and are relatively uncommon in newer facilities; (2) activated sludge systems, which rely on suspended biomass growth and solids separation by sedimentation; and (3) membrane bioreactors, which

المنارة للاستشارات
www.manaraa.com

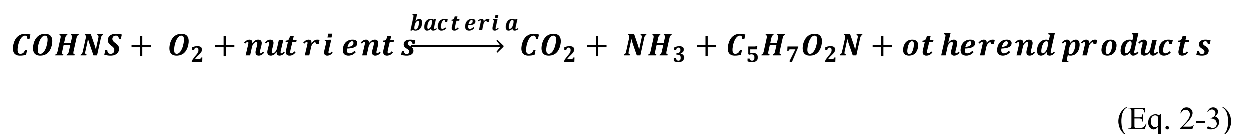
combine suspended biomass growth with solids separation by membranes. In activate sludge process, microorganisms, especially the heterotrophic bacteria, use dissolved oxygen to grow and consume BOD. A series of chemical compounds must be present to serve as the carbon source, electron donor, and electron acceptor, as summarized in Table 2-2.

Table 2-2- Classification of bacteria by electron donor, electron acceptor and source of carbon

Type of Bacteria	Carbon Source	Electron Donor	Electron Acceptor
Aerobic heterotrophic	Organic compounds	Organic compounds	O_2
Aerobic autotrophic	CO_2	NH_4^+, NO_2^-, Fe(II), H_2S, S, $S_2O_3^{2-}$	O_2
Facultative heterotrophic	Organic compounds	Organic compounds	NO_2^-, NO_3^-
Anaerobic heterotrophic	Organic compounds	Organic compounds	CO_2, SO_4^{2-}, Fe(III), Organic compounds
Anaerobic autotrophic	CO_2	NH_4^+	NO_2^-

For BOD removal, the biological treatment system must provide sufficient contact time between the wastewater and heterotrophic microorganisms, sufficient oxygen, and sufficient nutrients. In all aerobic oxidation processes, the conversion of organic compounds is carried out by mixed bacterial cultures in general accordance with the stoichiometry shown below (Metcalf and Eddy, 2014). In Eq. 2.3, organic matter in wastewater is presented as COHNS.

Oxidation and synthesis:



$$COHNS + O_2 + nutrients \xrightarrow{bacteria} CO_2 + NH_3 + C_5H_7O_2N + other end products$$

(Eq. 2-3)

Endogenous respiration:

المنارة للاستشارات
www.manaraa.com

$$C_5H_7O_2N + 5O_2 \xrightarrow{bacteria} 5CO_2 + 2H_2O + NH_3 + energy \qquad \text{(Eq. 2-4)}$$

The bacterial growth pattern in a batch reactor is characterized by four different phases. The first phase, which is known as the lag phase, represents the time required for the organisms to acclimate to their new environment. In the second phase—known as the exponential growth phase—bacterial cells multiply at their maximum rate due to the availability of substrate and nutrients. In the stationary phase, the biomass concentration remains relatively constant with time. In the death phase the biomass concentration decreases due to cell death, primarily because the substrate has been depleted.

Under steady state conditions, for which the influent flowrate and substrate concentration are relatively constant, the mixed liquor suspended solids (MLSS) and the mixed liquor volatile suspended solids (MLVSS) concentrations are also relatively constant in a reactor. The following equations show the rate of substrate utilization and biomass growth based on Monod kinetics and the Michaelis-Menten equation (Bailey and Ollis, 1986):

$$r_{su} = -\frac{kXS}{K_s+S} \qquad \text{(Eq. 2-5)}$$

$$r_g = Y\frac{kXS}{K_s+S} - k_d X \qquad \text{(Eq. 2-6)}$$

r_{su} = rate of substrate utilization, g/m^3.d
r_g = net biomass production rate, gVSS/m^3.d
k = maximum specific substrate utilization rate, g substrate/g microorganisms.d
X = biomass (microorganisms) concentration, g/m^3
S = growth-limiting substrate concentration in solution, g/m^3
K_s = half-velocity constant, g/m^3
Y = synthesis yield coefficient, g VSS/g bsCOD
k_d = endogenous decay coefficient, g VSS/g VSS.d

The biomass mass balance can be written as:

المنارة للاستشارات
www.manaraa.com

$$Accumulation = inflow - outflow + net\ growth \qquad \text{(Eq. 2-7)}$$

or

$$\frac{dX}{dt} = QX_0 - [(Q - Q_w)X_e] - (Q_w X_R) + r_x V \qquad \text{(Eq. 2-8)}$$

In steady-state condition (*dX/dt* = 0), and the Eq. 2-8 can be simplified to:

$$\frac{(Q-Q_w)X_e + Q_w X_R}{VX} = Y\frac{r_{su}}{X} - b \qquad \text{(Eq. 2-9)}$$

The inverse of the left-hand side of the Eq. 2-9 is defined as solids retention time. Therefore:

$$SRT = \frac{VX}{(Q-Q_w)X_e + Q_w X_R} \qquad \text{(Eq. 2-10)}$$

SRT = solids retention time, D
V = reactor volume, m^3
Q = influent flowrate, m^3/d
X = concentration of biomass in aeration tank, g VSS/m^3
Q_w = waste sludge flowrate, m^3/d
X_e = concentration of biomass in effluent, g VSS/m^3
X_R = concentration of biomass in the RAS line from the clarifier, $gVSS/m^3$

The substrate utilization rate (r_{su}) can be rewritten as the amount of substrate removed in the reactor divided by reactor volume, so:

$$r_{su} = \frac{Q(S_0 - S)}{V} \qquad \text{(Eq. 2-11)}$$

Finally, by combining Eq. 2-9 and Eq. 2-10:

$$\frac{1}{SRT} = \frac{YQ(S_0 - S)}{VX} - b \qquad \text{(Eq. 2-12)}$$

Some of the key operational parameters in the activated sludge process include the MLSS (typical values range from 1000 mg/L to 8000 mg/L), the hydraulic retention time (HRT), and the SRT. In simplest terms, SRT is the average amount of time the biomass remains in the reactor before being wasted. The selection of an appropriate SRT is related to the target growth

المنارة للاستشارات
www.manaraa.com

rate of microorganisms in the aeration tank. A longer SRT allows slowly growing microorganisms to enrich, which promotes the development of bacterial species capable of specific treatment objectives (e.g., nitrification).

In a typical wastewater treatment scenario, the substrate concentration is typically described by the BOD parameter but can also be described by other bulk organic surrogate parameters, including total or dissolved organic carbon (TOC or DOC) or fluorescence.

Figure 2-6 illustrates the relationship between total fluorescence and SRT.

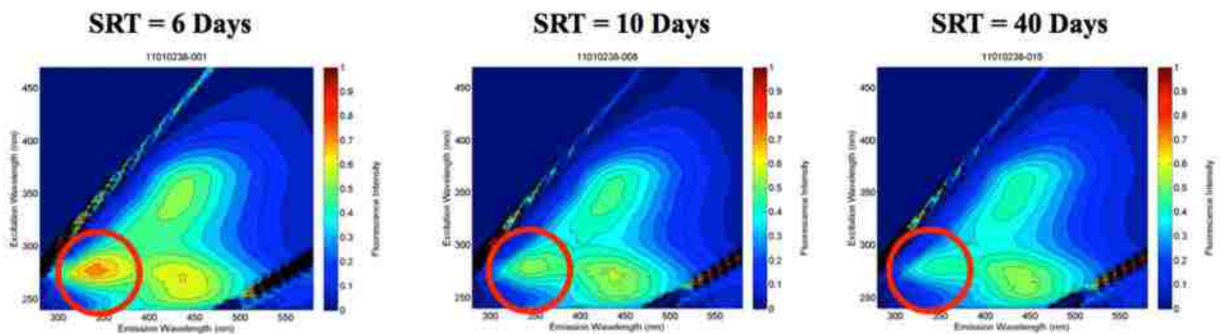


Figure 2-6. Relationship between total fluorescence and SRT (different full-scale facilities). The red circle highlights the organic matter fraction specific to protein and soluble microbial products

2.6.2 Antibiotic Resistance in Biofilm

Antibiotic resistance has also been observed in attached growth systems, such as in biofilms. A biofilm is a group of bacteria that attaches to a surface by producing a mixture of polymers consisting of polysaccharide and proteins. Antibiotic resistance in biofilms often involves different mechanisms than those observed in suspended growth systems (e.g., efflux pumps, modifying enzymes, and mutations) (Stewart and Costerton, 2001). Instead, the dominant mechanisms of resistance in biofilms are thought to be 1) poor antibiotic penetration, 2) nutrient limitation, and 3) formation of highly protected phenotypes (Stewart and Costerton, 2001).

المنارة للاستشارات
www.manaraa.com

Biofilm bacteria generate extracellular polymeric substances (EPS) that prevent the diffusion of certain antibiotics into the biofilm (Chadha, 2014). Antibiotics that are more hydrophilic and positively charged, like aminoglycosides, are hindered to a greater degree than other antibiotics (Chadha, 2014).

The bacteria in biofilm live close to each other, which facilitates the exchange of plasmids and free DNA in the case of environmental stresses (Chadha, 2014). The cells in the biofilm are known to use chemical communication tools known as quorum sensing. Quorum sensing enables bacteria to coordinate their metabolism and help them to adapt to ongoing changes in the environment (e.g., exposure to antibiotics) (Chadha, 2014). Bacteria are able to use quorum sensing to activate specific genes in response to chemical signals that they receive from other bacteria (Shih and Huang, 2002). Many of these chemical signals are homoserine lactones (HSLs). Due to the high cell densities in the biofilm, HSL-mediated gene expression may play an important role in biofilm formation and antibiotic resistance (Shih and Huang, 2002).

Shih and Huang (2002) used *P. aeruginosa* variants, wild-type PAO1, single mutants JP1 (Δ*las*I::Tn*10*, Tc^r) and PDO100 (Δ*rhl*I::Tn*501*, Hg^r), and double mutant JP2 (Δ*las*I::Tn*10*, Tc^r ; Δ*rhl*I::Tn*501*, Hg^r) for batch culture and continuous biofilm cultivation. Except the wild-type, the other strains are actually quorum sensing-deficient mutants. The biofilms were exposed to kanamycin for 2 hours at 1X, 5X and 10X the MIC of kanamycin for PAO1 (MIC is 10 mg/L). After treatment with kanamycin, viable cell and total cell densities were determined by plating on R2A agar and fluorescence microscope, respectively. Figure 2-7 shows surviving cell fractions between the wild-type and the mutants. As shown in Figure 2-7, PAO1 biofilms were not significantly affected by kanamycin, even at concentrations 10 times higher than the MIC (i.e., 100 mg/L), whereas quorum sensing-deficient mutants JP1 and JP2 were susceptible to all

المنارة للاستشارات
www.manaraa.com

kanamycin concentrations (i.e., 10, 50, and 100 mg/L). PDO100 mutants were susceptible to 100 mg/L. The results suggest that quorum sensing bacteria are more resistant to antibiotics.

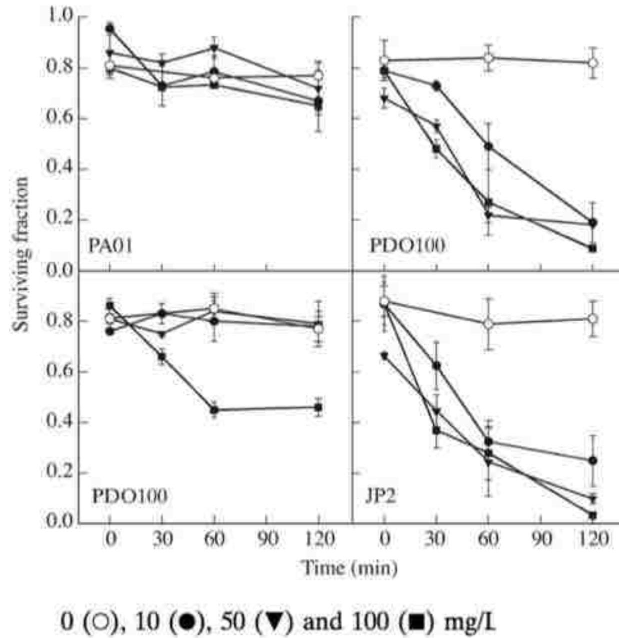


Figure 2-7 Surviving cell fractions of P. aeruginosa

P. aeruginosa PAO1, PDO100, JP1, and JP2 after treatment with kanamycin (Shih and Huang, 2002)

Infectious diseases caused by biofilms are difficult to treat because the bacteria are profoundly resistant to antibiotics. As noted earlier, one of the mechanisms by which bacteria in biofilms show resistance to antibiotics is slow or incomplete penetration of antibiotics into the biofilm. Interestingly, when the bacteria detach from the biofilm, they become sensitive to antibiotics again. Therefore, as long as the bacteria in the biofilm do not contribute to permanent AR proliferation in suspended bacteria, they may not pose a significant public health risk. However, more research is needed to clarify the role of biofilms in AR proliferation. This is beyond the scope of the current study, which focuses on AR in suspended growth applications.

المنارة للاستشارات
www.manaraa.com

2.7 TOrC Removal by Biological Treatment

Once the readily biodegradable compounds are depleted in engineered biological treatment applications, the microbial community experiences some degree of starvation, and only those bacteria with the ability to degrade recalcitrant compounds can survive. This selective pressure associated with longer SRTs may select for bacteria with the ability to degrade a wide variety of organic compounds, including some TOrCs. Recent studies have demonstrated the relationship between SRT and TOrC removal. Suarez et al. (2010) suggested that TOrC removal was linked to nitrification, while other studies reported that it was specifically related to SRT (Melcer and Klecka, 2011; Clara et al., 2005). Multiple studies identified "critical" SRTs for significant TOrC removal. Clara et al. (2005) identified a broadly applicable "critical" SRT of 10 days, while Oppenheimer et al. (2007) and Salveson et al. (2012) identified compound-specific "minimum" or "threshold" SRTs, respectively, as summarized in

Table 2-3.

Table 2-3. Threshold SRT for 80% TOrC degradation (Salveson et al., 2012)

TOrC	Threshold SRT
Acetaminophen	2
Caffeine	2
Ibuprofen	5
Naproxen	5
Bisphenol A	10
Triclosan	10
DEET	15
Gemfibrozil	15
Atenolol	15
BHA	15
Diphenhydramine	20
Benzophenone	20
Trimethoprim	30

المنارة للاستشارات
www.manaraa.com

Gerrity et al. (2013) examined the effects of solids retention time on standard wastewater parameters and the degradation of TOrCs in independent, full-scale activated sludge basins with SRTs ranging from 5.5-15 days. The results showed that biological process optimization strategies (e.g., longer SRTs) can be implemented to reduce reliance on advanced treatment technologies, such as advanced oxidation and reverse osmosis. Batt et al. (2007) was also able to evaluate the effects of SRT at a single facility, and that study also concluded that longer SRTs achieving nitrification conditions achieved greater removal of four antibiotics.

As mentioned earlier, substrate biodegradation can be modeled by the Michaelis-Menten equation, but the removal of TOrCs at the ng/L or lower μg/L level is slightly different because the removal of such compounds does not contribute significantly to biomass growth. Instead, these substances may be transformed by cometabolism. In cometabolism, microorganisms are able to simultaneously degrade a primary substrate as the growth substrate and a secondary substrate as the non-growth substrate (Nzila, 2013). The secondary substrates are recalcitrant to normal biodegradation since they are not considered a source of energy by bacteria, but they can be removed biologically as a result of this fortuitous event (Nzila, 2013). As an example, Chen and Aitken (1999) showed that benzo[*a*]pyrene can be converted to CO_2 while phenanthrene or salicylate is used as the primary substrate.

2.7.1 Occurrence of Indicator Trace Organic Compounds and Antibiotics

Many medications and personal care products are not completely metabolized or absorbed by the human body. Therefore, these compounds are excreted from the human body and are present in raw wastewater as a diverse mixture of TOrCs. Watkinson et al. (2009) examined the presence of

المنارة للاستشارات
www.manaraa.com

28 antibiotics in different hospital effluents, wastewater effluents, rivers, and finished drinking waters in Queensland, Australia. The β-lactams (e.g., amoxicillin, cephalexin, and penicillin) were by far the most commonly used antibiotics for human purposes, with macrolides (e.g., erythromycin) and sulfonamides (e.g., sulfasalazine and sulfamethoxazole) also used in significant quantities. The results showed that the median antibiotic concentrations in the municipal wastewater influent ranged from non-detect for 14 of the antibiotics to as high as 1.4 μg/L for amoxicillin. The median concentrations of the target antibiotics in the current study (sulfamethoxazole, trimethoprim, and tetracycline) in wastewater influent were 250 ng/L, 430 ng/L, and non-detect, and their maximum concentrations were 3.0 μg/L, 4.3 μg/L, and 100 ng/L, respectively. In general, the results showed that WWTPs are able to achieve greater than 80% removal of all targeted antibiotics present in the aqueous phase (Watkinson et al., 2009).

Gerrity et al. (2011) demonstrated the temporal variability in TOrC concentrations in primary effluent and finished effluent from a wastewater treatment plant in Las Vegas. Some compounds (e.g., atenolol) exhibited significant changes in concentration in the primary effluent over time, while others were relatively stable throughout the sampling period. The concentrations of sulfamethoxazole in the primary effluent were found to vary from approximately 750 ng/L to just over 1,500 ng/L, whereas the concentrations of trimethoprim were found to be stable around 600 ng/L on both sampling days. The MIC for sulfamethoxazole is 76 μg/ml, which is significantly higher than the MIC for trimethoprim at 4 μg/ml (CLSI, 2012). Therefore, higher concentration of sulfamethoxazole are expected in the primary effluent, as Gerrity et al. (2012) reported, because people are likely to be administered higher doses in accordance with the higher MIC. It should be noted that the biodegradability of the compounds, solubility, and stability of the compound in water also affect the concentration of antibiotics in wastewater. Gerrity et al.

www.manaraa.com

(2011) showed that many of the target compounds were removed to a high degree, except for the more recalcitrant compounds (e.g., sulfamethoxazole and TCEP). It is important to remember that high TOrC concentrations (e.g., antibiotics) may contribute to the proliferation of antibiotic resistance by imposing selective pressure on microorganisms in biological treatment systems.

Kim et al. (2005) explored the effect of SRT on the fate of tetracycline in the activated sludge process by using two SBRs. The SBRs were operated with ambient concentration and elevated concentration of tetracycline (0.25 mg/L). The research team employed 96-well plates employing a tetracycline-based enzyme-linked immunosorbent assay (ELISA) to monitor the tetracycline concentrations in the SBRs. The results showed that the removal efficiency of tetracycline at an SRT of 3 days was significantly lower than that of the 7-day SRT, thereby indicating that longer SRTs are able to achieve superior removal rates.

2.7.2 Modeling the Removal of Trace Organic Compounds in Biological Treatment Systems

Raw wastewater contains a wide variety of compounds, including natural and synthetic organic compounds. The biological treatment process is designed to remove biodegradable organic compounds in wastewater, but not the all of organic compounds are readily biodegradable (i.e., those that are refractory). There are also organic compounds that are not only resistant to biodegradation, but may also pose a threat to the environment due to their toxicity.

Recalcitrant compounds can be removed from wastewater by three main mechanisms: biodegradation, sorption or solids partitioning, and volatilization (Metcalf and Eddy, 2014). In aerobic biodegradation, with proper environmental and operational conditions, some of the refractory compounds can serve as growth substrates (Metcalf and Eddy, 2014). Heterotrophic

المنارة للاستشارات
www.manaraa.com

bacteria can consume some petroleum compounds, like benzene and toluene, non-halogenated solvents like alcohols and ketones, and some halogenated solvents such as methylene chloride. Some chlorinated compounds can be degraded in a process called cometabolic degradation. In this process, some bacteria will produce specific enzymes that mediate a reaction with oxygen and hydrogen, which finally change the structure of the compounds that make them easier to be degraded by other aerobic bacteria. Partitioning onto the biomass is another mechanism by which the compounds can be removed from wastewater. In order to describe solids partitioning, modified Freundlich isotherms can be used (n=1) for relatively low contaminant concentrations:

$$q = K_p S \quad \text{(Eq. 2-13)}$$

q = g organic adsorbed/g adsorbent
K_p = partition coefficient, L/g
S = concentration of organic compound in liquid, g/L

The amount of organic compounds removed by adsorption can be estimated by the following equation:

$$r_{ad} = r_{waste} K_p S \quad \text{(Eq. 2-14)}$$

r_{ad} = rate of organic compound removed via adsorption daily, g/d
r_{waste} = rate of solids wasted daily, g/d

The rate of solids wasted from a biological reactor is dependent on SRT, so;

$$r_{waste} = \frac{X_T V}{SRT} \quad \text{(Eq. 2-15)}$$

X_T =total MLVSS concentration in aeration tank, g VSS/m^3
V =Volume of reactor, m^3

By substituting Eq. 2-15 into Eq. 2-16, the equation for the mass loss rate would be:

المنارة للاستشارات
www.manaraa.com

$$r_{ad} = \frac{X_T V K_p S}{SRT} \quad \text{(Eq. 2-16)}$$

Mass loss by volatilization can be modeled by the mass transfer expression introduced by (Bielefeldt and Stensel, 1999):

$$r_{sv} = Q_g S_{g,VOC} = Q_g (H) S_{L,VOC} \left\{1 - exp\left[\frac{(\alpha K_L a_{,VOC}\, V)}{Q_g (H)}\right]\right\} \quad \text{(Eq. 2-17)}$$

Q_g = gas flowrate through reactor, m^3/d
$S_{g,VOC}$ = VOC content in the gas leaving the reactor, g/m^3
H = Henry's constant of the VOC at the reactor temperature, Lwater/Lair
$S_{L,VOC}$ = liquid concentration of the VOC, g/m^3
$K_L a_{,VOC}$ = VOC mass transfer coefficient, d^{-1}
α = ratio of mass transfer in reactor mixed liquor to that in clean water
V = reactor volume, m^3

Therefore, the steady-state mass balance for modeling the mass loss due to biodegradation, sorption, and volatilization would be:

$$0 = Influent - degradation - sorption - volatilization - effluent \quad \text{(Eq. 2-18)}$$

$$QS_0 = r_{su} + r_{ad} + r_{sv} + QS \quad \text{(Eq. 2-19)}$$

QS_0 =mass of compound in influent, g/d
r_{su} =biodegradation rate, g/d
r_{ad} =solids adsoption rate, g/d
r_{sv} =volatilization rate, g/d
QS = mass of compound in effluent, g/d

$$QS_0 = \left(\frac{1}{Y}\right) \frac{\mu_m S}{(K_s + s)} (X_s)(V) + \frac{X_T K_p V S}{SRT} + K_L a_s S V + QS \quad \text{(Eq. 2-20)}$$

X_s = biomass concentration cable of degrading the specific organic compound

Eq. 2-20 can also be used for the biological removal of trace organic compounds, but since the TOrC concentrations are very low compared to the growth substrate, a pseudo-first order

المنارة للاستشارات
www.manaraa.com

biodegradation model can be used instead. Also, the volatilization term can be neglected for most TOrCs. Therefore, the simplified biodegradation model is as follows:

$$r_{SU} = K_b X_{H,S}(E)V \qquad \text{(Eq. 2-21)}$$

r_{US} = specific compound removal rate by biodegradation, ng/d
K_b = first order degradation rate coefficient, m^3/g.d
$X_{H,S}$ = concentration of bacteria capable of degrading specific compound, g/m^3
E = reactor specific compound concentration, ng/m^3
V = reactor volume

Therefore, the steady-state mass balance is:

$$QS_0 = K_b X_{H,S}(E)V + QS \qquad \text{(Eq. 2-22)}$$

Antibiotics are typically found in domestic wastewater in the ng/L to μg/L range. These concentrations are considerably lower than minimum inhibitory concentrations (MICs). It seems that antibiotics at these concentrations have little or no effect on the operation of any biological reactor. So regardless of the presence of antibiotics, the biological reactors are able to achieve their goals in removing organic and inorganic pollutants. It should be noted that antibiotics at higher concentrations may inhibit the growth of some bacterial species and alter the microbial community, which would eventually reduce treatment efficiency.

المنارة للاستشارات
www.manaraa.com

3.0 An Evaluation of Bacterial Resistance to Trimethoprim and the Role of Wastewater as a Reservoir of Environmental Thymine and Thymidine

3.1 Abstract

The antibiotic trimethoprim acts by disrupting dihydrofolate reductase during nucleotide synthesis. Bacteria can grow in the presence of trimethoprim by expressing trimethoprim resistance genes or by acquiring thymine or thymidine from environmental reservoirs to facilitate nucleotide synthesis. The purpose of this study was to evaluate the impact of thymine or thymidine in activated sludge from a biological wastewater treatment process on the quantification of trimethoprim-resistant bacteria (TRB). The biological treatment process was also modified to assess the impacts of varying solids retention time (SRT) on trimethoprim concentrations, culturable trimethoprim-resistant bacteria, and multi-drug resistant bacteria. This is significant because longer SRTs are often employed to improve the quality of treated wastewater effluent. In the presence of trimethoprim at standard clinical concentrations, greater numbers of culturable bacteria were observed with (1) samples manually augmented with reagent-grade thymidine, (2) samples manually augmented with sonicated biomass (i.e., cell lysate), (3) samples manually augmented with activated sludge filtrate, and (4) activated sludge samples collected from reactors with longer SRTs. These observations suggest that longer SRTs may select for trimethoprim-resistant bacteria and/or result in false positives for trimethoprim resistance due to higher concentrations of free thymine or thymidine.

المنارة للاستشارات
www.manaraa.com

3.2 Introduction

Wastewater treatment plants (WWTPs) play an important role in protecting human and environmental health from wastewater-derived pollution, but they are also considered significant reservoirs of antibiotic resistance (AR) (Novo and Manaia, 2010). Previous studies have highlighted the selective pressure exerted on bacteria in wastewater matrices and the impact of wastewater treatment on antibiotic resistant bacteria (ARBs) and antibiotic resistance genes (ARGs) (Schwartz et al., 2003; Su et al., 2014; Guo et al., 2015; Zhang et al., 2015). Specifically, the engineered biological treatment systems in WWTPs are intended to maximize bacterial activity and growth. Coupled with continuous exposure to antibiotics, these systems have the potential to increase the concentration of ARBs by promoting horizontal gene transfer (Wang et al., 2011; Martinez, 2008) and/or inhibiting antibiotic susceptible bacteria (Lopatkin et al., 2016).

There is still no consensus as to whether WWTPs truly contribute to the proliferation of AR. Some studies suggest that WWTPs achieve a significant reduction in the number of ARBs (Guo et al., 2015; Huang et al., 2012), while other research indicates that WWTPs serve as major contributors of ARBs and ARGs (Kim et al., 2010). Luo et al. (2014) conducted a mass balance of the New Delhi metallo-β-lactamase (NDM-1) ARG in two wastewater treatment plants in China. The number of gene copies increased through the biological treatment process in both facilities, but the net loading in the finished effluent increased in only one of the two facilities. Such contradictory outcomes may arise when studies focus on different influent wastewater qualities, different treatment technologies and/or operational conditions, or different methodologies for the detection of ARBs and ARGs.

المنارة للاستشارات
www.manaraa.com

Biological treatment is common to nearly all municipal wastewater facilities, but the treatment objectives in each facility (e.g., biochemical oxygen demand (BOD) removal, nitrification/denitrification, phosphorus removal) may differ depending on a variety of factors. One of the critical operational parameters for biological wastewater treatment, specifically the activated sludge process, is solids retention time (SRT). In simplest terms, the SRT is the average amount of time the biomass is recycled within the system. SRT is also related to the growth rate of microorganisms: a longer SRT selects for more slowly growing microorganisms, which ultimately results in a more diverse microbial population. This is particularly important when nitrification is desired because slow-growing nitrifiers can be washed out of the system with SRTs shorter than ~5 days (Tai et al., 2006).

Longer SRTs have also been correlated with lower total organic carbon (TOC) (Leu et., 2012) and trace organic compound (TOrC) concentrations (Clara et al., 2005; Oppenheimer et al., 2007; Suarez et al., 2010; Melcer and Klecka, 2011; Salveson et al., 2012; Gerrity et al., 2013). TOrCs include various classes of over-the-counter and prescription pharmaceuticals, personal care products, pesticides, herbicides, flame retardants, etc., which are present in water and wastewater at trace levels (i.e., µg/L and ng/L). Gerrity et al. (2013) determined that lower effluent concentrations of the antibiotics sulfamethoxazole (SMX) and trimethoprim (TMP) could be achieved with longer SRTs in a full-scale activated sludge process. However, there are limited studies that directly evaluate the relationship between SRT and the prevalence of ARBs and/or ARGs. Therefore, additional studies are needed to assess the role of such operational conditions on AR occurrence.

SMX, which is a member of the sulfonamide antibiotic class, disrupts folate synthesis by inhibiting dihydropteroate synthetase (DHPS) (Bushby and Hitchings, 1968). TMP, which is

المنارة للاستشارات
www.manaraa.com

often administered in tandem with SMX, also targets folic acid synthesis by disrupting the conversion of dihydrofolate to tetrahydrofolate. Without tetrahydrofolate, thymidylate synthetase is unable to transfer a methyl group to deoxyuridine monophosphate (dUMP) to make deoxythymidine monophosphate (dTMP) (Molecular Genetics of Bacteria, 2013). In short, SMX and TMP achieve bacteriostatic disruption of nucleotide synthesis for both Gram positive and Gram negative bacteria. These pathways are summarized in Figure 3-1.

Bacteria may be able to bypass these pathways by obtaining thymidine or thymine from their environment. Amyes and Smith (1974) discovered that secondary reservoirs of thymidine or thymine, which are present at varying concentrations in different types of growth media, reduce the antibacterial efficacy of TMP. For this reason, TMP sensitivity tests require nutrient media with limited thymine/thymidine content [e.g., Mueller Hinton (MH) agar]. However, free thymine/thymidine in environmental samples may still result in overestimation of AR prevalence unless bacteria are separated from their matrix before assay (e.g., with membrane filtration). For example, longer SRTs achieve greater microbial diversity and treatment efficacy but also lead to higher rates of cell death and decay (Metcalf and Eddy, 2014). This can potentially result in a significant environmental reservoir of thymine/thymidine as the intracellular components are released from the cells.

المنارة للاستشارات
www.manaraa.com

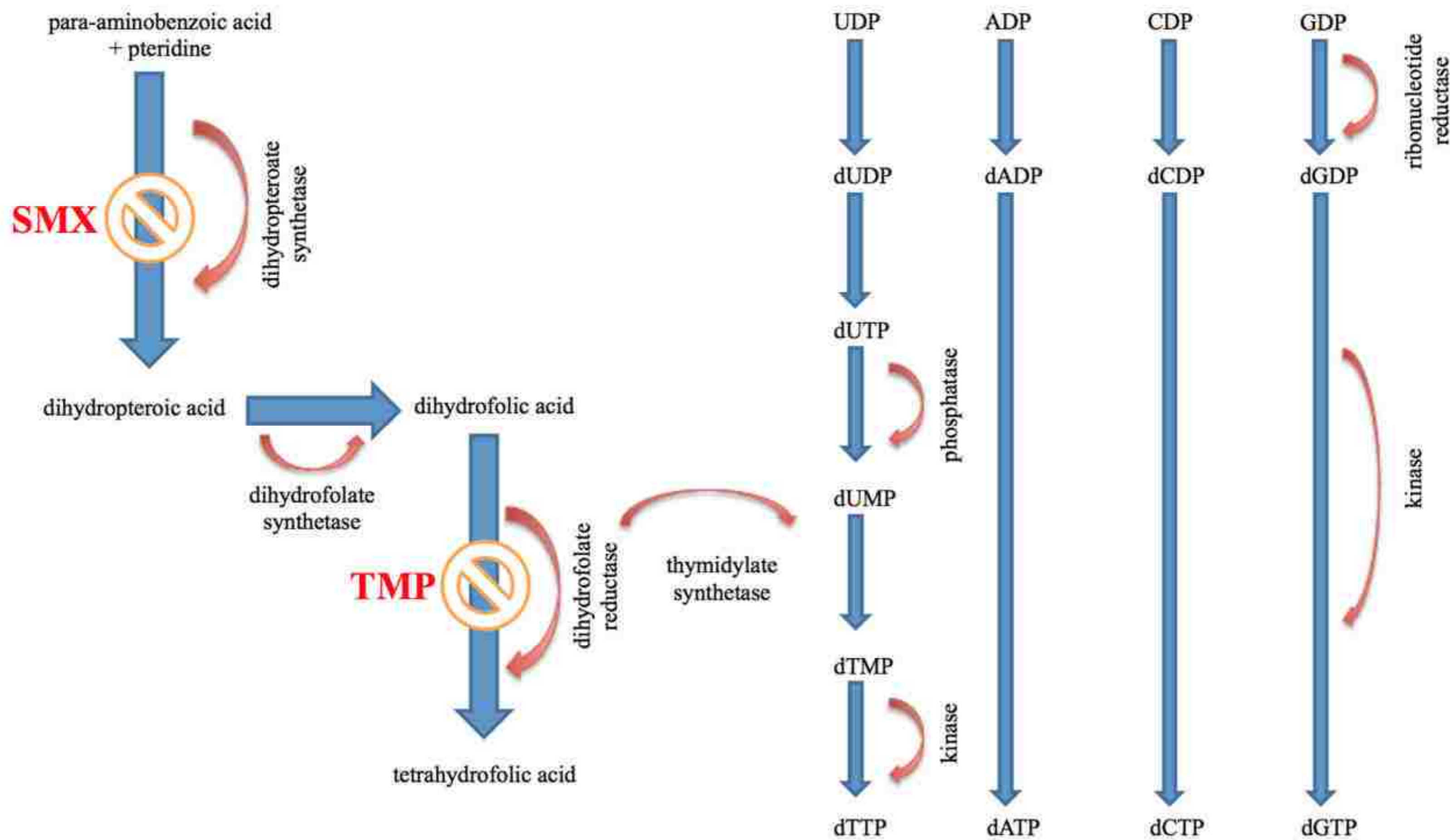


Figure 3-1. Pathways describing the disruption of DNA synthesis

Pathways describing the disruption of DNA synthesis with sulfamethoxazole (SMX) and/or trimethoprim (TMP). External sources of thymine/thymidine allow bacteria to bypass the conversion of dUMP to dTMP, thereby negating the effects of SMX and TMP

المنارة للاستشارات
www.manaraa.com

This research explores the impact of thymine/thymidine in environmental samples consisting of activated sludge from a biological wastewater treatment process. The wastewater samples were generated from laboratory-scale sequencing batch reactors (SBRs) fed with full-scale primary effluent and operated at varying SRTs. Experiments were performed to (1) confirm the effects of secondary thymidine reservoirs, (2) evaluate the effects of sonicated biomass (i.e., cell lysate), (3) evaluate the effects of cellular debris as a function of SRT, and (4) evaluate the effects of SRT on single- and multi-drug resistance and the removal of TOrCs, including TMP and SMX.

3.3 Materials and methods

3.3.1 Description of laboratory-scale sequencing batch reactors

The laboratory-scale activated sludge process was achieved with four parallel SBRs (Figure 3-2) fed with primary effluent from a full-scale WWTP in Las Vegas, Nevada. The acrylic SBRs had a total volume of 8 L and a working volume of 4 L after accounting for the volume of settled solids. Automation of the SBRs was achieved with a series of multi-station outlet timers, a peristaltic pump, electric actuated ball values, and solenoid valves. A MasterFlex peristaltic pump (Model 77200-62, Cole Parmer, Vernon Hills, IL) was used to transfer primary effluent from a wet well through a polytetrafluoroethylene/stainless steel strainer (Hach, Loveland, CO) and a 50-µm cartridge filter (Watts WPC50-975) prior to filling the reactors. The cartridge filters were replaced every two days to mitigate fouling and anaerobic conditions. A four-station irrigation timer (Orbit, Bountiful, UT) was used to control the volume fed to each reactor. Electric actuated solenoid valves (Parker Hannifin Corporation, Cleveland, OH) and an industrial grade air compressor (Porter-Cable PCFP02003; 3.5 gallons; 135 psi) were used to aerate the SBRs to achieve a relatively constant dissolved oxygen concentration of 3 to 4 mg/L. The

المنارة للاستشارات
www.manaraa.com

compressed air was passed through a pressure gauge and air flow meter before being fed into the SBRs via stone diffusers. Aeration was sufficient to achieve adequate mixing of the mixed liquor without the need for mechanical mixing. The target SRTs were achieved by wasting predetermined volumes of mixed liquor toward the end of each aeration phase, and this was accomplished with four electric actuated ball valves (W.E. Anderson, Michigan City, IN).

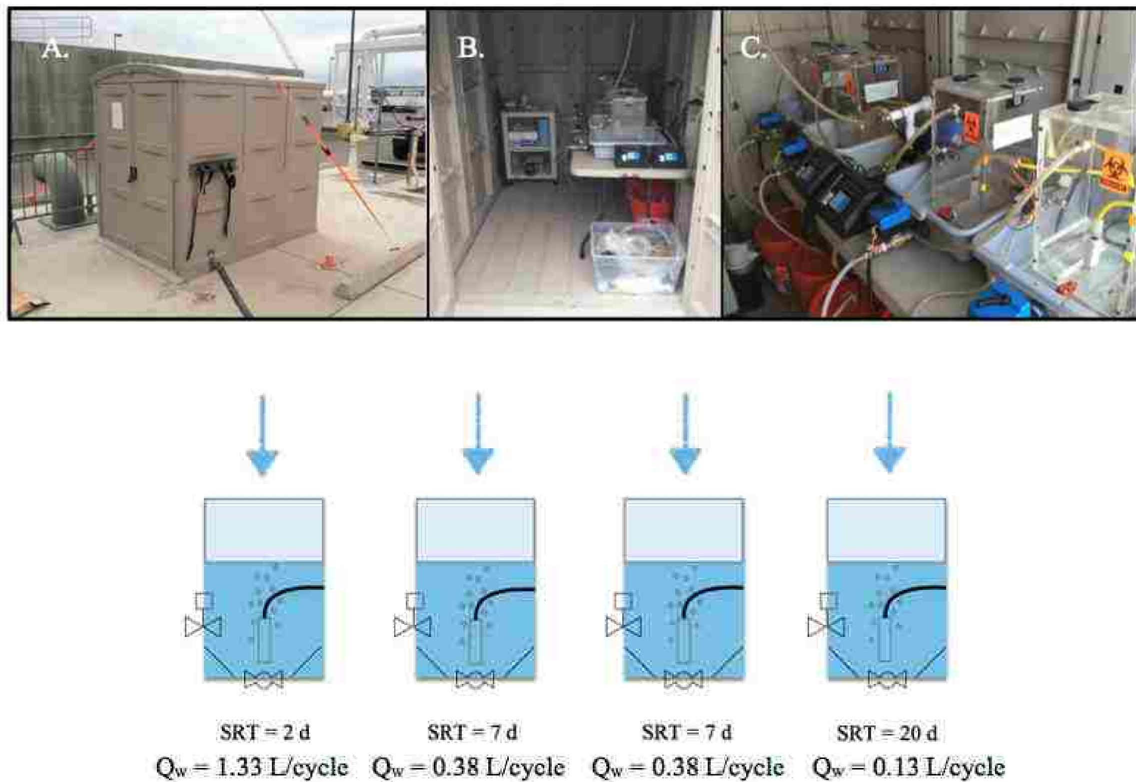


Figure 3-2. Sequencing batch reactors (varying SRT)

Top: Photos of the (A) exterior of the experimental shed, (B) interior of the shed, and (C) parallel sequencing batch reactors (SBRs). The reactors were set up at a full-scale wastewater treatment plant in Las Vegas, NV. The reactors were fed with primary effluent from the full-scale facility. *Bottom:* Schematic of the SBRs, target solids retention times (SRTs), and target waste activated sludge flow rates (Q_w)

The SBRs were initially seeded with return activated sludge (RAS) from the full-scale WWTP, which operates at an SRT of ~7 days. The SBRs were operated with a cycle time of 8 hours for 3 cycles per day over a period of 60 days. Each cycle consisted of the following five stages: (1)

المنارة للاستشارات
www.manaraa.com

filling with primary effluent for 29 minutes as the irrigation timer cycled through each reactor, (2) immediate aeration for 6.5 hours, (3) solids settling for 1 hour, (4) discharge of settled effluent for 30 minutes, and (4) idle for 1 minute. Again, solids wasting was performed toward the end of each aeration phase to minimize clogging of the ball valves. SRTs of 2 days, 7 days (in duplicate), and 20 days were targeted for this research (Figure 3-3). The corresponding waste activated sludge (WAS) flow rates (Q_w) were determined according to Eq. 3-1.

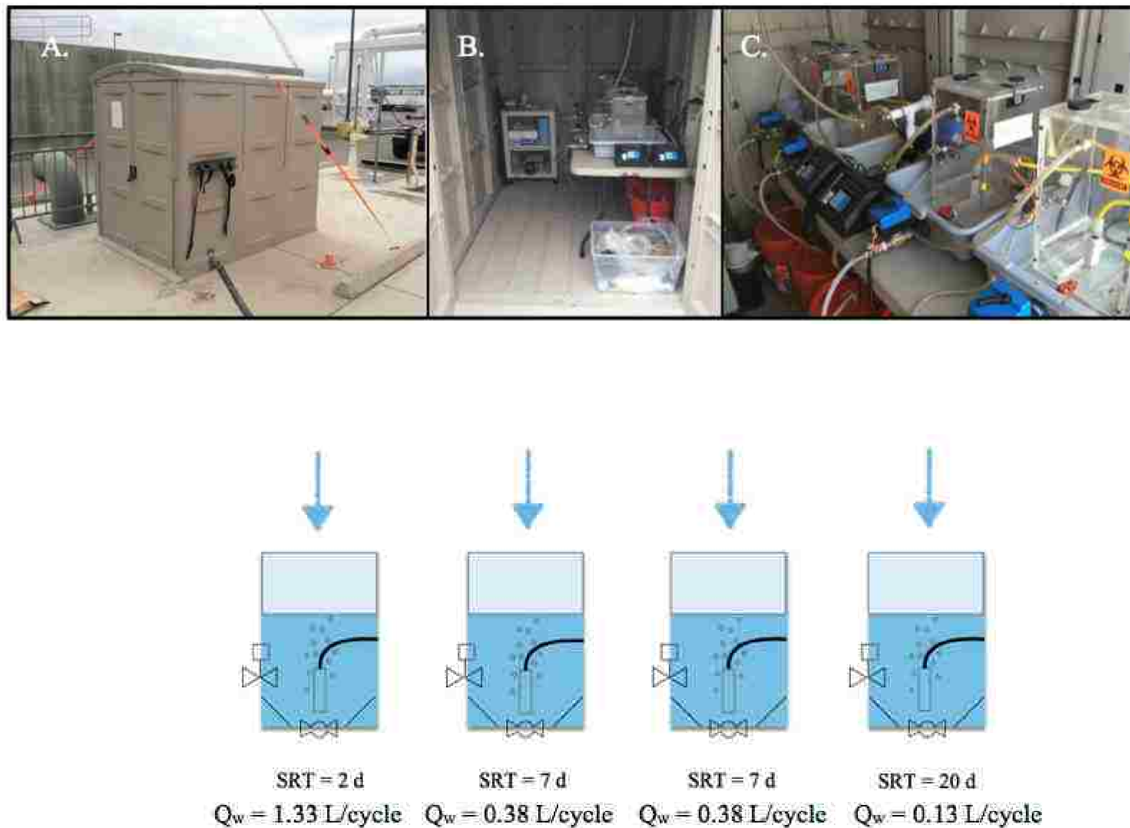


Figure 3-3. Sequencing batch reactors (varying antibiotic concentrations)

Top: Photos of the (A) exterior of the experimental shed, (B) interior of the shed, and (C) parallel sequencing batch reactors (SBRs). The reactors were set up at a full-scale wastewater treatment plant in Las Vegas, NV. The reactors were fed with primary effluent from the full-scale facility. *Bottom:* Schematic of the SBRs, target solids retention times (SRTs), and target waste activated sludge flow rates (Q_w)

www.manaraa.com

$$Q_w = \left(\frac{1}{f}\right)\left(\frac{V_R}{\theta_c} - \frac{Q_e * C_e}{C}\right) = \frac{V_R}{f\ \theta_C}\ (\text{when } C_e \approx 0) \quad \text{(Eq. 3-1)}$$

Q_w = WAS flow rate, L/cycle

f = frequency, cycles/day

V_R = volume of the SBR, L = 8 L

θ_C = SRT, days

Q_e = effluent flow rate, L/day

C_e = total suspended solids in settled effluent, mg/L

C = mixed liquor suspended solids, mg/L.

3.3.2 Preparation of trimethoprim stock solution

Trimethoprim (Sigma Aldrich, St. Louis, MO) was used for this study. The antibiotic stock solution was prepared based on the Clinical and Laboratory Standards Institute (CLSI, 2012). Appropriate solvent was used for trimethoprim, which was 90% volume of sterile nanopure water with 10% volume of 0.05 M hydrochloric acid. The antibiotic stock solution was then passed through a acrodisc syringe filter to be sterilized. The stock solution was stored in refrigerator at at 4±2°C and was used within 48 hours.

3.4 Analytical methods

3.4.1 General water quality parameters

A series of general water quality parameters was monitored for the duration of the study to ensure the SBRs were properly mimicking a full-scale activated sludge system. These tests included pH, mixed liquor suspended solids (MLSS) concentration, mixed liquor volatile suspended solids (MLVSS) concentration, soluble chemical oxygen demand (sCOD), nitrogen speciation (i.e., ammonia, nitrate, and nitrite), and dissolved oxygen (DO). Standard methods

المنارة للاستشارات
www.manaraa.com

were employed when applicable; a summary of the analyses and associated methods is provided in Table 3-1.

3.4.2 Trace organic compounds

To demonstrate the relationship between SRT and TOrC removal, samples were analyzed for ambient levels of the target antibiotics (TMP and SMX) and the beta-blocker atenolol, which served as an indicator compound. Primary and secondary effluent (i.e., settled effluent) samples from the four SBRs were collected in 1-L, silanized, amber glass bottles preserved with sodium azide (1 g/ L) and ascorbic acid (50 mg/L). Samples were immediately placed on ice and held at 4°C for up to 14 days until further processing, which consisted of filtration with 0.7-µm glass fiber filters and on-line solid phase extraction (SPE). The samples were then analyzed for the target compounds by liquid chromatography tandem mass spectrometry (LC-MS/MS) with isotope dilution according to previously published methods (Vanderford and Snyder, 2006). The method reporting limits (i.e., 3-5x the method detection limits) were determined to be 5 ng/L.

Table 3-1. Summary of methods for water quality parameters

Measurement	Sampling and Measurement Method	Analysis Method	Sample Container/ Quantity of Sample	Preservation/ Storage	Hold Time
pH	Orion Model 720A pH meter	Standard Method 4500-H B	20 mL glass vials/10 mL	None	Immediate analysis
MLSS	0.45-µm glass fiber filters, 25-mL baking crucibles, 105ºC oven, analytical balance	Standard Methods 2540 D	50 mL centrifuge tube/10 mL	Refrigeration/ Store @ 4±2°C	7 d
MLVSS	0.45-µm glass fiber filters, 25-mL baking crucibles, 550ºC oven, analytical balance	Standard Methods 2540 D,E	50 mL centrifuge tube/10 mL	Refrigeration/ Store @ 4±2°C	7 d

المنارة للاستشارات
www.manaraa.com

Measurement	Sampling and Measurement Method	Analysis Method	Sample Container/ Quantity of Sample	Preservation/ Storage	Hold Time
NH_3	Hach DR/5000 spectrophotometer, Salicylate Method	Hach Method 10031	150 mL amber glass bottle/100 µL	HCl addition to pH<2 / Store @ 4±2°C	28 d
NO_3	Hach DR/5000 spectrophotometer, Cadmium Reduction Method	Hach Method 8039	150 mL amber glass bottle/10 mL	Filter / Store @ 4±2°C	48 h
NO_2	Hach DR/5000 spectrophotometer, Diazotization Method	Hach Method 8507	150 mL amber glass bottle/10 mL	Filter / Store @ 4±2°C	48 h
DO	O_2 electrode probe	Standard Method 4500-O G	40 mL glass vials/20 mL	None	Immediate analysis
Soluble COD (sCOD)	Hach DR/5000 spectrophotometer, Reactor Digestion Method	U.S. EPA method 410.4, Hach Method 8000	20 mL glass vials/2 mL	H_2SO_4 addition to pH<2 / Store @ 4±2°C	28 d
Spread Plates	Spread plate on MH agar (w/ and w/o antibiotics)	Described in main text	50 mL conical tube/100 µL per plate	None	8 h
Trimethoprim	LC-MS/MS, API 4000 triple-quadrupole mass spectrometer	Vanderford and Snyder (2006)	500 mL pre-cleaned amber bottle	1 g/L NaN_3 and 50 mg/L Ascorbic Acid / Store @ 4±2°C	28 days

Reference: Vanderford, B.J., Snyder, S.A., 2006. Analysis of pharmaceuticals in water by isotope dilution liquid chromatography/tandem mass spectrometry. *Environmental Science and Technology* 40 (23): 7312-20.

The TOrC sampling was performed at the conclusion of the testing period (i.e., after 60 days of operation). Two independent sets of samples were collected to assess variability in compound occurrence and removal between cycles. Primary effluent was collected in duplicate on both days. For the first sample set, secondary effluent from the SBR operating with a 2-day SRT was collected in duplicate. For the second sample event, secondary effluent from the SBR operating with a 20-day SRT was collected in duplicate. For the 7-day SRT, two independent reactors were

المنارة للاستشارات
www.manaraa.com

operated under similar conditions, and only one set of samples was collected from each reactor on both days.

3.5 Microbiological methods

The microbiology components of the study were divided into four sets of experiments, as summarized in Table 3-2: (1) manual augmentation of thymidine with reagent-grade chemical, (2) manual augmentation of thymidine via cell lysing, (3) varying of SRT in the SBRs to evaluate the effects of cellular debris, and (4) varying of SRT in the SBRs to evaluate single- and multi-drug resistance. In each of these experiments, spread plates were prepared with 100 µL of sample on MH agar (Thermo scientific, Waltham, MA) supplemented with 4 µg/mL of trimethoprim (Sigma Aldrich, St. Louis, MO). This trimethoprim concentration represents the published standard minimum inhibitory concentration (MIC) according to the Clinical and Laboratory Standards Institute (CLSI, 2014). Final samples were serially diluted in 0.01% phosphate buffered saline (PBS) to target plate counts of 25 to 250 colony forming units (CFU). Plates were incubated at 35±0.5°C for 36±2 hours. The results were reported as the mean of triplicate plates ±1 standard deviation in CFU/100 µL. Modifications specific to each set of experiments are described in the sections below.

المنارة للاستشارات
www.manaraa.com

Table 3-2. Summary of experimental conditions

Experiment	Description	Media Supplements[1]	SRT of plated MLSS
1	Manual augmentation of thymidine with reagent-grade chemical	0, 20, 60, or 100 μg/mL of thymidine	7 days
2	Manual augmentation of thymidine[2] via cell lysing	0%, 0.01%, 0.1%, or 1% sonicated MLSS (SRT = 7 days)	7 days
3	Varying of SRT in the SBRs to evaluate effects of cellular debris	0%, 0.01%, 0.1%, or 1% MLSS filtrate (SRT = 7 days)	2, 7, 20 days
4	Varying of SRT in the SBRs to evaluate the effects of multi-drug resistance among Gram positive bacteria	76 μg/mL of sulfamethoxazole and Staph/Strep supplement	2, 7, 20 days

[1]Base media = Mueller Hinton agar with 4 μg/mL of trimethoprim
[2]Thymidine, among other intracellular components, assumed to be present in the cell lysate

3.5.1 Manual augmentation of thymidine with reagent-grade chemical

Three sets of grab samples of mixed liquor suspended solids (MLSS) (i.e., activated sludge) were collected in sterile conical tubes from one of the SBRs operating with a 7-day SRT. The samples were immediately transported on ice to the laboratory for same-day processing and analysis. Samples were serially diluted and plated as described above, except that the MH agar was also supplemented with 0, 20, 60, or 100 μg/mL of reagent-grade thymidine (Sigma Aldrich). Two sets of negative controls were used: (1) MH agar with TMP (no MLSS or thymidine) and (2) MH agar with thymidine (no MLSS or TMP). Both were negative for bacterial growth. Another set of controls consisting of MLSS and varying concentrations of thymidine (no TMP) confirmed that manual augmentation of thymidine had no significant impact on bacterial growth in the absence of TMP ($p>0.05$; Figure 3-4).

www.manaraa.com

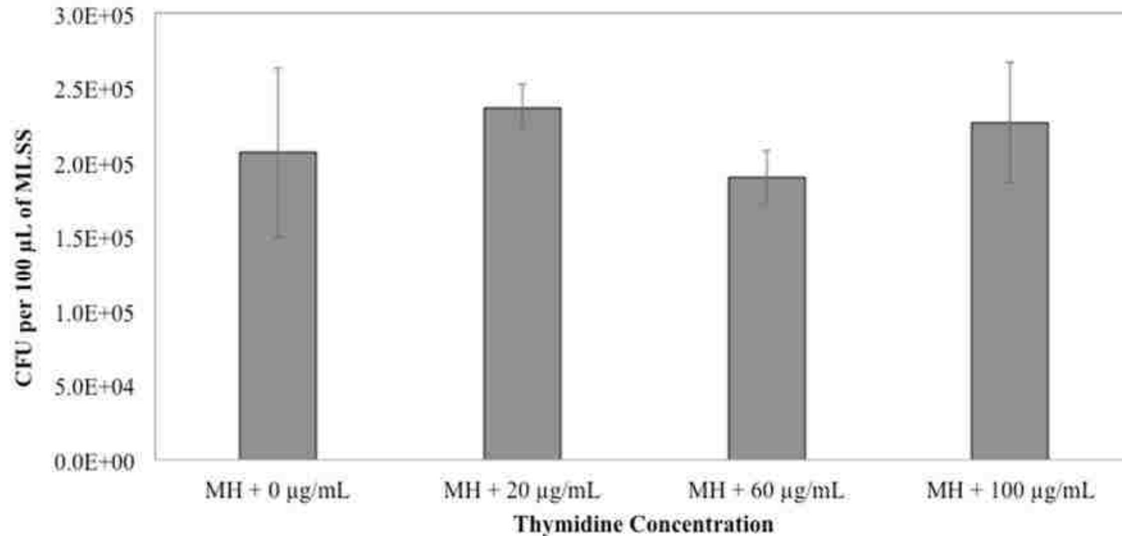


Figure 3-4. The effect of thymidine on total bacterial growth

The effect of thymidine on total bacterial growth on MH agar in the absence of TMP. Manual augmentation of thymidine had no significant effect on plate counts ($p>0.05$). Columns represent the mean values of triplicate plates, and error bars represent ±1 standard deviation

3.5.2 Manual augmentation of thymidine via cell lysing

Three sets of grab samples of MLSS were collected in sterile conical tubes from one of the SBRs operating with a 7-day SRT. The samples were immediately transported on ice to the laboratory for same-day processing and analysis. 10 mL of each sample were passed through a 0.45-µm filter (Whatman) to collect the suspended solids and eliminate aqueous thymine and thymidine. The filters were then placed in sterile conical tubes containing 50 mL of 0.9% sodium chloride solution and shaken at 400 rpm for 2 hours at 20°C to resuspend the solids. Then, 12 mL of each suspension were transferred to a 15-mL conical tube for sonication. Sonication was performed with a Branson 450 Sonifier (VWR, Radnor, PA) at a constant frequency of 20 kHz and power input of 160 W (13 kW/L) for 4 min, which resulted in a specific energy of ~900 kWh/m^3. Sonication was performed in an ice batch to avoid increases in temperature. Sonicated samples were then passed through 0.1-µm Acrodisc syringe filters (PALL, Ann Arbor, MI) to remove

المنارة للاستشارات
www.manaraa.com

large cellular debris and intact bacterial cells, and the resulting filtrate served as the final solution of dissolved intracellular components (e.g., free thymine/thymidine). MLSS samples—also from the 7-day SRT—were serially diluted and plated as described above, except that each plate was supplemented with 0, 1.2, 12, or 120 μL of post-sonication filtrate. These volumes correspond with concentrations of 0%, 0.01%, 0.1%, and 1% by volume (based on 12 mL of MH agar). Two sets of negative controls were used to ensure no bacterial contamination was present: (1) MH agar supplemented with TMP (no MLSS or post-sonication filtrate) and (2) MH agar supplemented with 0.01%, 0.1%, and 1% post-sonication filtrate (no MLSS or TMP). Both were negative for bacterial growth.

3.5.3 Varying of SRT to evaluate the effects of cellular debris

The SBRs were operated with SRTs of 2 days, 7 days, and 20 days for a period of ~30 days prior to these experiments. Three sets of grab samples of MLSS were collected in sterile conical tubes from each of the SBRs. The samples were immediately transported on ice to the laboratory for same-day processing and analysis. MLSS samples from each SBR were serially diluted and plated as described above, except that the MH agar was supplemented with 0%, 0.01%, 0.1%, or 1% MLSS filtrate (by volume) from the SBR operated with a 7-day SRT. To prepare the stock filtrates, the MLSS was passed through 0.1-μm Acrodisc syringe filters (PALL, Ann Arbor, MI). The filtrate, which presumably contained intracellular components from lysed cells, was then used to supplement the media based on the aforementioned concentrations. Therefore, these experiments simultaneously evaluated the effects of SRT and varying concentrations of intracellular components on the prevalence of TMP resistant bacteria. Two sets of negative controls were used to ensure no bacterial contamination was present: (1) MH agar supplemented

المنارة للاستشارات
www.manaraa.com

with TMP and (2) MH agar supplemented with 0.01%, 0.1%, or 1% MLSS filtrate. Both were negative for bacterial growth.

3.5.4 Varying of SRT to evaluate multi-drug resistance

The SBRs were operated with SRTs of 2 days, 7 days (in duplicate), and 20 days. 50-mL grab samples of primary effluent (i.e., feed to the SBRs) and MLSS were collected in sterile conical tubes. Three separate sample events were performed over a period of 60 days: 3 days after seeding/startup (9 total cycles), 30 days after startup (90 total cycles), and 60 days after startup (180 total cycles). Each set of samples was immediately transported on ice to the laboratory for same-day processing and analysis. Samples were serially diluted and plated as described above, except that the plates were also supplemented with 76 µg/mL of SMX (CLSI, 2014) to target multidrug-resistant bacteria and Staph/Strep supplement (Pro-Lab Diagnostics, Ontario, Canada) consisting of 1 mg/L of colistin sulfate and 1.5 mg/L of nalidixic acid to select for Gram positive *Staphylococcus* and *Streptococcus*. The addition of SMX was warranted because TMP is often administered in conjunction with SMX in clinical applications due to their complementary inhibition of DNA synthesis (Figure 3-1). The addition of Staph/Strep supplement was warranted because vancomycin was also added in independent samples (data not shown), to which Gram negative bacteria are intrinsically resistant. Therefore, this particular set of experiments focused on Gram positive bacteria as the target microorganisms.

3.6 Statistical analysis

Single factor analysis of variance (ANOVA) with post-hoc Tukey's test was performed with XLSTAT (Addinsoft, NY) at a significance level of 0.05.

المنارة للاستشارات
www.manaraa.com

3.7 Results and discussion

3.7.1 General water quality

The general water quality of the SBRs was monitored on an approximately weekly basis to validate the performance of the activated sludge process. The average pH of the primary effluent was 6.4±0.2, and the pH of the secondary effluent was relatively constant, regardless of SRT, with an average of 6.9±0.2. During the aeration phase, the average DO concentration was relatively constant in the four SBRs with an average of 4.7±0.5 mg/L and no reading lower than 3.7 mg/L.

The principal treatment objectives of the activated sludge process are the removal of organic matter and nitrogen (sometimes phosphorus as well). Reductions in BOD are typically used to verify the removal of organic matter, although TOC or COD can also be used as a surrogate in some applications (Christian et al., 2016). Figure 3-5 illustrates the average sCOD, total suspended solids (or MLSS), and volatile suspended solids (or MLVSS) in the primary and secondary effluents as a function of SRT. Consistent with full-scale activated sludge systems, there was a clear trend in sCOD removal in that longer SRTs resulted in lower and more consistent effluent sCOD concentrations. There was also a positive correlation between SRT and MLSS/MLVSS because of the greater recycle ratio for longer SRTs.

To further validate the performance of the reactors, nitrogen speciation was performed to determine the extent of nitrification in each reactor (Figure 3-6).

المنارة للاستشارات
www.manaraa.com

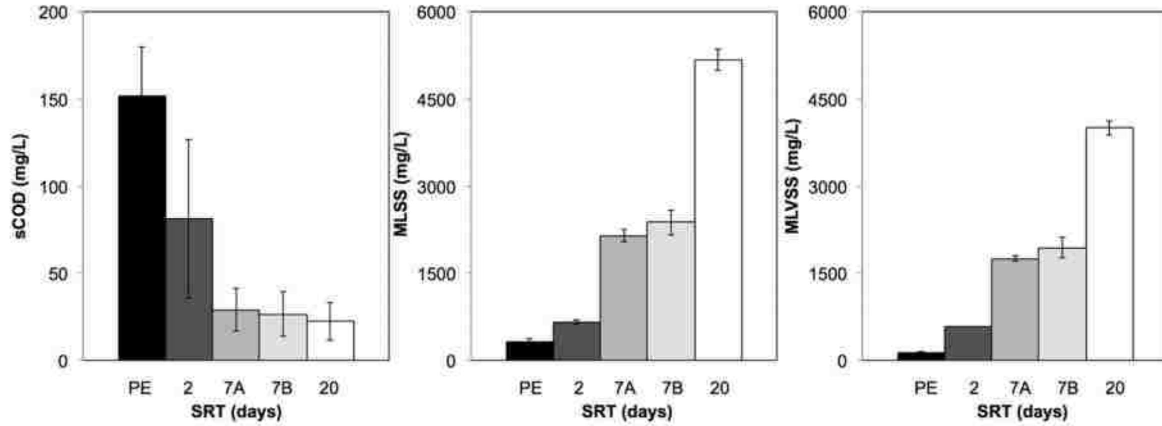


Figure 3-5. Average concentrations of sCOD, MLSS and MLVSS in the SBRs

Average concentrations of sCOD, MLSS and MLVSS in the SBRs as a function of SRT. The primary effluent (PE) represents the feed water quality prior to biological treatment in the SBRs. Columns represent the mean values for 5 sample events over 60 days of operation of the SBRs, and error bars represent ±1 standard deviation

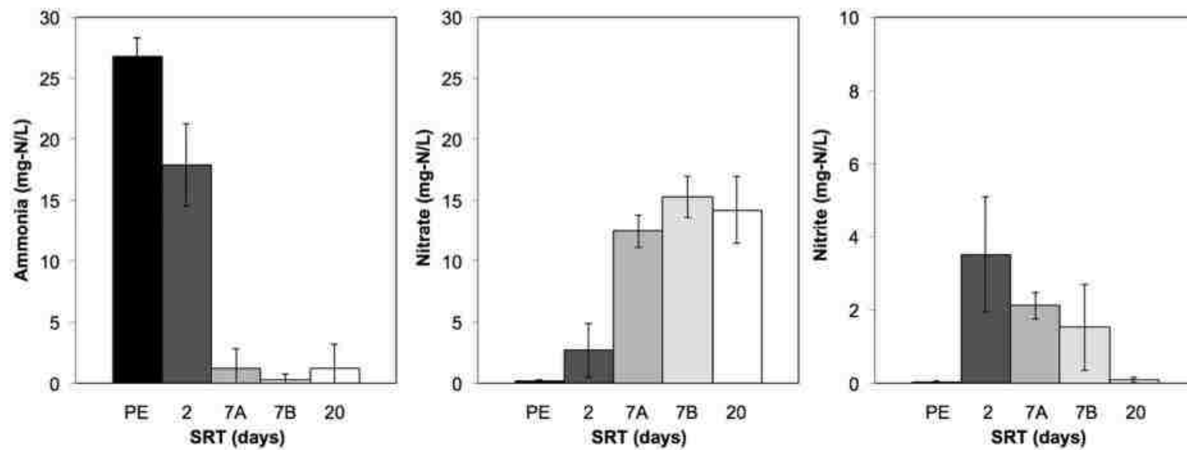


Figure 3-6. Average concentrations of ammonia, nitrate, and nitrite in the SBRs

Average concentrations of ammonia, nitrate, and nitrite in the SBRs as a function of SRT. The primary effluent (PE) represents the feed water quality prior to biological treatment in the SBRs. Columns represent the mean values for 3 sample events over 60 days of operation of the SBRs, and error bars represent ±1 standard deviation. The final 2 sample events were excluded because of temperature effects

As expected, the nitrogen in the primary effluent was almost entirely in the form of ammonia, and the extent of nitrification increased with longer SRTs. Activated sludge systems with SRTs <5 days are typically assumed to be deficient in nitrifying bacteria (Tai et al., 2006), which limits the conversion of ammonia to nitrite and nitrate. Assuming sufficient oxygen input, longer SRTs lead to the development of more mature microbial communities that are capable of converting

المنارة للاستشارات
www.manaraa.com

nearly all of the ammonia to nitrate, nitrite, and/or nitrogen gas, depending on the exact operational conditions. In the current study, the longer SRTs achieved nearly complete nitrification and also appeared to achieve partial denitrification based on an estimation of the nitrogen mass balance.

As the experiment progressed, the ambient temperature at the study site decreased from approximately 32°C down to less than 10°C, thereby causing the water temperature to decrease and hindering the removal of organic matter and the extent of nitrification (Head and Oleszkiewicz, 2004). These trends were observed for sCOD, ammonia, and nitrate, as shown in Figure 3-7. The sCOD in the secondary effluents increased only slightly at lower temperatures, but nitrification was clearly impeded, as indicated by the increasing ammonia concentrations and decreasing nitrate concentrations in the secondary effluents. Despite the change in water quality, the target SRTs were still achieved throughout the study period so the experimental objectives were not compromised, and the change in temperature allowed for an additional evaluation of temperature effects on antibiotic resistance.

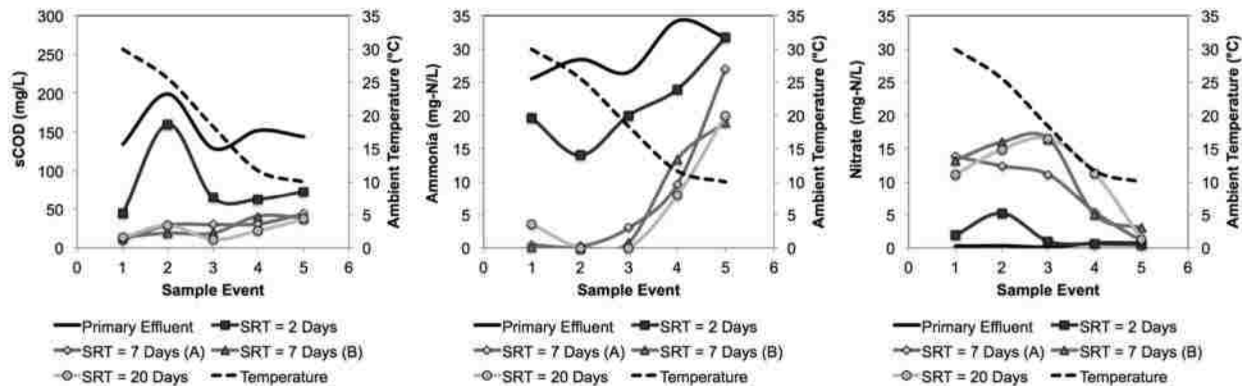


Figure 3-7. Effect of temperature on sCOD, ammonia, and nitrate

Effect of temperature on sCOD, ammonia, and nitrate as a function of SRT over 60 days of operation of the SBRs

www.manaraa.com

3.7.2 Trace organic compounds

There is some disagreement in the literature regarding the dominant removal mechanisms (i.e., sorption, biodegradation, volatilization) for various TOrCs. For example, Li and Zhang (2010) demonstrated that TMP and SMX removals were primarily attributable to adsorption, while Salveson et al. (2012) suggested that their removals were primarily linked to biodegradation. Regardless, longer SRTs will lead to higher MLSS concentrations (suggesting greater adsorption potential), higher MLVSS concentrations (suggesting more abundant biomass available for biodegradation), and a more diverse microbial community. This synergism between higher solids concentrations and a more abundant and diverse microbial community will lead to greater removals of hydrophobic and/or biodegradable compounds. However, as the bacteria are recycled within these systems, they will be repeatedly exposed to antibiotics in the primary effluent, and those effects might be magnified for hydrophobic compounds that accumulate on solid surfaces.

المنارة للاستشارات
www.manaraa.com

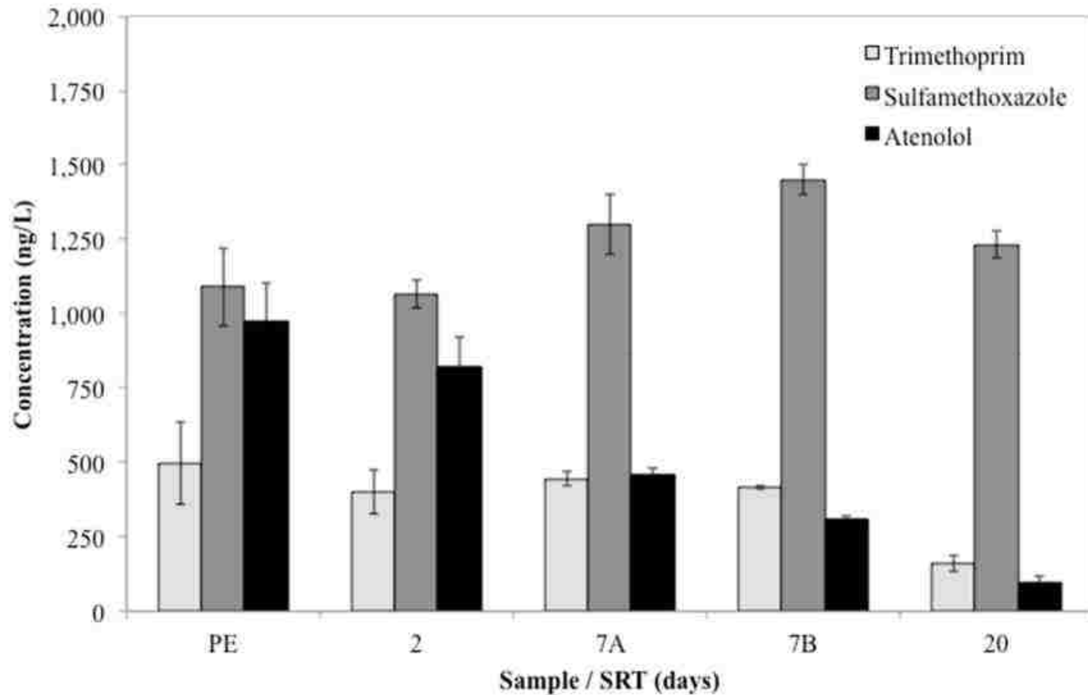


Figure 3-8. Concentration of trimethoprim, sulfamethoxazole, and atenolol

Aqueous concentrations of trimethoprim, sulfamethoxazole, and atenolol in the primary and secondary effluents as a function of SRT. Columns represent the mean values of replicate samples (2-4 replicates), and error bars represent ±1 standard deviation or the span of the data, depending on the number of replicates

Figure 3-8 summarizes the occurrence and removal of TMP, SMX, and atenolol in the SBRs. The corresponding raw data are provided in Table A1 (Appendix A). Consistent with the literature (Salveson et al., 2012; Gerrity et al., 2013), the removal of the biodegradable compound atenolol was positively correlated with SRT, with removals of 8-13%, 44-73%, and 90-92% for SRTs of 2, 7, and 20 days, respectively. TMP concentrations remained relatively constant in the primary and secondary effluents until the SRT was increased to 20 days, at which point the reactor consistently achieved 70% removal. This is supported by Salveson et al. (2012), which identified a 'threshold SRT' of 30 days to achieve 80% removal of TMP. Other studies reported TMP removals of 20-40% with SRTs ranging from 10-16 days (Göbel et al., 2007; Radjenovic et al., 2009), and Pérez et al. (2005) attributed TMP removal to aerobic nitrifiers that should be present in systems operated with longer SRTs. SMX exhibited no consistent trend as a

www.manaraa.com

function of SRT and actually increased in concentration by as much as 53%. Radjenovic et al. (2009) noted that increases in SMX have been reported previously and may be related to reformation of the parent compound during biological wastewater treatment. Therefore, the extent and mechanism of removal may be compound-specific, but longer SRTs have the potential to achieve lower concentrations of certain TOrCs, including the antibiotic TMP. But as noted earlier, the recycled biomass will be repeatedly exposed to antibiotics in the primary effluent and those accumulated on the solids. For this study, the primary effluent concentrations ranged from 370-710 ng/L for TMP and 970-1,300 ng/L for SMX, but solids partitioning was not quantified.

3.7.3 Estimation of thymidine concentration

One of the primary goals of this study was to monitor the level of TMP resistant bacteria in the absence/presence of TMP and different concentrations (0, 20, 60, and 100 µg/mL) of reagent-grade thymidine. Subsequent experiments assessed the impacts of intracellular components released from biomass either through laboratory sonication or through natural cell lysis (i.e., cell death and decay). As a basis for comparison, the total amount of thymine/thymidine present in the SBRs as a function of SRT can be estimated based on the following assumptions: DNA comprises 3% of the total dry weight of the bacterial population (Physiology of the bacterial cell: a molecular approach, 1990) and thymine comprises 25% of the total bases. The resulting estimates of thymine/thymidine content are summarized in Table 3-3. The thymidine contents listed in Table 3-3 was then used as a starting point to select a range of concentrations for manual augmentation of thymidine and the other two experiments.

المنارة للاستشارات
www.manaraa.com

Table 3-3. Summary of calculation of thymidine concentration in samples

SRT (d)	Active biomass (mg/L)	Inert biomass (mg/L)	Weight ratio (DNA/DW[1]) (%)	Thymidine content[2] (%)	Intracellular thymidine content[3] (mg/L)	Extracellular thymidine content[4] (mg/L)	Total thymidine content (mg/L)
2	310	240	3	0.75	2.3	1.8	4.1
7	1050	1100	3	0.75	7.9	8.3	8.3
20	2050	3400	3	0.75	15	25	40

[1]DW = Dry Weight; [2]Thymidine content assumes AT ratio of 50%; [3]Intracellular estimated from active biomass; [4]Extracellular estimated from inert biomass.

3.7.4 Manual augmentation of thymidine with reagent-grade chemical

In the first set of microbiological experiments, the level of TMP resistance within the microbial community was evaluated in the absence/presence of TMP and varying concentrations (0, 20, 60, and 100 μg/mL) of reagent-grade thymidine. The plate counts are summarized in Figure 3-9, and relative TMP resistance is summarized in Figure 3-10.

Over the three sample sets, the total bacterial count in the absence of TMP and thymidine was an average of $(2.22\pm0.39)\times10^5$ CFU per 100 μL of MLSS from the 7-day SRT. As noted earlier, there was no significant impact of thymidine on bacterial counts in the absence of TMP ($p>0.05$; Figure S3). With the addition of 4 μg/mL of TMP, the bacterial count decreased significantly because of the TMP sensitivity of a large portion (~90%) of the microbial community. However, the apparent TMP resistance increased from 11% to 44% with increasing concentrations of thymidine. The corresponding average plate counts were $(2.39\pm0.65)\times10^4$ CFU/100 μL, $(4.18\pm0.74)\times10^4$ CFU/100 μL, $(5.99\pm0.61)\times10^4$ CFU/100 μL, and $(9.79\pm0.91)\times10^4$ CFU/100 μL for thymidine concentrations of 0, 20, 60, and 100 μg/mL, respectively. The data for thymidine concentrations of 0 and 20 μg/mL proved to be statistically similar to each other, but all other pairings were found to be statistically different ($p<0.05$; Table A2).

المنارة للاستشارات
www.manaraa.com

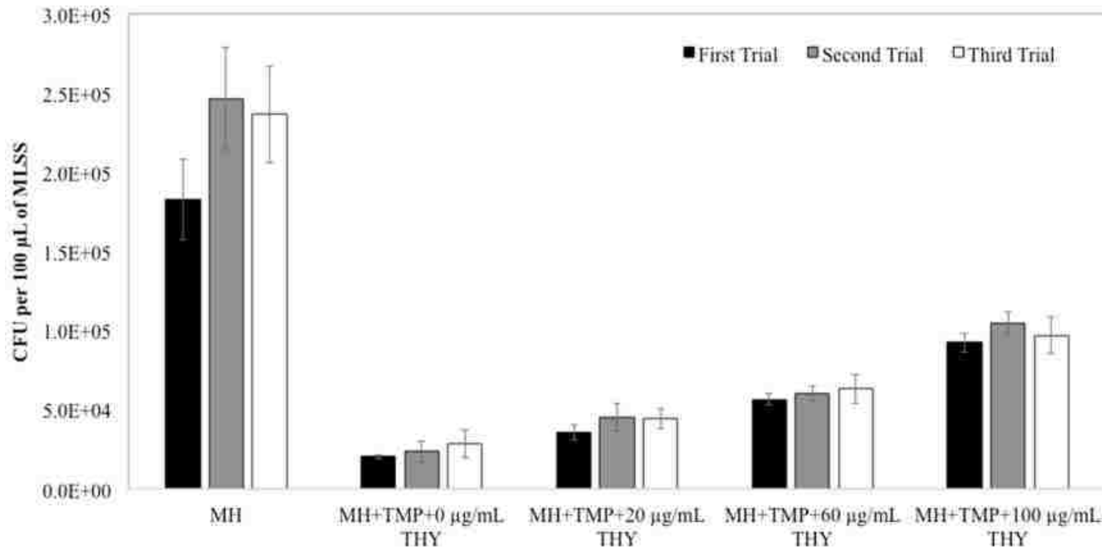


Figure 3-9. Bacterial growth in the presence of different thymidine concentrations

Bacterial growth in the presence of different thymidine concentrations and in the presence/absence of trimethoprim. Columns represent the mean values of triplicate plates, and error bars represent ±1 standard deviation

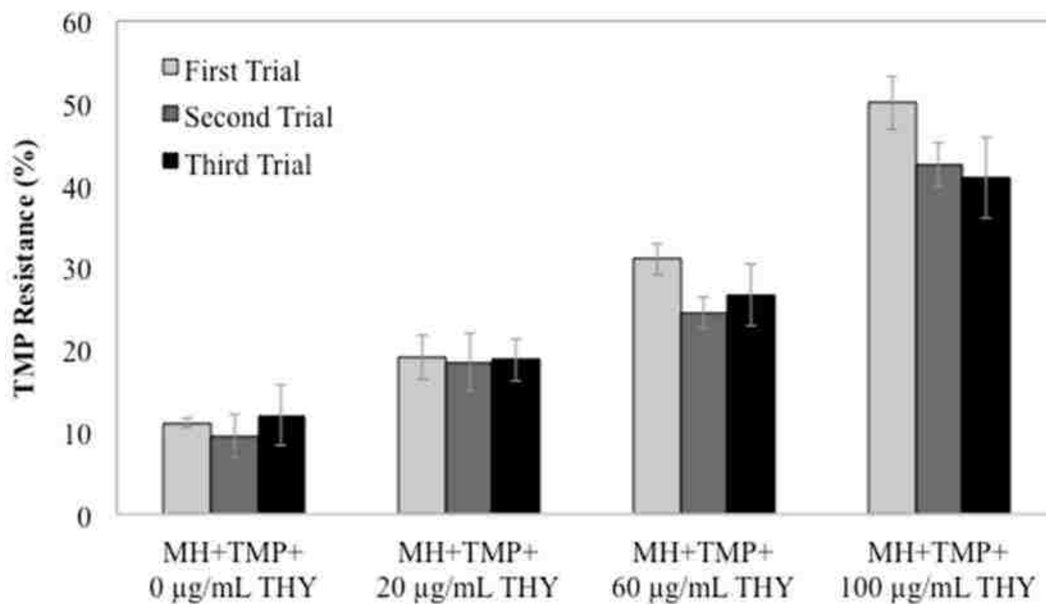


Figure 3-10. Apparent TMP resistance in the presence of thymidine

Apparent TMP resistance within the microbial community in the presence of 4 µg/mL of TMP and varying concentrations of thymidine. TMP resistance is reported as the percentage of bacteria that grew in the presence of TMP relative to the total culturable count in the absence of TMP. Columns represent the mean values of triplicate plates, and error bars represent ±1 standard deviation

These data suggest that a baseline level of ~10% of the culturable microbial community is either truly resistant to TMP or is able to access environmental reservoirs of thymine/thymidine.

المنارة للاستشارات
www.manaraa.com

Furthermore, manually augmenting the concentration of thymidine increases apparent TMP resistance within the microbial community in a linear fashion. This supports the findings of Amyes and Smith (1974) and highlights the importance of media selection for antibiotic susceptibility testing. The following sections expand on this concept by illustrating how the sample matrix can also cause significant interferences.

3.7.5 Manual augmentation of thymidine via cell lysing

When bacterial cells enter the death phase, they lyse and release their cellular contents (e.g., thymine and thymidine) into their surrounding environment. In theory, more cellular debris may result in higher concentrations of free thymine and thymidine in a biological reactor, and as shown in the previous section, free thymidine has the potential to increase apparent resistance to TMP. Therefore, samples with greater cellular debris may exhibit greater TMP resistance due to water quality rather than actual changes in the microbial community. In the second set of microbiological experiments, the level of TMP resistance within the microbial community was evaluated in the presence of 0%, 0.01%, 0.1%, and 1% post-sonication filtrate. The plate counts are summarized in Figure 3-11, and relative TMP resistance is summarized in Figure 3-12.

المنارة للاستشارات
www.manaraa.com

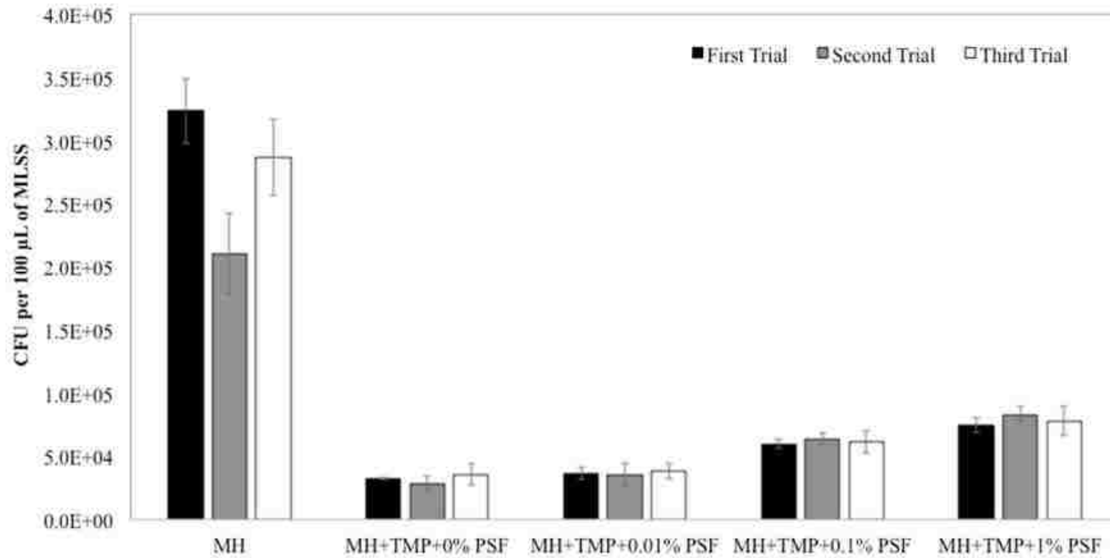


Figure 3-11. Bacterial growth in the presence of post-sonication filtrate

Bacterial growth in the presence of different concentrations of post-sonication filtrate. Columns represent the mean values of triplicate plates, and error bars represent ±1 standard deviation

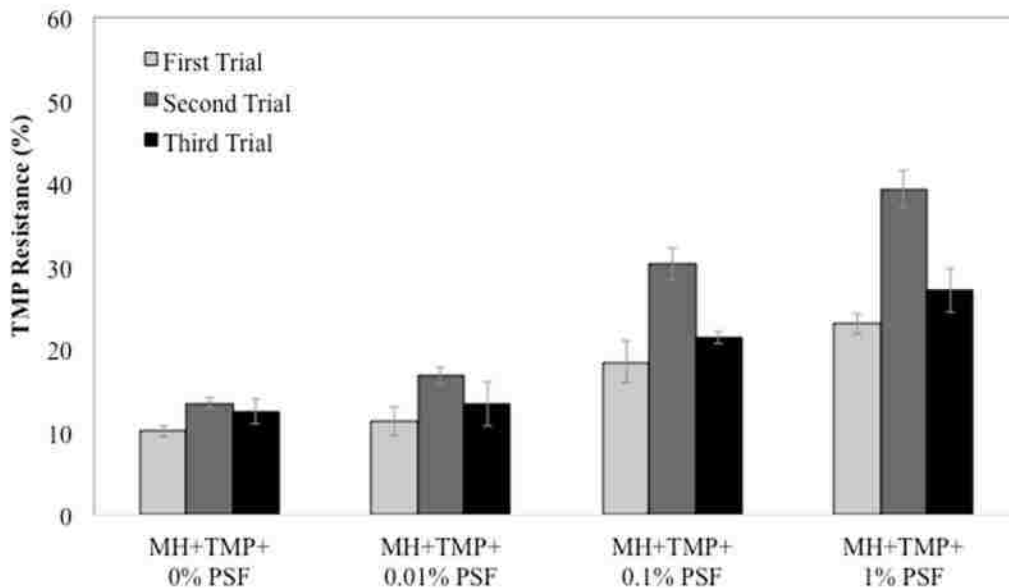


Figure 3-12. Apparent TMP resistance in the presence of PSF

Apparent TMP resistance within the microbial community in the presence of 4 µg/mL of TMP and varying concentrations of post-sonication filtrate (PSF). TMP resistance is reported as the percentage of bacteria that grew in the presence of TMP relative to the total culturable count in the absence of TMP. Columns represent the mean values of triplicate plates, and error bars represent ±1 standard deviation

المنارة للاستشارات
www.manaraa.com

Over the three sample sets, the total bacterial count in the absence of TMP and post-sonication filtrate was an average of $(2.73\pm0.56)\times10^5$ CFU per 100 µL of MLSS from the 7-day SRT. With the addition of 4 µg/mL of TMP, the bacterial count decreased by ~90% due to bacterial sensitivity to TMP. These baseline total and resistant counts were consistent with those observed during the thymidine testing phase. With the addition of the post-sonication filtrate, the apparent TMP resistance increased from 12% to 30%. The corresponding average plate counts were $(3.22\pm0.40)\times10^4$ CFU/100 µL, $(3.68\pm0.50)\times10^4$ CFU/100 µL, $(6.16\pm0.49)\times10^4$ CFU/100 µL, and $(7.83\pm0.60)\times10^4$ CFU/100 µL for post-sonication filtrate concentrations of 0%, 0.01%, 0.1%, and 1%, respectively. Adjacent pairings of post-sonication filtrate (i.e., 0% and 0.01%, 0.01% and 0.1%, and 0.1% and 1%) proved to be statistically similar to each other, but all other pairings were found to be statistically different ($p<0.05$; Table A3).

3.7.6 Varying of SRT to evaluate the effects of cellular debris

The previous experiments indicated that TMP sensitivity is affected by thymidine and the addition of bacterial intracellular components. The next set of experiments assessed whether cellular debris naturally present in the MLSS matrix affects TMP sensitivity and whether those effects are also related to SRT. Metcalf and Eddy (2014) describes the MLVSS production rate in activated sludge systems as a function of substrate removed, influent nonbiodegradable VSS (nbVSS), and a series of kinetic coefficients (Eq. 3-2):

$$\boldsymbol{P_{X,VSS} = \frac{QY(S_0-S)}{1+(k_d)SRT} + \frac{(f_d)(k_d)YQ(S_0-S)SRT}{1+(k_d)SRT} + QX_{o,i}} \qquad \text{(Eq. 3-2)}$$

$P_{X,VSS}$ = daily VSS production rate, g VSS/d

S_0 = influent substrate concentration, mg COD/L

S = effluent substrate concentration, mg COD/L

Q = influent flowrate, m^3/d

المنارة للاستشارات
www.manaraa.com

f_d = fraction of cell mass remaining as cell debris, g VSS/g VSS

Y = synthesis yield coefficient for heterotrophic bacteria, g VSS/g COD

$X_{o,i}$ = nbVSS concentration in influent, g/m^3

k_d = specific endogenous decay coefficient, d^{-1}

The second term in Eq. 3-2 represents the amount of cellular debris generated in the activated sludge process each day, and it also indicates there is a strong relationship between SRT and rates of bacterial death and decay. After normalizing to flow rate, Eq. 3-2 can be used to determine the corresponding concentration of cellular debris as a function of SRT, as shown in Figure 3-13. The SRTs in the current study correspond with theoretical cellular debris concentrations of 1.9, 4.7, and 7.6 mg/L, which suggests there may be higher concentrations of free thymidine at longer SRTs.

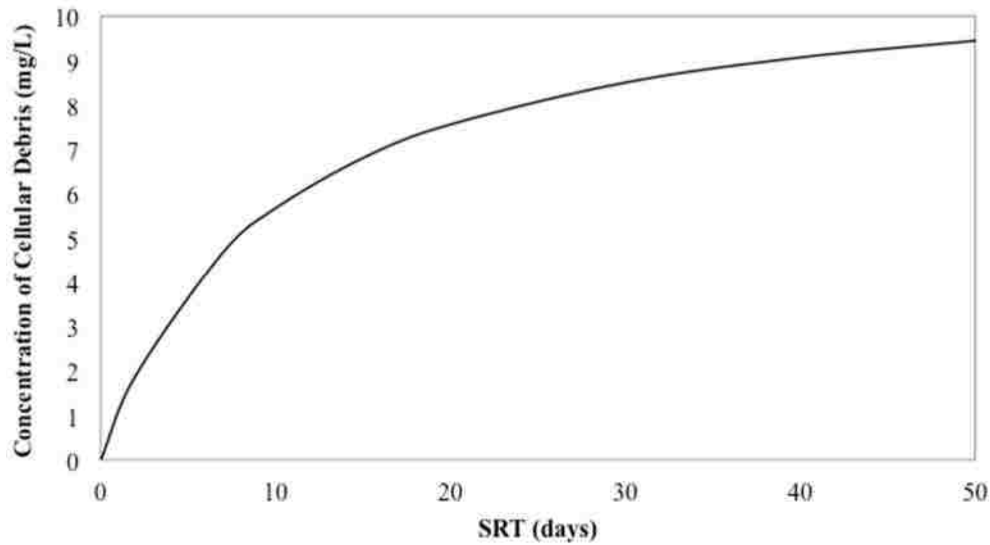


Figure 3-13. Relationship between theoretical concentration of cellular debris

Relationship between theoretical concentration of cellular debris (see Eq. 3 in main text) and SRT. Typical values for microbial kinetics were assumed (see below). Eq. 3 actually determines the daily volatile suspended solids production rate in g/day, but Eq. 3 can be normalized to flow rate to determine the corresponding concentration of cellular debris in mg/L, as shown in the figure. In other words, the concentration of cellular debris is independent of flow rate. Assumed parameters: k_d =0.1 d^{-1}; Y = 0.4 g VSS/g COD; f_d = 0.15 g/g; S_0 = 190 g COD/m^3; S_f = 1 g COD/m^3.

المنارة للاستشارات
www.manaraa.com

The objectives of these experiments were to (1) evaluate whether the addition of MLSS filtrate impacts apparent TMP resistance and (2) evaluate whether apparent TMP resistance varies as a function of SRT. By definition, longer SRTs require less ‘wasting’ of solids and higher biomass concentrations. Therefore, plating of fixed sample volumes may result in higher colony counts simply due to the higher biomass concentrations as a function of SRT. Reporting TMP resistance in relative terms (i.e., as a percentage of the total culturable bacteria) corrects for this issue. MLSS samples from the 2-day, 7-day, and 20-day SRTs were plated in the presence of 0%, 0.01%, 0.1%, and 1% MLSS filtrate from the 7-day SRT. The aggregated data are summarized in Figure 3-14, and raw data are shown in Figures 3-15 - 3-20.

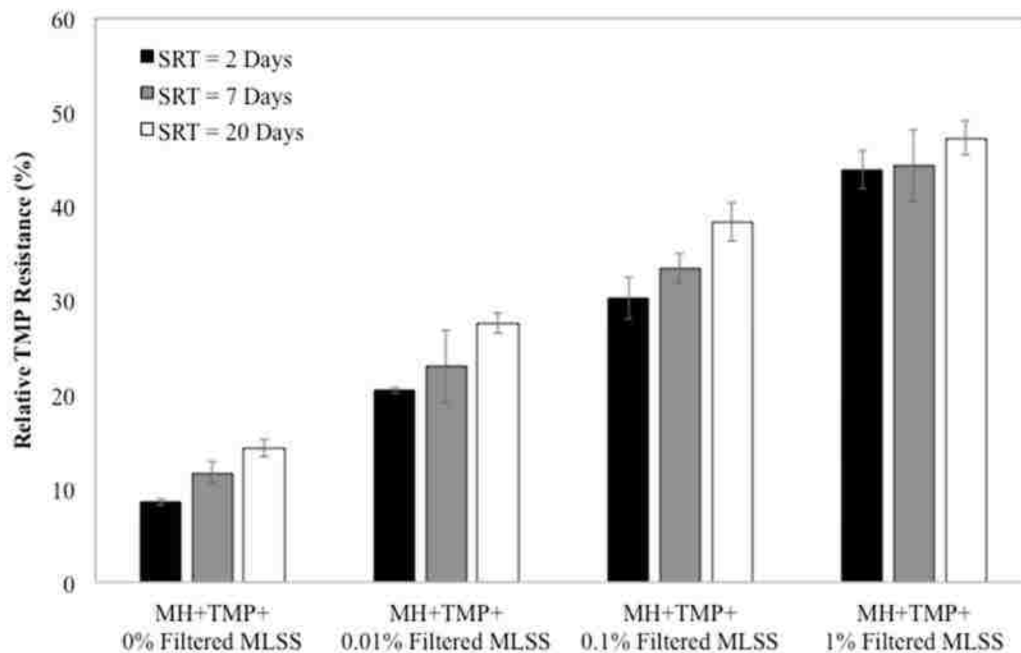


Figure 3-14. Apparent TMP resistance in the presence of filtered MLSS

within the microbial community as a function of SRT in the presence of 4 μg/mL of TMP and varying concentrations of MLSS filtrate. TMP resistance is reported as the percentage of bacteria that grew in the presence of TMP relative to the total culturable count in the absence of TMP. Columns represent the mean values of triplicate plates over three samples events, and error bars represent ±1 standard deviation

المنارة للاستشارات
www.manaraa.com

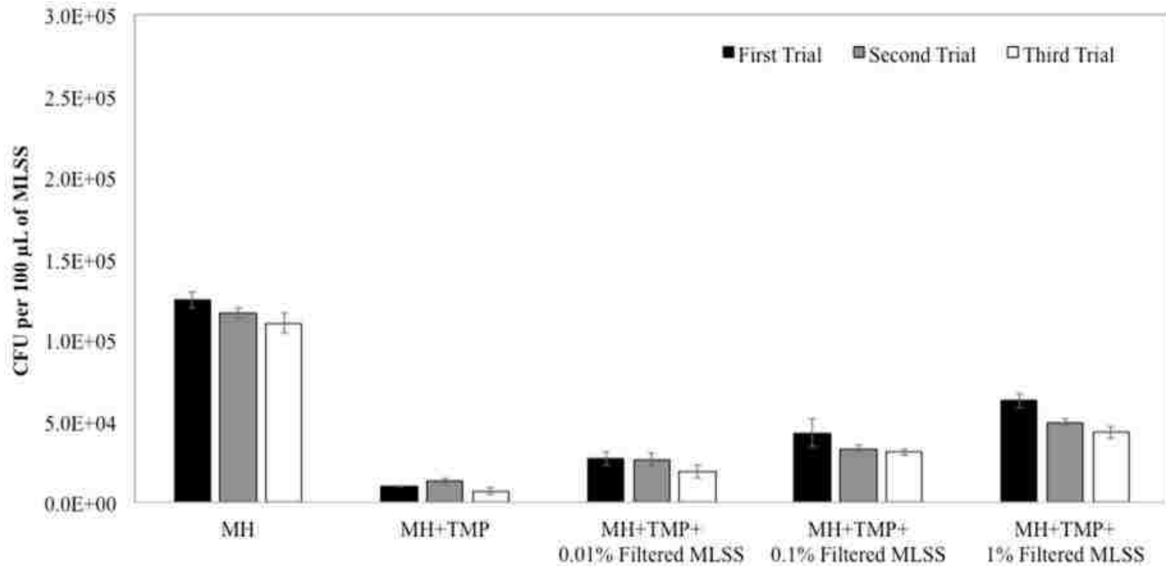


Figure 3-15. Bacterial growth in the presence of different concentrations of MLSS filtrate (2-day SRT)

Bacterial growth in the presence of different concentrations of MLSS filtrate collected from the 7-day SRT. The bacteria represent MLSS collected from the 2-day SRT. Columns represent the mean values of triplicate plates, and error bars represent ±1 standard deviation

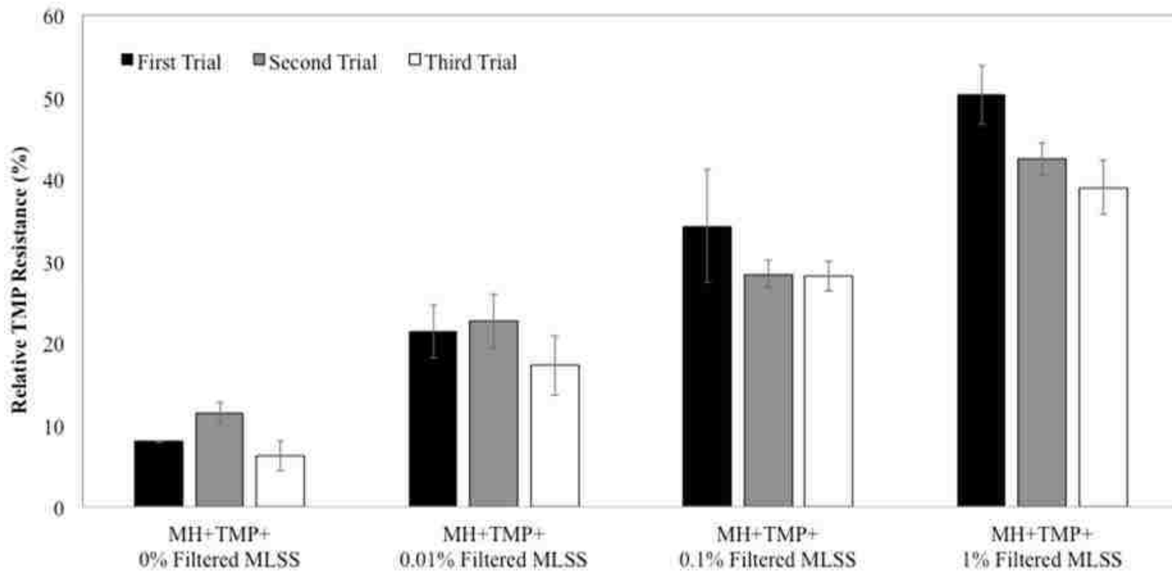


Figure 3-16. Apparent TMP resistance in the presence of MLSS filtrate (2-day SRT)

Apparent TMP resistance within the microbial community in the presence of 4 µg/mL of TMP and varying concentrations of MLSS filtrate (7-day SRT). The bacteria represent MLSS collected from the 2-day SRT. TMP resistance is reported as the percentage of bacteria that grew in the presence of TMP relative to the total culturable count in the absence of TMP. Columns represent the mean values of triplicate plates, and error bars represent ±1 standard deviation

المنارة للاستشارات
www.manaraa.com

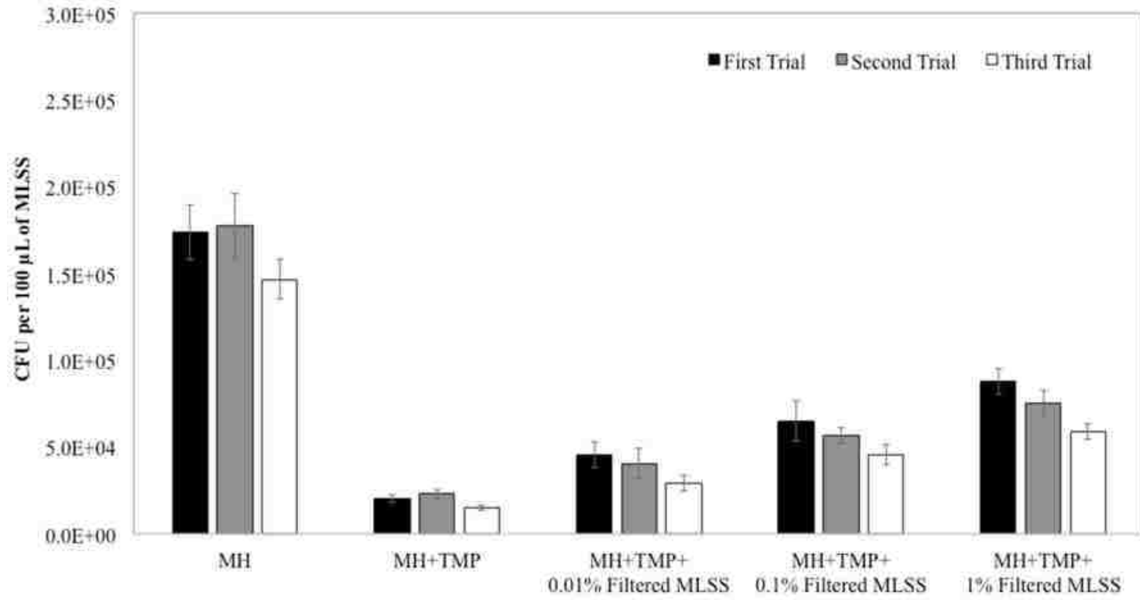


Figure 3-17. Bacterial growth in the presence of different concentrations of MLSS filtrate (7-day SRT)

Bacterial growth in the presence of different concentrations of MLSS filtrate collected from the 7-day SRT. The bacteria represent MLSS collected from the 7-day SRT. Columns represent the mean values of triplicate plates, and error bars represent ±1 standard deviation

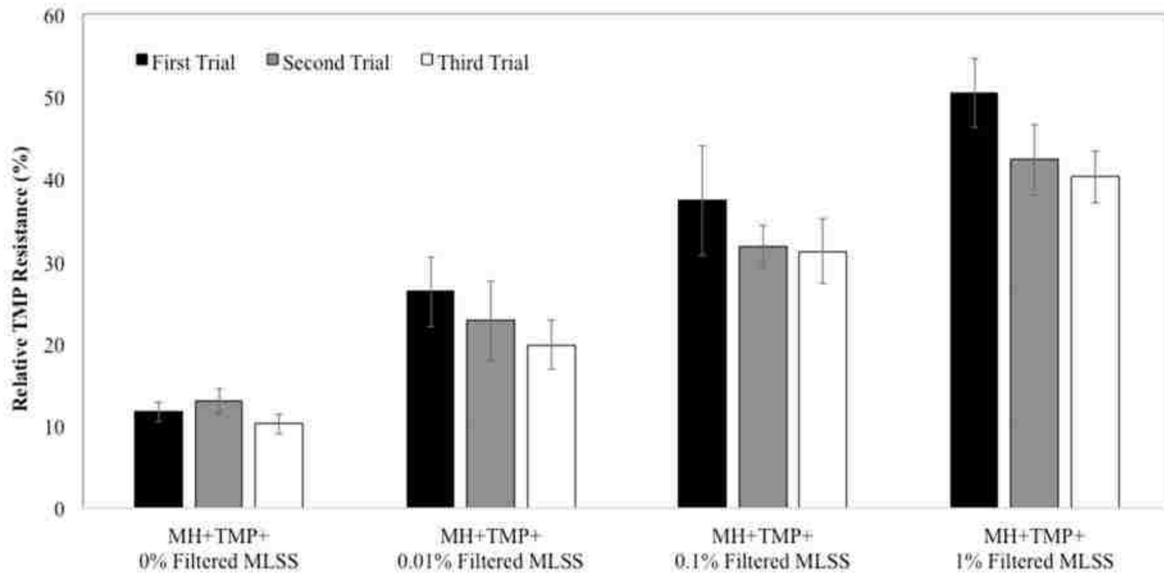


Figure 3-18. Apparent TMP resistance in the presence of MLSS filtrate (7-day SRT)

Apparent TMP resistance within the microbial community in the presence of 4 µg/mL of TMP and varying concentrations of MLSS filtrate (7-day SRT). The bacteria represent MLSS collected from the 7-day SRT. TMP resistance is reported as the percentage of bacteria that grew in the presence of TMP relative to the total culturable count in the absence of TMP. Columns represent the mean values of triplicate plates, and error bars represent ±1 standard deviation

المنارة للاستشارات
www.manaraa.com

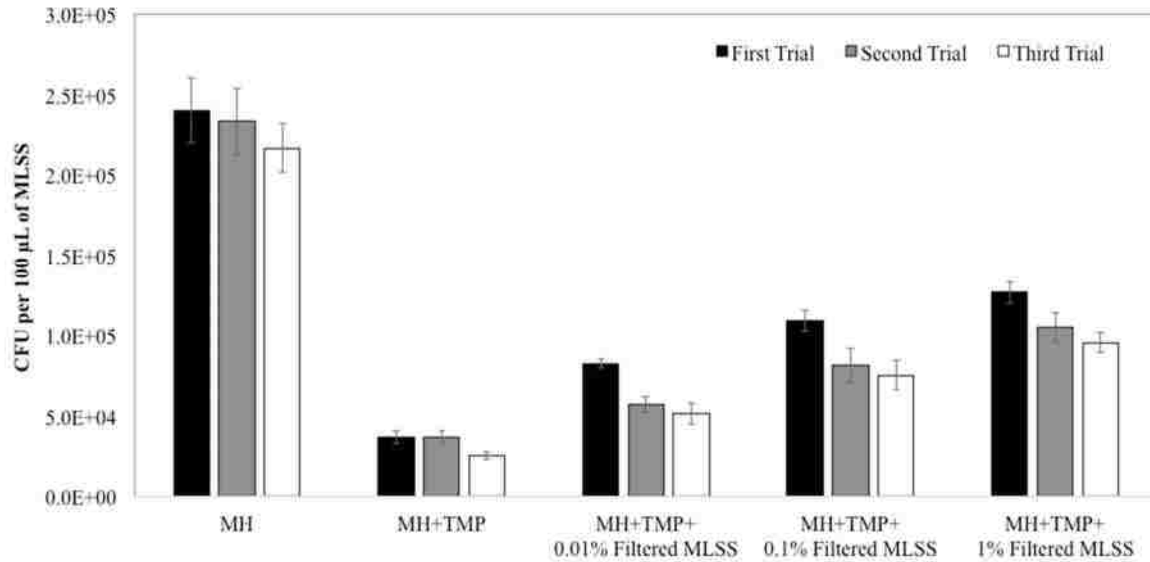


Figure 3-19. Bacterial growth in the presence of different concentrations of MLSS filtrate (20-day SRT)

Bacterial growth in the presence of different concentrations of MLSS filtrate collected from the 7-day SRT. The bacteria represent MLSS collected from the 20-day SRT. Columns represent the mean values of triplicate plates, and error bars represent ±1 standard deviation

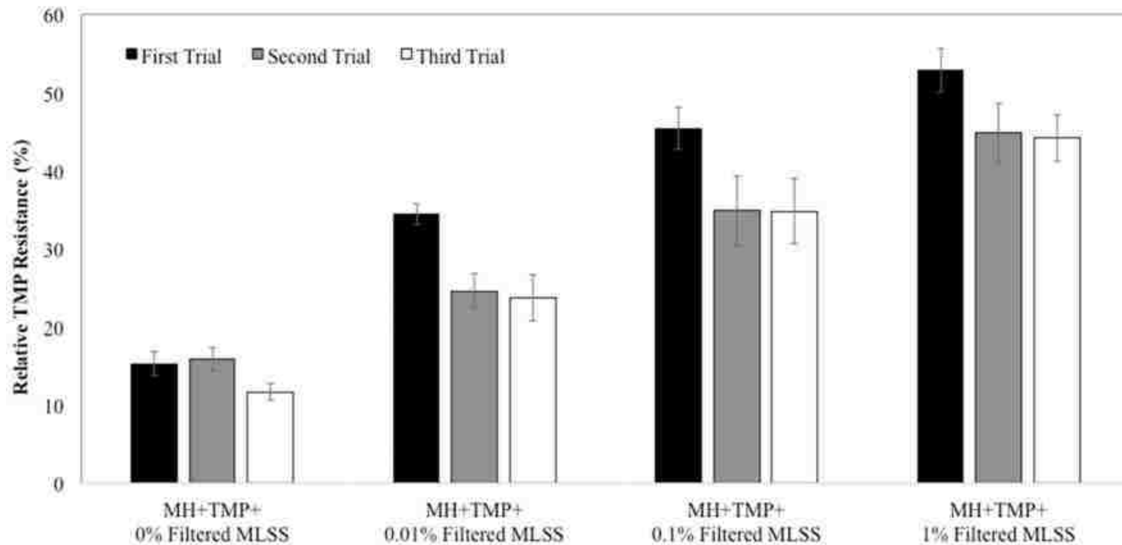


Figure 3-20. Apparent TMP resistance in the presence of MLSS filtrate (20-day SRT)

Apparent TMP resistance within the microbial community in the presence of 4 µg/mL of TMP and varying concentrations of MLSS filtrate (7-day SRT). The bacteria represent MLSS collected from the 20-day SRT. TMP resistance is reported as the percentage of bacteria that grew in the presence of TMP relative to the total culturable count in the absence of TMP. Columns represent the mean values of triplicate plates, and error bars represent ±1 standard deviation

المنارة للاستشارات
www.manaraa.com

Over the three sample sets, the average bacterial counts in the absence of TMP and MLSS filtrate were $(1.17\pm0.08)\times10^5$ CFU/100 µL, $(1.66\pm0.20)\times10^5$ CFU/100 µL, and $(2.30\pm0.19)\times10^5$ CFU/100 µL for SRTs of 2, 7, and 20 days, respectively. As expected, the bacterial counts were positively correlated with SRT—consistent with the higher MLSS and MLVSS concentrations. With the addition of 4 µg/mL of TMP, relative TMP resistance averaged (8.5±0.3)%, (11.6±1.1)%, and (14.3±0.9)% for SRTs of 2, 7, and 20 days, respectively. The adjacent SRTs proved to be statistically similar for this experiment, but the SRTs of 2 and 20 days proved to be statistically different ($p<0.05$; Table A7).

The addition of MLSS filtrate led to consistent increases in relative TMP resistance—up to 44-47% resistance for the 1% dosing condition. In each dosing scenario, the SRT effect was still apparent in that longer SRTs were correlated with greater prevalence of resistance. For the MLSS collected from the 2-day SRT, all but one adjacent filtrate dose pairing (i.e., 0.01% and 0.1%) proved to be statistically different ($p<0.05$; Table A4). For the 7-day SRT, all dose pairings proved to be statistically different ($p<0.05$; Table A5), and for the 20-day SRT, all but two adjacent dose pairings (i.e., 0.01% and 0.1%; 0.1% and 1%) proved to be statistically different ($p<0.05$; Table A6). Therefore, these data suggest that there are positive correlations between (1) SRT and relative TMP resistance and (2) exposure to dissolved constituents and relative TMP resistance. One possible explanation for the SRT effect might be the higher concentrations of cellular debris present at longer SRTs.

3.7.7 Varying of SRT to evaluate multidrug resistance among Gram positive bacteria

The last set of experiments highlighted several issues: (1) multi-druge resistance, (2) the general impact of biological treatment (i.e., primary effluent vs. MLSS), (3) the impact of SRT, and (4)

www.manaraa.com

the impact of temperature. In contrast with the previous experiments, these analyses focused on a subset of the microbial community, specifically culturable Gram positive *Staphylococcus* and *Streptococcus*. Over the three sample events, the average *Staph*/*Strep* count in the primary effluent was approximately $(3.34 \pm 1.69) \times 10^4$ CFU/100 µL, and the counts increased by a factor of 1.5-3.6 in the MLSS, with a positive correlation with SRT. For sample event 1, it is important to note that the microbial communities may not have completely stabilized prior to sampling. The SBRs were seeded with RAS from the full-scale facility, which operates at an SRT of ~7 days. Considering that the first set of samples was collected three days post-startup, the SBRs operating at the 2-day and 20-day SRTs may have still been acclimating, although the SBRs with a 7-day SRT should have been relatively stable. Figure 3-21 illustrates the relative SMX/TMP resistance as a function of SBR operation time, SRT, and temperature. The first feature that can be noted is the apparent increase in the relative SMX/TMP resistance during biological treatment. In other words, the primary effluent samples exhibited the lowest levels of resistance, but those levels increased dramatically, particularly in the first sample event, due to the biological treatment process. Furthermore, consistent with the SRT experiments with TMP only, the data exhibited a positive correlation between SRT and relative SMX/TMP resistance, although the statistical significance of those differences varied over time (Table A9). The 2-day SRT was always significantly different from the 20-day SRT, and it was often significantly different from the 7-day SRTs. Both 7-day SRTs were always statistically similar to each other and often statistically similar to the 20-day SRT.

The other clear feature of Figure 3-21 is the decrease in relative SMX/TMP resistance over time, which corresponds with the decrease in ambient temperature. In fact, the SRT differences were least significant for the last sample event, which exhibited the lowest levels of relative resistance

المنارة للاستشارات
www.manaraa.com

and the lowest temperature. This does not appear to be an artifact of microbial community acclimation to the target SRTs because the SBRs operating with a 7-day SRT did not require any acclimation period. Previous studies observed increases in ARG abundance, including the *sul*1 ARG that encodes resistance to SMX, during cold storage of biosolids (Miller et al., 2013; Miller et al., 2014). However, those studies focused only on molecular detection of AR and did not evaluate the expression of those genes or employ culture methods. Therefore, additional studies are needed to further evaluate the influence of temperature and the potential differences between culture and molecular methods in characterizing AR.

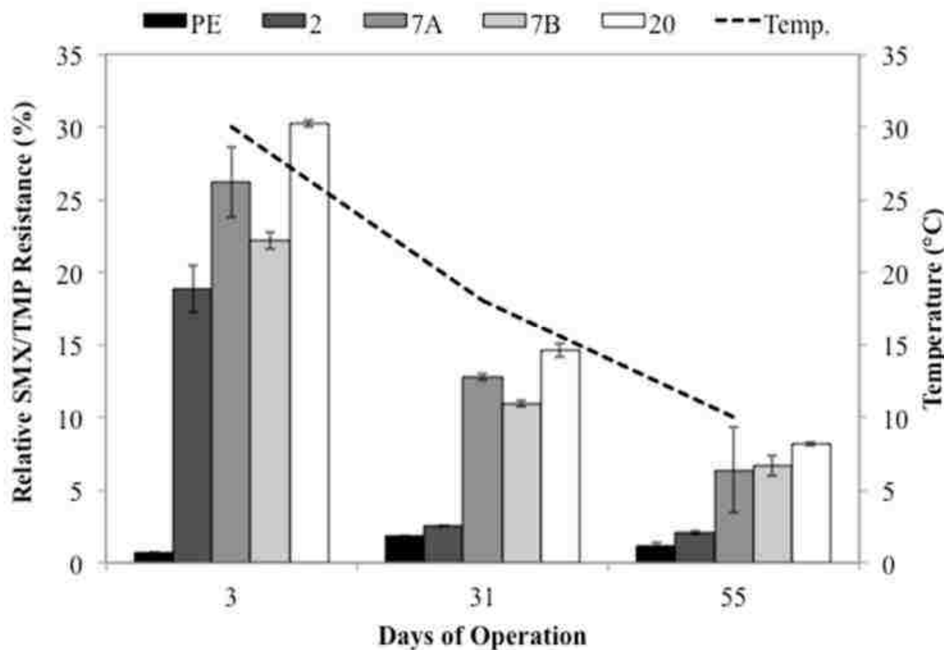


Figure 3-21. Ambient air temperature and apparent SMX/TMP resistance

Ambient air temperature and apparent SMX/TMP resistance within the microbial community as a function of SRT over the duration of the study. Relative resistance is reported as the percentage of bacteria that grew in the presence of the antibiotics relative to the total culturable count in the absence of the antibiotics. Columns represent the mean values of triplicate plates, and error bars represent ±1 standard deviation

www.manaraa.com

3.8 Conclusions

Bacterial antibiotic resistance is becoming an increasingly important issue for the municipal wastewater industry because treated wastewater effluent is known to contain numerous antibiotics and antibiotic resistance elements. Treated wastewater effluents are increasingly being used to augment conventional water supplies for various industrial, commercial, agricultural, and municipal applications. Therefore, stakeholders and decision makers must now address concerns related to wastewater-derived antibiotic resistance amidst uncertainty related to its potential public and environmental health impacts.

One operational change that can be implemented to improve the quality of wastewater effluent is increasing the SRT of activated sludge systems. This study demonstrated that longer SRTs are effective in improving water quality with respect to reductions in bulk organic matter, nitrogen, and some trace organics. However, this study also demonstrated that longer SRTs are associated with higher rates of cell death and decay and higher concentrations of cellular debris. This cellular debris may consist of dissolved intracellular components such as thymine or thymidine, which bacteria can use to negate the bacteriostatic effects of some antibiotics, including TMP and SMX. Increases in thymidine concentration—either from culture media or from environmental reservoirs—increase apparent bacterial resistance to TMP. In this study, these increases were achieved through manual thymidine addition, lysing of bacterial cells, and by supplementing with dissolved constituents present in mixed liquor.

Finally, this study demonstrated that biological treatment promotes single- and multi-drug resistance among non-specific heterotrophic bacteria and also among *Staphylococcus* and *Streptococcus*. The effect is also exacerbated by longer SRTs. However, it is not clear whether the SRT effect is due to selective pressure or higher concentrations of cellular debris. On the

المنارة للاستشارات
www.manaraa.com

other hand, decreasing temperature appears to reduce the prevalence of antibiotic resistance based on culture methods. Future studies should explain the role of temperature in promoting or attenuating AR, and studies must also consider potential differences between molecular and culture-based methods in quantifying AR.

المنارة للاستشارات
www.manaraa.com

4.0 IMPACT OF SOLIDS RETENTION TIME AND ANTIBIOTIC LOADING IN ACTIVATED SLUDGE SYSTEMS ON MICROBIAL COMMUNITY STRUCTURE AND BULK AND TRACE ORGANIC MATTER

4.1 Abstract

Solids retention time (SRT) is one of the most important factors in designing and operating activated sludge systems for biological wastewater treatment. Longer SRTs have been shown to alter the structure and function of microbial communities, thereby leading to improved treatment efficacy with respect to bulk and trace organics, nutrient removal, and membrane fouling. However, research has also shown that longer SRTs lead to increased prevalence of antibiotic resistant bacteria, perhaps due to increased exposure to antibiotics present in influent wastewater. The purpose of this study was to characterize changes in microbial community structure in a laboratory-scale activated sludge system as a function of SRT (2-20 days) and influent concentrations (1x-100x ambient concentrations) of five antibiotics: ampicillin, sulfamethoxazole, tetracycline, trimethoprim, and vancomycin. Changes in microbial community structure were evaluated based on traditional plating methods and 16s rDNA sequencing, and microbial community function was evaluated based on changes in effluent water quality, including bulk organic matter characterization and antibiotic concentrations. The results indicated that SRT—but not antibiotic loading—had a significant impact on microbial community structure (e.g., reduction in relative prevalence of *Acinetobacter* and *Arcobacter*) and effluent water quality. Therefore, spikes in influent antibiotics (at sub-therapeutic concentrations) are not expected to adversely impact biological wastewater treatment.

www.manaraa.com

4.2 Introduction

Due to the importance of potable reuse throughout the world, municipalities are increasingly turning to advanced wastewater treatment trains to achieve stringent water quality criteria (Gerrity et al., 2013). However, the more conventional aspects of wastewater treatment, particularly biological processes, are sometimes overlooked, despite the fact that they play critical roles in effluent water quality and operational performance (Leu et al., 2012). For example, rapid membrane fouling at an advanced treatment facility in California was attributed to the use of non-nitrified secondary wastewater effluent (Trussell et al., 2000). Instead of modifying the biological treatment process, the facility was upgraded with pre-ozonation to achieve bulk organic matter transformation to reduce membrane fouling rates (Stanford et al., 2011) but the pre-ozone process also resulted in significant increases in direct *N*-nitrosodimethylamine (NDMA) formation (Gerrity et al., 2015). Similar reductions in fouling with net reductions in NDMA could likely be achieved with optimized biological treatment (Leu et al., 2012; Ouyang and Liu, 2009; Sharp et al., 2010; Wang et al., 2014), potentially leading to reduced costs and energy consumption (Gerrity et al., 2014).

The objectives of secondary biological treatment at municipal wastewater treatment plants (WWTPs) vary considerably between facilities (e.g., biochemical oxygen demand, nitrification/denitrification, phosphorus). Solids retention time (SRT), which is defined as the average amount of time biomass is recycled within an activated sludge system, is critically important in determining whether these treatment objectives can be achieved. For example, systems with shorter SRTs (<5 days) are known to be deficient in nitrifiers, as these organisms are washed out of the system due to their relatively slow growth rates (Li and Wu, 2014). Systems with longer SRTs select for more slowly growing microorganisms, such as nitrifying

www.manaraa.com

microorganisms (Roh and Chu, 2011) and estrogen degrading bacteria (Roh and Chu, 2011), thereby resulting in a more diverse microbial community (Roh and Chu, 2011) potentially capable of emerging treatment needs such as trace organic compound (TOrC) mitigation.

TOrCs are ubiquitous in untreated and treated wastewater (Luo et al., 2014) and are frequently detected in surface water (Kolpin et al., 2002; Pal et al., 2010) and groundwater supplies (Lapworth et al., 2012) impacted by wastewater effluent. As a result, some municipalities are considering operational changes or treatment upgrades for TOrC mitigation (Gerrity et al., 2013). Numerous studies have documented the relationship between SRT and reductions in TOrC concentrations (Clara et al., 2005; D. Gerrity et al., 2013; Melcer and Klecka, 2011; Oppenheimer et al., 2007; Suarez et al., 2010; Leu et al., 2012; Salveson et al., 2012; Vuono et al., 2016) For example, Vuono et al. (2016) reported increases in rRNA/rDNA ratios for rare taxa associated with longer SRTs, possibly indicating higher rates of TOrC degradation by these rare taxa. In general, longer SRTs may lead to lower TOrC concentration, however due to the complexity of removal mechanisms, outcomes may vary between studies. Sorption on primary sludge might be the main mechanisms for micropollutants removal in primary treatment, while a range of processes like biodegradation/biotransformation, sorption, and volatilization may be responsible for TOrC removal during secondary treatment (Luo et al., 2014). Biological reactors operating with longer SRTs contain higher mixed liquor suspended solids (MLSS) concentration, therefore lower TOrC concentration in longer SRTs may be correlated with higher biomass concentration in these reactors. Previous studies have shown that diclofenac and galaxolide (Clara et al., 2011) and triclosan (Samaras et al., 2013) can be removed by sorption mechanism.

In aerobic biodegradation, with proper environmental and operational conditions, some of the refractory compounds can serve as growth substrates (Metcalf and Eddy, 2014). Some

المنارة للاستشارات
www.manaraa.com

chlorinated compounds can be degraded in a process called cometabolic degradation. In this process, some bacteria will produce specific enzymes that mediate a reaction with oxygen and hydrogen, which finally change the structure of the compounds that make them easier to be degraded by other aerobic bacteria (Metcalf and Eddy, 2014). Also, biological treatment systems with longer SRTs may select for slowly growing bacteria, which promote degradation of some compounds (Roh and Chu., 2011).

In addition to general concerns related to antibiotic occurrence and exposure, recent studies suggest a link between wastewater treatment and the occurrence of antibiotic resistant bacteria and antibiotic resistance genes (Auerbach et al., 2007; Zhang et al., 2015). Although longer SRTs often result in lower effluent TOrC concentrations, there is evidence to suggest that longer SRTs may proliferate antibiotic resistance (Neyestani et al., 2016). The mechanism of the observed increase in antibiotic resistance at longer SRTs is still unclear, but it may be related to longer exposure to antibiotics in a system that is conducive to microbial growth and horizontal gene transfer (Baquero et al., 2008) .Furthermore, concentrations of antibiotics vary considerably over time, either due to typical intraday variability (Gerrity et al., 2011) or unique discharges (e.g., hospital wastewater effluent; (Coutu et al., 2013)). The occurrence of antibiotics in hospital and urban wastewater have been investigated by previous studies. For some antibiotics such as ciprifloxacin and ofloxacin, higher concentrations (at least one order of magnitude) were detected in hospital wastewater compared with urban wastewater since fluoroquinolones frequently used antibiotics in hospitals (Rodriguez-Mozaz et al., 2015). In contrast, other group of antibiotics like cephalosporins, which include cefazolin and cefotaxime, were detected in lower concentrations in hospital wastewaters compared to those detected in domestic wastewater (Gros et al., 2013). Therefore, it is important to understand typical microbial community

المنارة للاستشارات
www.manaraa.com

structure and how the community responds to varying SRTs and antibiotic loadings. This will provide critical information for evaluations of treatment efficacy and antibiotic resistance, including the most appropriate subpopulations to target for antibiotic resistance studies.

Culture-dependent techniques have traditionally been used to characterize microbial communities, but these techniques may result in bias considering that more than 99% of environmental microorganisms cannot be detected by culture-dependent methods (Hugenholtz, 2002). Culture-independent microbial community characterization based on 16s rDNA sequencing has become increasingly accessible in recent years, leading to an abundance of published literature on the topic. For example, bacterial communities among different WWTPs with different technological configurations have been investigated previously. Gonzales-Martinez et al. (2016) explored bacterial communities among 10 different WWTPs and between conventional and highly loaded A-stage activated sludge systems. Zhang et al. (2012) investigated the bacterial communities among different WWTPs in Asia and North America. Although these studies explored bacterial communities among different WWTPs with different treatment technologies and operational conditions, they reported a core of genera was consistently shared between activated sludge samples (Gonzalez-Martinez et al., 2016; Zhang et al., 2012; Wanger and Loy., 2002). The same pattern was also monitored among all the secondary influent samples (Gonzales-Martinez et al., 2016). However, microbial community characterization and assessments of operational variables are often performed on samples collected from different systems (Gonzales-Martinez et al., 2016; Zhang et al., 2012), which may introduce confounding factors. Few studies have evaluated changes in microbial community structure within the same system (Ahmed et al., 2007; Vuono et al., 2016).

المنارة للاستشارات
www.manaraa.com

The current research explores the impacts of varying SRT (2-20 days) on microbial community structure in controlled laboratory-scale sequencing batch reactors (SBRs) fed with primary effluent (PE) from a full-scale municipal WWTP. Influent antibiotic concentrations (1x-100x ambient concentrations) are also discussed in the context of microbial community structure and treatment efficacy, specifically bulk water quality parameters and effluent antibiotic concentrations. This information will aid researchers in identifying important subpopulations for studies of TOrC mitigation, antibiotic resistance proliferation, and other issues related to operational performance and public health.

4.3 Materials and methods

4.3.1 Automation and operation of the laboratory-scale sequencing batch reactors

The laboratory-scale activated sludge process was achieved with four parallel SBRs fed with PE from a full-scale WWTP in Las Vegas, Nevada (Figure 3-2). The acrylic SBRs had a total volume of 8 L and a working volume of 4 L, after accounting for the volume of settled solids. Automation of the SBRs was achieved with a series of multi-station outlet timers, a pump, and electric actuated ball and solenoid valves. A MasterFlex peristaltic pump (Model 77200-62, Cole Parmer, Vernon Hills, IL) was used to pump PE from a wet well through a polytetrafluoroethylene/stainless steel strainer (Hach, Loveland, CO) and a 50-µm cartridge filter (Watts WPC50-975) prior to filling the reactors. The cartridge filters were replaced every two days to mitigate fouling and anaerobic conditions. A four-station irrigation timer (Orbit, Bountiful, UT) was used to control the volume fed to each reactor. Electric actuated solenoid valves (Parker Hannifin Corporation, Cleveland, OH) and an industrial grade air compressor (Porter-Cable PCFP02003; 3.5 gallons; 135 psi) were used to aerate the SBRs to achieve a

المنارة للاستشارات
www.manaraa.com

relatively constant dissolved oxygen (DO) concentration of 3 to 4 mg/L. The compressed air was passed through a pressure gauge and air flow meter before being fed into the SBRs via stone diffusers. Aeration was sufficient to achieve adequate mixing of the mixed liquor without the need for mechanical mixing. The target SRTs were achieved by wasting predetermined volumes of mixed liquor toward the end of each aeration phase, and this was accomplished with four electric actuated ball valves (W.E. Anderson, Michigan City, IN).

The SBRs were initially seeded with return activated sludge (RAS) from the full-scale facility, which typically operates at an SRT of ~7 days. The SBRs were operated with a total cycle time of 8 hours for 3 cycles per day over 2 experimental phases—60 days of operation for the SRT experiments and 60 days of operation for the antibiotic loading experiments. Each cycle consisted of the following five stages: (1) filling with PE for 29 minutes as the irrigation timer cycled through each reactor, (2) aeration for 6.5 hours (from the start of the filling cycle), (3) solids settling for 1 hour, (4) discharge of settled effluent for 30 minutes, and (4) idle for 1 minute. Again, solids wasting was performed toward the end of each aeration phase to minimize clogging of the ball valves.

In phase 1, the SBRs were operated with SRTs of 2 days, 7 days (in duplicate), and 20 days and fed with PE and ambient concentrations of antibiotics. The corresponding waste activated sludge (WAS) flow rates (Q_w) (Figure 3-2) were determined according to Eq. 3-1. The primary and secondary effluents from phase 1A were tested for bulk water quality parameters and TOrCs, and the PE and MLSS were analyzed with 16S rDNA sequencing.

At the conclusion of phase 1, the system was restarted by seeding different volumes of RAS in each SBR to target final MLSS concentrations of 1000 mg/L, 2000 mg/L, 3000 mg/L, and 4000 mg/L. These MLSS concentrations are characteristic of SRTs of 3, 6, 10, and 15 days, although

المنارة للاستشارات
www.manaraa.com

the microbial community of the full-scale RAS is characteristic of a ~7-day SRT. Therefore, the intent of this experiment was to simulate the MLSS effect (i.e., varying solids concentration and biomass abundance) while controlling for microbial community structure. The primary and secondary effluents from phase 1B were tested for TOrC concentrations.

In phase 2, the SRT was held constant at ~7 days, but the antibiotic concentrations in the PE were varied between 1x (ambient), ~10x (in duplicate), and ~100x. The antibiotic spike solutions were stored at the study site in coolers and replaced every 3 days. The antibiotic spike solutions were delivered to the SBRs with 12V DC, timer-controlled peristaltic pumps during the filling phase. The spiking levels (Table 4-1) were determined based on TOrC data collected during the SRT testing in phase 1.

Table 4-1. Summary of Spiked Antibiotic Concentrations in the SBRs

Antibiotic	Units	1x	10x	100x	MIC[1]	100x/MIC
Ampicillin	μg/L	0.2 (spiked)[2]	2	20	32,000	0.06%
Sulfamethoxazole	μg/L	1 (ambient)	10	100	76,000	0.13%
Tetracycline	μg/L	0.1 (spiked)[2]	1	10	16,000	0.06%
Trimethoprim	μg/L	0.5 (ambient)	5	50	4,000	1.25%
Vancomycin	μg/L	0.5 (ambient)	5	50	4,000	1.25%

[1]MIC = minimum inhibitory concentration (CLSI, 2014)
[2]Ampicillin and tetracycline were spiked at 2×MRL because they were <MRL during phase 1 testing

The table also shows the corresponding minimum inhibitory concentrations (MICs; i.e., clinical standards) to illustrate that the concentrations still represent sub-inhibitory levels (CLSI, 2014). The highest concentrations as a percentage of the MIC are for trimethoprim and vancomycin, which were spiked at 1.25% of the MIC. Because the concentrations of ampicillin and tetracycline were <method report limit (MRL) in the PE (described later), the 1x concentrations were actually spiked at twice the analytical MRL. The primary and secondary effluents from

المنارة للاستشارات
www.manaraa.com

phase 2 were tested for bulk water quality parameters and TOrCs, and the PE and MLSS were analyzed with 16S rDNA sequencing.

4.3.2 Preparation of antibiotic stock solutions

As mentioned earlier, five different antibiotics were selected for this project including: ampicillin sodium salt (Sigma Aldrich, St. Louis, MO), sulfamethoxazole (Sigma Aldrich, St. Louis, MO), tetracycline hydrochloride (Sigma Aldrich, St. Louis, MO), vancomycin hydrochloride (Sigma Aldrich, St. Louis, MO), and trimethoprim (Sigma Aldrich, St. Louis, MO). All antibiotic stock solutions were prepared based on the Clinical and Laboratory Standards Institute (CLSI, 2012). Appropriate Solvents were used for each antibiotic including: 0.01 M PBS for ampicillin, sterile nanopure hot water and minimal amount of 2.5 M NaOH for sulfamethoxazole, 90% volume of sterile nanopure water with 10% volume of 0.05 M hydrochloric acid for trimethoprim, and sterile nanopure water for tetracycline and vancomycin. The antibiotic stock solutions were then passed through acrodisc syringe filters to be sterilized. All stock solutions were stored in refrigerator at at 4±2°C and were used within 48 hours.

4.3.3 General water quality parameters

To ensure the SBRs were operating as intended and were properly mimicking a full-scale activated sludge system, a series of general water quality parameters were monitored for the duration of the study. These tests included temperature, pH, MLSS, mixed liquor volatile suspended solids (MLVSS), soluble chemical oxygen demand (sCOD), nitrogen speciation (i.e., ammonia, nitrate, and nitrite), and DO. Standard methods were employed when applicable; a summary of the analyses and associated methods is provided in Table B1 (Appendix B).

www.manaraa.com

4.3.4 UV-Vis and fluorescence spectroscopy

Bulk organic matter was also characterized according to previously published methods (Chen et al., 2003; Christian et al., 2016; Gerrity et al., 2012). Briefly, UV-Vis and fluorescence spectroscopy were performed following laboratory filtration with 0.7-µm glass fiber syringe filters (GD/X, Whatman, GE Healthcare Bio-Sciences, Pittsburgh, PA). The analysis was performed with an Aqualog spectrofluorometer (Horiba, Edison, NJ) equipped with a 150-watt, short-arc xenon lamp with an excitation range from 230 to 1200 nm. Excitation was limited to $\lambda \geq 240$ nm, and emissions were collected over 3 seconds of integration time. Data processing included corrections for blank response, the spectral sensitivity of the lamp, the inner filter effect, and Rayleigh masking, all of which were performed within the instrument software, and excitation emission matrices (EEMs) were also prepared with Matlab (Natick, MA). The fluorescence data were normalized to an average Raman peak area, which was based on excitation at 350 nm and emission from 380 to 410 nm in deionized water. Fluorescence regional integration was performed according to Chen et al. (2003) to calculate the regional and total fluorescence intensities. Integration was based on three regions representing (1) microbial byproducts, proteins, and biopolymers; (2) fulvic-like substances; and (3) humic-like substances.

4.3.5 Trace organic compounds

Primary and secondary effluent samples from the four SBRs were collected in 1-L silanized amber glass bottles preserved with sodium azide (1 g/ L) and ascorbic acid (50 mg/L). Samples were immediately placed on ice and transported to the laboratory where they were refrigerated at 4°C for up to 14 days. Sample processing consisted of filtration with 0.7-µm glass fiber filters and on-line solid phase extraction (SPE). The samples were then analyzed for the five target

www.manaraa.com

antibiotics (ampicillin, sulfamethoxazole, tetracycline, trimethoprim, and vancomycin) and nine indicator TOrCs [acetaminophen, caffeine, ibuprofen, atenolol, gemfibrozil, triclosan, primidone, sucralose, and tris(2-chloroethyl) phosphate (TCEP)] by liquid chromatography tandem mass spectrometry (LC-MS/MS) with isotope dilution according to previously published methods (Trenholm et al., 2006; Vanderford and Snyder, 2006; Salveson et al., 2012). Method reporting limits (MRLs) were set at 3-5x the corresponding method detection limits.

4.3.6 Molecular-based microbial community characterization of SBRs

Four sets of samples were collected from the laboratory-scale SBRs for 16s rDNA sequencing—two sets of samples during phase 1 SRT testing and two sets of samples during phase 2 antibiotic concentration testing. The samples included PE and MLSS from each SBR and were collected on consecutive days toward the end of each 60-day testing phase. The DNA was extracted and purified using the PowerBiofilm DNA isolation kit (MO BIO, Carlsbad, CA), following manufacturer's protocol. The extracted DNA was shipped to Research and Testing Laboratory (Lubbock, TX) for further analysis. Briefly, amplicons were sequenced on the Illumina MiSeq platform using MiSeq Reagent Kits V3 2×300 (Illumina, San Diego, CA). Initial amplification was performed with universal primers for Bacteria (28F and 388R). Quality assurance/quality control (QA/QC) procedures included denoising and chimera checks to eliminate potentially erroneous data. The remaining sequences were processed through the USEARCH global alignment program for diversity analysis and taxonomic classification. Statistical analyses were performed for the top 10 most abundant genera in the samples by principal component analysis (PCA) with XLSTAT (Addinsoft, NY).

المنارة للاستشارات
www.manaraa.com

4.4 Results and discussion

4.4.1 General water quality parameters

General water quality parameters were monitored on an approximately biweekly basis to validate the performance of the SBRs. The average pH of the PE was 6.4 in phase 1 and 6.8 in phase 2, and the average pH of the SBR effluents was approximately 6.9 in phase 1 and 7.1 in phase 2. During the aeration cycle, the average DO concentrations ranged from 4.5-5.2 mg/L in phase 1 and 3.7-3.8 in phase 2. As described later, one parameter that proved to be highly significant during long-term testing was temperature, which decreased from 30°C to 10°C in phase 1 (September to November) but increased from 22°C to 37°C during phase 2 (March to May). During phase 1, the average MLSS concentrations were 654±33, 2142±104, 2374±219, and 5172±182, and the average MLVSS concentrations were 575±5, 1747±50, 1937±174, and 4000±120 for SRTs of 2, 7A, 7B, and 20, respectively. During phase 2, the SBRs were operated at a constant SRT of 7 days so the MLSS and MLVSS concentrations were relatively constant at 2274±43 and 1840±53, respectively.

Figures 4-1A and 4-1B illustrate the average sCOD and ammonia concentrations in the primary and secondary effluents during phase 1 as a function of SRT. Figures 4-1C and 4-1D illustrate the same parameters as a function of influent antibiotic concentration during phase 2. The corresponding data for nitrate and nitrite are shown in Figure 4-2. As expected, sCOD removal and the extent of nitrification were positively correlated with SRT, and there was no significant difference in sCOD or nitrification when the SBRs were spiked with higher concentrations of antibiotics. As noted earlier, the temperature decreased to 10°C toward the end of phase 1. This resulted in a ~15% reduction in sCOD removal for the longer SRTs (i.e., decrease from 85-90%

المنارة للاستشارات
www.manaraa.com

removal to 69-74% removal), and it severely inhibited nitrification for all SRTs (i.e., decrease from 33-99% conversion to 0-40% conversion), as summarized in Table 4-2 and illustrated in Figure 4-3. Previous research has documented reductions in nitrification rates at lower temperatures (Head and Oleszkiewicz, 2004).

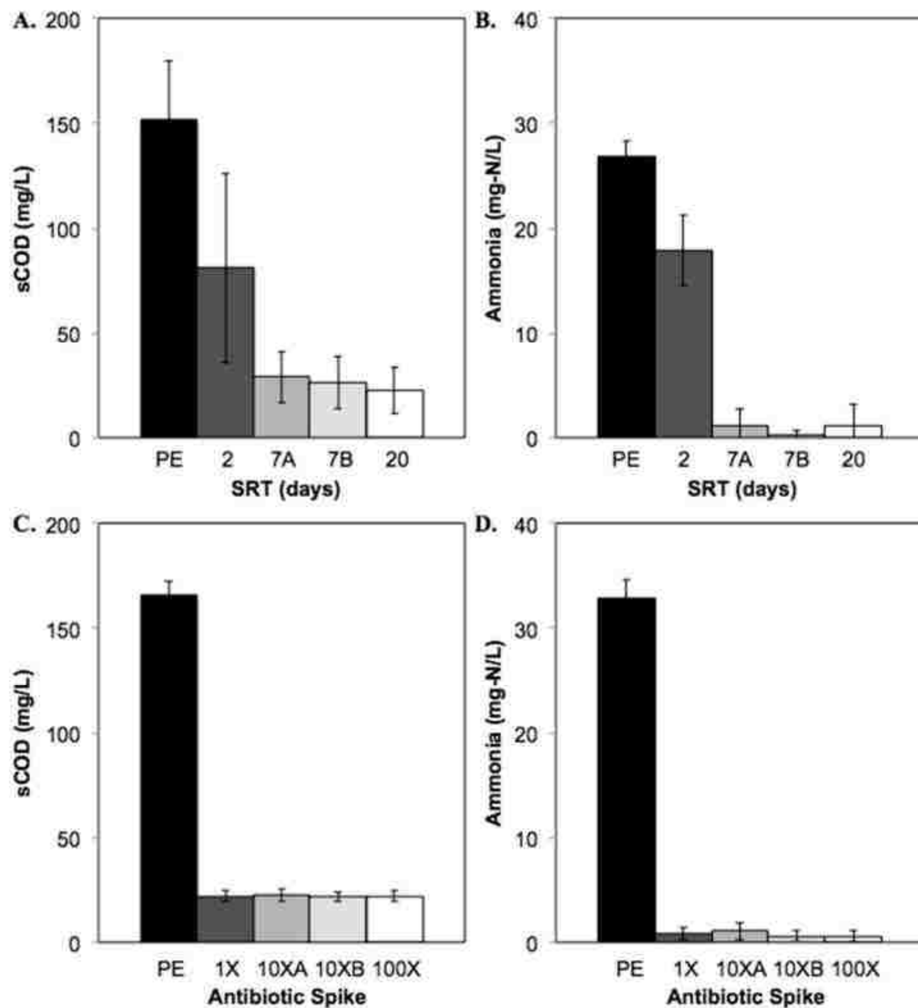


Figure 4-1. Average concentrations of (A) sCOD and (B) ammonia

Average concentrations of (A) sCOD and (B) ammonia in the primary (PE) and secondary effluents as a function of SRT during phase 1 testing. Columns represent the mean values of 5 and 3 sample events, respectively. Fewer sample events are included for ammonia due to temperature effects (described in main text). Average concentrations of (C) sCOD and (D) ammonia in the PE and secondary effluents as a function of influent antibiotic concentration during phase 2 testing (mean values of 3 sample events). Error bars represent ±1 standard deviation

المنارة للاستشارات
www.manaraa.com

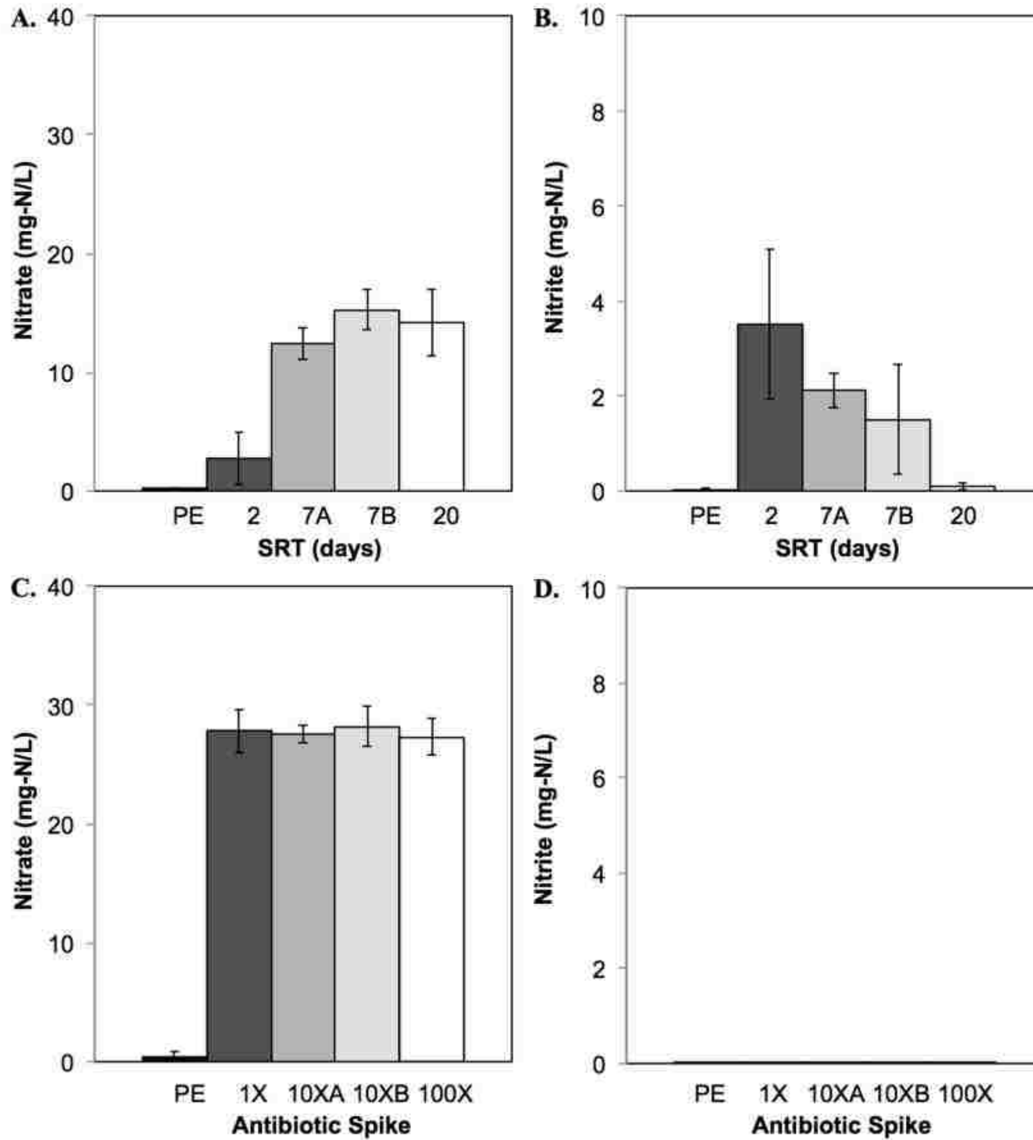


Figure 4-2. Average concentrations of (A) nitrate and (B) nitrite

Average concentrations of (A) nitrate and (B) nitrite in the primary (PE) and secondary effluents as a function of SRT during phase 1 testing (mean values of 3 sample events). Average concentrations of (C) nitrate and (D) nitrite in the PE and secondary effluents as a function of influent antibiotic concentration during phase 2 testing (mean values of 3 sample events). Nitrite concentrations were consistently <0.02 mg-N/L during phase 2 testing. Error bars represent ±1 standard deviation

المنارة للاستشارات
www.manaraa.com

Table 4-2. Extent of nitrification and apparent denitrification

Phase	Sample	Nitrification[4]	Denitrification[5]
1A[1]	**PE**	--	--
	SRT = 2 d	33%	11%
	SRT = 7 d (A)	96%	42%
	SRT = 7 d (B)	99%	37%
	SRT = 20 d	96%	43%
1B[2]	**PE**	--	--
	SRT = 2 d	0%	0%
	SRT = 7 d (A)	15%	12%
	SRT = 7 d (B)	40%	31%
	SRT = 20 d	37%	34%
2[3]	**PE**	--	--
	AB = 1X	98%	14%
	AB = 10X (A)	97%	14%
	AB = 10X (B)	98%	13%
	AB = 100X	98%	16%

Extent of nitrification and apparent denitrification during (top) first three sample events of phase 1 (moderate temperature), (middle) final sample event of phase 1 (cold temperature), and (bottom) three sample events from phase 2 (moderate temperature)

[1]Averages from first three sample events for phase 1A (average temperature = 25°C)

[2]Data from final sample event for phase 1B (temperature = 10°C)

[3]Averages from three sample events for phase 2 (average temperature = 28°C)

[4]% removal = $([Ammonia]_{PE} - [Ammonia]_{SBR}) / [Ammonia]_{PE} \times 100$

[5]% removal = $([TIN]_{PE} - [TIN]_{SBR}) / [TIN]_{PE} \times 100$

المنارة للاستشارات

www.manaraa.com

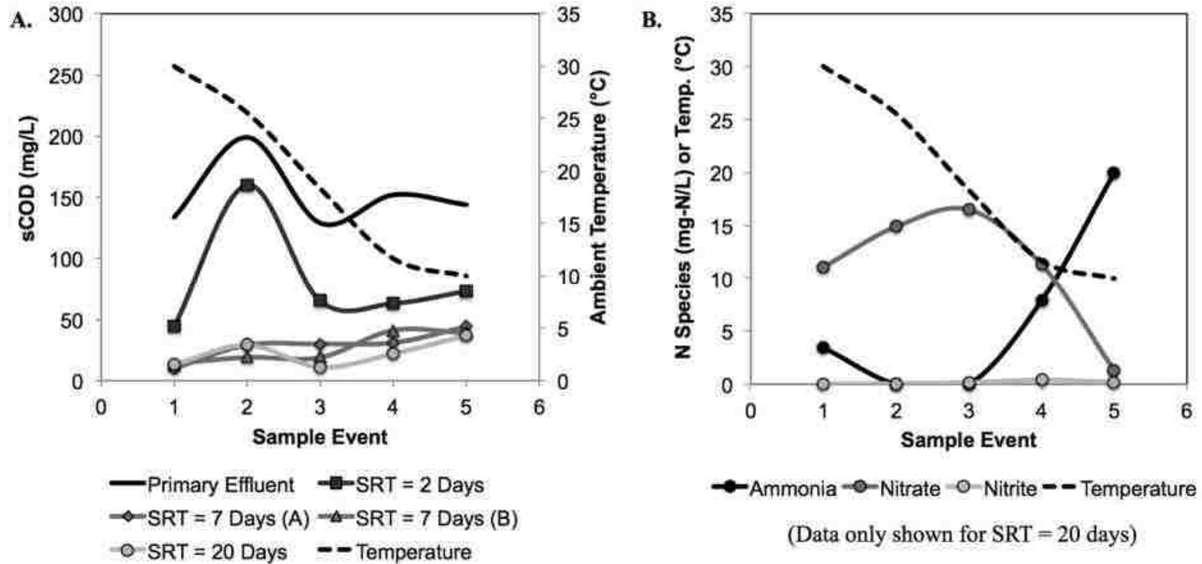


Figure 4-3. Temporal variability in the sCOD, ammonia, nitrate, and nitrire

(A) Temporal variability in the sCOD concentrations relative to ambient temperature in the primary effluent and SBR effluents as a function of SRT. (B) Temporal variability in effluent ammonia, nitrate, and nitrite concentrations relative to ambient temperature from the SBR operating with a 20-day SRT. These data are provided as examples of the effects of decreasing temperature during phase 1 testing

4.4.2 UV-Vis and fluorescence spectroscopy

As noted earlier, more extensive transformation and/or removal of bulk organic matter may yield significant benefits for advanced treatment with respect to membrane fouling (Stanford et al., 2011), ozone efficacy due to reductions in dissolved organic carbon concentrations (Lee et al., 2013), and the efficacy of ultraviolet (UV) disinfection, photolysis, or advanced oxidation due to increases in UV transmittance (Lee et al., 2016). The current study evaluated changes in fluorescence, as measured by EEMs, peak fluorescence, and regional fluorescence, and also changes in UV absorbance to describe how biological treatment might impact downstream treatment efficacy.

The EEMs in Figure 4-4 illustrate that bulk organic matter transformation and/or removal was positively correlated with SRT, while the EEMs in Figure 4-5 indicate that there were no

المنارة للاستشارات
www.manaraa.com

apparent differences in fluorescence resulting from varying influent antibiotic concentrations. The peak fluorescence data in Figure 4-6 demonstrate that effluents from systems with shorter SRTs may be characterized by more variable fluorescence signatures, consistent with the greater variability in sCOD values presented earlier. In fact, the 2-day SRT sometimes exhibited increases in fluorescence associated with humic-like peak C, while fulvic-like peak A exhibited more consistent reductions ranging from 15% to 36% for SRTs of 2 and 20 days, respectively. Protein-like peak T exhibited the most consistent reductions and was less dependent on SRT, with reductions ranging from 74% to 85%, respectively. Although these compounds are typically considered as organic foulant for microfiltration, ultrafiltration, and reverse osmoses, however the role of each component on membrane fouling is based on other factors such as pH (Ang and Elimelech, 2007), water quality constituents (e.g., calcium) (Ang and Elimelech, 2007), and type of membrane (e.g., hydrophilic or hydrophobic) (Lee et al., 2006). Literatures suggest that effluent organic matter (EfOM) contribute to organic fouling of ultrafiltration, microfiltration, and RO systems (Jarusutthirak et al., 2002; Jarusutthirak and Amy, 2001; Zhao et al., 2010) and any reduction in EfOM could possibly lead to alleviating membrane fouling issues. In fact, longer SRTs are associated with lower membrane fouling (Farias et al., 2014; Van den Broeck et al., 2012) probably due to reduction in EfOM. Additional fluorescence data are summarized in Table 4-3 – 4-6 and in Figure 4-7.

المنارة للاستشارات
www.manaraa.com

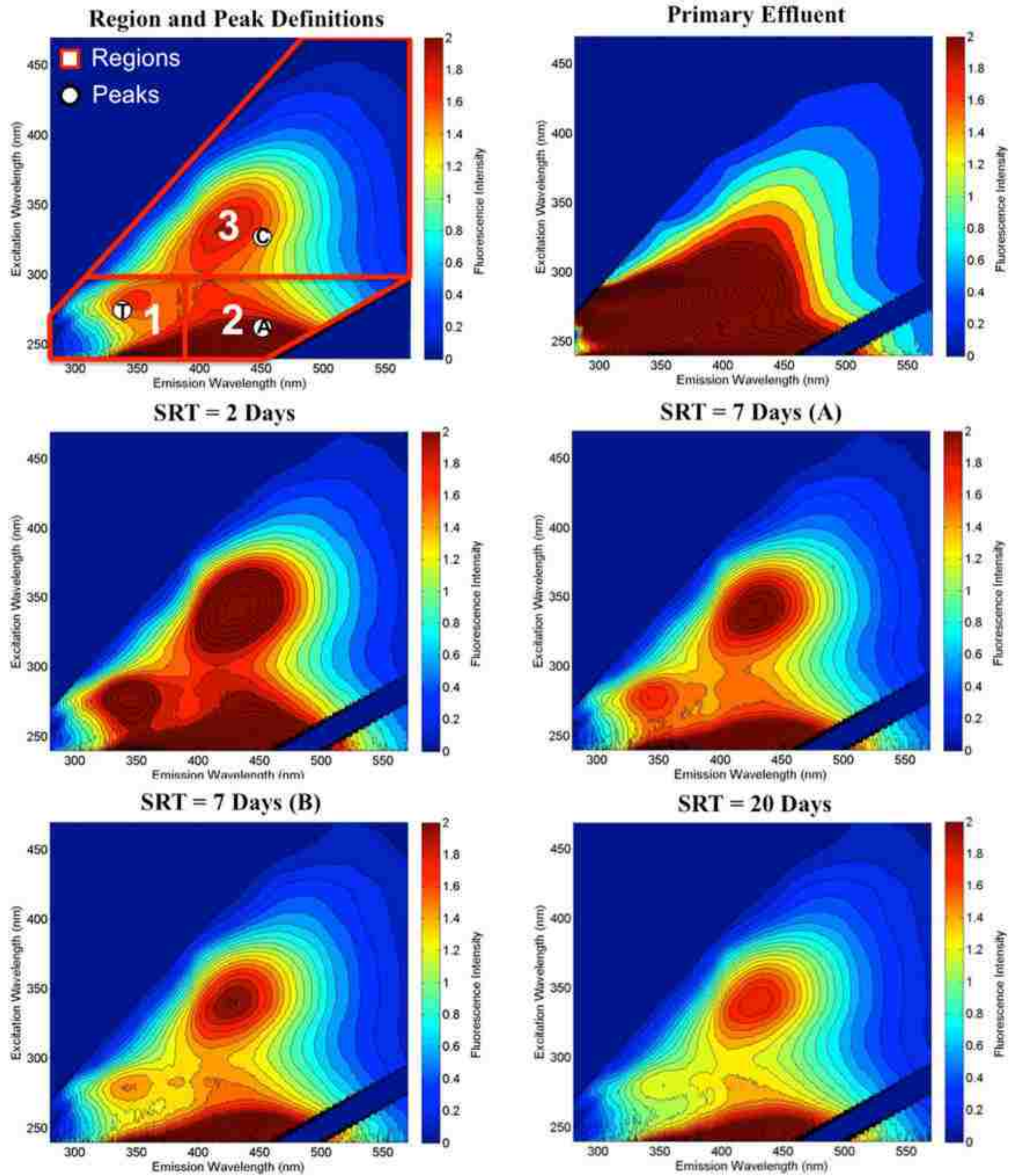


Figure 4-4. Representative EEMs as a function of SRT

Representative EEMs to illustrate changes in fluorescence as a function of SRT. The EEM in the top left defines the peaks and regions typically discussed in the literature, and the remaining EEMs illustrate the fluorescence of the primary and secondary effluents from the SBRs during the first sample event (similar results observed for subsequent sample events)

المنارة للاستشارات
www.manaraa.com

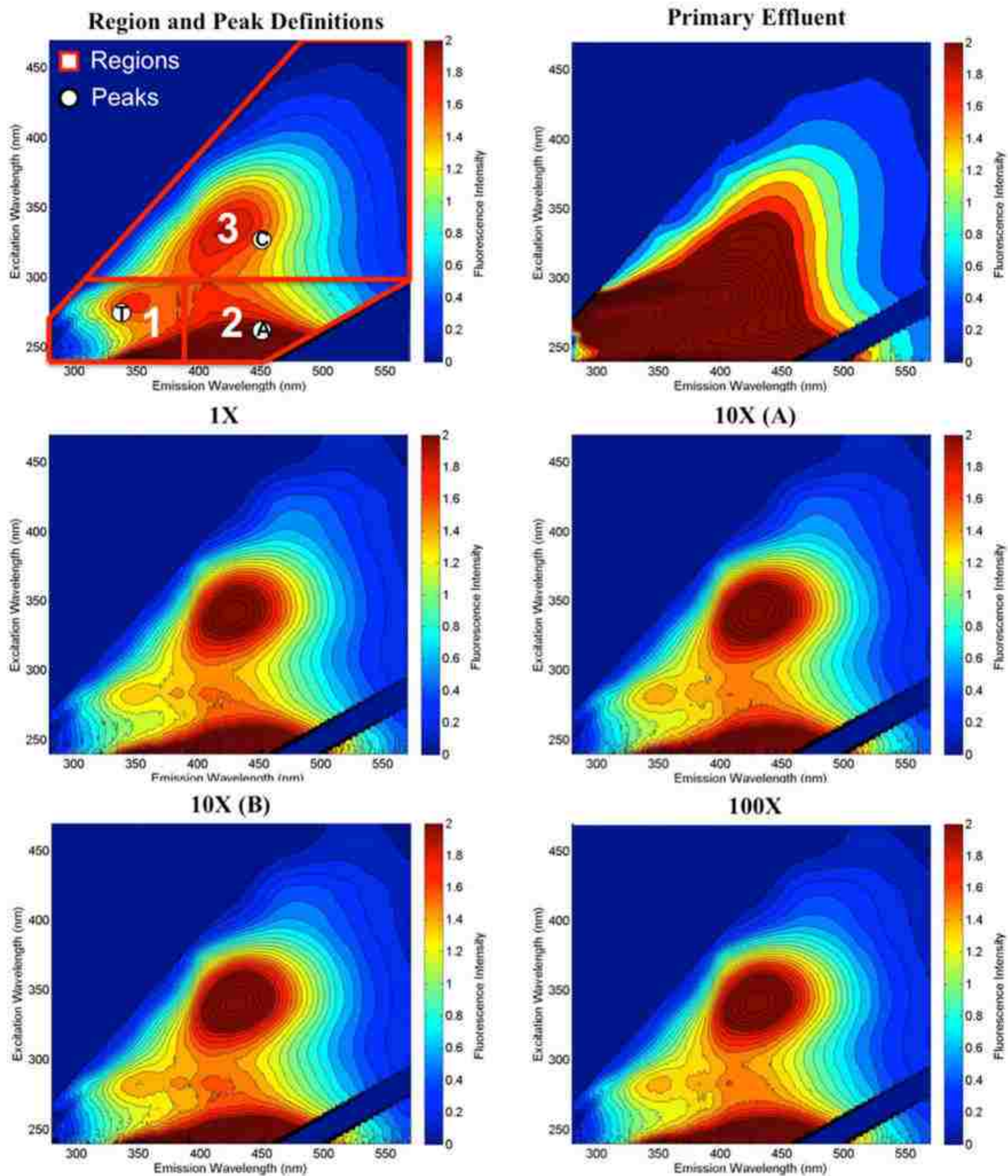


Figure 4-5. Representative EEMs as a function of influent antibiotic concentration

Representative EEMs to illustrate changes in fluorescence as a function of influent antibiotic concentration. There were no apparent differences in the EEMs for the SBRs spiked with different concentrations of antibiotics

www.manaraa.com

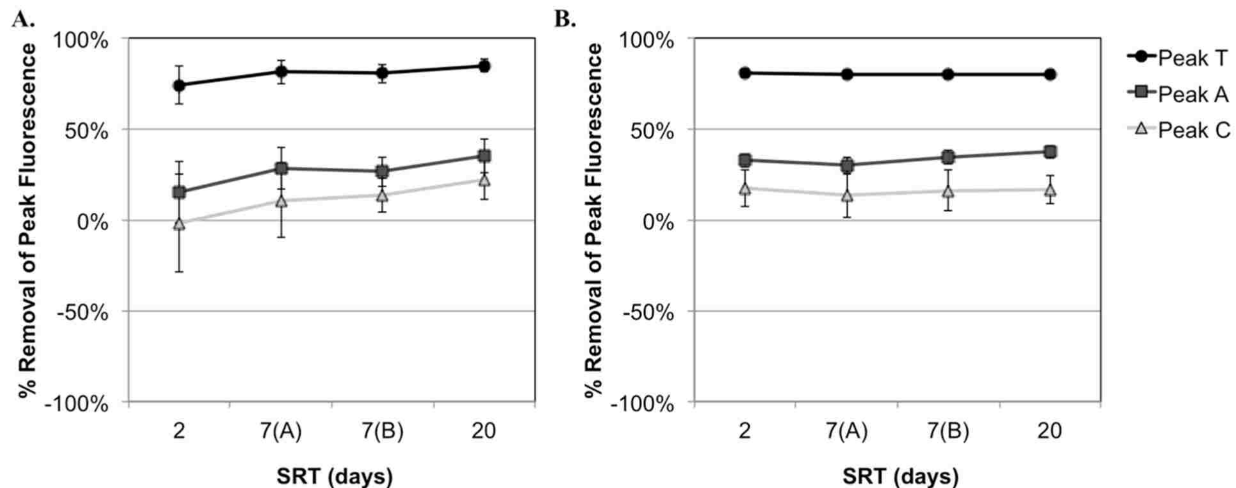


Figure 4-6. Average reductions in peak fluorescence

Average reductions in peak fluorescence during (A) phase 1 SRT testing and (B) phase 2 antibiotic testing. Error bars represent ±1 standard deviation

Table 4-3. Average peak fluorescence values during phase 1 SRT testing (5 sample events)

Sample	Peak T (AFU)	Peak A (AFU)	Peak C (AFU)
Primary Effluent	9.01 ± 2.23	2.98 ± 0.46	2.08 ± 0.30
SRT = 2 Days	2.16 ± 0.41	2.48 ± 0.19	2.07 ± 0.26
SRT = 7 Days (A)	1.59 ± 0.20	2.10 ± 0.05	1.82 ± 0.27
SRT = 7 Days (B)	1.69 ± 0.30	2.16 ± 0.09	1.78 ± 0.08
SRT = 20 Days	1.30 ± 0.12	1.89 ± 0.05	1.60 ± 0.14

Table 4-4. Average peak fluorescence values during phase 2 antibiotic testing (3 sample events)

Sample	Peak T (AFU)	Peak A (AFU)	Peak C (AFU)
Primary Effluent	6.77 ± 0.84	2.99 ± 0.19	2.15 ± 0.07
AB = 1X	1.31 ± 0.04	2.02 ± 0.20	1.78 ± 0.26
AB = 10X (A)	1.36 ± 0.03	2.09 ± 0.24	1.87 ± 0.31
AB = 10X (B)	1.32 ± 0.09	1.95 ± 0.20	1.81 ± 0.29
AB = 100X	1.32 ± 0.03	1.87 ± 0.15	1.80 ± 0.21

Table 4-5. Average regional fluorescence values during phase 1 SRT testing (5 sample events)

Sample	Region 1 (AFU)	Region 2 (AFU)	Region 3 (AFU)	Total Fluorescence (AFU)
Primary Effluent	99,337 ± 20,992	47,972 ± 7,224	14,787 ± 2,372	162,096 ± 27,130
SRT = 2 Days	27,514 ± 3,493	30,069 ± 2,021	13,988 ± 1,376	71,571 ± 5,810
SRT = 7 Days (A)	22,272 ± 1,996	26,079 ± 1,096	12,322 ± 1,471	60,673 ± 3,847
SRT = 7 Days (B)	22,902 ± 2,947	26,660 ± 980	12,275 ± 543	61,837 ± 3,958
SRT = 20 Days	19,079 ± 1,410	23,679 ± 900	11,074 ± 799	53,921 ± 1,707

Table 4-6. Average regional fluorescence values during phase 2 antibiotic testing (3 events)

Sample	Region 1 (AFU)	Region 2 (AFU)	Region 3 (AFU)	Total Fluorescence (AFU)
Primary Effluent	78,725 ± 8,231	46,863 ± 3,753	15,510 ± 462	141,097 ± 11,482
AB = 1X	19,536 ± 993	25,266 ± 2,721	12,299 ± 1,598	57,101 ± 5,259
AB = 10X (A)	20,285 ± 1,032	26,348 ± 2,684	12,864 ± 1,701	59,496 ± 5,315
AB = 10X (B)	19,679 ± 1,315	24,800 ± 2,392	12,206 ± 1,551	56,685 ± 5,181
AB = 100X	19,468 ± 863	23,945 ± 1,921	11,629 ± 1,231	55,042 ± 3,927

www.manaraa.com

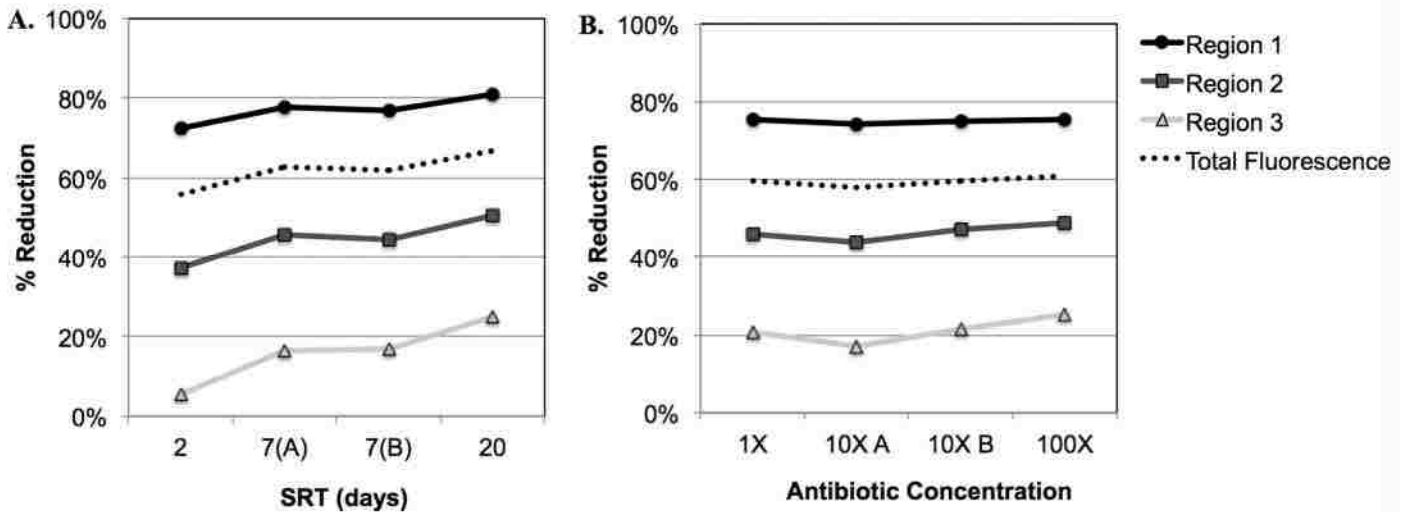


Figure 4-7. Average reductions in regional fluorescence during (A) phase 1 and (B) phase 2

Based on the UV absorbance data in Table 4-7, there is a clear benefit to operating with longer SRTs for systems employing low pressure UV processes for disinfection, photolysis, or contaminant oxidation. Based on the same reactor assumptions in Lee et al. (2016) including an electrical efficiency of 30% and a path length of 10 cm, the average UV_{254} absorbance of 0.171 cm^{-1} for the 2-day SRT results in energy consumption values of 0.030 kWh/m^3 and 0.372 kWh/m^3 for UV doses of 80 mJ/cm^2 and 1000 mJ/cm^2. These UV doses were selected to represent typical conditions in disinfection and photolysis or advanced oxidation applications, respectively. In contrast, the average UV_{254} absorbance of 0.135 cm^{-1} for the 20-day SRT results in lower energy consumption values of 0.024 kWh/m^3 and 0.301 kWh/m^3, respectively. Therefore, potential cost savings associated with the UV process (and other forms of tertiary treatment) may help offset the additional costs associated with longer SRTs (Canales et al., 1994; Li and Wu, 2014). Finally, the reductions in UV_{280} absorbance, which is sometimes used as a measure of the protein content of a water sample, were also positively correlated with SRT, consistent with the reductions in fluorescence associated with protein-like peak T.

المنارة للاستشارات
www.manaraa.com

Table 4-7. Reductions in regional fluorescence and UV absorbance

Sample	UV_{254} Absorbance (cm^{-1})	UVT_{254}	Low Dose UV Energy Consumption [1,3] (kWh/m^3)	High Dose UV Energy Consumption [2,3] (kWh/m^3)	UV_{280} Absorbance (cm^{-1})
PE	0.510 ± 0.109	31%	0.087	1.09	0.418 ± 0.110
SRT = 2 d	0.171 ± 0.009	67%	0.030	0.37	0.129 ± 0.008
SRT = 7 d (A)	0.149 ± 0.008	71%	0.026	0.33	0.112 ± 0.005
SRT = 7 d (B)	0.153 ± 0.010	70%	0.027	0.34	0.115 ± 0.009
SRT = 20 d	0.135 ± 0.008	73%	0.024	0.30	0.099 ± 0.006
PE	0.427 ± 0.033	37%	0.073	0.91	0.328 ± 0.032
AB = 1X	0.146 ± 0.003	71%	0.026	0.32	0.107 ± 0.003
AB = 10X (A)	0.148 ± 0.006	71%	0.026	0.33	0.110 ± 0.006
AB = 10X (B)	0.144 ± 0.005	72%	0.025	0.32	0.106 ± 0.003
AB = 100X	0.143 ± 0.005	72%	0.025	0.32	0.106 ± 0.003

Reductions in regional fluorescence and UV absorbance as a function of SRT (5 sample events) and influent antibiotic concentration (3 sample events). Error bars represent ±1 standard deviation

[1]Assumes UV dose = 80 mJ/cm^2 for disinfection applications (NWRI/WRF, 2012)
[2]Assumes UV dose = 1000 mJ/cm^2 for advanced oxidation applications
[3]Based on Lee et al. (2016): Energy Consumption (kWh/m^3) = $\frac{H`}{L \times \eta_{UV} \times 3600} \times \frac{2.303 \times UV\, A_{254} \times L}{(1-10^{-UV\, A_{254} \times L})}$
UVT = UV transmittance; H` = target UV dose (mJ/cm^2); L = pathlength (cm); η_{UV} = electrical efficiency of lamp

4.4.3 Trace organic compounds

I. Phase 1 SRT testing

Monitoring of antibiotics and indicator TOrCs during phase 1 focused on two different objectives: (1) verifying previously documented relationship between SRT and TOrC removal and (2) identifying target concentrations for phase 2 antibiotic testing. Primary effluent and SBR effluent samples were collected on consecutive days at the end of phase 1. Therefore, treatment performance was characteristic of cold temperature conditions in which the extent of nitrification ranged from 0% to 37% and the extent of denitrification ranged from 0% to 34% for SRTs of 2 and 20 days, respectively (Table 4-2). The resulting TOrC concentrations are summarized in Table 4-8.

In phase 1, the antibiotics ampicillin and tetracycline were the only compounds with concentrations lower than their respective MRLs in all samples, although it is important to note

المنارة للاستشارات
www.manaraa.com

that their respective MRLs were also higher than some of the other target compounds. Other studies also reported undetected or very low concentrations of penicillin and tetracycline families of antibiotics (Graham et al., 2011; Hirsch et al., 1999; Rodriguez-Mozaz et al., 2015). Very low concentration of ampicillin was expected since ß-lactam rings are readily susceptible to hydrolysis (Hirsch et al., 1999). Tetracyclines also tend to form stable complexes with calcium or similar ions and can bind to suspended matter and sediment (Hirsch et al., 1999). In contrast, the concentration of the antibiotic sulfamethoxazole was approximately 1 μg/L in the PE and actually increased in concentration in some of the SBR effluents, as has been previously described by Radjenovic et al. (2009). Trimethoprim and vancomycin were present in the PE at concentrations of approximately 0.5 μg/L, but trimethoprim eventually exhibited an overall decrease in concentration for the 20-day SRT while vancomycin exhibited an increase in concentration similar to sulfamethoxazole. The trimethoprim data support Salveson et al. (2012), which reported that SRTs of approximately 30 days were required for 80% removal of trimethoprim. Vancomycin, which is described as a 'last resort' antibiotic, is less commonly described in the literature. A recent study by Qiu et al. (2016) reported vancomycin removal of up to 99% and attributed that removal to biodegradation. However, that study focused on the biological processes at two vancomycin-producing facilities with influent concentrations of approximately 50 mg/L. Therefore, the microbial community may have been better acclimated to vancomycin degradation. Also, the effluent vancomycin concentrations were still on the order of 240-500 μg/L, which is three orders of magnitude higher than the SBR effluents in the current study.

المنارة للاستشارات
www.manaraa.com

Table 4-8. Summary of TOrC concentrations during phase 1 (variation of SRT)

Group	Antibiotic	Unit	Day 1							Day 2						
			PE	PE[1]	SRT 2	SRT 2[1]	SRT 7A	SRT 7B	SRT 20	PE	PE[1]	SRT 2	SRT 7A	SRT 7B	SRT 20	SRT 20[1]
Antibiotics	Ampicillin	ng/L	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100
	Sulfamethoxazole	ng/L	990	970	1100	1000	1400	1500	1300	1100	1300	1100	1200	1400	1200	1200
	Tetracycline	ng/L	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
	Trimethoprim	ng/L	380	370	370	330	420	420	120	530	710	500	470	410	180	180
	Vancomycin	ng/L	420	470	670	890	980	760	770	450	460	740	1000	770	820	770
High Removal	Acetaminophen	ng/L	95000	94000	<10000	<10000	<10000	<10000	<10000	93000	100000	<10000	<10000	<10000	<10000	<10000
	Caffeine	ng/L	67000	68000	50000	51000	<50	<50	<50	66000	73000	43000	<50	61	<50	<50
	Ibuprofen	ng/L	19000	19000	15000	16000	220	110	17	23000	24000	16000	89	250	<10	<10
Moderate Removal	Atenolol	ng/L	880	830	790	710	480	320	71	1100	1100	960	440	300	110	110
	Gemfibrozil	ng/L	1800	1700	1500	1500	1700	1300	72	1400	1700	1600	1500	1100	140	140
	Triclosan	ng/L	140	140	400	360	160	71	59	210	220	470	190	72	58	67
Low Removal	Primidone	ng/L	180	180	200	190	210	200	210	210	200	210	210	220	220	200
	Sucralose	ng/L	42000	41000	54000	47000	49000	49000	47000	45000	45000	51000	48000	40000	40000	42000
	TCEP	ng/L	260	250	260	260	260	260	260	300	300	310	280	280	330	330

المنارة للاستشارات
www.manaraa.com

The nine indicator TOrCs were divided into three groups based on their relative removals. The compounds with high removal (acetaminophen, caffeine, and ibuprofen) were all present at relatively high concentrations (20-100 μg/L) in the PE. The 2-day SRT achieved moderate reductions in these compounds, but the concentrations were still in the μg/L level. The 7-day SRTs achieved up to 99% removal of these compounds, and with the exception of ibuprofen on day 1, the 20-day SRT achieved the MRLs for all three compounds. For the second group of compounds, the concentrations in the PE were significantly lower, and two of the compounds exhibited steady decreases in concentration with longer SRTs, although the compounds were still detectable even for the 20-day SRT. The third compound—triclosan—actually consistently increased in concentration for the 2-day SRT but generally exhibited a net decrease in concentrations for SRTs of 7 and 20 days. Triclosan is a highly sorbing and biodegradable compound (Salveson et al., 2012). Therefore, it is assumed that triclosan desorbed from the solids in the SBR with a 2-day SRT, and the immature microbial community was unable to biodegrade the compound. On the other hand, the higher solids concentrations and desorption potential for the 7-day and 20-day SRTs was likely offset by higher biodegradation rates, thereby resulting in net decreases in concentration. Finally, the third group, which contained compounds with low sorption potential and low biodegradability, exhibited no consistent change in concentration regardless of SRT.

II. Phase 1 MLSS testing

To evaluate the effects of solids concentration while controlling for the microbial community, a follow-up experiment was performed with different volumes of seeded RAS. The volumes were selected to simulate the MLSS concentrations associated with SRTs ranging from 3 to 15 days (Table 4-9). It is important to note that the SBRs were seeded with RAS from a full-scale system

المنارة للاستشارات
www.manaraa.com

with a ~7-day SRT, which suggests that the initial microbial community was more mature than that of a system with a truly short SRT (e.g., 3 days). Furthermore, the initial set of TOrC samples was collected from a laboratory-scale system that had been operating for ~60 days, whereas the new samples were collected from a system that had been seeded with 'full-scale' microorganisms immediately prior to the experiment.

In contrast with the first sample set, tetracycline was actually detected in two of the SBR effluents at concentrations of 12 ng/L and 14 ng/L, but this was likely due to the lower detection limits achieved for those particular samples. For trimethoprim and the 'moderate removal' TOrCs, there was a clear improvement in water quality for MLSS concentrations greater than 1000 mg/L, but there was no clear distinction between MLSS concentrations of 2000, 3000, or 4000 mg/L. Atenolol, gemfibrozil, and trimethoprim have been described as having low sorption

Table 4-9. Summary of TOrC concentrations during phase 1 (variation of MLSS concentration)

Group	Antibiotic	k_{bio} (L/g_{ss}.d)	Unit	MLSS (mg/L)[1] PE	1,000	2,000	3,000	4,000
				Theoretical SRT (days)[2] 0	3	6	10	15
Antibiotics	Ampicillin	<0.1	ng/L	<100	<100	<100	<100	<100
	Sulfamethoxazole	(0.06-0.3)	ng/L	1800	1400	1400	1300	1300
	Tetracycline	<0.1	ng/L	<5	12	14	<100	<100
	Trimethoprim	(0.09-1.4)	ng/L	480	330	140	68	130
	Vancomycin	<0.1	ng/L	880	1300	1200	1100	1200
High Removal	Acetaminophen	(1.8-10.5)	ng/L	72000	<100	<100	<5	<5
	Caffeine	(1.7-6.8)	ng/L	48000	<100	<100	<100	<100
	Ibuprofen	(1.7-7.3)	ng/L	15000	12	<20	<20	<20
Moderate Removal	Atenolol	(0.3-4.3)	ng/L	770	190	27	<20	<20
	Gemfibrozil	(0.3-5.8)	ng/L	1100	330	7	3	3
	Triclosan	(0.2-1.8)	ng/L	210	68	40	28	33
Low Removal	Primidone	<0.1	ng/L	190	200	190	190	200
	Sucralose	<0.1	ng/L	47000	52000	47000	54000	52000
	TCEP	<0.1	ng/L	220	290	250	260	250

[1]Reactor seeded with RAS from full-scale WWTP with ~7-day SRT

www.manaraa.com

potentials (Salveson et al., 2012) so their greater removal at the higher MLSS concentrations may be attributable to faster kinetics resulting from more abundant biomass. Triclosan has a higher sorption potential (Salveson et al., 2012) so its removal is likely a combination of sorption and biotransformation. For gemfibrozil, the removal efficiency in the MLSS experiment was greater than what was observed during the previous SRT testing. This may be attributable to differences in the microbial community that developed during long-term operation of the SBRs versus the microbial community of the RAS seed from the full-scale system. Therefore, greater TOrC removal at longer SRTs appears to be due to a combination of (1) greater physical removal due to higher MLSS concentrations and (2) greater biotransformation due to (a) more abundant biomass and (b) more mature microbial communities. Additional MLSS experiments with RAS seeds from full-scale facilities with a wider range of SRTs would be needed to verify this theory. The second order rate constants listed in Table 4-9 were calculated based on the initial and final concentrations of the compounds and MLSS concentration. The results showed that the calculated rate constants differed as a function of MLSS concentrations.

III. Phase 2 antibiotic concentration testing

For phase 2, the antibiotic spiking concentrations corresponding with 1x, 10x, and 100x ambient levels were determined based on the TOrC data from phase 1 and were summarized previously in Table 1. For the TOrC analyses, PE and SBR effluent samples were collected on consecutive days at the end of phase 2. Therefore, treatment performance was characteristic of warm temperature conditions in which the extent of nitrification was ~98% and the extent of denitrification was ~15% for all SBRs (Table 4-2). The resulting TOrC concentrations are summarized in Table 4-10.

المنارة للاستشارات

www.manaraa.com

As summarized in Table 4-11, the observed concentrations for sulfamethoxazole, trimethoprim, and vancomycin were relatively consistent with the target concentrations. Deviations from the target concentrations were primarily due to temporal variability in ambient concentrations (i.e., 1x) and presumably adsorption onto suspended solids (e.g., for vancomycin). On the other hand, the observed concentrations of ampicillin and tetracycline were significantly different from the target concentrations. In fact, ampicillin was <MRL in all samples, even when spiked at 20 μg/L. With respect to the PE samples, tetracycline was only present at reportable concentrations in the 100x sample, and even though the antibiotic was spiked at 10 μg/L, the observed concentration was only 0.3 μg/L. Tetracycline was reliably detected in several secondary effluents (i.e., after biological treatment), even when the PE had not been spiked. This suggests that matrix interference may have been a significant factor.

Factors such as adsorption and hydrolysis may have also affected the observed concentrations of ampicillin and tetracycline. Gao et al. (2012) detected tetracycline in PE (164 ng/L) but not in secondary effluent. Extracted solids resulted in consistent tetracycline detection (750 μg/kg dw), thereby suggesting that the compound preferentially adsorbed to solids and was removed in the clarification process. As described earlier, other studies in the literature describe a wide range of values for tetracycline, and there are some studies reporting tetracycline at <MRL (Watkinson et al., 2009). With respect to ampicillin, there is a general paucity of data describing typical concentrations in wastewater, but Li and Zhang. (2011) noted that both ampicillin and tetracycline rapidly adsorb onto solids. Therefore, one can conclude that a combination of analytical and natural limitations likely hindered reliable detection of ampicillin and tetracycline, despite the fact that these compounds were spiked at high concentrations. Regardless, based on the other three antibiotics, the antibiotic delivery system appeared to work as intended.

المنارة للاستشارات
www.manaraa.com

For phase 2, the primary objective of the TOrC sampling was to evaluate whether the elevated antibiotic concentrations hindered the biotransformation capabilities of the microbial community. The general water quality parameters, particularly with respect to sCOD removal, nitrification, and bulk organic matter transformation, provided a preliminary indication that the spiked antibiotics had no significant impact on treatment efficacy. The SBR effluent concentrations for the 'high removal' (i.e., acetaminophen, caffeine, and ibuprofen) and 'moderate removal' (i.e., atenolol, gemfibrozil, and triclosan) compounds further support this conclusion, as those concentrations were significantly attenuated via biotransformation even in the 100x SBR. This is supported by Li and Zhang. (2011) who observed significant caffeine attenuation via biotransformation even with a suite of antibiotics spiked at the high µg/L level. Therefore, elevated antibiotic concentrations (up to 1.25% of the standard MIC) do not appear to impact the functional capacity of the microbial community in wastewater treatment applications.

With respect to antibiotic removal, the concentrations of ampicillin were always <MRL so it was not possible to assess its treatment efficacy. For the primary effluents, tetracycline was detected only in the 100x samples, but it was consistently detected in the secondary effluents, partially due to the lower MRLs. The tetracycline concentrations in the 10x secondary effluents were consistently in the 16-30 ng/L range (theoretical C_0 = 1 µg/L) but increased to 160 ng/L in the 100x secondary effluents (theoretical C_0 = 10 µg/L). In contrast with the phase 1 testing, the SBRs were able to achieve net reductions in sulfamethoxazole concentrations of 29% for the 1x samples, 46% for the 10x samples, and 42% for the 100x samples. Despite the high relative removal, the effluent concentrations of sulfamethoxazole were still 50-60 µg/L for the 100x SBRs due to the higher spiking levels. The vancomycin data were inconsistent considering that removals of 30-68% were achieved on day 1, while all of the day 2 samples resulted in increases

المنارة للاستشارات
www.manaraa.com

in concentration. Finally, trimethoprim was the only compound that exhibited consistent decreases in relative removal efficiency as the antibiotic spiking level increased from 1x (85% removal) to 10x (70% removal) to 100x (53% removal).

المنارة للاستشارات
www.manaraa.com

Table 4-10. Summary of TOrC concentrations during phase 2 (variation of antibiotic concentrations)

Antibiotic	Unit	Day 1									Day 2								
		PE1[1]	1X	1X[2]	PE2[1]	10XA	PE3[1]	10XB	PE4[1]	100X	PE1[1]	1X	PE2[1]	10XA	PE3[1]	10XB	PE4[1]	100X	100X[2]
Ampicillin	**ng/L**	<100	<100	<100	<100	<100	<10000	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100
Sulfamethoxazole	**ng/L**	2700	2000	2000	12000	6300	11000	6100	92000	51000	2300	1500	11000	5600	11000	5900	100000	59000	60000
Tetracycline	**ng/L**	<100	23	<100	<100	26	<10000	25	390	160	<100	16	<100	24	<100	30	140	160	160
Trimethoprim	**ng/L**	980	110	89	4800	1300	5000	1300	45000	20000	910	220	4800	1500	4300	850	48000	23000	23000
Vancomycin	**ng/L**	1200	1100	1100	2100	2000	6500	2100	33000	23000	920	1300	1700	2300	1800	2200	28000	26000	28000
Acetaminophen	**ng/L**	100000	<100	<100	96000	<100	100000	<100	98000	<100	90000	<100	91000	<100	90000	<100	94000	<100	<100
Caffeine	**ng/L**	67000	<5	<5	66000	<100	64000	<100	63000	<100	70000	<100	66000	<100	64000	<100	65000	<100	<100
Ibuprofen	**ng/L**	24000	14	17	22000	14	23000	23	23000	27	22000	23	22000	14	22000	13	22000	11	11
Atenolol	**ng/L**	1200	120	120	1200	130	1200	160	1200	130	1100	<2000	1100	<2000	1100	<2000	1100	<2000	<2000
Gemfibrozil	**ng/L**	1600	7.6	7.5	1600	31	1700	61	1700	41	1500	420	1600	18	1600	16	1600	33	33
Triclosan	**ng/L**	240	36	33	230	46	220	54	210	41	270	150	190	100	240	50	230	42	40
Primidone	**ng/L**	210	210	200	220	210	240	210	220	170	190	190	190	200	180	190	190	190	180
Sucralose	**ng/L**	38000	44000	45000	40000	43000	40000	46000	41000	37000	42000	51000	40000	45000	37000	46000	43000	47000	39000
TCEP	**ng/L**	330	310	300	320	300	290	290	280	280	160	250	160	240	160	230	160	230	240

[1]Target antibiotic concentrations summarized in Table 1

[2]Duplicate sample collected

Table 4-11. Comparison of target and observed concentrations in phase 2

Antibiotic	Units	1X		10x		100x	
		Target	Actual	Target	Actual	Target	Actual
Ampicillin	µg/L	0.2	<0.1	2	<0.1	20	<0.1
Sulfamethoxazole	µg/L	1	2.5	10	11	100	96
Tetracycline	µg/L	0.1	<0.1	1	<0.1	10	0.3
Trimethoprim	µg/L	0.5	0.9	5	4.7	50	47
Vancomycin	µg/L	0.5	1.1	5	3.0	50	31

Comparison of target and observed concentrations in phase 2 primary effluent based on averages from sampling on consecutive days

المنارة للاستشارات
www.manaraa.com

The trimethoprim removal efficacy for the 1x SBR (with a 7-day SRT) was also significantly greater than the removal efficacy for the 7-day SRT in phase 1. In phase 1, the low removal was initially attributed to the immature microbial community, as supported by the literature, but considering the 85% removal in phase 2, the phase 1 result may have actually been due to the lower temperature.

4.4.4 Molecular characterization of the microbial community

I. Phase 1 SRT testing

Improvements in water quality at longer SRTs are sometimes attributed to a diversification of the microbial community (Roh and Chu, 2011). The previous sections indicated that improvements in water quality parameters (sCOD, absorbance/fluorescence, nitrogen, and TOrC concentrations) were correlated with longer SRTs, while higher influent concentrations of antibiotics had a minimal impact on treatment performance. This section explores how SRT and antibiotic loading impacted the microbial community as a means of potentially linking changes in water quality with community diversification.

Goods coverage plot for Phase 1 is presented in Figure 4-8. Goods coverage is a number between 0 and 1, with 1 indicating that all expected species have been observed. Goods numbers in Table 4-12 indicate that no additional sequencing was required. The curves on the plot also reached asymptote indicating that the sample size were adequate for all the samples to be considered representative of the whole community. Species richness and diversity indices including ACE, Chao1, Goods, Shannon, and Simpson were calculated and are presented in Table 4-12. Species richness indices are numbers associated with only the count of species in samples. Species diversity not only accounts for spices count, but also considers the relative abundance of species,

المنارة للاستشارات
www.manaraa.com

which is a parameter that incorporates richness and evenness. Diversity indices increased at longer SRTs, thereby indicating that microbial community diversity increased for longer SRTs.

Tables 4-13 and 4-14 summarize genera with relative abundances greater than 0.5% in the first and second sample sets, respectively, during phase 1. The results of the first and second sample sets are presented together in this text. Figures 4-9 and 4-10 illustrate the same data and offer a direct visual comparison between samples. *Arcobacter, Bacteroides, Tolumonas, Aeromonas*, *Acinetobacter,* and *Acidovorax* were the most abundant genera in PE and they comprised (33.2%-29.5%), (7.67%-11.5%), (3.13%-4.54%), (2.49%-3.67%), (3.24%-2.90%), and (3.84%-1.97%) of the total microbial community, respectively. A similar study conducted by McLellan et al. (2010) identified *Acinetobacter*, *Aeromonas*, and *Arcobacter* among the most abundant taxa in sewage profile. The results from McLellan et al. (2010) study were from two full-scale WWTPs in Milwaukee metropolitan area. Gonzalez-Martinez et al. (2016) also analyzed the microbial community structure of wastewater influent among 10 WWTPs in Netherlands. At the genus level, the authors detected *Aeromonas* (2.5-13%), *Arcobacter* (3-42%), and *Bacteroides* (5.05-19.5%) in all samples. The literature suggests these heterotrophic bacteria are all capable of biochemical oxygen demand (BOD) degradation, although *Arcobacter* is aerobic (Collado et al., 2011), *Aeromonas* is a facultative anaerobe (Igbinosa et al., 2012), and *Bacteroides* is anaerobic (Ueki et al., 2008). These bacteria were generally outcompeted during biological treatment, thereby leading to reduced relative prevalence in the SBR effluents. *Arcobacter* was still abundant after biological treatment, although its relative prevalence decreased from ~30% to <10%. When the samples were collected during the current study, limited nitrification or denitrification was observed for the 2-day SRT (Table S3), presumably due to a combination of

المنارة للاستشارات
www.manaraa.com

the short SRT and low temperature, however *Acidovorax* was reported to have an important role in denitrification (Hoshino et al., 2005; Kniemeyer et al., 1999; Heylen et al, 2008).

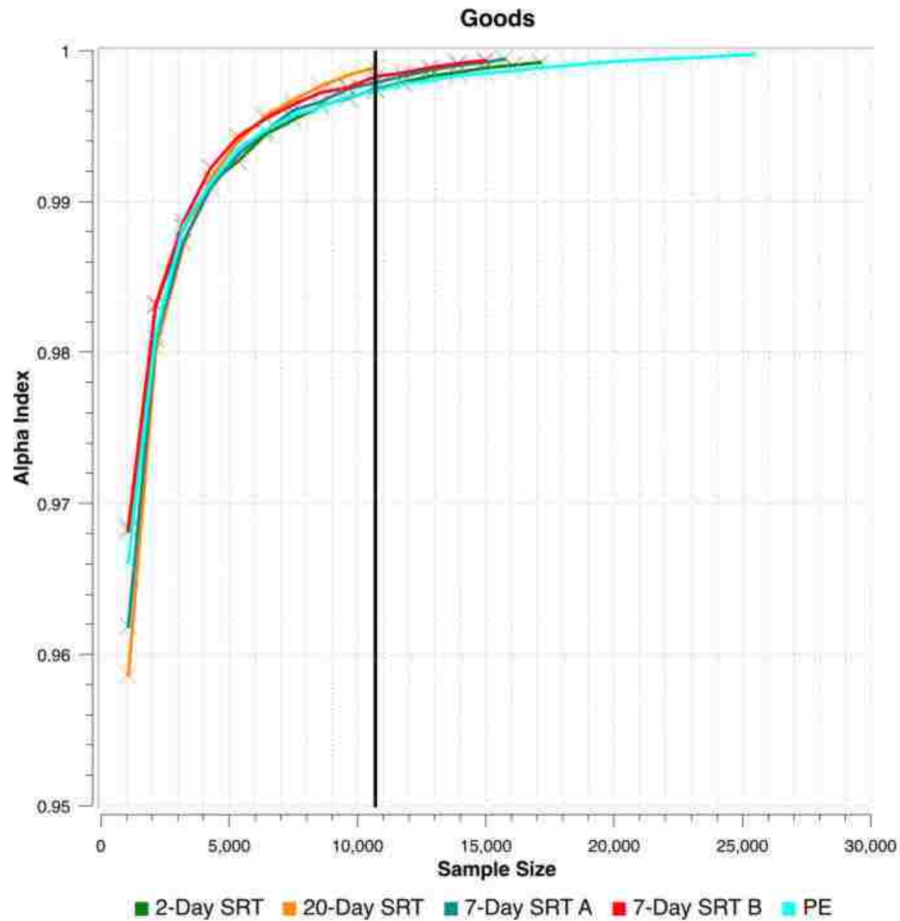


Figure 4-8. Goods coverage plot for phase 1

Table 4-12. Diversity and richness indices

	PE	2-Day SRT	7-Day SRT A	7-Day SRT B	20-Day SRT
Shannon	4.607	4.442	5.168	5.749	5.813
Simpson	0.121	0.122	0.066	0.032	0.036
ACE	230.897	178.939	207.739	205.565	219.101
Chao1	230.925	175.020	206.049	205.245	218.042
Goods	0.998	0.998	0.999	0.998	0.999

Diversity and richness indices including Shannon, Simpson, ACE, Chao1, and Goods mean values of all samples (Average of two trials are reported)

www.manaraa.com

Table 4-13. Relative abundance of microbial community structure (first sample set)

Phylum	Class	Order	Family	Genus	PE	2 Days	7A Days	7B Days	20 Days
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	7.67%	1.10%	0.20%	0.17%	0.48%
	Flavobacteriia	Bacteroidales	Prevotellaceae	*Prevotella*	0.92%	0.27%	0.06%	0.01%	0.09%
		Flavobacteriales	Cryomorphaceae	*Fluviicola*	0.00%	0.82%	0.52%	2.99%	0.90%
			Flavobacteriaceae	*Cloacibacterium*	0.61%	0.48%	0.04%	0.10%	0.12%
				Flavobacterium	0.11%	0.95%	1.69%	2.56%	2.71%
	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	*Sediminibacterium*	0.00%	0.09%	0.87%	1.18%	0.00%
			Saprospiraceae	*Haliscomenobacter*	0.02%	0.00%	0.20%	0.02%	0.59%
			Sphingobacteriaceae	*Sphingobacterium*	0.00%	0.00%	1.65%	0.06%	0.27%
Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	*Agitococcus*	0.00%	4.26%	14.73%	7.52%	2.31%
			Streptococcaceae	*Streptococcus*	1.14%	0.10%	0.25%	0.10%	0.11%
Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	*Nitrospira*	0.00%	0.01%	0.00%	0.04%	1.18%
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	*Acidovorax*	3.84%	4.77%	1.76%	0.76%	1.22%
				Hydrogenophaga	0.01%	2.92%	0.58%	0.14%	0.03%
				Simplicispira	0.20%	0.46%	1.85%	5.24%	5.84%
		Neisseriales	Neisseriaceae	*Uruburuella*	0.57%	0.31%	0.45%	0.13%	0.27%
		Rhodocyclales	Rhodocyclaceae	*Thauera*	0.10%	0.06%	3.49%	3.79%	10.14%
				Zoogloea	0.07%	2.11%	0.61%	0.39%	0.05%
	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	*Arcobacter*	33.21%	8.15%	3.01%	1.82%	4.90%
	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	*Aeromonas*	2.49%	0.99%	0.29%	0.27%	0.47%
				Tolumonas	3.13%	0.73%	0.27%	0.08%	0.37%
		Enterobacteriales	Enterobacteriaceae	*Salmonella*	0.00%	0.11%	2.55%	3.42%	1.34%
		Pseudomonadales	Moraxellaceae	*Acinetobacter*	3.24%	20.42%	1.46%	0.72%	1.33%
				Moraxella	0.75%	0.49%	0.30%	0.26%	0.31%
		Pseudomonadales	Pseudomonadaceae	*Cellvibrio*	0.00%	0.00%	0.57%	1.42%	0.30%
				Pseudomonas	0.70%	0.86%	1.96%	1.52%	1.23%
		Thiotrichales	Thiotrichaceae	*Thiothrix*	0.08%	5.88%	3.27%	7.04%	0.00%
Spirochaetes	Spirochaetia	Spirochaetales	Leptospiraceae	*Leptonema*	0.00%	0.00%	0.80%	0.39%	0.05%
				Leptospira	0.00%	0.12%	4.21%	0.04%	0.00%
Other	Other	Other	Other	Other	41.13%	42.86%	52.34%	57.84%	63.38%

Microbial community structure in the primary effluent and SBR effluents as a function of SRT (first sample set). A particular genus was included in the table if its relative abundance was >0.5% in at least one sample

المنارة للاستشارات
www.manaraa.com

Table 4-14. Relative abundance of microbial community (second sample set)

Phylum	Class	Order	Family	Genus	PE	SRT = 2	SRT = 7A	SRT = 7B	SRT = 20
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	11.52%	0.49%	0.04%	0.09%	0.07%
			Porphyromonadaceae	*Parabacteroides*	2.41%	0.07%	0.10%	0.07%	0.05%
			Prevotellaceae	*Prevotella*	2.26%	0.13%	0.02%	0.02%	0.11%
	Cytophagia	Cytophagales	Cytophagaceae	*Leadbetterella*	0.00%	0.00%	0.43%	0.04%	1.05%
	Flavobacteriia	Flavobacteriales	Cryomorphaceae	*Fluviicola*	0.00%	0.02%	0.31%	2.32%	1.06%
			Flavobacteriaceae	*Flavobacterium*	0.07%	1.29%	2.44%	2.47%	4.01%
	Sphingobacteriia	Sphingobacteriales	Saprospiraceae	*Haliscomenobacter*	0.00%	0.00%	0.11%	0.02%	0.75%
Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	*Agitococcus*	0.00%	0.40%	2.62%	4.30%	2.98%
			Enterococcaceae	*Enterococcus*	0.08%	0.00%	0.04%	0.77%	0.05%
			Streptococcaceae	*Streptococcus*	0.86%	0.18%	0.13%	0.49%	0.27%
	Clostridia	Clostridiales	Ruminococcaceae	*Ruminococcus*	0.82%	0.04%	0.00%	0.07%	0.13%
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Phenylobacterium*	0.00%	0.00%	0.22%	0.78%	0.00%
		Rhodobacterales	Rhodobacteraceae	*Rhodobacter*	0.00%	0.02%	0.41%	0.75%	0.52%
		Sphingomonadales	Sphingomonadaceae	*Novosphingobium*	0.00%	0.00%	0.37%	0.64%	0.00%
	Betaproteobacteria	Burkholderiales	Burkholderiaceae	*Chitinimonas*	0.00%	0.00%	0.03%	2.28%	0.53%
			Comamonadaceae	*Acidovorax*	1.97%	0.02%	1.60%	1.41%	1.01%
				Alicycliphilus	0.01%	0.06%	0.72%	1.98%	0.64%
				Comamonas	0.70%	0.40%	0.04%	0.05%	0.14%
				Hydrogenophaga	0.04%	0.80%	0.48%	0.88%	0.02%
				Simplicispira	0.09%	1.26%	2.28%	4.78%	6.88%
		Neisseriales	Chromobacteriaceae	*Aquaspirillum*	0.20%	2.85%	0.00%	0.00%	0.00%
		Rhodocyclales	Rhodocyclaceae	*Dechloromonas*	0.20%	1.14%	0.88%	1.13%	1.35%
				Rhodocyclus	0.13%	0.03%	0.27%	0.77%	0.42%
				Thauera	0.06%	0.28%	14.89%	7.04%	8.97%
				Uliginosibacterium	0.04%	0.01%	0.31%	1.25%	2.03%
				Zoogloea	0.43%	1.22%	1.49%	2.26%	1.14%
	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	*Arcobacter*	29.45%	2.48%	0.86%	0.55%	1.34%
				Sulfurospirillum	1.04%	0.06%	0.02%	0.00%	0.00%
	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	*Aeromonas*	3.67%	0.87%	0.09%	0.34%	0.42%
				Tolumonas	4.54%	0.21%	0.00%	0.04%	0.01%
		Enterobacteriales	Enterobacteriaceae	*Enterobacter*	0.53%	0.11%	0.03%	0.08%	0.15%

المنارة للاستشارات
www.manaraa.com

				Klebsiella	0.73%	0.11%	0.01%	0.01%	0.03%
				Salmonella	0.00%	0.04%	1.77%	1.36%	2.04%
		Oceanospirillales	Halomonadaceae	*Halomonas*	0.00%	0.01%	0.16%	0.00%	0.71%
		Pasteurellales	Pasteurellaceae	*Haemophilus*	0.50%	0.04%	0.00%	0.00%	0.00%
		Pseudomonadales	Moraxellaceae	*Acinetobacter*	2.90%	4.50%	0.58%	1.04%	1.06%
				Moraxella	1.01%	0.39%	0.08%	0.31%	0.15%
			Pseudomonadaceae	*Cellvibrio*	0.01%	0.00%	0.30%	1.07%	0.77%
				Pseudomonas	0.80%	2.27%	1.82%	1.65%	1.94%
		Thiotrichales	Thiotrichaceae	*Thiothrix*	0.10%	5.05%	6.23%	1.32%	0.04%
		Competibacteraceae	Competibacteraceae	*Candidatus Competibacter*	0.00%	0.00%	0.11%	0.18%	0.90%
		Xanthomonadales	Xanthomonadaceae	*Aquimonas*	0.01%	0.00%	0.36%	0.82%	0.00%
				Arenimonas	0.00%	0.00%	0.09%	1.56%	0.00%
				Thermomonas	0.02%	0.17%	1.48%	0.95%	0.86%
Spirochaetes	Spirochaetia	Spirochaetales	Leptospiraceae	*Leptonema*	0.00%	0.74%	0.26%	0.57%	0.00%
Other	Other	Other	Other	Other	32.80%	72.22%	55.51%	51.49%	55.45%

Microbial community structure in the primary effluent and SBR effluents as a function of SRT (second sample set). A particular genus was included in the table if its relative abundance was >0.5% in at least one sample

www.manaraa.com

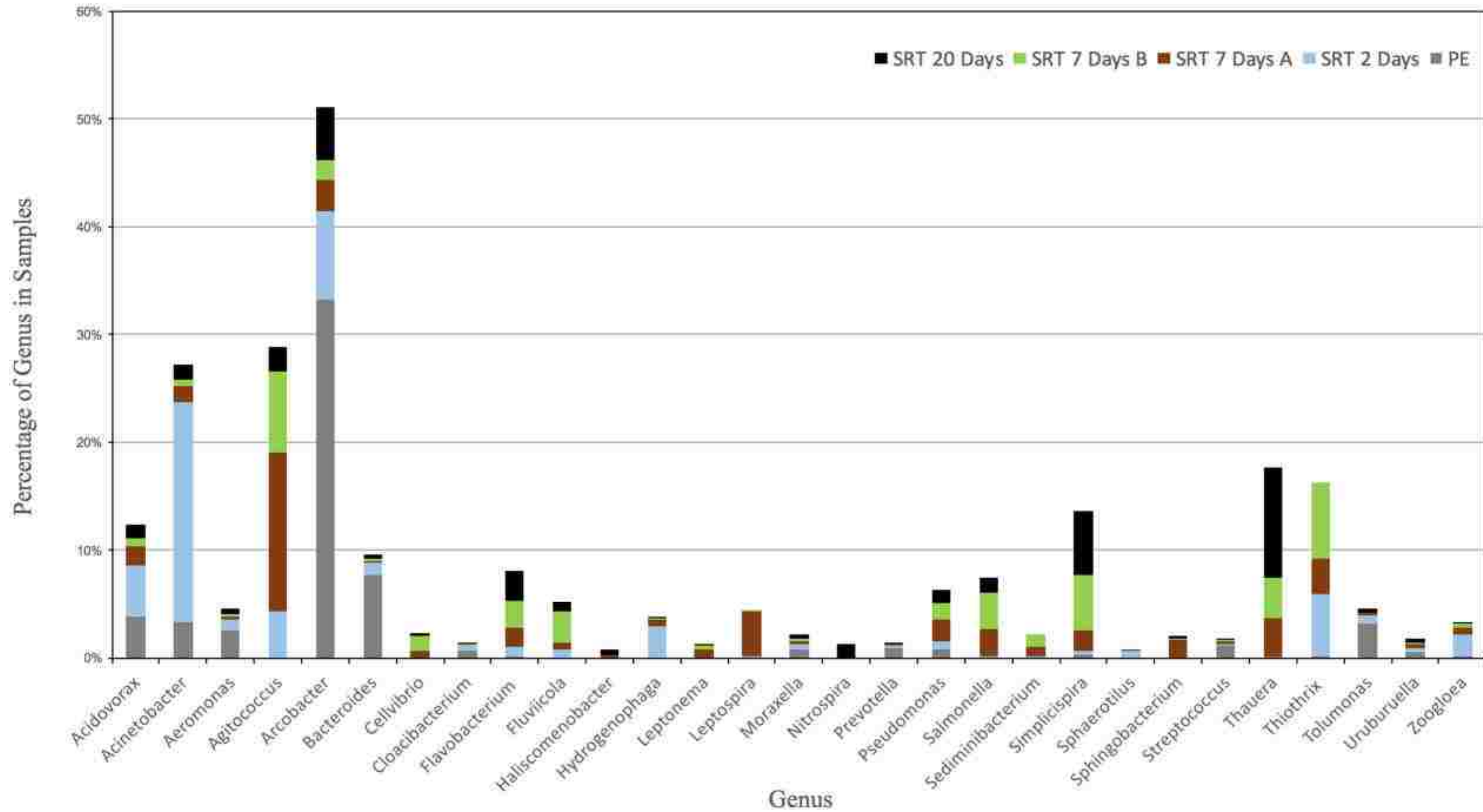


Figure 4-9. Microbial community structure (first sample set)

Microbial community structure in the primary effluent and SBR effluents as a function of SRT (first sample set). Each column represents a genus with relative abundance >0.5% in at least one sample. The individual percentages represent the relative abundance in each sample

المنارة للاستشارات
www.manaraa.com

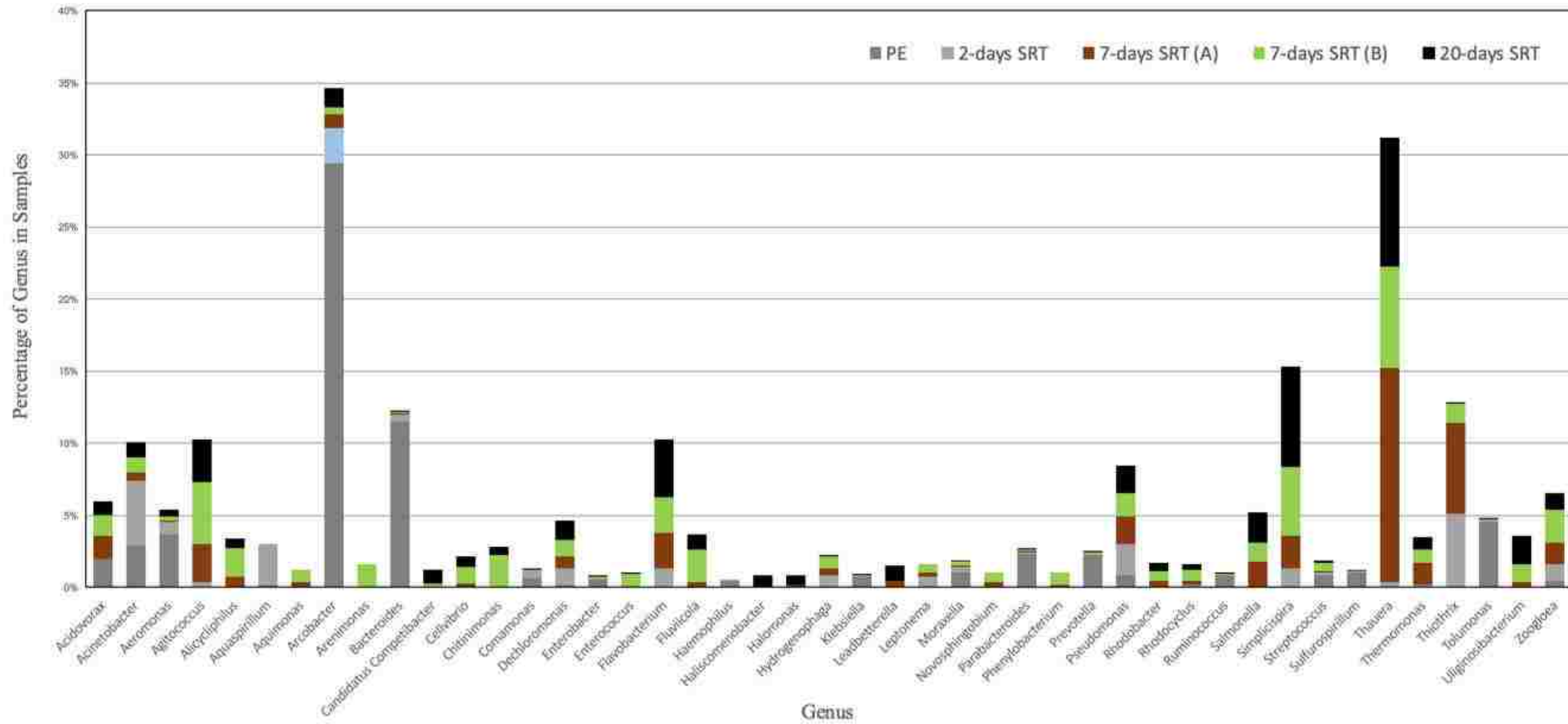


Figure 4-10. Microbial community structure (second sample set)

Microbial community structure in the primary effluent and SBR effluents as a function of SRT (second sample set). Each column represents a genus with relative abundance >0.5% in at least one sample. The individual percentages represent the relative abundance in each sample

المنارة للاستشارات
www.manaraa.com

Tolumonas and *Arcobacer* are speculated to have a role on biodegradation of antibacterial and anti-inflammatory organic matter (Wang et al., 2016). Bacteria of *Acinetobacter* is reported to play a role in biodegradation of organic and inorganic hazardous waste including xenobiotics and halogens pollutants (Abdel-el-haleem, 2003). Although *Acinetobacter* is associated with biodegradation of hazardous waste pollutants, this genus has emerged as a significant nosocomial pathogen probably because of intensive consumption of broad-spectrum antibiotics in hospitals (Towner, 2009).

For the 2-day SRT, *Acinetobacter, Arcobacter, Thiothrix, Acidovorax*, *Agitococcus,* and *Dechloromonas* were the most abundant genera with (20.4%-4.50%), (8.15%-2.48%), (5.9%-5.05%) , (4.77%-0.02%), (4.26%-0.40%), and (2.92%-1.14%) of total microbial community, respectively. Gonzalez-Martinez et al. (2016) studied the structure of the microbial communities in three bioreactors operated with SRTs of less than 1 day. The results showed that *Acidovorax* (2.5-7.5%), *Aeromonas* (3.3-6.0%), *Arcobacter* (1-25%), *Dechloromonas* (1-7%), and *Rhodoferax* (1.1-11.6%) were among the most abundant genera in their bioreactors. Therefore, *Acidovorax, Arcobacter, Aeromonas,* and *Dechloromonas* were common between the studies; *Agitococcus, Acinetobacter,* and *Thiothrix* were relatively abundant in the current study but not in Gonzalez-Martinez et al. (2016); and *Rhodoferax* was relatively abundant in Gonzalez-Martinez et al. (2016) but not in the current study.

For the two reactors with a 7-day SRT, *Agitococcus* (14.73%-2.62%)*, Thauera* (3.49%-14.89%), *Thiothrix* (7.04%-1.32%), *Simplicispira* (1.85%-5.24%), *Salmonella* (3.42%-1.36%)*,* and *Arcobacter* (3.01%-0.55%) were the most abundant genera, and for the 20-day SRT, *Thauera* (10.14%-8.97%), *Simplicispira* (5.84%-6.88%), *Arcobacter* (4.90%-1.34%), and *Falovobacterium* (2.71%-4.01%) were the most abundant genera. *Salmonella* is particularly

www.manaraa.com

interesting because some species are responsible for one million foodborne illnesses in the United States, with 19,000 hospitalizations and 380 deaths (Centers for Disease Control and Prevention, 2016). Espigares et al. (2006) reported no significant difference in *Salmonella* prevalence before and after activated sludge systems, but the current study suggests a positive correlation between *Salmonella* abundance and SRTs ≥7 days. *Falovobacterium* and *Zoogloea* (0.05%-2.26%) has been linked to biomass floc formation (Wastewater Bacteria, 2007). Both genera are abundant in longer SRTs, while *Falovobacterium* is most abundant in 20 day SRT and *Zoogloea* is most abundant in 7 day SRT. *Zoogloea* also plays a role in floc formation (Shao et al., 2009) and biodegradation of antibacterial organic matter (Wang et al., 2016). *Thiothrix,* which was abundant in 7-day SRT, can oxidize sulfides as an energy source (Wastewater Bacteria, 2007).

A PCA can be used to identify statistically significant changes in the core genera of microbial community (top 10 genera), or conclude that communities are statistically similar. Figure 4-11A summarizes the results of the PCA (combining both sample sets) for phase 1. The positioning of the samples (red dots) and genera (blue dots) relative to each other provide a statistical representation of their similarities. For example, there were apparent differences in the PE and 2-day SRT, but they were more similar to each other than the longer SRTs of 7 and 20 days. The PCA also confirms that the two sample sets and the duplicate reactors with 7-day SRTs proved to be statistically similar. With respect to the genera, the PCA indicates that *Arcobacter* is closely linked to PE, while *Thauera*, *Simplicispira*, and *Agitococcus* were more representative of 7-day and 20-day SRT. Genera in the lower left corner of Figure 4-11A were typically found in all the samples. Those genera that are close to the center of diagram, are typically found in all the

المنارة للاستشارات
www.manaraa.com

samples but with varying relative abundance (e.g., *Acinetobacter*, *Bacteroides* and *Aeromonas* in the lower left corner of Figure 4-11A).

Although many studies, including the current research, have focused on the core genera of microbial communities (Ahmed et al., 2007; Gonzalez-Martinez et al., 2016), some studies have explored rare microorganisms as well Vuono et al. (2016) explored both abundant and rare microorganisms in activated sludge systems. Their results showed that rare microorganisms may have an unrecognized role based on their higher protein synthesis. It should be noted that the role of rare microorganisms in the Vuono et al. (2016) study might be highlighted due to the presence of anaerobic, anoxic, and aerobic treatment systems. For example, phosphate accumulating organisms (PAOs) were determined to be among the rare taxa with significantly high rRNA/rDNA ratio. It is well known that the combination of anaerobic and aerobic cycles will result in higher accumulation of polyphosphates in PAOs within their cells during aerobic cycle, which is not the case for conventional activated sludge systems with aerobic cycle only. Therefore, the functionality of PAOs may be highlighted in this scenario, although they are not among the core genera of microbial community.

المنارة للاستشارات
www.manaraa.com

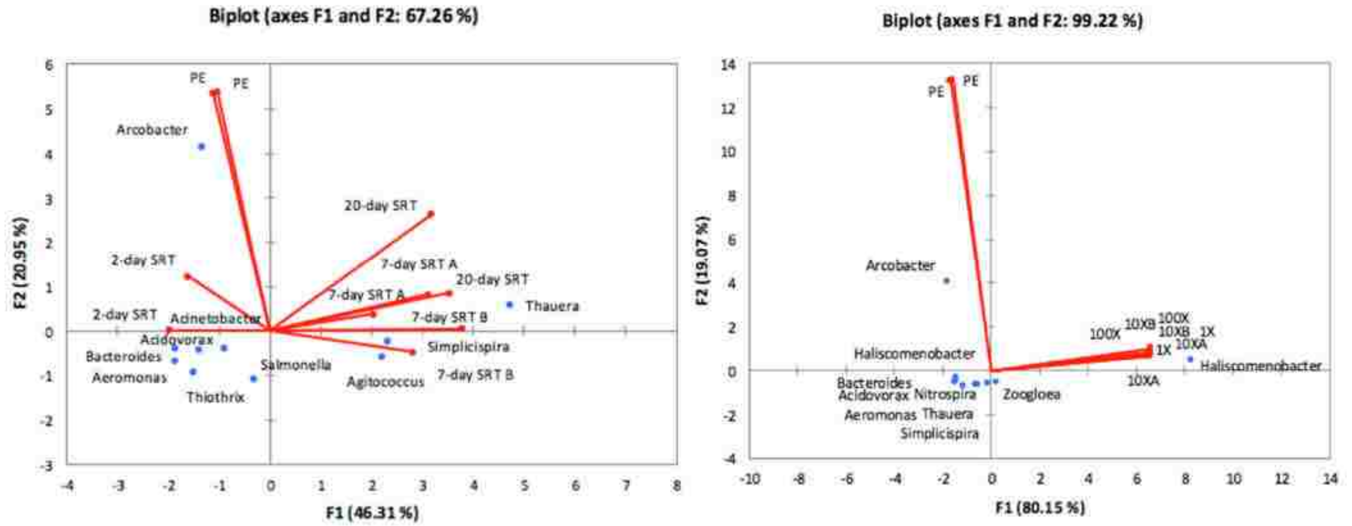


Figure 4-11. Results of principal component analyses for phase 1 and phase 2

Results of principal component analyses for A) phase 1 (left picture) and B) phase 2 (right picture) of the core genera of the microbial community (top 10 genera). Each figure reflects the data from both sample events during each phase. Red dots represent the different samples (i.e., primary effluent vs. SRTs and primary effluent vs. relative antibiotic concentrations), and blue dots represent the different genera

Lu et al. (2015) presented their microbial community data in the context of relative abundance of pathogens, including *Arcobacter butzieri*, *Salmonella enterica*, *Aeromonas hydrophila*, and *Escherichia coli*. Table 8 summarizes the relative abundances of these pathogens in the current study as a function of SRT. According to Table 15, *Acrobacter butzieri*, *Aeromonas hydrophila* were detected in PE and longer SRTs reduce the abundancy of these two species. However, *Salmonella enterica* was not detected in PE and was found to be increasingly abundant in longer SRTs. Escherichia coli was also not detected in PE, 2-days SRT, and 7-day SRT, however, it was found in 20-day SRT.

www.manaraa.com

Table 4-15. Comparison of the relative abundance of potential pathogens

Species	Phase 1 SRT (days) (%)					Phase 2 Relative Antibiotic Concentration (%)				
	PE	2	7 (A)	7 (B)	20	PE	1X	10X (A)	10X (B)	100X
Salmonella enterica	0.00	0.07	2.15	2.38	1.68	0.00	0.00	0.00	0.00	0.00
Arcobacter butzieri	0.13	0.02	0.00	0.00	0.01	0.25	0.002	0.00	0.00	0.00
Aeromonas hydrophila	2.10	0.78	0.27	0.26	0.37	0.00	0.00	0.00	0.00	0.00
Escherichia coli	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.00

Comparison of the relative abundance of potential pathogens in the primary effluent and SBR effluents as a function of SRT. (Average of two trials are reported in each Phase)

II. Phase 2 antibiotic concentration testing

Figure 4-11B shows the effect of ambient antibiotic concentrations on microbial community structure in PE and the SBRs. As previously described, all the SBRs were operated at the same SRT (~7 days) at this stage. *Arcobacter* was found to be dominant in PE and *Zoogloea*, *Nitrospira*, and *Thauera* were mostly abundant in the the SBRs. *Acidovorax*, and *Bacteroides* were found in all samples, however they were more abundant in PE. The results revealed that targeted antibiotic concentrations did not change the microbial community structure and function. All samples from the SBRs were analyzed to be similar for the top most abundant genera. Of the list of potential pathogens presented earlier, *Acrobacter butzieri* was found only in PE, and none of the pathogens were found in the MLSS samples from the SBRs (Table 15).

4.4.5 Microbial community analysis with respect to the treatment aspects

Longer SRTs are associated with lower micropollutants concentrations and higher nutrient removal. Microbial community analysis can be used to determine the relative abundance of microorganisms that are able to degrade trace organic compounds. Wojcieszyńska et al, (2014)

المنارة للاستشارات
www.manaraa.com

listed *Pseudomonas*, *Sphingomonas*, and *Stenotrophomonas* as genera capable of degrading naproxen. These genera were also found in this study. The abundance of these genera were varied among the biological reactors, however for *Pseudomonas* was relatively higher in 20-day (1.93%) compared to 7-day SRT (1.83%) and PE (0.79%). *Sphingomonas* was only detected in 20-day and 7-day SRT in low percentages (<0.1%). *Pseudomonas* was also reported as genus capable of using caffeine as sole carbon source (Summers et al., 2015). Wu et al, (2012) reported some species of *Stenotrophomonas, Pseudomonas,* and *Burkholderia* that are able to degrade paracetamol (Acetaminophen). The relative abundance of *Burkholderia* was lower than 0.5%, however, it was only detected in 20-day SRT (0.13%). It should be noted that not all of the species of a genus can degrade a specific compound, however, the data can be used as baseline to determine the possible relations between abundancy of microorganisms and the concentrations of micropollutants.

4.5 Conclusions

SRT is a key parameter in designing biological wastewater treatment processes. Depending on the treatment objectives, SRT can be varied to maximize the treatment efficiencies. In this study, it was shown that SRT can change the microbial community structures in the SBRs. Long SRTs are associated with lower TOrCs and total organic carbon (TOC) and better treated effluent quality. Microbial community structure in PE was shown to be different than biological treatment process. In PE, *Acinetobacter* and *Arcobacter* were among the most abundant genera, however they were outcompeted in the SBRs. Nitrospira, a nitrifying genus, was seen to be more dominant in longer SRTs, indicating the role of SRT on changing microbial communities. Furthermore, higher concentrations of antibiotics (up to 100 times of typical concentrations in wastewater) was shown to have minimal effect on changing microbial communities and the

www.manaraa.com

performance of the SBRs. This really important in case of accidental release of industrial wastewater into domestic wastewater. Biological wastewater treatment process was able to handle the higher concentrations of antibiotics and the results showed that the SBRs performance did not changed considerably.

Therefore, longer SRTs are generally beneficial in terms of effluent water quality, and higher (but still subclinical) influent antibiotic concentrations have minimal impact on treatment performance. However, the literature suggests that these conditions may also proliferate antibiotic resistance during biological wastewater treatment. More studies are needed to further clarify the role of biological wastewater treatment with respect to antibiotic resistance, and operational decision making must not only consider conventional water quality parameters but also contaminants of emerging concern, including pharmaceuticals and antibiotic resistance elements (e.g., antibiotics, antibiotic resistant bacteria, and antibiotic resistance genes). Molecular tools will continue to play a significant role in developing a comprehensive understanding of microbial community structure and function, specifically related to water quality.

المنارة للاستشارات
www.manaraa.com

5.0 EFFECT OF SOLIDS RETENTION TIME AND ELEVATED ANTIBIOTIC CONCENTRATIONS ON THE FATE OF ANTIBIOTIC RESISTANCE AND MICROPOLLUTANTS REMOVAL DURING BIOLOGICAL WASTEWATER TREATMENT

5.1 Abstract

Solids retention time (SRT) is a key factor in designing biological wastewater treatment processes. Longer SRTs can lead to reductions in trace organic compound (TOrC) concentrations, facilitate nutrient removal, reduce oxidant scavenging, and mitigate membrane fouling. However, longer SRTs may contribute to antibiotic resistance (AR) proliferation. Also, higher antibiotic concentrations in biological treatment systems may negatively impact the performance of reactors by inhibiting sensitive bacteria or contribute to AR proliferation by exerting a selective pressure. This research aimed to characterize the role of SRT and elevated antibiotic concentrations on AR proliferation in biological treatment processes. Spread plate technique was used to determine the number of Gram positive *Staphylococcus/Streptococcus* strains. The extent of AR was also determined based on minimum inhibitory concentrations (MICs) of resistant isolates. The results revealed that longer SRTs select for antibiotic resistant bacteria (ARBs). The results also showed that higher concentration of antibiotics also led to higher rate of AR.

5.2 Introduction

Antibiotic resistant bacteria (ARBs) and antibiotic resistance genes (ARGs) are now considered contaminants of emerging concern (CECs) that pose a threat to public health (Pruden et al., 2006). Although dissemination and proliferation of ARBs and ARGs are governed by very

المنارة للاستشارات
www.manaraa.com

complex environmental pathways, but it seems that wastewater treatment plants (WWTPs) have an important role in mitigation or proliferation of ARBs and ARGs. WWTPs are engineered quality solutions to water pollution in environment, but they are also considered significant reservoirs for antibiotic resistance (AR) (Novo and Manaia, 2010). Previous studies investigated the role of wastewater treatment plants in the proliferation or mitigation of antibiotic resistant bacteria (ARBs) and antibiotic resistance genes (ARGs) (Guo et al., 2015; Su et al., 2014; Zhang et al., 2015). Previous studies highlighted the selection pressure exerted on bacteria in wastewater matrices (Schwartz et al., 2003). Specifically, the presence of antibiotics can form a selective pressure that increases the concentration of ARBs by inhibiting antibiotic-susceptible bacteria. This medium also increases the chance of mutation and horizontal gene transfer (HGT) (Martínez, 2008; Wang and Schaffner, 2011).

Data reported in previous publications are sometimes inconsistent and contradictory. For example, Aminov et al. (2001) and Auerbach et al. (2007) showed that due to the continuous exposure of bacteria to sub-inhibitory concentrations of antibiotics, wastewater treatment plants provide an environment that is potentially suitable for proliferation of ARGs and ARBs. However, (Suller and Russell, 2000) showed that continuous exposure of a triclosan-sensitive *Staphylococcus aureus* strain to sub-inhibitory concentrations of triclosan did not promote any changes in triclosan susceptibility or to other targeted antibiotics.

Despite the efforts to elucidate the role of wastewater treatment plans (WWTPs) in relation to antibiotic resistance, there is still no clear evidence that WWTPs, especially the biological treatment processes, are contributing to the proliferation of AR. Some studies suggest that WWTPs achieve a significant reduction in the number of ARBs (Guo et al., 2015; Huang et al., 2012), while other research indicates that WWTPs serve as major contributors of ARBs and

المنارة للاستشارات
www.manaraa.com

ARGs (Kim et al., 2010). These uncertainties may arise from research evaluating different treatment technologies, operational conditions, influent wastewater quality or wastewater constituents, and different methodologies for the detection of ARBs and ARGs. Therefore, additional studies and analyses are needed to assess the role of wastewater treatment processes on proliferation and mitigation of antibiotic resistance.

ARGs in wastewater are of interest due their ability to escape from advanced treatment systems. (Wang et al., 2015) monitored the fate of 10 subtypes of ARGs for sulfonamide, tetracycline, β-lactam class, and macrolide resistance and the class 1 integrase gene (*intI*1) across each stage of 5 full-scale pharmaceutical WWTPs in China. The results showed that the WWTPs can reduce the number of ARGs by 0.5-2.5 orders of magnitude in the aqueous phase, but a significant amount of ARGs are discharged in dewatered sludge. The total load of ARGs in dewatered sludge was 7-fold to 308-fold higher than raw influent and 16-fold to 638-fold higher than final effluent. The results also showed the proliferation of ARGs in the biological treatment processes. Chen and Zhang, (2013) conducted research to evaluate the removal rate of ARGs in WWTPs in China. Three WWTPs with different advanced treatment systems (biological aerated filter, constructed wetland, and UV disinfection) were selected to quantify the concentration of ARGs. In this study, the concentrations of 16S rRNA genes, *tetM*, *tetO*, *tetQ*, *sulI*, *sulII* and *intI*1 were measured in wastewater and biosolids. The results revealed that ARGs concentration decreased by 1.3 - 2.1 orders of magnitude in the constructed wetland and by 1.0-1.2 orders of magnitude in the biological aerated filter. However, only small changes were observed for the targeted ARGs between influent and effluent of the UV disinfection system. The same observation was made by McKinney and Pruden, (2012) regarding the limited potential of UV disinfection to damage ARGs in wastewater effluents.

المنارة للاستشارات
www.manaraa.com

Amador et al. (2015) evaluated the role of hospitals and wastewater treatment plants as contributors of AR in Portugal. The ampicillin-resistant *Enterobacteriaceae* were enumerated and isolated and tested for antimicrobial susceptibility using the disk diffusion method. The study measured the resistance to the β-lactam group of antibiotics, including cefoxitin and the combination of amoxicillin and calvulanic acid, and the non-β-lactam group, including tetracycline and the combination of trimethoprim and sulfamethoxazole. The results showed that wastewater treatment plant effluent contained a higher rate of multidrug resistance compared with the untreated influent. A similar study was performed by Nagulapally et al. (2009) to examine the occurrence of ciprofloxacin, trimethoprim/sulfamethoxazole, and vancomycin resistant bacteria in a wastewater treatment plant. The results revealed that a significant number of fecal coliforms, *E. coli*, and enterococci exhibited resistance to the target antibiotics in municipal treatment plants.

Although many of the recent AR studies focus on molecular methods, some studies are assessing AR occurrence and fate through culture-based methods. (Zhang et al., 2015b) studied AR among heterotrophic bacteria using traditional spread plating and streaking techniques. The bacterial isolates were tested for susceptibility to 12 different antibiotics based on the standard concentrations identified by the CLSI. One of the major findings from the study was that wastewater treatment plants typically reduced the extent of multi-drug resistance in the treated effluent. In other words, bacteria present in the effluent were resistant to fewer antibiotics than bacteria present earlier in the treatment train. Through sequencing, they also discovered that Gram negative bacteria dominated the wastewater influent, while Gram positive bacteria dominated the effluent.

المنارة للاستشارات
www.manaraa.com

Wastewater is a source of constituents of concern, including pathogenic bacteria, nutrients, heavy metals, and trace organic contaminants (TOrCs), including antibiotics. Therefore, wastewater treatment plants, especially those that employ longer SRTs, have the potential to continuously expose bacteria to sub-inhibitory concentrations of a wide range of antimicrobial compounds (Aminov et al., 2001; Auerbach et al., 2007). On the other hand, longer SRTs may select for bacteria with the ability to degrade a wide variety of organic compounds, including some TOrCs. Once the readily biodegradable compounds are depleted in engineered biological treatment applications, the microbial community experiences some degree of starvation, and only those bacteria with the ability to degrade recalcitrant compounds can survive. Recent studies have demonstrated the relationship between SRT and TOrC removal. Suarez et al. (2010) suggested that TOrC removal was linked to nitrification, while other studies reported that it was specifically related to SRT (Clara et al., 2005; Melcer and Klecka, 2011). Multiple studies identified "critical" SRTs for significant TOrC removal. Clara et al. (2005) identified a broadly applicable "critical" SRT of 10 days, while Oppenheimer et al. (2007) and Salveson et al. (2012) identified compound-specific "minimum" or "threshold" SRTs, respectively.

A typical WWTP usually has three major treatment steps: (1) preliminary/primary, (2) secondary treatment, and (3) tertiary/advanced treatment. During primary treatment, large solids and grit are physically removed by screening and sedimentation. In secondary treatment, a major portion of the biodegradable organic matter, or biochemical oxygen demand (BOD), is removed via cellular respiration by native biomass. In addition to BOD removal via aeration, the biological process can be engineered to achieve nitrification (aerobic), denitrification (anoxic), and phosphorus removal (sequential anerobic and aerobic). The secondary process also involves physical removal of the biomass by sedimentation in secondary clarifiers or by membranes in

المنارة للاستشارات
www.manaraa.com

membrane bioreactors. In many WWTPs, secondary effluent is then subjected to tertiary treatment involving filtration and disinfection.

In particular, biological treatment processes in WWTPs may provide an ideal environment for the proliferation of AR. Bacteria in these systems are exposed to sub-inhibitory concentrations of a suite of antibiotics and other AR inducing elements and compounds (Aminov et al., 2001; Auerbach et al., 2007). Depending on the operational conditions, bacteria remain in the bioreactors for varying amounts of time depending on the treatment target (i.e., BOD removal or BOD/nutrient removal). Solids retention time (SRT) is one of the key operational parameters in a suspended growth bioreactor and refers to the average amount of time the bacteria stay in the system before being 'wasted.' With longer SRTs, bacteria may have a greater chance of obtaining antibiotic resistance elements through horizontal gene transfer mechanisms. Those elements can then be propagated via vertical gene transfer, or bacterial replication. Despite the potential for AR transfer, it is not yet clear whether the biological treatment process actually contributes to the proliferation of AR or whether it actually provides some level of mitigation due to AR bacteria being outcompeted.

This research was conducted to provide a better understanding of the effect of varying SRT on AR proliferation and the fate of antibacterial compounds. SRT is a fundamental parameter in designing biological treatment systems and may greatly influence the fate of AR in wastewater treatment plants. Developing a correlation between SRT and AR may help environmental engineers and policy makers to make an inform decision regarding wastewater treatment design and operation. This research also explores the effect of elevated antibiotic concentrations in biological treatment systems. Higher concentration of antibiotics may negatively impact the

المنارة للاستشارات
www.manaraa.com

performance of biological treatment and proliferate AR. This study investigates the relationship between ambient antibiotic concentrations and AR proliferation.

5.3 Materials and methods

5.3.1 Description of laboratory-scale sequencing batch reactors

The laboratory-scale activated sludge process was achieved with four parallel SBRs (Figures 3-2 and 3-3) fed with primary effluent from a full-scale WWTP in Las Vegas, Nevada. The acrylic SBRs had a total volume of 8 L and a working volume of 4 L after accounting for the volume of settled solids. Automation of the SBRs was achieved with a series of multi-station outlet timers, a peristaltic pump, electric actuated ball values, and solenoid valves. A MasterFlex peristaltic pump (Model 77200-62, Cole Parmer, Vernon Hills, IL) was used to transfer primary effluent from a wet well through a polytetrafluoroethylene/stainless steel strainer (Hach, Loveland, CO) and a 50-µm cartridge filter (Watts WPC50-975) prior to filling the reactors. The cartridge filters were replaced every two days to mitigate fouling and anaerobic conditions. A four-station irrigation timer (Orbit, Bountiful, UT) was used to control the volume fed to each reactor. Electric actuated solenoid valves (Parker Hannifin Corporation, Cleveland, OH) and an industrial grade air compressor (Porter-Cable PCFP02003; 3.5 gallons; 135 psi) were used to aerate the SBRs to achieve a relatively constant dissolved oxygen concentration of 3 to 4 mg/L. The compressed air was passed through a pressure gauge and air flow meter before being fed into the SBRs via stone diffusers. Aeration was sufficient to achieve adequate mixing of the mixed liquor without the need for mechanical mixing. The target SRTs were achieved by wasting predetermined volumes of mixed liquor toward the end of each aeration phase, and this was accomplished with four electric actuated ball valves (W.E. Anderson, Michigan City, IN).

المنارة للاستشارات
www.manaraa.com

The SBRs were initially seeded with return activated sludge (RAS) from the full-scale WWTP, which typically operates at an SRT of ~7 days. The SBRs were operated with a cycle time of 8 hours for 3 cycles per day over a period of 60 days. Each cycle consisted of the following five stages: (1) filling with primary effluent for 29 minutes as the irrigation timer cycled through each reactor, (2) immediate aeration for 6.5 hours, (3) solids settling for 1 hour, (4) discharge of settled effluent for 30 minutes, and (4) idle for 1 minute. Again, solids wasting was performed toward the end of each aeration phase to minimize clogging of the ball valves. SRTs of 2 days, 7 days (in duplicate), and 20 days were targeted for this phase (Figure 3-2).

In order to evaluate the effect of influent antibiotic concentration, the SRTs of the four reactors remained constant at ~7 days, but the reactor influent (i.e., primary effluent) contained target antibiotics at concentrations of 1x (ambient concentrations), ~10x (in duplicate), and ~100x (Figure 3-3). Based on the initial round of TOrC sampling (results shown later in Table 5-4) the antibiotic concentrations shown in Table 5-1 were selected for this phase of the research. The table also shows the corresponding MICs to illustrate that the concentrations still represent sub-inhibitory levels. The highest concentrations as a percentage of the MIC are for trimethoprim and vancomycin, which were spiked at 1.25% of the MIC. Because the concentrations of ampicillin and tetracycline were <MRL in the primary effluent (Table 5-1), the 1x concentrations were actually spiked at twice the analytical MRL to achieve detections during the second round of TOrC sampling.

Table 5-1. Summary of Spiked Antibiotic Concentrations

Antibiotic	Units	1X	10x	100x	MIC	100x/MIC
Ampicillin	µg/L	0.2 (spiked)	2	20	32,000	0.06%
Sulfamethoxazole	µg/L	1 (ambient)	10	100	76,000	0.13%
Tetracycline	µg/L	0.1 (spiked)	1	10	16,000	0.06%
Trimethoprim	µg/L	0.5 (ambient)	5	50	4,000	1.25%
Vancomycin	µg/L	0.5 (ambient)	5	50	4,000	1.25%

المنارة للاستشارات
www.manaraa.com

5.3.2 Preparation of antibiotic stock solutions

As mentioned earlier, five different antibiotics were selected for this project including: ampicillin sodium salt (Sigma Aldrich, St. Louis, MO), sulfamethoxazole (Sigma Aldrich, St. Louis, MO), tetracycline hydrochloride (Sigma Aldrich, St. Louis, MO), vancomycin hydrochloride (Sigma Aldrich, St. Louis, MO), and trimethoprim (Sigma Aldrich, St. Louis, MO). All antibiotic stock solutions were prepared based on the Clinical and Laboratory Standards Institute (CLSI, 2012). Appropriate Solvents were used for each antibiotic including: 0.01 M PBS for ampicillin, sterile nanopure hot water and minimal amount of 2.5 M NaOH for sulfamethoxazole, 90% volume of sterile nanopure water with 10% volume of 0.05 M hydrochloric acid for trimethoprim, and sterile nanopure water for tetracycline and vancomycin. The antibiotic stock solutions were then passed through acrodisc syringe filters to be sterilized. All stock solutions were stored in refrigerator at at 4±2°C and were used within 48 hours.

5.3.3 Analytical methods

I. General water quality parameters

A series of general water quality parameters was monitored for the duration of the study to ensure the SBRs were properly mimicking a full-scale activated sludge system. These tests included pH, MLSS concentration, mixed liquor volatile suspended solids (MLVSS) concentration, soluble chemical oxygen demand (sCOD), nitrogen speciation (i.e., ammonia, nitrate, and nitrite), and dissolved oxygen (DO). Standard methods were employed when applicable; a summary of the analyses and associated methods is provided in Table B1 (Appendix B).

II. Trace organic compounds

المنارة للاستشارات
www.manaraa.com

The target antibiotics include ampicillin (AMP), sulfamethoxazole (SMX), trimethoprim (TMP), tetracycline (TC), and vancomycin (VA). The target antibiotics were selected based on their frequency of clinical administration and previous reports of the occurrence of the antibiotics or their associated antibiotic resistance genes in water and wastewater. A suite of indicator TOrCs was identified to complement the target antibiotics. The indicator TOrCs include compounds with varying susceptibility to biodegradation and sorption in addition to compounds with antimicrobial properties, including triclocarban and triclosan. These compounds were selected based on their ubiquity in wastewater, their utility in evaluating process performance, and the project team's experience and familiarity with their analysis and occurrence, specifically as part of a previous Water Environment Research Foundation project (WERF-CEC4R08; Salveson et al., 2012). Although the primary objective of this study was to evaluate the effects of SRT on AR, the inclusion of the indicator TOrCs allowed the team to expand on its previous work evaluating the relationship between SRT and TOrC removal (Gerrity et al., 2013; Salveson et al., 2012)

Primary and secondary effluent (i.e., settled effluent) samples from the four SBRs were collected in 1-L, silanized, amber glass bottles preserved with sodium azide (1 g/ L) and ascorbic acid (50 mg/L). Samples were immediately placed on ice and held at 4°C for up to 14 days until further processing, which consisted of filtration with 0.7-μm glass fiber filters and on-line solid phase extraction (SPE). The samples were then analyzed for the target compounds by liquid chromatography tandem mass spectrometry (LC-MS/MS) with isotope dilution according to previously published methods (Vanderford and Snyder, 2006).

المنارة للاستشارات
www.manaraa.com

5.3.4 Microbiological methods

I. Detection of culturable antibiotic resistant bacteria and minimum inhibitory concentrations (MICs)

For the spread plate analyses, a selective supplement was used to isolate for Gram positive bacteria, specifically *Staphylococcus* and *Streptococcus*. Gram positive bacteria were selected as the target microorganisms due to their importance for emerging multidrug resistance. In addition, bacteria with intrinsic resistance to the target antibiotics might have significantly confounded the results. Therefore, the culture methods focused on Gram positive bacteria.

Mueller Hinton agar containing Staph/Strep selective supplement was used to identify the total culturable Gram positive bacteria in samples. MH media has a high buffering capacity, which reduces the possibility of chemical transformations. Also, as mentioned before, MH media contains minimum amount of thymidine and thymine. Therefore, any interference due to the presence of thymidine and thymine is minimized. Plating was performed by using spread plate technique. In order to ensure data quality and limit the number of plates per sample event, the microbiological sampling was divided into two phases. Early in the week, samples were collected and tested for resistance to AMP and SMX/TMP. A second set of samples were collected later in the week and tested for resistance to TC and VA. All reactors and sampling locations were tested simultaneously for the two sets of samples. Three set of samples were collected for these tasks over the two months. In order to isolate resistant strains, bacteria were exposed to the target antibiotics (supplemented in growth media) at their reported minimum inhibitory concentrations (MICs). MICs which were used in this project are listed in Table 5-2 (CLSI, 2012).

المنارة للاستشارات
www.manaraa.com

Table 5-2. Summary of minimum inhibitory concentrations (MICs) for each antibiotic

Antibiotic	MIC (μg/mL)
AMP	32
SMX/TMP[a]	76/4
TC	16
VA	4

A 50 mL grab sample was collected in a sterile conical tube during the each SBR draw phase. A total of five samples from the SBRs were transported on ice to the laboratory. All samples were processed within 8 hours. Samples were transferred onto 0.9% sodium chloride for the first dilution and then were serially diluted in 0.01M phosphate buffer saline (PBS) to get 25 to 250 colony forming unit (CFU) per plate. 100 μL of each sample was aseptically transferred onto each plate containing MH agar supplemented with different concentrations of target antibiotics and Staph/Strep selective supplement. Each target dilution was plated in triplicate. Plates were incubated at 35±0.5°C for 24±2 hours. The results were reported as the mean of triplicate plates ± one standard deviation in CFU/100 μL. Negative controls were used to verify the absence of foreign contamination. An analysis schematic is provided in Figure 5-1.

Visible colonies on MH agar with the presence of antibiotics and staph/strep supplement were considered antibiotic resistant Staph/Strep bacteria. A total of eight random isolates were harvested from each set of triplicate plates. The isolates were transferred into culture tubes containing MH broth for overnight incubation. These antibiotic resistant pure cultures were then sent to the University of Arizona for the MIC assay.

In MIC assay, a series of wells in a 96-well tray were spiked with 50 μL of a pure culture in addition to serial dilutions of the corresponding target antibiotic (0 to 32 times the standard MIC). The minimum concentration at which growth is inhibited (based on absorbance at 600

المنارة للاستشارات
www.manaraa.com

nm) was reported as the sample MIC. Growth is determined by a BioTek Synergy HT Multi-Mode Microplate reader. The MIC assay is depicted in Figure 5-2.

5.3.5 Statistical analysis

Single factor analysis of variance (ANOVA) with post-hoc Tukey's test was performed with XLSTAT (Addinsoft, NY) at a significance level of 0.05.

5.3.6 Limitations

As indicated earlier, the mail goal of this study was to characterize the role of solids retention time and antibiotic concentrations on proliferation of antibiotic resistance. Therefore, the SBRs were operated in two phases during Fall 2015 (September through November) and Spring 2016 (March through May). Although the SBRs were Installed in a shed, but the temperature changed drastically over time (from September to November for first phase and March to May for the second phase. With respect to the size of the SBRs, temperature variation was higher in the SBRs compared to a biological system in a full scale plant. In the next few sections, the effect of temperature on the fate of AR in the SBRs will be discussed, however it should be remembered that the temperature variation in a full scale plant is much lower than a small SBR.

المنارة للاستشارات
www.manaraa.com

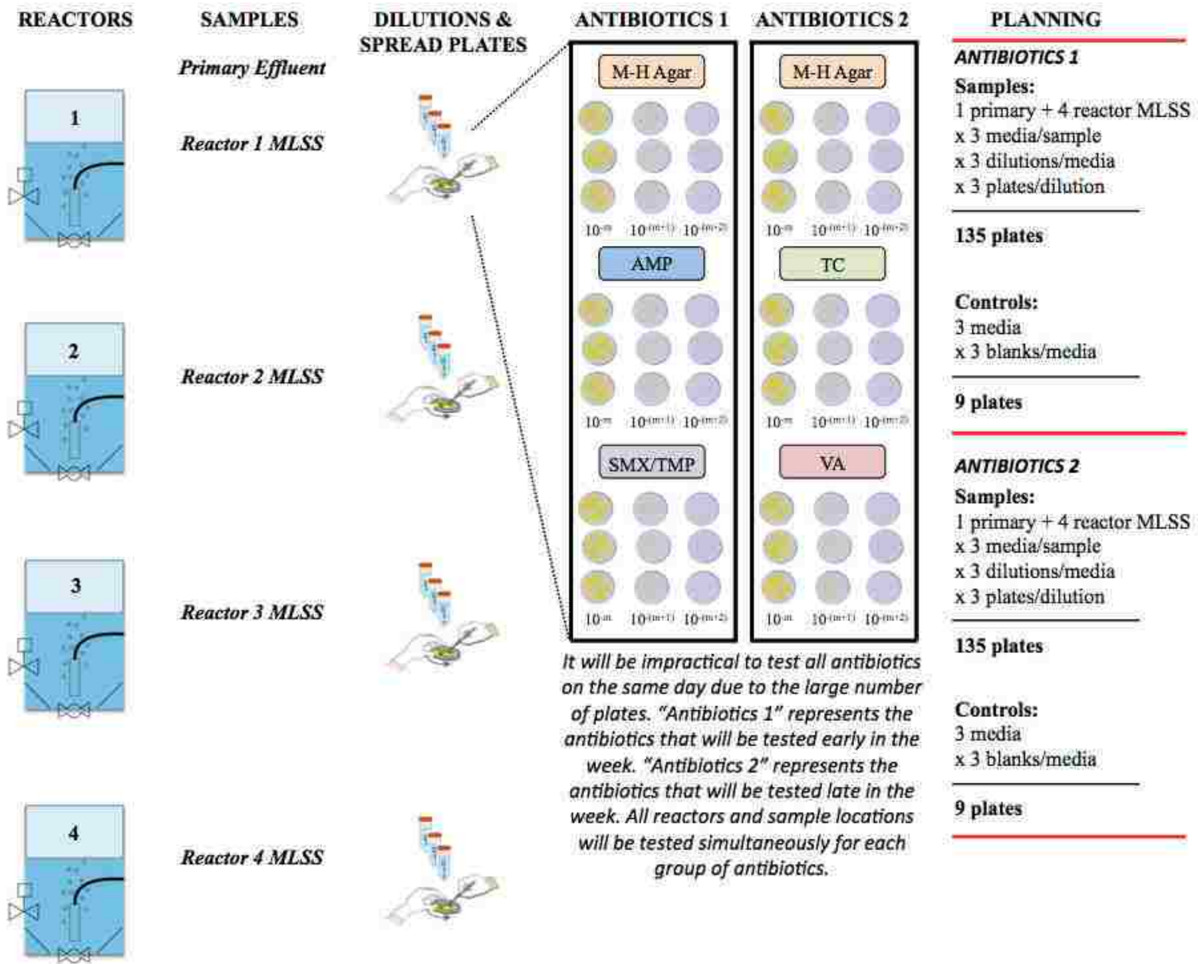


Figure 5-1. Schematic of spread plate analysis

Schematic of spread plate analysis for Staph/Strep bacteria (M-H agar contained Staph/Strep selective supplement for the selection of Staph/Strep)

www.manaraa.com

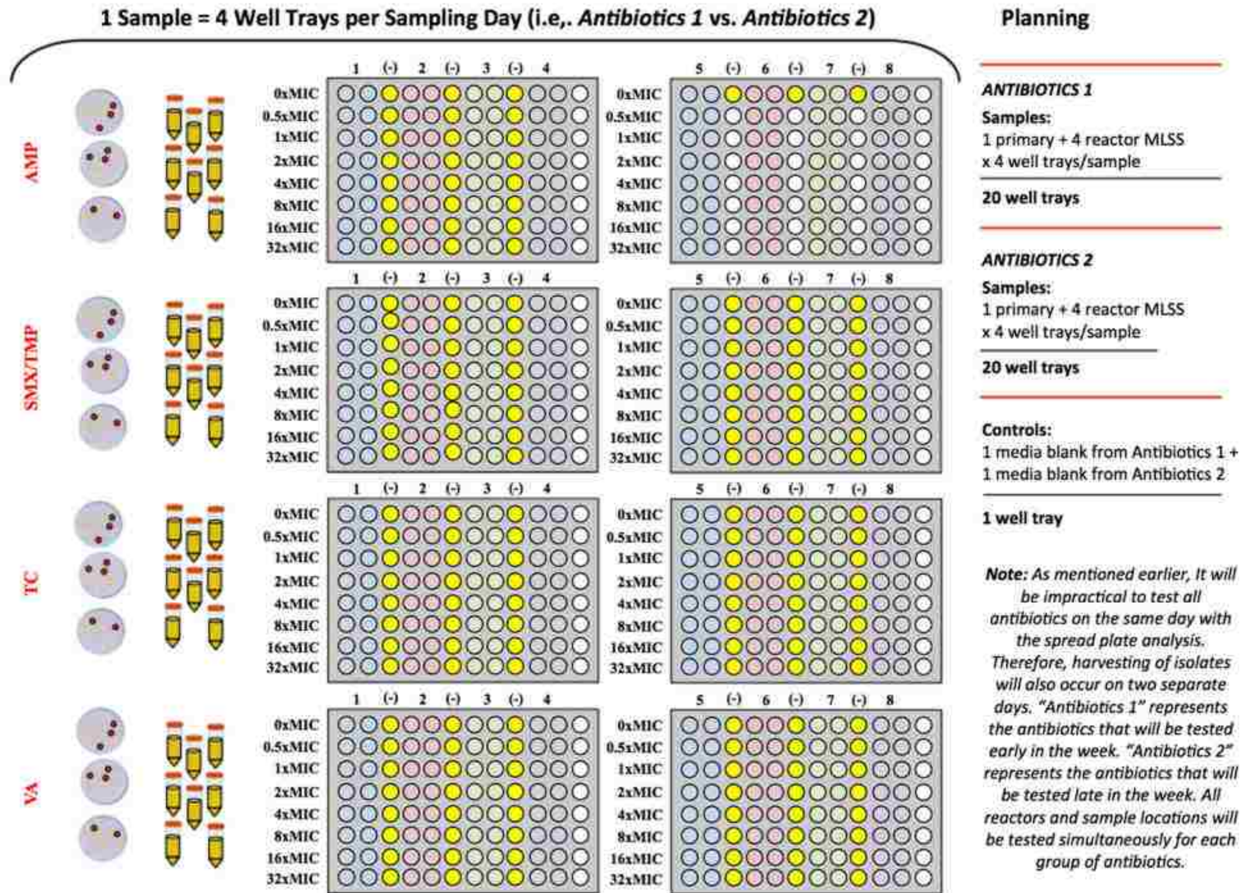


Figure 5-2. Schematic of minimum inhibitory concentration analysis

المنارة للاستشارات
www.manaraa.com

5.4 Results and discussion

5.4.1 The effect of varying solids retention time on sequencing batch reactor performance

The general water quality of the SBRs was monitored on an approximately weekly basis to validate the performance of the activated sludge process. The average pH of the primary effluent was 6.4±0.2, and the pH of the secondary effluent was relatively constant, regardless of SRT, with an average of 6.9±0.2. During the aeration phase, the average DO concentration was relatively constant in the four SBRs with an average of 4.7±0.5 mg/L and no reading lower than 3.7 mg/L.

The principal treatment objectives of the activated sludge process are the removal of organic matter and nitrogen (sometimes phosphorus as well). Reductions in BOD are typically used to verify the removal of organic matter, although TOC or sCOD can also be used as a surrogate in some applications (Christian et al., 2016). Figure 5-3 illustrates the average sCOD, total suspended solids (or MLSS), and volatile suspended solids (or MLVSS) in the primary and secondary effluents as a function of SRT. Consistent with full-scale activated sludge systems, there was a clear trend in sCOD removal in that longer SRTs resulted in lower and more consistent effluent COD concentrations. There was also a positive correlation between SRT and MLSS/MLVSS because of the greater 'recycle ratio' for longer SRTs.

To further validate the performance of the reactors, nitrogen speciation was performed to determine the extent of nitrification in each reactor (Figure 5-4). As expected, the nitrogen in the primary effluent was almost entirely in the form of ammonia, and the extent of nitrification increased with longer SRTs. Activated sludge systems with SRTs <5 days are typically assumed to be deficient in nitrifying bacteria (Tai et al., 2006), which limits the conversion of ammonia to

المنارة للاستشارات
www.manaraa.com

nitrite and nitrate. Assuming sufficient oxygen input, longer SRTs lead to the development of more mature microbial communities that are capable of converting nearly all of the ammonia to nitrate, nitrite, and/or nitrogen gas, depending on the exact operational conditions. The longer SRTs achieved nearly complete nitrification and also appeared to achieve partial denitrification based on an estimation of the nitrogen mass balance.

As this experiment progressed, the ambient temperature at the study site decreased from approximately 32°C down to less than 10°C, thereby causing the water temperature to decrease and hindering the removal of organic matter and the extent of nitrification (Head and Oleszkiewicz, 2004). These trends were observed for sCOD, ammonia, and nitrate, as shown in Figure 5-5. The sCOD in the secondary effluents increased only slightly at lower temperatures, but nitrification was clearly impeded, as indicated by the increasing ammonia concentrations and decreasing nitrate concentrations in the secondary effluents.

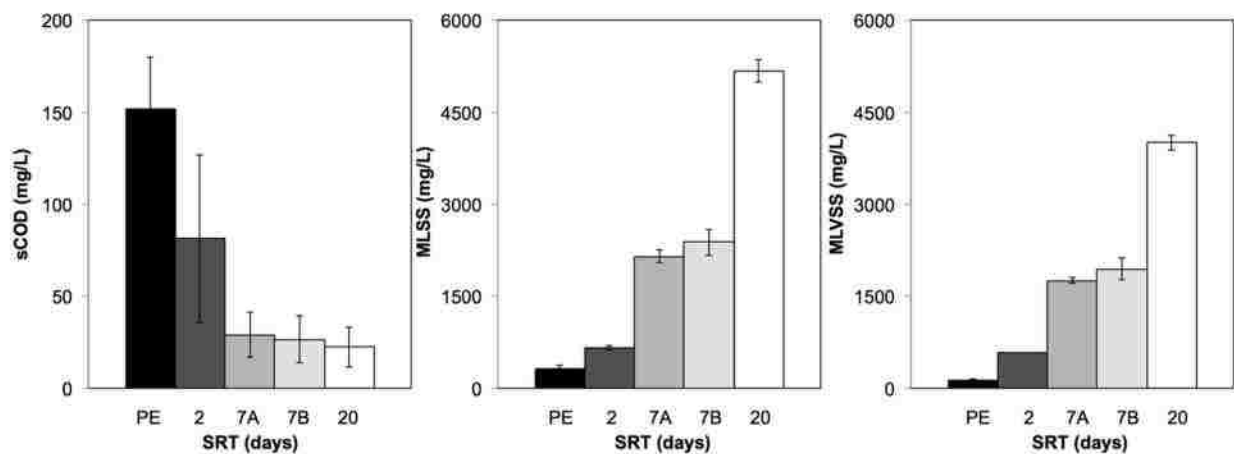


Figure 5-3. Average concentrations of sCOD, MLSS and MLVSS

Average concentrations of sCOD, MLSS and MLVSS in the SBRs as a function of SRT. The primary effluent (PE) represents the feed water quality prior to biological treatment in the SBRs. Columns represent the mean values for 5 sample events over 60 days of operation of the SBRs, and error bars represent ±1 standard deviation

www.manaraa.com

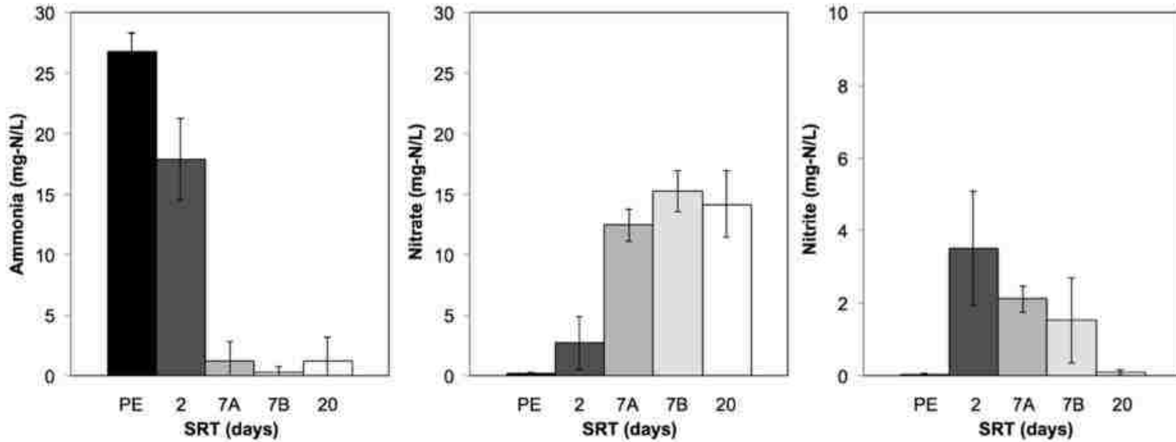


Figure 5-4. Average concentrations of ammonia, nitrate, and nitrite (varying SRT)

Average concentrations of ammonia, nitrate, and nitrite in the SBRs as a function of SRT. The primary effluent (PE) represents the feed water quality prior to biological treatment in the SBRs. Columns represent the mean values for 3 sample events over 60 days of operation of the SBRs, and error bars represent ±1 standard deviation. The final 2 sample events were excluded because of temperature effects (described in main text and illustrated in Figure 3)

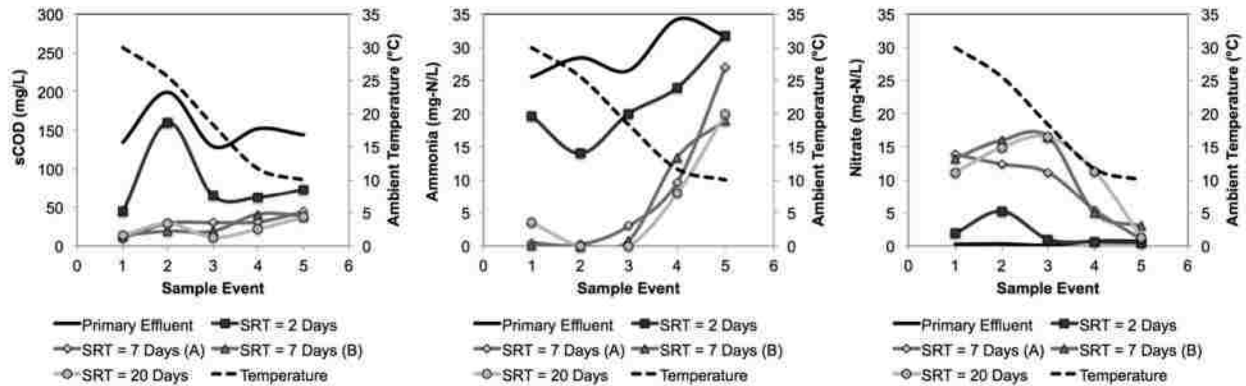


Figure 5-5. Effect of temperature on sCOD, ammonia, and nitrate (varying SRT)

Effect of temperature on sCOD, ammonia, and nitrate as a function of SRT over 60 days of operation of the SBRs

5.4.2 The effect of elevated antibiotic concentrations on the sequencing batch reactors performance

Over the duration of running the SBRs with elevated antibiotic concentrations, the pH of the primary effluent was consistently 6.8, and the pH of the four reactors was consistently 7.1-7.2. The average DO concentrations were 3.7-3.8 in the four reactors, with no reading lower than 3.4 mg/L.

المنارة للاستشارات
www.manaraa.com

Figure 5-6 illustrates the average sCOD in the primary effluent and the four secondary effluents, and it also illustrates the average total and volatile suspended solids concentrations in the primary effluent and mixed liquors. In contrast with the last experiment, for which the varying SRTs had a significant impact on general water quality parameters, all the reactors were designed to work with SRT of ~7 days. As a result, the MLSS and MLVSS values were nearly identical in the four reactors, with an overall average MLSS of 2,274±43 mg/L and an overall average MLVSS of 1,840±53 mg/L. The stable operation of the SBRs resulted in an overall average sCOD reduction of 87% and an overall average sCOD of 22±0.40 mg/L in the secondary effluent. These consistencies between the reactors provide a preliminary indication that the varying antibiotic concentrations in the SBR feeds were not impacting overall treatment performance. Also, when the samples were collected for the general water quality analyses, the ambient temperature ranged from 72°F-99°F (22°C-37°C). Therefore, there were no significant changes in system performance as there were when the temperature dropped to less than 50°F (10°C) during this phase.

Similar to sCOD, there were no significant differences between the reactors with respect to nitrification. Again, all reactors were operated with an SRT of ~7 days, which is sufficient to maintain a stable population of nitrifiers. As a result, the ammonia in the primary effluent (average of 32.8±1.8 mg-N/L) was consistently converted to nitrate (overall average of 27.7±0.5 mg-N/L) and, to a much lesser extent, nitrite (overall 0.01±0.00 mg-N/L) (Figure 5-7). The residual ammonia concentration in the four reactors was an average of 0.7±0.2 mg-N/L.

المنارة للاستشارات
www.manaraa.com

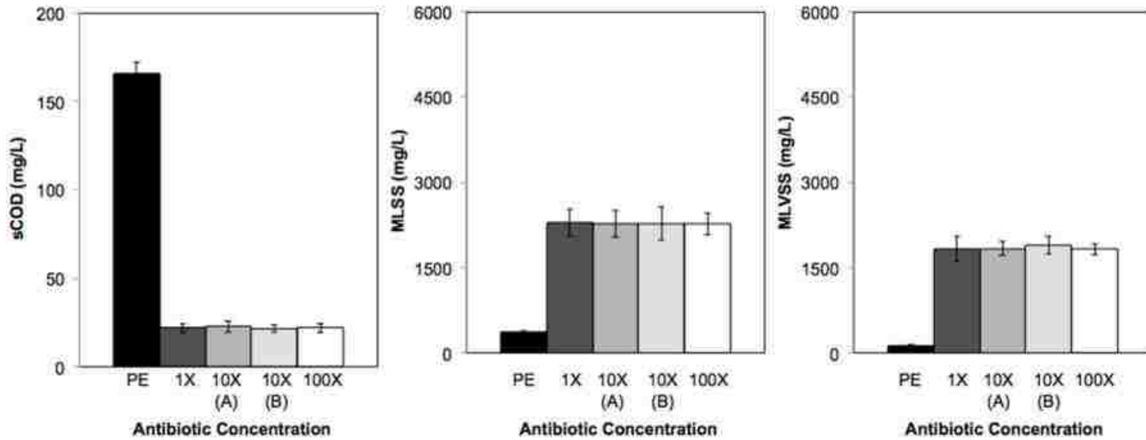


Figure 5-6. Average concentrations of sCOD, MLSS and MLVSS (elevated antibiotic concentrations)

Average concentrations of sCOD, MLSS and MLVSS in the SBRs with elevated antibiotic concentrations. The primary effluent (PE) represents the feed water quality prior to biological treatment in the SBRs. Columns represent the mean values for 5 sample events over 60 days of operation of the SBRs, and error bars represent ±1 standard deviation

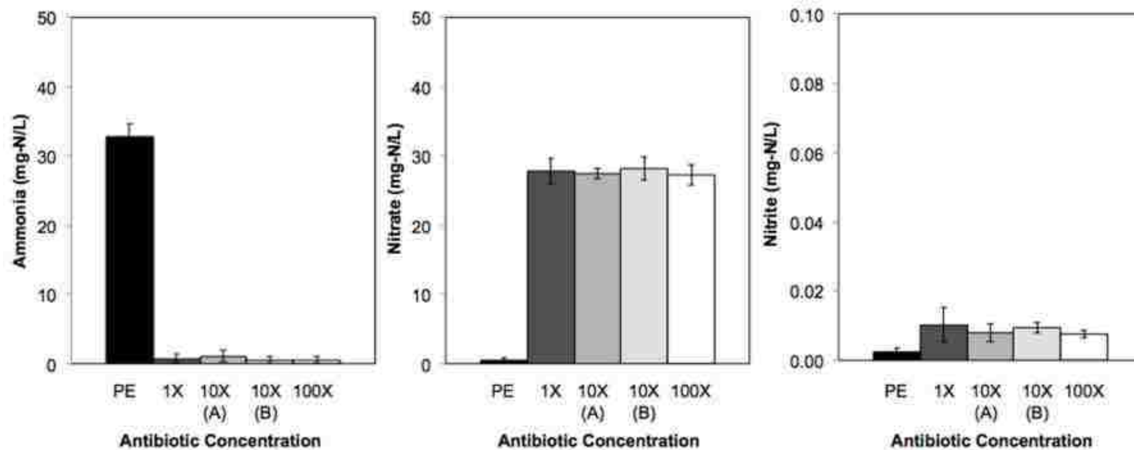


Figure 5-7. Average concentrations of ammonia, nitrate (elevated antibiotic concentrations)

Average concentrations of ammonia, nitrate, and nitrite in the SBRs with elevated antibiotic concentrations. The primary effluent (PE) represents the feed water quality prior to biological treatment in the SBRs. Columns represent the mean values for 3 sample events over 60 days of operation of the SBRs, and error bars represent ±1 standard deviation

5.4.3 The effect of varying SRT on mitigation of micropollutants

Sampling for indicator trace organic compounds and the target antibiotics was performed at the end of 2 months of operation to ensure that a stable microbial population had developed in the reactors prior to testing. Two independent sets of samples were collected to assess variability in

المنارة للاستشارات
www.manaraa.com

TOrC mitigation between cycles. TOrCs were analyzed in the primary effluent and in the secondary effluent from each of the SBRs.

The exact mechanism of bioattenuation of trace organic compounds in activated sludge systems is not completely understood, although multiple studies have demonstrated a positive correlation between solids retention time and TOrC elimination. For the more biodegradable compounds, it is unclear whether the benefit of longer SRTs is actually due to the higher solids concentration (i.e., a greater amount of biomass) or the greater diversity of the microbial community, more specifically the proliferation of microbes capable of specific biological processes (e.g., nitrification and/or denitrification). Again, it is important to note that the TOrC samples in the current study were collected toward the end of 60-day operation of the SBRs when the temperatures in the reactors had decreased to the approximate threshold for nitrification. Therefore, any reductions in TOrC concentrations in the SBRs were achieved during periods of limited nitrification and slightly reduced metabolic activity.

A summary of the TOrC concentrations for this phase is provided in Table 5-3. The target compounds for this study were selected to encompass a wide range of treatability in terms of biodegradation and sorption. The attenuation of the target compounds was generally in agreement with these TOrC properties, particularly with respect to biodegradability. The most biodegradable compounds (e.g., caffeine, ibuprofen, naproxen) achieved—or at least approached—their respective method reporting limits (MRLs) at longer SRTs, while the least biodegradable compounds (e.g., TCEP and carbamazepine) experienced little change in concentration as a result of biological treatment. Interestingly, the most hydrophobic compounds (e.g., triclocarban and fluoxetine) actually increased in concentration after biological treatment,

المنارة للاستشارات
www.manaraa.com

presumably due to some type of chemical equilibrium phenomenon that caused these compounds to desorb over time. These general observations are illustrated in Figure 5-8.

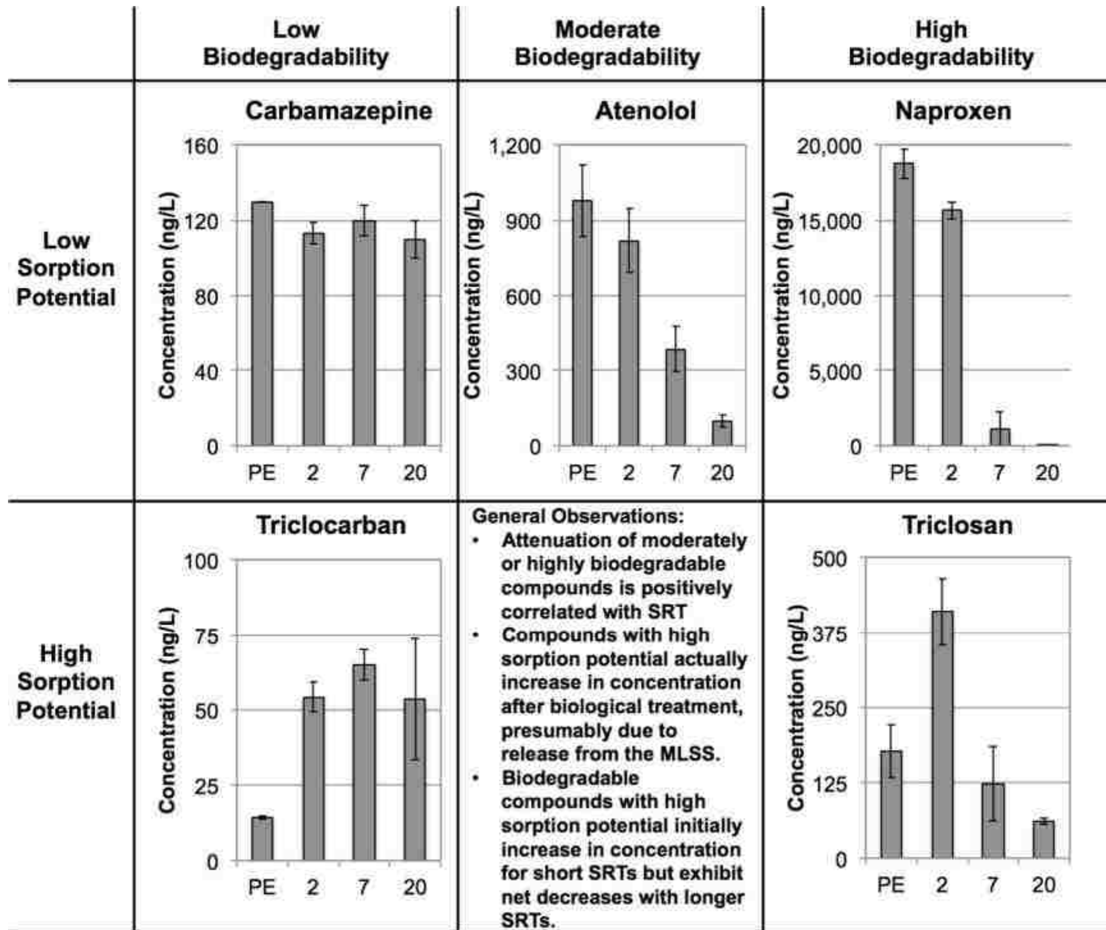


Figure 5-8. Summary of TOrC mitigation as a function of SRT

With respect to the target antibiotics, only trimethoprim exhibited a significant level of attenuation, and that was only observed for an SRT of 20 days. Sulfamethoxazole remained relatively constant during biological treatment at concentrations ranging from 1 to 1.5 μg/L.

المنارة للاستشارات
www.manaraa.com

Table 5-3. Summary of TOrC concentrations in the PE and effluent from the SBRs as a function of SRT

TOrC		Day 1 (11/20/2015)							Day 2 (11/21/2015)						
	SRT	PE	PE[1]	2	2[1]	7A	7B	20	PE	PE[1]	2	7A	7B	20	20[1]
Ampicillin	ng/L	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100
Sulfamethoxazole	ng/L	990	970	1100	1000	1400	1500	1300	1100	1300	1100	1200	1400	1200	1200
Tetracycline	ng/L	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
Trimethoprim	ng/L	380	370	370	330	420	420	120	530	710	500	470	410	180	180
Vancomycin	ng/L	420	470	670	890	980	760	770	450	460	740	1000	770	820	770
Acetaminophen	ng/L	95000	94000	<10000	<10000	<10000	<10000	<10000	93000	100000	<10000	<10000	<10000	<10000	<10000
Atenolol	ng/L	880	830	790	710	480	320	71	1100	1100	960	440	300	110	110
Caffeine	ng/L	67000	68000	50000	51000	<50	<50	<50	66000	73000	43000	<50	61	<50	<50
Carbamazepine	ng/L	130	130	110	120	120	120	120	130	130	110	110	130	110	100
DEET	ng/L	260	260	250	250	270	220	160	320	370	310	300	230	180	180
Fluoxetine	ng/L	8.8	16	7.9	7.8	26	22	20	16	26	7.6	28	23	22	22
Gemfibrozil	ng/L	1800	1700	1500	1500	1700	1300	72	1400	1700	1600	1500	1100	140	140
Ibuprofen	ng/L	19000	19000	15000	16000	220	110	17	23000	24000	16000	89	250	<10	<10
Meprobamate	ng/L	750	780	940	900	850	400	890	940	1100	1300	1000	420	1000	1000
Naproxen	ng/L	18000	18000	16000	16000	2600	200	18	19000	20000	15000	1400	330	21	23
Primidone	ng/L	180	180	200	190	210	200	210	210	200	210	210	220	220	200
Sucralose	ng/L	42000	41000	54000	47000	49000	49000	47000	45000	45000	51000	48000	40000	40000	42000
TCEP	ng/L	260	250	260	260	260	260	260	300	300	310	280	280	330	330
Triclocarban	ng/L	14	15	49	55	64	72	77	14	14	59	64	60	42	42
Triclosan	ng/L	140	140	400	360	160	71	59	210	220	470	190	72	58	67

المنارة للاستشارات
www.manaraa.com

Similar to Watkinson et al. (2009), who studied antibiotic occurrence in Australia, tetracycline concentrations were <MRL in all samples in the current study. A separate study of spiked tetracycline noted that removal was significantly correlated with SRT, while varying HRT (between 7 and 24 hours) had no significant impact on tetracycline removal (Kim et al., 2005). In the current study, the 'last resort' antibiotic vancomycin was consistently detected in primary effluent and biologically treated samples, and similar to hydrophobic compounds like triclocarban, vancomycin actually increased in concentration (up to 1 μg/L) after biological treatment.

5.4.4 The effect of elevated antibiotic concentrations on the SBRs performance with respect to trace organic compounds

The TOrC experiments in this phase had two primary objectives: (1) verify the spiking levels of the target antibiotics and (2) evaluate the effects of elevated antibiotic concentrations on treatment performance. TOrC samples were collected on consecutive days to evaluate the reproducibility of the data. The resulting concentrations are summarized in Table 5-4, and a direct comparison with the target antibiotic concentrations is provided in Table 5-5.

With respect to the first objective, the actual concentrations observed in the primary effluent pumped to each SBR were generally consistent with the target concentrations (i.e., 1x, 10x, or 100x) for sulfamethoxazole, trimethoprim, and vancomycin. Differences between the observed and target concentrations of these antibiotics, particularly for vancomycin, may have been due to rapid adsorption onto the suspended solids in the primary effluent. On the other hand, the observed concentrations of ampicillin and tetracycline were significantly different from the target concentrations.

المنارة للاستشارات
www.manaraa.com

Table 5-4. Summary of TOrC concentrations in the PE and effluent from the SBRs with elevated antibiotic concentrations

TOrC		Day 1 (5/12/2016)									Day 2 (5/13/2016)								
		PE1	1X	1X[1]	PE2	10XA	PE3	10XB	PE4	100X	PE1	1X	PE2	10XA	PE3	10XB	PE4	100XA	100XB
Ampicillin	ng/L	<100	<100	<100	<100	<100	<10000	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100
Sulfamethoxazole	ng/L	2700	2000	2000	12000	6300	11000	6100	92000	51000	2300	1500	11000	5600	11000	5900	100000	59000	60000
Tetracycline	ng/L	<100	23	<100	<100	26	<10000	25	390	160	<100	16	<100	24	<100	30	140	160	160
Trimethoprim	ng/L	980	110	89	4800	1300	5000	1300	45000	20000	910	220	4800	1500	4300	850	48000	23000	23000
Vancomycin	ng/L	1200	1100	1100	2100	2000	6500	2100	33000	23000	920	1300	1700	2300	1800	2200	28000	26000	28000
Acetaminophen	ng/L	10000	<100	<100	96000	<100	100000	<100	98000	<100	90000	<100	91000	<100	90000	<100	94000	<100	<100
Atenolol	ng/L	1200	120	120	1200	130	1200	160	1200	130	1100	<2000	1100	<2000	1100	<2000	1100	<2000	<2000
Caffeine	ng/L	67000	<5.0	<5.0	66000	<100	64000	<100	63000	<100	70000	<100	66000	<100	64000	<100	65000	<100	<100
Carbamazepine	ng/L	180	180	180	160	170	170	170	170	130	160	150	150	160	150	160	150	160	130
DEET	ng/L	1000	630	620	1100	600	1000	660	1000	500	510	470	490	480	480	390	470	400	340
Fluoxetine	ng/L	<1000	19	18	<1000	16	<1000	16	<1000	16	<1000	13	<1000	22	<1000	21	<1000	22	15
Gemfibrozil	ng/L	1600	7.6	7.5	1600	31	1700	61	1700	41	1500	420	1600	18	1600	16	1600	33	33
Ibuprofen	ng/L	24000	14	17	22000	14	23000	23	23000	27	22000	23	22000	14	22000	13	22000	11	11
Meprobamate	ng/L	780	930	920	800	980	820	950	870	760	710	870	720	910	720	860	750	870	760
Naproxen	ng/L	21000	15	13	20000	14	21000	23	21000	38	19000	36	19000	10	19000	14	19000	19	18
Primidone	ng/L	210	210	200	220	210	240	210	220	170	190	190	190	200	180	190	190	190	180
Sucralose	ng/L	38000	44000	45000	40000	43000	40000	46000	41000	37000	42000	51000	40000	45000	37000	46000	43000	47000	39000
TCEP	ng/L	330	310	300	320	300	290	290	280	280	160	250	160	240	160	230	160	230	240
Triclocarban	ng/L	15	19	12	21	12	20	16	24	28	19	40	16	16	22	18	20	26	28
Triclosan	ng/L	240	36	33	230	46	220	54	210	41	270	150	190	100	240	50	230	42	40

المنارة للاستشارات
www.manaraa.com

Table 5-5. Comparison of Target and Observed Antibiotic Concentrations in Primary Effluent

Antibiotic	Units	1X		10x		100x	
		Target	Actual	Target	Actual	Target	Actual
Ampicillin	µg/L	0.2	<0.1	2	<0.1	20	<0.1
Sulfamethoxazole	µg/L	1	2.5	10	11	100	96
Tetracycline	µg/L	0.1	<0.1	1	<0.1	10	0.3
Trimethoprim	µg/L	0.5	0.9	5	4.7	50	47
Vancomycin	µg/L	0.5	1.1	5	3.0	50	31

In fact, ampicillin was <MRL in all samples, even when spiked at 20 µg/L. With respect to the primary effluent samples, tetracycline was only present at reportable concentrations in the 100x sample, and even though the antibiotic was spiked at 10 µg/L, the observed concentration was only 0.3 µg/L. Tetracycline was reliably detected in several secondary effluents (i.e., after biological treatment), even when the primary effluent had not been spiked. This suggests that matrix interference may have been a significant factor.

Factors such as adsorption and hydrolysis may have also affected the observed concentrations of ampicillin and tetracycline. Gao et al. (2012) detected tetracycline in primary effluent (164 ng/L) but not in secondary effluent. Extracted solids resulted in consistent tetracycline detection (750 µg/kg dw), thereby suggesting that the compound preferentially adsorbed to solids and was removed in the clarification process. Other studies in the literature describe a wide range of values for tetracycline, and there are several studies reporting tetracycline at <MRL (Watkinson et al., 2009). With respect to ampicillin, there is a general paucity of data describing typical concentrations in wastewater, but Li and Zhang, (2010) noted that both ampicillin and tetracycline rapidly adsorb onto solids. Therefore, one can conclude that a combination of analytical and natural limitations likely hindered reliable detection of ampicillin and tetracycline, despite the fact that these compounds were spiked at high concentrations. Regardless, based on the other three antibiotics, the antibiotic delivery system appeared to work as intended.

المنارة للاستشارات
www.manaraa.com

The second objective of the TOrC sampling was to evaluate whether the elevated antibiotic concentrations might hinder the biodegradation capabilities of the microbial community. The general water quality parameters, particularly with respect to sCOD removal and nitrification, provided a preliminary indication that the spiked antibiotics had no significant impact on treatment efficacy. The SBR effluent concentrations for the most biodegradable compounds (e.g., acetaminophen, caffeine, naproxen, and ibuprofen) further support this conclusion, as those concentrations were significantly attenuated via biodegradation even in the 100x SBR. This is supported by Li and Zhang (2010) who observed significant caffeine attenuation via biodegradation even with a suite of antibiotics spiked at the high μg/L level. Therefore, elevated antibiotic concentrations (up to 1.25% of the standard MIC) do not appear to impact the functional capacity of the microbial community in wastewater treatment applications.

5.4.5 The effect of varying SRT on culturable antibiotic resistant bacteria

The geometric mean plate counts and standard deviations for the three sample events are summarized in Figure 5-9 through Figure 5-11. As mentioned earlier, each sample event for the antibiotic resistance testing was divided into two different groups to limit the plating on each day. Therefore, each data set includes two different primary effluent samples. The first sample event was performed 3 and 5 days post-startup, which corresponds to 9 and 15 treatment cycles, respectively. Sample event 2 was performed 31 and 33 days post-startup (93 and 99 treatment cycles), and sample event 3 was performed 53 and 55 days post-startup (159 and 165 treatment cycles). For sample events 1 and 2, ampicillin and sulfamethoxazole/trimethoprim were tested as part of group 1, and tetracycline and vancomycin were tested as part of group 2. The groupings were then swapped for sample event 3 to eliminate any sample/analysis bias.

المنارة للاستشارات
www.manaraa.com

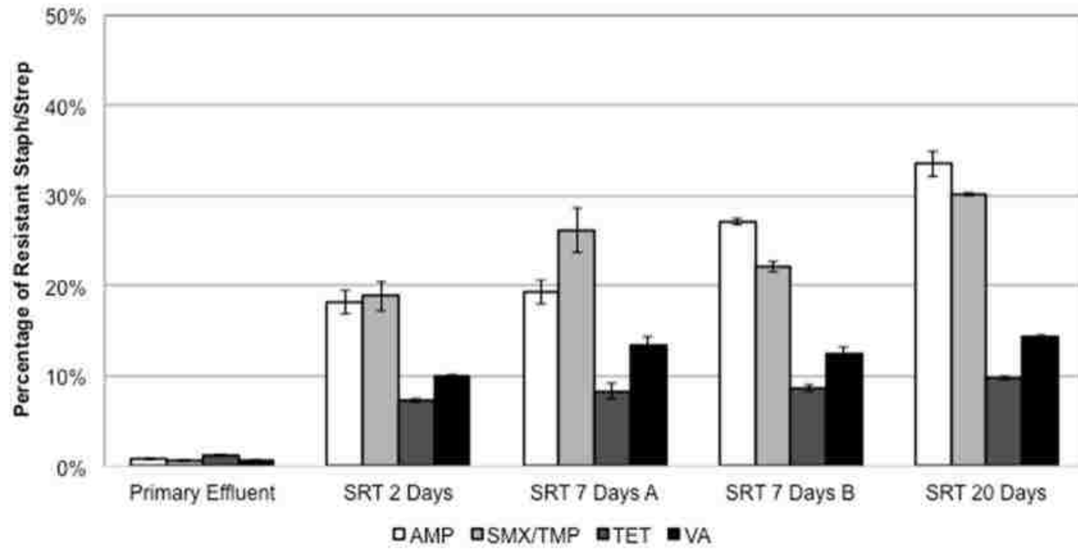


Figure 5-9. Percentage of Resistant Staph/Strep in the SBRs with varying SRT (Sample Event 1)

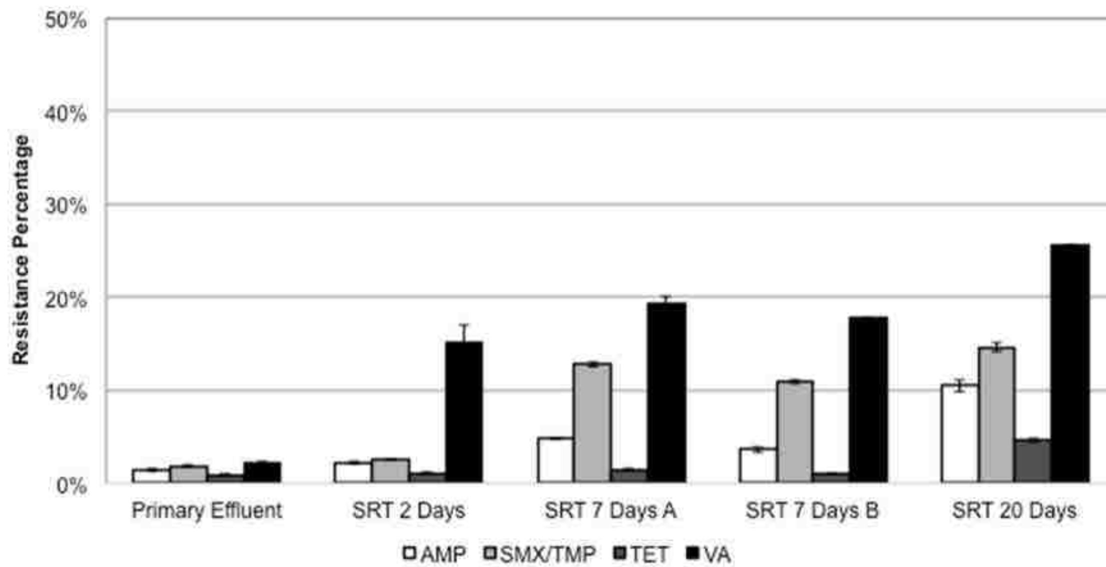


Figure 5-10. Percentage of Resistant Staph/Strep in the SBRs with varying SRT (Sample Event 2)

www.manaraa.com

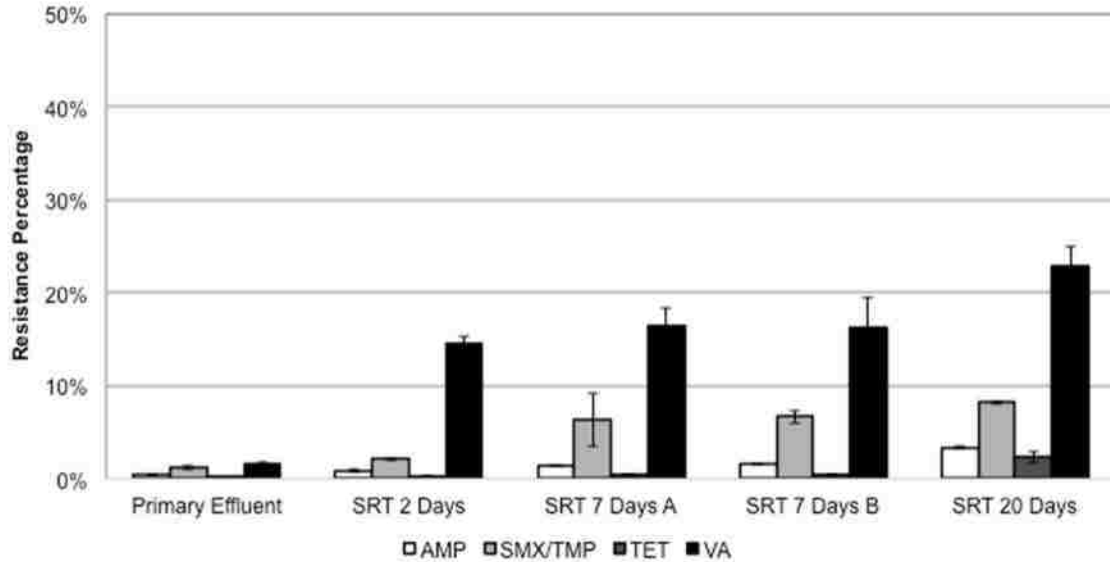


Figure 5-11. Percentage of Resistant Staph/Strep in the SBRs with varying SRT (Sample Event 3)

The plate counts were used to calculate the ratios of total Staph/Strep in each MLSS sample to total Staph/Strep in the primary effluent (Table 5-6 through Table 5-8). The values were generally a function of SRT in that longer SRTs with greater 'recycle ratios' led to higher levels of Staph/Strep in the MLSS. To calculate the ratio of antibiotic resistant bacteria, the plate counts on media supplemented with each antibiotic (i.e., MH + Staph/Strep supplement + antibiotic) were divided by the plate counts for that same sample on media that did not contain antibiotics (i.e., MH + Staph/Strep supplement).

The data suggest that biological treatment appears to select for antibiotic resistant bacteria. This is based on the fact that the antibiotic resistant Staph/Strep comprise a greater percentage of the total Staph/Strep population in the MLSS compared to the primary effluent. Furthermore, the data suggest that longer SRTs also select for antibiotic resistance, as indicated by the higher percentages for SRTs of 7 and 20 days. For sample event 1, it is important to note that the microbial community in the SBR targeting the longest SRT was not exactly representative of a

المنارة للاستشارات
www.manaraa.com

20-day SRT at this point in the study. The SBRs were seeded with RAS from the full-scale facility, which operates at a moderate SRT of ~6-7 days. Considering that the first set of samples were collected 3- and 5-days post-startup, the SRT in the last reactor had not yet reached 20 days, although it was longer than the other three reactors. In the first sample event, the antibiotic resistance effect appeared to be more pronounced for ampicillin and sulfamethoxazole/trimethoprim than for tetracycline and vancomycin (Figure 5-9).

For sample event 2 (Figure 5-10), there was still a consistent positive correlation between the ratio of antibiotic resistant Staph/Strep and SRT, but the actual percentages dramatically decreased compared to sample event 1, except for vancomycin which actually increased. This trend continued into sample event 3 in which the percentage of antibiotic resistant Staph/Strep decreased to less than 10% for ampicillin, sulfamethoxazole/trimethoprim, and tetracycline (Figure 10). The percentages for vancomycin decreased slightly but remained relatively consistent with sample event 2. Despite the decrease in percentages, the level of antibiotic resistance was consistently higher in the MLSS and for longer SRTs. The temporal trends across the three sample events are illustrated in Figure 5-12.

المنارة للاستشارات
www.manaraa.com

Table 5-6. The effect of varying SRT on culturable Staph/Strep resistant bacteria (Sample Event 1)

Sample	MH+S/S (CFU/100 µL)	MH+S/S+AMP (CFU/100 µL)	MH+S/S+ SMX/TMP (CFU/100 µL)	MH+S/S (CFU/100 µL)	MH+S/S+TET (CFU/100 µL)	MH+S/S+VA (CFU/100 µL)
Primary Effluent	$(8.69\pm0.46)x10^3$	$(7.23\pm0.15)x10^1$	$(6.12\pm0.59)x10^1$	$(4.86\pm0.40)x10^3$	$(6.32\pm0.49)x10^1$	$(3.46\pm0.15)x10^1$
SRT = 2 Days	$(5.57\pm0.72)x10^3$	$(1.02\pm0.06)x10^3$	$(1.05\pm0.05)x10^3$	$(1.90\pm0.06)x10^4$	$(1.38\pm0.08)x10^3$	$(1.90\pm0.10)x10^3$
SRT = 7 Days (A)	$(7.10\pm0.86)x10^3$	$(1.37\pm0.08)x10^3$	$(1.86\pm0.05)x10^3$	$(2.61\pm0.40)x10^4$	$(2.16\pm0.12)x10^3$	$(3.52\pm0.31)x10^3$
SRT = 7 Days (B)	$(5.10\pm0.17)x10^3$	$(1.38\pm0.03)x10^3$	$(1.13\pm0.07)x10^3$	$(2.77\pm0.06)x10^4$	$(2.40\pm0.17)x10^3$	$(3.42\pm0.32)x10^3$
SRT = 20 Days	$(3.12\pm0.29)x10^4$	$(1.05\pm0.05)x10^4$	$(9.44\pm0.81)x10^3$	$(8.36\pm0.35)x10^4$	$(8.20\pm0.17)x10^3$	$(1.20\pm0.03)x10^4$

Table 5-7. The effect of varying SRT on culturable Staph/Strep resistant bacteria (Sample Event 2)

Sample	MH+S/S (CFU/100 µL)	MH+S/S+AMP (CFU/100 µL)	MH+S/S+ SMX/TMP (CFU/100 µL)	MH+S/S (CFU/100 µL)	MH+S/S+TET (CFU/100 µL)	MH+S/S+VA (CFU/100 µL)
Primary Effluent	$(1.63\pm0.40)x10^4$	$(2.32\pm0.32)x10^2$	$(2.93\pm0.57)x10^2$	$(2.27\pm0.44)x10^4$	$(2.05\pm0.29)x10^2$	$(4.97\pm0.62)x10^2$
SRT = 2 Days	$(1.89\pm0.17)x10^4$	$(4.29\pm0.76)x10^2$	$(4.85\pm0.51)x10^2$	$(2.50\pm0.71)x10^4$	$(2.70\pm0.50)x10^2$	$(3.77\pm0.60)x10^3$
SRT = 7 Days (A)	$(5.54\pm0.61)x10^4$	$(2.66\pm0.25)x10^3$	$(7.08\pm0.66)x10^3$	$(2.87\pm0.46)x10^4$	$(4.13\pm0.29)x10^2$	$(5.54\pm0.64)x10^3$
SRT = 7 Days (B)	$(5.30\pm0.27)x10^4$	$(1.92\pm0.25)x10^3$	$(5.79\pm0.40)x10^3$	$(3.55\pm0.40)x10^4$	$(3.77\pm0.53)x10^2$	$(6.31\pm0.68)x10^3$
SRT = 20 Days	$(8.48\pm0.69)x10^4$	$(8.93\pm0.15)x10^3$	$(1.24\pm0.06)x10^4$	$(3.92\pm0.32)x10^4$	$(1.82\pm0.25)x10^3$	$(1.00\pm0.08)x10^4$

Table 5-8. The effect of varying SRT on culturable Staph/Strep resistant bacteria (Sample Event 3)

Sample	MH+S/S (CFU/100 µL)	MH+S/S+AMP (CFU/100 µL)	MH+S/S+ SMX/TMP (CFU/100 µL)	MH+S/S (CFU/100 µL)	MH+S/S+TET (CFU/100 µL)	MH+S/S+VA (CFU/100 µL)
Primary Effluent	$(3.59\pm0.85)x10^4$	$(1.36\pm0.21)x10^2$	$(4.22\pm0.40)x10^2$	$(3.87\pm0.60)x10^4$	$(3.87\pm0.60)x10^1$	$(6.22\pm0.50)x10^2$
SRT = 2 Days	$(5.56\pm0.32)x10^4$	$(4.80\pm1.00)x10^2$	$(1.16\pm0.15)x10^3$	$(5.95\pm0.47)x10^4$	$(2.06\pm0.25)x10^2$	$(8.62\pm1.15)x10^3$
SRT = 7 Days (A)	$(6.69\pm0.36)x10^4$	$(9.35\pm0.60)x10^2$	$(4.26\pm2.20)x10^3$	$(8.42\pm0.59)x10^4$	$(3.71\pm0.50)x10^2$	$(1.38\pm0.27)x10^4$
SRT = 7 Days (B)	$(6.56\pm0.45)x10^4$	$(1.02\pm0.07)x10^3$	$(4.39\pm0.76)x10^3$	$(8.59\pm0.46)x10^4$	$(4.22\pm0.45)x10^2$	$(1.40\pm0.35)x10^4$
SRT = 20 Days	$(1.25\pm0.06)x10^5$	$(4.23\pm0.31)x10^3$	$(1.03\pm0.06)x10^4$	$(9.96\pm0.25)x10^4$	$(2.38\pm0.67)x10^3$	$(2.29\pm0.27)x10^4$

المنارة للاستشارات
www.manaraa.com

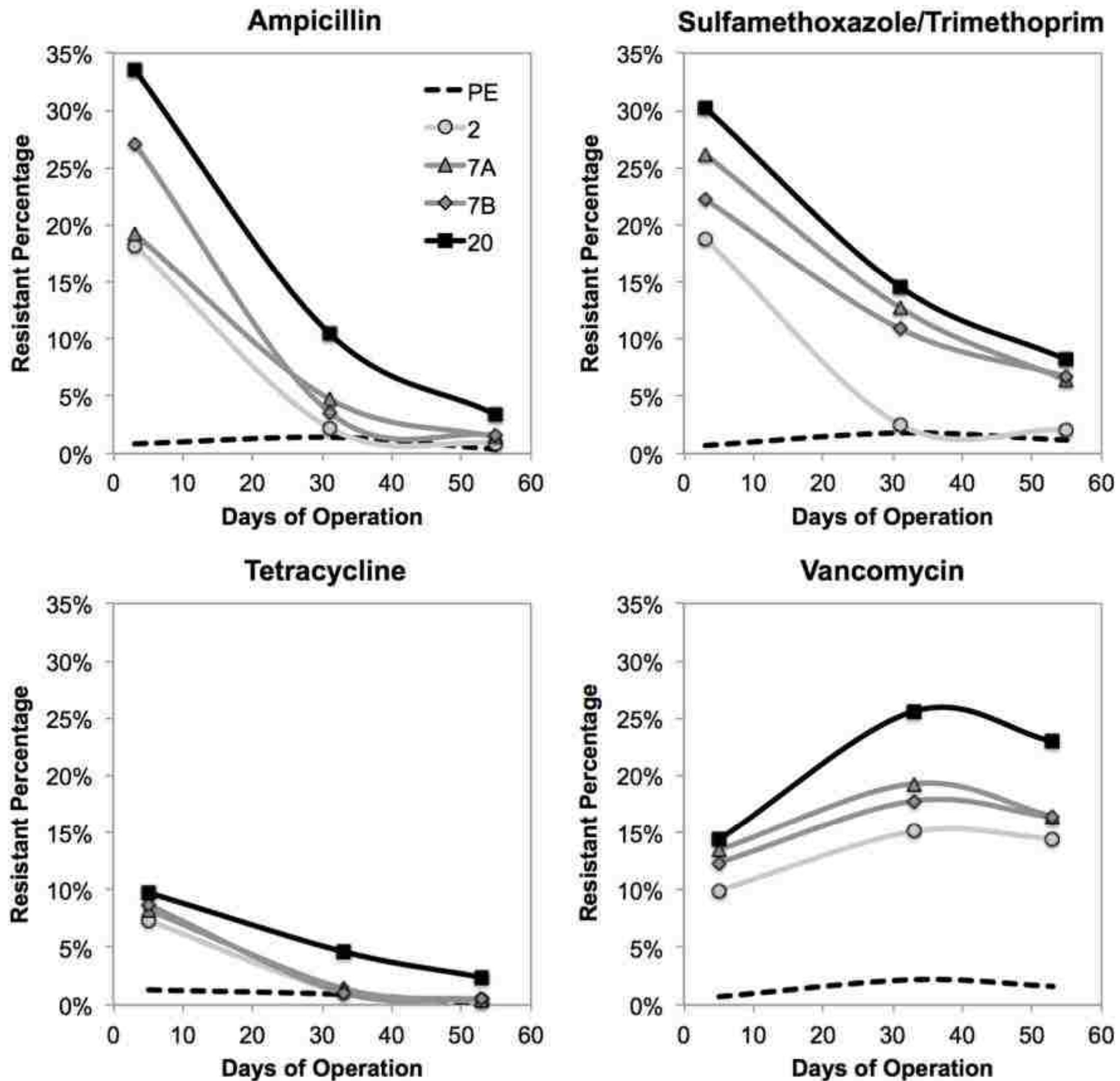


Figure 5-12. Temporal trend in antibiotic resistant percentage (Varying SRT)

One potential hypothesis for the decrease observed in Figure 5-12 is that the resistance mechanisms became too ‘expensive’ for the bacteria to maintain after experiencing environmental stress (i.e., cold temperatures). Environmental stressors such as temperature, pH, salinity can induce structural and physiological responses among certain species of bacteria (Beales, 2004). McMahon et al. (2007) studied the responses of Gram negative *E. coli* and *Salmonella enterica* and Gram positive *Staphylococcus aureus* to exposure to cold temperatures

المنارة للاستشارات
www.manaraa.com

(10°C for *E. coli* and *Salmonella* and 21°C for *Staphylococcus*). The level of AR exhibited by *E. coli* remained relatively constant after exposure to cold temperatures, but the level of AR exhibited by *Salmonella enterica* decreased significantly, particularly for trimethoprim. *Staphylococcus* exhibited a decrease in AR for one of the three antibiotics tested (i.e., oxacillin but not gentamicin or erythromycin), although the lowest temperatures tested was 21°C. On the other hand, Miller et al. (2014) reported an increase in *intI*1 and *sul*1 when biosolids were stored at temperatures less than 10°C. Therefore, temperature effects appear to be species- and antibiotic-specific and might also differ for culture versus molecular methods. More specifically, changes in temperature might induce changes in gene prevalence but not necessarily gene expression. This topic requires further investigation to fully explain the effects of temperature on AR prevalence.

5.4.6 The effect of elevated antibiotic concentrations on culturable antibiotic resistant bacteria

Three sample events were performed to evaluate the potential proliferation of AR during biological treatment with elevated antibiotic concentrations. Each sample event was divided into two subsamples to yield a more manageable number of plates on a given day. Resistance to ampicillin and sulfamethoxazole/trimethoprim was evaluated in the first subsample, and resistance to tetracycline and vancomycin was evaluated in the second subsample. The first sample event was performed 3 and 5 days after startup (i.e., after 9 and 15 SBR cycles, respectively), and the second sample event was performed 31 and 33 days after startup (i.e., after 93 and 99 SBR cycles, respectively. The third sample event was scheduled to be performed after approximately 60 days of operation, but an unforeseen scheduling issue forced the reactors to be shut down after 50 days of operation. For the third sample event, the SBRs were restarted and

المنارة للاستشارات
www.manaraa.com

allowed to operate for an additional 14-16 days prior to sample collection (i.e., after 42 and 48 SBR cycles, respectively). Over the duration of the SBRs in this phase, the ambient temperature increased from 59°F to 77°F (15°C to 25°C).

The plate counts for sample events 1 to 3 are summarized in Table 5-9 to Table 5-11. These plate counts were used to determine the ratios of culturable Staph/Strep in the four mixed liquors relative to the culturable Staph/Strep in the primary effluent. This calculation was used as a means to characterize the change in the overall Staph/Strep population during biological treatment.

The plate counts were also used to determine the percentage of culturable Staph/Strep that were resistant to the target antibiotics spiked at their standard MICs. These data are summarized in Figure 5-13 to Figure 5-15. The three sample events are directly compared in Figure 5-16 to illustrate the temporal trend in antibiotic resistance. These data yielded the following general observations:

- Consistent with previous phase (varying SRT), biological treatment appears to increase the prevalence of culturable AR bacteria with respect to all of the target antibiotics.
- The relative prevalence of AR bacteria in the mixed liquor ranged from as low as 2% for 1x tetracycline to as high as 32% for 100x vancomycin.
- There was a slight positive correlation between influent antibiotic concentrations and the prevalence of culturable AR bacteria.

Consistent with previous task (varying SRT), there appears to be a positive correlation between temperature and culture-based antibiotic resistance. In varying SRT phase, AR decreased sharply as the temperature decreased, and in elevated antibiotic concentrations phase, AR increased with increasing temperature.

المنارة للاستشارات
www.manaraa.com

Single factor analysis of variance (ANOVA) with post-hoc Tukey's test was also performed and the results are summarized in Appendix C. As it was expected, two 7-day SRT reactors were not significantly different ($p>0.5$) for most of the samples. Except for vancomycin and tetracycline, AR rates were significantly different among 2-day, 7-day and 20-day SRT ($p<0.5$). For ampicillin and tetracycline, only one sampling event showed no significant difference between 2-day, 7-day, and 20-day SRT. According to the results from statistical analysis, longer SRTs may promote antibiotic resistance at least for some antibiotics.

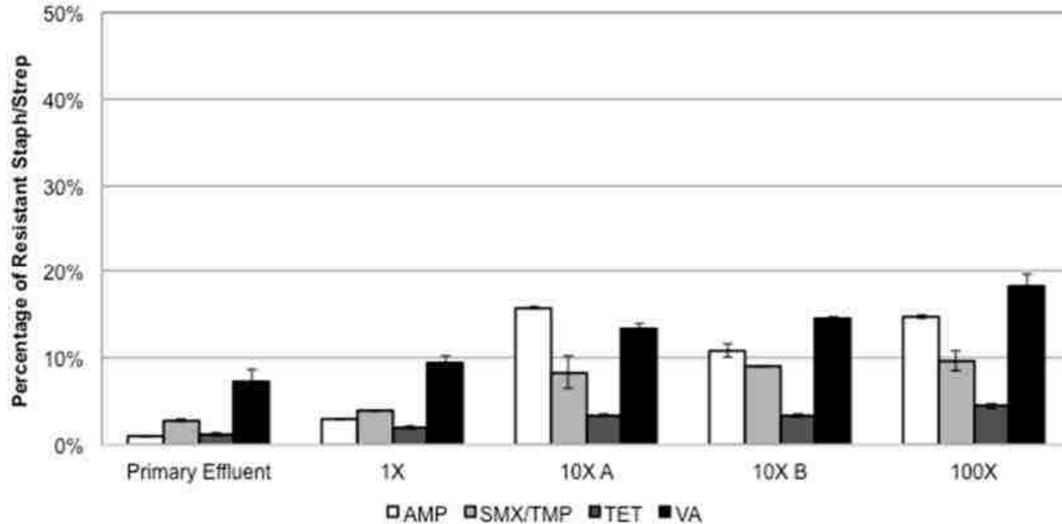


Figure 5-13. Percentage of Resistant Staph/Strep in the SBRs with elevated antibiotic concentrations (Sample Event 1)

www.manaraa.com

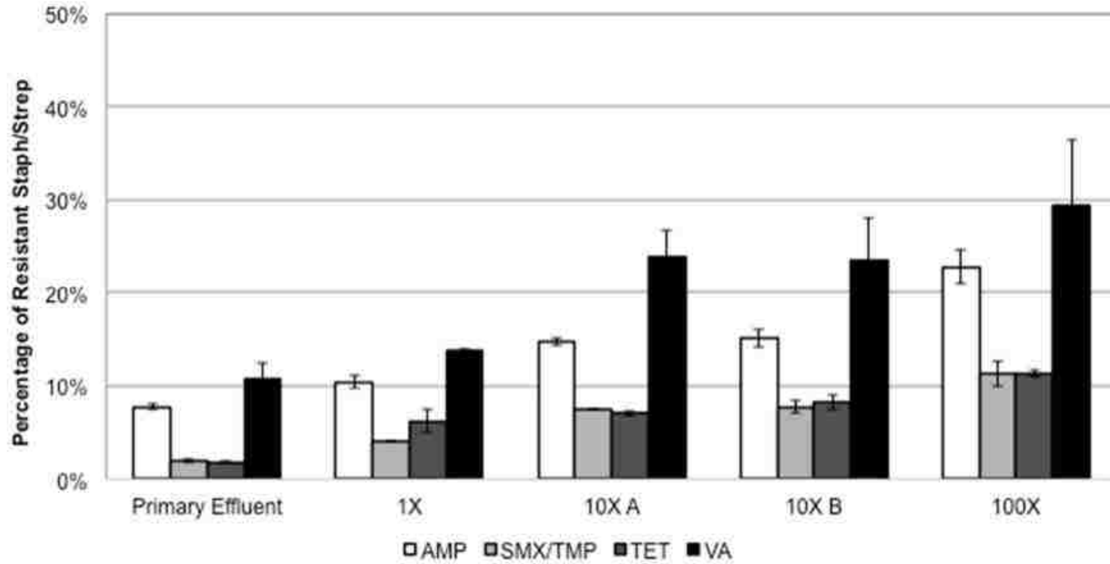


Figure 5-14. Percentage of Resistant Staph/Strep in the SBRs with elevated antibiotic concentrations (Sample Event 2)

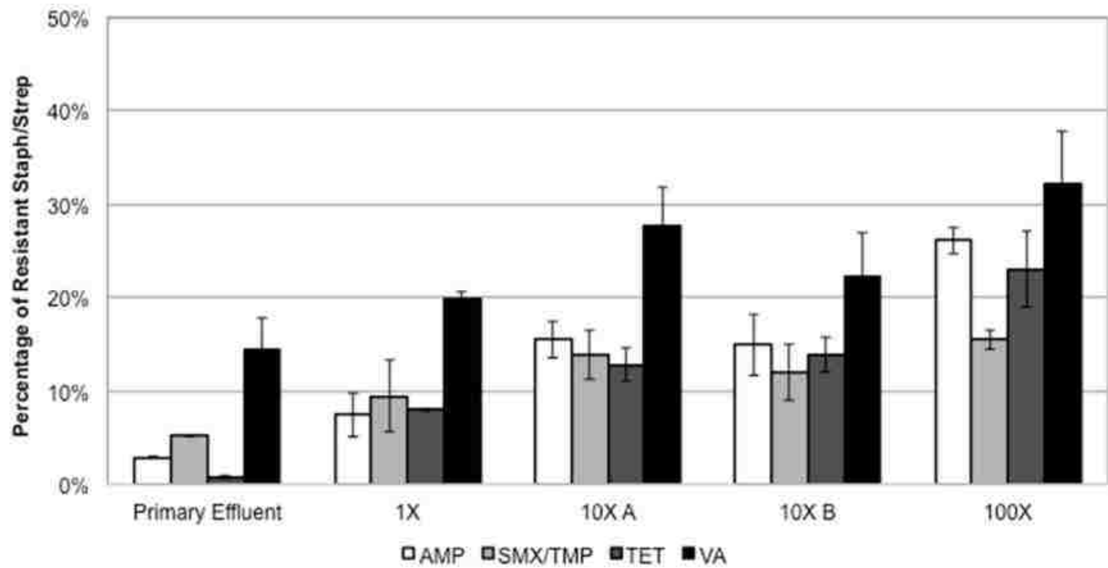


Figure 5-15. Percentage of Resistant Staph/Strep in the SBRs with elevated antibiotic concentrations (Sample Event 3)

المنارة للاستشارات
www.manaraa.com

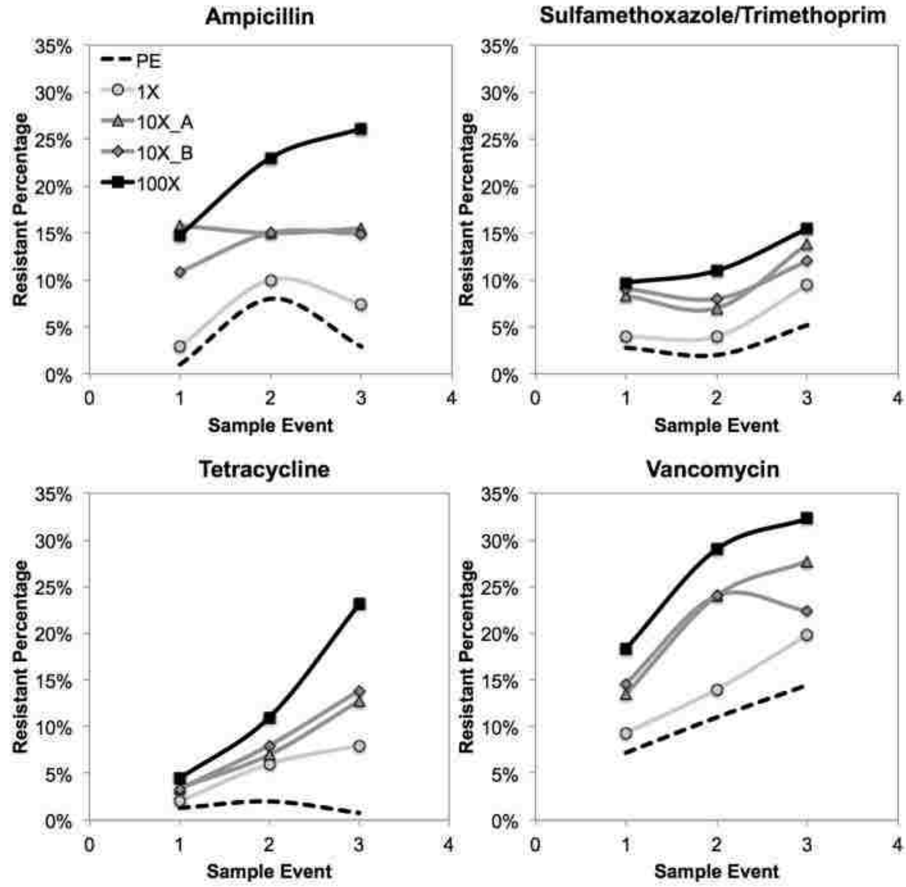


Figure 5-16. Temporal trend in antibiotic resistant percentage (Elevated antibiotic concentrations)

المنارة للاستشارات
www.manaraa.com

Table 5-9. The effect of elevated antibiotic concentrations on culturable Staph/Strep resistant bacteria (Sample Event 1)

Sample	MH+S/S (CFU/100 µL)	MH+S/S+AMP (CFU/100 µL)	MH+S/S+ SMX/TMP (CFU/100 µL)	MH+S/S (CFU/100 µL)	MH+S/S+TET (CFU/100 µL)	MH+S/S+VA (CFU/100 µL)
Primary Effluent	$(2.00\pm0.10)x10^{4}$	$(1.86\pm0.15)x10^{2}$	$(5.65\pm0.58)x10^{2}$	$(1.34\pm0.35)x10^{4}$	$(1.66\pm0.21)x10^{2}$	$(9.62\pm0.55)x10^{2}$
1X	$(1.86\pm0.12)x10^{4}$	$(5.39\pm0.36)x10^{2}$	$(7.39\pm0.53)x10^{2}$	$(2.20\pm0.49)x10^{4}$	$(4.38\pm0.52)x10^{2}$	$(2.06\pm0.25)x10^{3}$
10X (A)	$(1.83\pm0.15)x10^{4}$	$(2.89\pm0.27)x10^{3}$	$(1.52\pm0.47)x10^{3}$	$(2.23\pm0.15)x10^{4}$	$(7.65\pm0.58)x10^{2}$	$(3.00\pm0.10)x10^{3}$
10X (B)	$(1.83\pm0.06)x10^{4}$	$(1.99\pm0.20)x10^{3}$	$(1.67\pm0.06)x10^{3}$	$(2.16\pm0.12)x10^{4}$	$(7.21\pm0.70)x10^{2}$	$(3.16\pm0.21)x10^{3}$
100X	$(1.96\pm0.15)x10^{4}$	$(2.90\pm0.17)x10^{3}$	$(1.91\pm0.38)x10^{3}$	$(1.96\pm0.21)x10^{4}$	$(8.69\pm0.36)x10^{2}$	$(3.59\pm0.65)x10^{3}$

Table 5-10. The effect of elevated antibiotic concentrations on culturable Staph/Strep resistant bacteria (Sample Event 2)

Sample	MH+S/S (CFU/100 µL)	MH+S/S+AMP (CFU/100 µL)	MH+S/S+ SMX/TMP (CFU/100 µL)	MH+S/S (CFU/100 µL)	MH+S/S+TET (CFU/100 µL)	MH+S/S+VA (CFU/100 µL)
Primary Effluent	$(4.05\pm0.50)x10^{3}$	$(3.14\pm0.51)x10^{2}$	$(7.99\pm0.36)x10^{1}$	$(7.56\pm1.53)x10^{3}$	$(1.37\pm0.36)x10^{2}$	$(8.06\pm0.21)x10^{2}$
1X	$(6.95\pm1.00)x10^{3}$	$(7.23\pm1.53)x10^{2}$	$(2.78\pm0.40)x10^{2}$	$(1.32\pm0.25)x10^{4}$	$(8.19\pm3.21)x10^{2}$	$(1.82\pm0.32)x10^{3}$
10X (A)	$(7.65\pm0.58)x10^{3}$	$(1.13\pm0.12)x10^{3}$	$(5.69\pm0.44)x10^{2}$	$(1.35\pm0.25)x10^{4}$	$(9.52\pm2.08)x10^{2}$	$(3.23\pm0.21)x10^{3}$
10X (B)	$(8.24\pm1.53)x10^{3}$	$(1.24\pm0.31)x10^{3}$	$(6.42\pm0.57)x10^{2}$	$(1.24\pm0.31)x10^{4}$	$(1.03\pm0.35)x10^{3}$	$(2.93\pm0.15)x10^{3}$
100X	$(7.56\pm1.53)x10^{3}$	$(1.72\pm0.21)x10^{3}$	$(8.58\pm0.70)x10^{2}$	$(1.27\pm0.35)x10^{4}$	$(1.44\pm0.35)x10^{3}$	$(3.73\pm0.12)x10^{3}$

Table 5-11. The effect of elevated antibiotic concentrations on culturable Staph/Strep resistant bacteria (Sample Event 3)

Sample	MH+S/S (CFU/100 µL)	MH+S/S+AMP (CFU/100 µL)	MH+S/S+ SMX/TMP (CFU/100 µL)	MH+S/S (CFU/100 µL)	MH+S/S+TET (CFU/100 µL)	MH+S/S+VA (CFU/100 µL)
Primary Effluent	$(9.25\pm1.53)x10^{3}$	$(2.67\pm0.46)x10^{2}$	$(4.79\pm0.75)x10^{2}$	$(5.85\pm1.73)x10^{3}$	$(4.44\pm0.61)x10^{1}$	$(8.46\pm0.50)x10^{2}$
1X	$(7.83\pm3.51)x10^{3}$	$(5.83\pm0.78)x10^{2}$	$(7.40\pm0.30)x10^{2}$	$(8.32\pm0.58)x10^{3}$	$(6.65\pm0.58)x10^{2}$	$(1.65\pm0.18)x10^{3}$
10X (A)	$(6.21\pm1.53)x10^{3}$	$(9.62\pm1.16)x10^{2}$	$(8.59\pm0.46)x10^{2}$	$(8.51\pm2.08)x10^{3}$	$(1.09\pm0.42)x10^{3}$	$(2.36\pm0.23)x10^{3}$
10X (B)	$(6.80\pm2.00)x10^{3}$	$(1.01\pm0.08)x10^{3}$	$(8.16\pm0.31)x10^{2}$	$(8.96\pm1.00)x10^{3}$	$(1.24\pm0.31)x10^{3}$	$(2.01\pm0.64)x10^{3}$
100X	$(5.65\pm0.58)x10^{3}$	$(1.48\pm0.07)x10^{3}$	$(8.73\pm0.32)x10^{2}$	$(9.78\pm2.65)x10^{3}$	$(2.26\pm0.21)x10^{3}$	$(3.16\pm0.31)x10^{3}$

المنارة للاستشارات
www.manaraa.com

Single factor analysis of variance (ANOVA) with post-hoc Tukey's test was performed to determine if the differences between AR resistance rates for the four SBRs were statistically significant. The results are summarized in Appendix C. Significant differences were expected between the 2-, 7-, and 20-day SRTs, but no significant differences were expected between the two reactors operating with a 7-day SRT. This was true for the two latter sample events, which showed no significant difference ($p>0.5$) for the 7-day SRTs for all antibiotics except AMP. In contrast, the first sample event exhibited significant differences, although these samples were collected only three days post-start-up when the microbial communities in the SBRs had not yet stabilized with respect to their corresponding SRTs. For the second and third sample events, when the temperature decreased considerably, the rate of AR decreased for all SRTs. Therefore, no significant differences were observed between the 7-day and 20-day SRTs. However, the AR rates between the 2-day and 20-day SRTs were still significantly different ($p<0.5$). Regression analysis was also performed to compare the rate of antibiotic resistance between predicated and experimental values. The regression analysis simultaneously considered all variables assumed to impact AR, including SRT, influent antibiotic concentrations, and temperature. Figure 5-17 shows the correlation between predicted values and experimental observations of target antibiotic resistance bacteria. According to the results, all the three variables including temperature, SRT, and influent antibiotic concentrations were significant in predicting AMP resistant bacteria ($P<0.5$), while temperature for predicting VA resistant, SRT for predicting TC, and influent antibiotic concentrations for predicting SMX/TMP resistant bacteria were insignificant variables ($p>0.5$). Regression analysis also helped to determine the best coefficient for each variable in each antibiotic. The following equations are presented to predict AR for each antibiotic:

المنارة للاستشارات
www.manaraa.com

1) AMP resistant = 0.5 × (SRT) + 0.5 × (Temp) + 6.5 × (Antibiotic Conc.)

2) SMX/TMP resistant = 0.5 × (SRT) + 0.5 × (Temp)

3) TC resistant = 0.5 × (Temp) + 4.5 × (Antibiotic Conc.)

4) VA resistant = 0.5 × (SRT) + 5.5 × (Antibiotic Conc.)

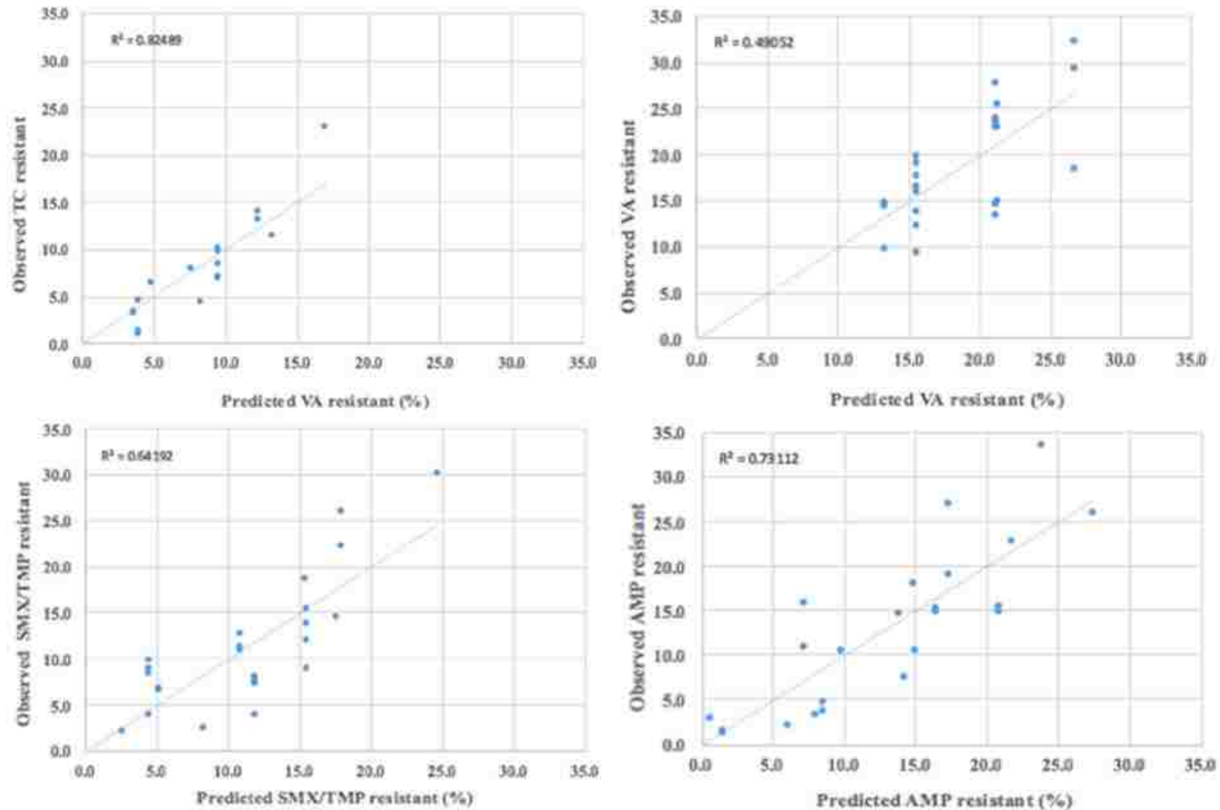


Figure 5-17. Linear correlation between predicted values and experimental observations of target antibiotic resistant bacteria

5.4.7 The effect of varying SRT on minimum inhibitory concentration among antibiotic resistant bacteria

Eight isolates from each of the primary effluent and mixed liquor plates (according to Figure 5-2) were transferred to 2-mL sample tubes containing MH broth with no additional supplements or antibiotics, incubated overnight, and then stored at 4°C until they were shipped on ice to the

المنارة للاستشارات
www.manaraa.com

University of Arizona the following week. Log phase samples of each pure culture were prepared at the University of Arizona and used to perform the MIC assay. This was repeated three times to evaluate the effects of varying SRT on the extent of AR exhibited by the tested isolates. Table 5-12 summarizes the antibiotic concentrations used in the well trays for the MIC assay.

Table 5-12. Comparison of target antibiotic concentrations used in the well trays for the MIC assay

MIC	AMP	SMX/TMP	TET	VA
0X	0	0	0	0
0.5X	16	38/2	8	2
1X	32	76/4	16	4
2X	64	152/8	32	8
4X	128	304/16	64	16
8X	256	608/32	128	32
16X	512	1216/64	256	64
32X	1024	2432/128	512	128

The results of the MIC assays are summarized in Table 5-13 through Table 5-17. Each table indicates (1) the percentage of isolates that were successfully revived at the University of Arizona, (2) the percentage of revived isolates that were antibiotic resistant (i.e., those that grew in the presence of the target antibiotic at concentrations greater than or equal to the CLSI MIC), (3) the observed MICs and corresponding number of isolates, (4) a weighted score for each set of 8 isolates, and (5) an average score and standard deviation for each SRT. The intent of the weighted score was to provide a basis for directly comparing the results from the various SRTs. The weighted score was determined as follows:

$$\textbf{Weighted Score} = \frac{n_1 \times 0.5 + n_2 \times 1 + n_3 \times 2 + n_4 \times 4 + n_5 \times 8 + n_6 \times 16 + n_7 \times 32 + n_8 \times 64}{n_1 + n_2 + n_3 + n_4 + n_5 + n_6 + n_7 + n_8} \quad \textbf{(Eq. 1)}$$

where, n_x = the number of isolates observed at each concentration.

www.manaraa.com

By definition, any isolates inhibited at 0.5x or 1x were assumed to be susceptible to antibiotics. Furthermore, any isolates inhibited at 0.5x were reported to have an MIC “<1x” because they may have been inhibited at concentrations even lower than 0.5X. A weighting factor of 64 was used for isolates that grew at the highest concentration used in the MIC assay. For these isolates, their true MIC is >32x.

In total, 480 isolates were harvested, processed, and shipped to the University of Arizona for the MIC assay. Of the 480 total harvested isolates, 415 (86%) were successfully revived for the MIC assay. The isolates that were pre-screened for resistance to ampicillin (70% revival rate) or sulfamethoxazole/trimethoprim (80% revival rate) were more difficult to revive in MH broth compared to those pre-screened for resistance to tetracycline (98% revival rate) or vancomycin (98% revival rate). 100% the revived isolates proved to be antibiotic resistant. This is expected considering that the isolates were previously grown on agar supplemented with the target antibiotic at the CLSI MIC.

370 isolates (~90% of the total revived isolates) grew in the presence of their respective target antibiotics at concentrations 32 times higher than the MIC. The MICs for the remaining 45 revived isolates were spread relatively evenly throughout the 2x-32x concentration range.

Average weighted scores may have been artificially attenuated due to low revival percentage (13%; 1 isolate) coupled with low observed MIC (2x) in one instance each for the primary effluent and 2-day SRT.

The isolates from the reactor with the 20-day SRT generated the highest average weighted score and the lowest standard deviation when the data for all antibiotics were aggregated, perhaps suggesting a positive correlation between SRT and extent of AR. However, the primary effluent and shorter SRTs were relatively similar to each other and exhibited high standard deviations,

المنارة للاستشارات
www.manaraa.com

which makes a true relationship uncertain. In addition, the data for individual antibiotics demonstrated no clear relationship between extent of AR and SRT. Considering that ~90% of the isolates grew at the highest tested concentration, even higher antibiotic concentrations (i.e., >32x) would have to be tested to get a more precise representation of extent of AR.

المنارة للاستشارات
www.manaraa.com

Table 5-13. Summary of Task 1 MIC Data for Primary Effluent

SRT	Antibiotic	Sample Event	% Revi-ved	% AR	CLSI MIC (µg/mL)	Observed MIC and Number of Isolates <1X	1X	2X	4X	8X	16X	32X	>32X	Weight-ed Score	Avg. Score	St. Dev.	Avg. Score	St. Dev.
Primary Effluent	AMP	1	100%	100%	32	0	0	0	0	0	0	0	8	64.0	58.8	8.9	54.3	19.0
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	50%	100%		0	0	1	0	0	0	0	3	48.5				
	SMX/TMP	1	75%	100%	76/4	0	0	2	1	0	0	0	3	33.3	33.1	31.0		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	13%	100%		0	0	1	0	0	0	0	0	2.0				
	TET	1	100%	100%	16	0	0	1	0	0	0	0	7	56.3	61.4	4.5		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	88%	100%		0	0	0	0	0	0	0	7	64.0				
	VA	1	100%	100%	4	0	0	0	0	0	0	0	8	64.0	64.0	0.0		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				

Table 5-14. Summary of Task 1 MIC Data for 2-Day SRT (Varying SRT)

SRT (d)	Antibiotic	Sample Event	% Revi-ved	% AR	CLSI MIC (µg/mL)	Observed MIC and Number of Isolates <1X	1X	2X	4X	8X	16X	32X	>32X	Weight-ed Score	Avg. Score	St. Dev.	Avg. Score	St. Dev.
2	AMP	1	88%	100%	32	0	0	0	0	0	0	0	7	64.0	43.3	35.8	54.8	20.9
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	13%	100%		0	0	1	0	0	0	0	0	2.0				
	SMX/TMP	1	100%	100%	76/4	0	0	0	0	0	0	0	8	64.0	64.0	0.0		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	88%	100%		0	0	0	0	0	0	0	7	64.0				
	TET	1	100%	100%	16	0	0	0	0	0	0	0	8	64.0	64.0	0.0		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				
	VA	1	100%	100%	4	0	0	0	0	0	0	0	8	64.0	48.0	24.3		
		2	100%	100%		0	0	2	1	3	0	0	2	20.0				
		3	100%	100%		0	0	0	0	0	0	1	7	60.0				

المنارة للاستشارات
www.manaraa.com

Table 5-15. Summary of Task 1 MIC Data for 7-Day A SRT (Varying SRT)

SRT (d)	Antibiotic	Sample Event	% Revi-ved	% AR	CLSI MIC (μg/mL)	Observed MIC and Number of Isolates <1X	1X	2X	4X	8X	16X	32X	>32X	Weight-ed Score	Avg. Score	St. Dev.	Avg. Score	St. Dev.
7A	AMP	1	100%	100%	32	0	0	1	0	0	0	0	7	56.3	61.4	4.5	56.1	13.5
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	25%	100%		0	0	0	0	0	0	0	2	64.0				
	SMX/TMP	1	100%	100%	76/4	0	0	0	0	0	0	0	8	64.0	57.1	11.9		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	38%	100%		0	0	1	0	0	0	0	2	43.3				
	TET	1	88%	100%	16	0	0	1	0	0	0	0	6	55.1	59.0	4.5		
		2	100%	100%		0	0	0	0	0	1	0	7	58.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				
	VA	1	100%	100%	4	0	0	2	0	0	0	0	6	48.5	41.7	26.4		
		2	100%	100%		0	0	2	4	0	1	0	1	12.5				
		3	75%	100%		0	0	0	0	0	0	0	6	64.0				

Table 5-16. Summary of Task 1 MIC Data for 7-Day B SRT (Varying SRT)

SRT (d)	Antibiotic	Sample Event	% Revi-ved	% AR	CLSI MIC (μg/mL)	Observed MIC and Number of Isolates <1X	1X	2X	4X	8X	16X	32X	>32X	Weight-ed Score	Avg. Score	St. Dev.	Avg. Score	St. Dev.
7B	AMP	1	50%	100%	32	0	0	0	0	0	0	0	4	64.0	58.8	8.9	56.8	11.5
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	50%	100%		0	0	1	0	0	0	0	3	48.5				
	SMX/TMP	1	88%	100%	76/4	0	0	0	0	0	0	0	7	64.0	57.1	11.9		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	75%	100%		0	0	2	0	0	0	0	4	43.3				
	TET	1	100%	100%	16	0	0	2	1	0	0	0	5	41.0	56.3	13.3		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				
	VA	1	100%	100%	4	0	0	3	0	0	0	0	5	40.8	45.3	16.8		
		2	100%	100%		0	0	1	0	1	3	0	3	31.3				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				

www.manaraa.com

Table 5-17. Summary of Task 1 MIC Data for 20-Day SRT (Varying SRT)

SRT (d)	Antibiotic	Sample Event	% Revi-ved	% AR	CLSI MIC (µg/mL)	Observed MIC and Number of Isolates								Weight-ed Score	Avg. Score	St. Dev.	Avg. Score	St. Dev.
						<1X	1X	2X	4X	8X	16X	32X	>32X					
20	AMP	1	38%	100%	32	0	0	0	0	0	0	0	3	64.0	64.0	0.0	61.8	4.6
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	38%	100%		0	0	0	0	0	0	0	3	64.0				
	SMX/TMP	1	75%	100%	76/4	0	0	0	0	0	0	0	6	64.0	64.0	0.0		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	50%	100%		0	0	0	0	0	0	0	4	64.0				
	TET	1	100%	100%	16	0	0	0	0	0	0	1	7	60.0	62.7	2.3		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				
	VA	1	100%	100%	4	0	0	1	0	1	0	0	6	49.3	56.6	7.4		
		2	100%	100%		0	0	0	1	0	0	0	7	56.5				
		3	88%	100%		0	0	0	0	0	0	0	7	64.0				

www.manaraa.com

5.4.8 The effect of elevated antibiotic concentrations on minimum inhibitory concentration among antibiotic resistant bacteria

Similar to the previous phase (MIC assay for varying SRT), the results were analyzed and the weighted score for each antibiotic concentration were reported. In total, 480 isolates were harvested, processed, and shipped to the University of Arizona for the MIC assay. Of the 480 total harvested isolates, 456 (95%) were successfully revived for the MIC assay. The isolates that were pre-screened for resistance to ampicillin (93% revival rate) or sulfamethoxazole/trimethoprim (93% revival rate) were more difficult to revive in MH broth compared to those prescreened for resistance to tetracycline (97% revival rate) or vancomycin (98% revival rate). This is consistent with Task 1. However, the differences in Task 2 were minimal, and the revival rates in Task 2 were significantly higher. 3 out of 456 revived isolates (0.7%) were found to be susceptible to antibiotics, specifically two isolates for ampicillin and one isolate for vancomycin, at or below the standard MIC. In comparison, the antibiotic resistance rate was 100% for the revived isolates in Task 1. Although 100% resistance was expected in Task 2 as well, the three isolates represent a very small fraction of the population.

438 isolates (~96% of the total revived isolates) grew in the presence of their respective target antibiotics at concentrations 32 times higher than the MIC. The MICs for the remaining 18 isolates were spread relatively evenly throughout the 0.5x-32x concentration range.

In this phase, the primary effluent exhibited the highest weighted score. This suggests the Staph/Strep in the primary effluent were characterized by the greatest extent of AR and that biological treatment did not promote resistance to higher concentrations of antibiotics. This contradicts the results in varying SRT experiment. Also, due to the relatively consistent growth

www.manaraa.com

even at 32x, it was not possible to reliably differentiate the MICs in the four SBRs. Again, concentrations higher than 32x would have to be tested to better characterize the effects of different operational conditions, such as influent antibiotic concentrations.

Consistent with previous phase (MIC assay with varying SRT), vancomycin exhibited the lowest average weighted score after aggregating all of the samples. Therefore, vancomycin proved to be the most effective antibiotic, although the level of resistance was still high.

المنارة للاستشارات
www.manaraa.com

Table 5-18. Summary of Task 2 MIC Data for Primary Effluent

Sam-ple	Antibiotic	Sample Event	% Revi-ved	% AR	CLSI MIC	Observed MIC and Number of Isolates								Weight-ed Score	Avg. Score	St. Dev.	Avg. Score	St. Dev.
					(µg/mL)	<1X	1X	2X	4X	8X	16X	32X	>32X					
Primary Effluent	AMP	1	100%	100%	32	0	0	0	0	0	0	0	8	0	64.0	0.0	63.7	1.2
		2	88%	100%		0	0	0	0	0	0	0	7	0				
		3	100%	100%		0	0	0	0	0	0	0	8	0				
	SMX/TMP	1	100%	100%	76/4	0	0	0	0	0	0	1	7	0	62.7	2.3		
		2	100%	100%		0	0	0	0	0	0	0	8	0				
		3	88%	100%		0	0	0	0	0	0	0	7	0				
	TET	1	100%	100%	16	0	0	0	0	0	0	0	8	0	64.0	0.0		
		2	75%	100%		0	0	0	0	0	0	0	6	0				
		3	100%	100%		0	0	0	0	0	0	0	8	0				
	VA	1	100%	100%	4	0	0	0	0	0	0	0	8	0	64.0	0.0		
		2	100%	100%		0	0	0	0	0	0	0	8	0				
		3	88%	100%		0	0	0	0	0	0	0	7	0				

Table 5-19. Summary of Task 2 MIC Data for 1X Antibiotic Concentrations

Sam-ple	Antibiotic	Sample Event	% Revi-ved	% AR	CLSI MIC	Observed MIC and Number of Isolates								Weight-ed Score	Avg. Score	St. Dev.	Avg. Score	St. Dev.
					(µg/mL)	<1X	1X	2X	4X	8X	16X	32X	>32X					
1X	AMP	1	88%	100%	32	0	0	0	0	0	0	0	7	64.0	61.0	5.2	61.3	5.0
		2	88%	86%		1	0	0	0	0	0	0	6	54.9				
		3	75%	100%		0	0	0	0	0	0	0	6	64.0				
	SMX/TMP	1	100%	100%	76/4	0	0	0	0	0	0	0	8	64.0	64.0	0.0		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				
	TET	1	100%	100%	16	0	0	0	0	0	0	0	8	64.0	64.0	0.0		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				
	VA	1	100%	88%	4	0	1	0	0	0	0	1	6	52.1	56.0	6.9		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	100%	100%		0	0	0	0	0	2	0	6	52.0				

المنارة للاستشارات
www.manaraa.com

Table 5-20. Summary of Task 2 MIC Data for 10X (A) Antibiotic Concentrations

Sample	Antibiotic	Sample Event	% Revived	% AR	CLSI MIC (µg/mL)	Observed MIC and Number of Isolates <1X	1X	2X	4X	8X	16X	32X	>32X	Weighted Score	Avg. Score	St. Dev.	Avg. Score	St. Dev.
10X (A)	AMP	1	100%	100%	32	0	0	0	0	0	0	0	8	64.0	64.0	0.0	61.3	6.3
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				
	SMX/TMP	1	100%	100%	76/4	0	0	0	0	0	0	0	8	64.0	64.0	0.0		
		2	88%	100%		0	0	0	0	0	0	0	7	64.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				
	TET	1	88%	100%	16	0	0	1	1	0	0	0	5	46.6	58.2	10.1		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				
	VA	1	100%	100%	4	0	0	0	2	0	0	0	6	49.0	59.0	8.7		
		2	88%	100%		0	0	0	0	0	0	0	7	64.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				

Table 5-21. Summary of Task 2 MIC Data for 10X (B) Antibiotic Concentrations

Sample	Antibiotic	Sample Event	% Revived	% AR	CLSI MIC (µg/mL)	Observed MIC and Number of Isolates <1X	1X	2X	4X	8X	16X	32X	>32X	Weighted Score	Avg. Score	St. Dev.	Avg. Score	St. Dev.
10X (B)	AMP	1	100%	100%	32	0	0	0	0	0	0	0	8	64.0	56.5	13.0	61.7	6.5
		2	100%	88%		1	0	0	1	1	0	0	5	41.6				
		3	88%	100%		0	0	0	0	0	0	0	7	64.0				
	SMX/TMP	1	75%	100%	76/4	0	0	0	0	0	0	0	6	64.0	64.0	0.0		
		2	75%	100%		0	0	0	0	0	0	0	6	64.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				
	TET	1	100%	100%	16	0	0	0	0	0	0	0	8	64.0	64.0	0.0		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				
	VA	1	100%	100%	4	0	0	0	0	0	0	0	8	64.0	62.5	2.6		
		2	88%	100%		0	0	0	0	0	0	1	6	59.4				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				

المنارة للاستشارات
www.manaraa.com

Table 5-22. Summary of Task 2 MIC Data for 100X Antibiotic Concentrations

Sam-ple	Antibiotic	Sample Event	% Revi-ved	% AR	CLSI MIC (µg/mL)	Observed MIC and Number of Isolates								Weight-ed Score	Avg. Score	St. Dev.	Avg. Score	St. Dev.
						<1X	1X	2X	4X	8X	16X	32X	>32X					
100X	AMP	1	75%	100%	32	0	0	0	0	0	0	0	6	64.0	64.0	0.0	61.5	4.9
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	88%	100%		0	0	0	0	0	0	0	7	64.0				
	SMX/TMP	1	75%	100%	76/4	0	0	0	0	0	0	0	6	64.0	61.4	4.5		
		2	100%	100%		0	0	1	0	0	0	0	7	56.3				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				
	TET	1	100%	100%	16	0	0	0	0	0	0	0	8	64.0	64.0	0.0		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	88%	100%		0	0	0	0	0	0	0	7	64.0				
	VA	1	100%	100%	4	0	0	1	1	0	0	0	6	48.8	56.6	7.6		
		2	100%	100%		0	0	0	0	1	0	0	7	57.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				

المنارة للاستشارات
www.manaraa.com

5.5 Conclusions

Solids retention time (SRT) is one of the most important factors in designing and operating activated sludge systems for biological wastewater treatment. Longer SRTs have been shown to alter the structure and function of microbial communities, thereby leading to improved treatment efficacy with respect to bulk and trace organics, nutrient removal, and membrane fouling. However, research has also shown that longer SRTs lead to increased prevalence of antibiotic resistant bacteria, perhaps due to increased exposure to antibiotics present in influent wastewater. In this study, the goal was to develop a baseline understanding of antibiotics and antibiotic resistant bacteria during wastewater treatment. According to the results, longer SRTs are associated with lower TOrCs concentrations and higher antibiotic resistance rate. First of all, it should be noted that any changes in the rate of AR in biological treatment systems does not necessarily indicates higher rate of AR in the product water. In fact, most of the MLSS are separated from wastewater through clarifiers. Then, recycled water is subjected to advanced treatment processes (e.g., disinfection, filtration) in many WWTPs. Furthermore, focusing on antibiotic resistance does not emphasize its greater importance over TOrCs. In fact, the results showed that biological treatment systems even in low SRTs select for AR. Therefore, more research projects need to be done to explore the risks associated with AR and TOrCs. In ideal scenario, research projects may focus on alternative approaches that minimize the proliferation of AR and maximize the removal of TOrCs removal. The generated results from this study can be used for further investigation of the safety of treated municipal wastewater.

المنارة للاستشارات
www.manaraa.com

6.0 CONCLUSIONS

According to the existing literature, biological treatment systems in WWTPs are considered significant reservoirs of AR. The results from this study provide further support for that statement and even suggest that biological treatment systems select for ARBs. This was concluded because of the higher rate of ARBs in biological reactors compared to the rates in primary effluent. SRT also plays an important role in the fate of ARBs in biological treatment systems. Longer SRTs are favorable since they can lead to reductions in trace organic compound (TOrC) and nutrient concentrations. However, according to the results from this study, employing longer SRTs may contribute to the proliferation of antibiotic resistance. Further studies are needed in this area to identify the optimum SRT, which simultaneously addresses both concerns (i.e., maximize TOrC removal while minimizing AR proliferation). It should be noted again that the outcomes of this study aimed to develop a baseline understanding of antibiotics and antibiotic resistant bacteria during wastewater treatment. Although this work explained their fate during biological treatment, it does not address the human health impacts of the use of recycled water or the presence of antibiotic resistant bacteria in the product water. However, the generated results can be used for further investigation of the safety of treated municipal wastewater.

Despite efforts to understand the fate of antibiotic resistance in WWTPs, specifically in biological treatment systems, there are still many uncertainties regarding this issue. Contradictory outcomes may arise when studies focus on different influent wastewater qualities, different treatment technologies and/or operational conditions, and even different methodologies for assessment of microbial community structure. The results from Chapter 3 actually proved that the understanding of antibiotic resistance patterns is more complicated than expected.

المنارة للاستشارات
www.manaraa.com

Considering that longer SRTs are associated with higher rates of cell death and decay and higher concentrations of cellular debris, the resulting dissolved intracellular components, such as thymine or thymidine, may be used by bacteria to negate the bacteriostatic effects of some antibiotics, including TMP and SMX. Therefore, free thymine/thymidine in environmental samples may still result in overestimation of AR prevalence unless bacteria are separated from their matrix before assay (e.g., with membrane filtration). More studies are recommended in this area to identify possible compounds that negate the effects of antibiotics, which may lead to overestimation of AR prevalence.

With respect to the microbial community in biological treatment systems, there is strong evidence that SRT impacts microbial biodiversity. However, it is still unclear whether the higher rates of antibiotic resistance observed at longer SRTs are caused by changes in microbial community structure and/or changes in the composition of the wastewater matrix. With increasing rates of antibiotic production and consumption or due to accidental releases, it is quite possible that antibiotic concentrations in raw wastewaters will increase over time. At first, it was hypothesized that higher concentrations of antibiotics would increase the risk of biological treatment failure and adversely impact microbial community structure. However, the results actually showed that higher antibiotic concentrations had minimal effects on the performance of the biological reactors and microbial community structure, but higher rates of antibiotic resistant bacteria.

المنارة للاستشارات
www.manaraa.com

7.0 APPENDIX A

المنارة للاستشارات
www.manaraa.com

Table A1. Summary of TOrC concentrations in the primary effluent and secondary effluent from the SBRs as a function of SRT.

Day	Sample / SRT	TMP (ng/L)	%[a] Removal	SMX (ng/L)	%[a] Removal	Atenolol (ng/L)	%[a] Removal
1	PE	380	--	990	--	880	--
	PE (duplicate)	370	--	970	--	830	--
	2 days	370	1	1,100	-12	790	8
	2 days (duplicate)	330	12	1,000	-2	710	17
	7 days (reactor A)	420	-12	1,400	-43	480	44
	7 days (reactor B)	420	-12	1,500	-53	320	63
	20 days	120	68	1,300	-33	71	92
2	PE	530	--	1,100	--	1,100	--
	PE (duplicate)	710	--	1,300	--	1,100	--
	2 days	500	19	1,100	8	960	13
	7 days (reactor A)	470	24	1,200	0	440	60
	7 days (reactor B)	410	34	1,400	-17	300	73
	20 days	180	71	1,200	0	110	90
	20 days (duplicate)	180	71	1,200	0	110	90

[a] % removal calculated based on average PE concentration for each day

المنارة للاستشارات
www.manaraa.com

Table A2. ANOVA and Tukey's test for manual augmentation of thymidine with reagent-grade chemical.

	Thymidine Addition			
	(1)_TRB	(2)_TRB in 20 µg/mL	(3)_TRB in 60 µg/mL	(4)_TRB in 100 µg/mL
Trial 1	12%	21%	33%	48%
Trial 2	10%	16%	29%	54%
Trial 3	11%	21%	31%	49%
Trial 4	6%	15%	26%	39%
Trial 5	11%	21%	22%	44%
Trial 6	11%	19%	26%	44%
Trial 7	8%	16%	24%	46%
Trial 8	15%	19%	31%	38%
Trial 9	13%	21%	25%	38%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1)_TRB	9	0.97	0.1077778	0.000694444
(2)_TRB in 20 µg/mL	9	1.69	0.1877778	0.000619444
(3)_TRB in 60 µg/mL	9	2.47	0.2744444	0.001377778
(4)_TRB in 100 µg/ml	9	4	0.4444444	0.003002778

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.562075	3	0.1873583	131.6078049	4.44E-18	2.90112
Within Groups	0.0455556	32	0.0014236			
Total	0.6076306	35				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.08	0.08365	Means Not Significantly Different
(1) to (3)	0.1666667	0.08365	Means Significantly Different
(1) to (4)	0.3366667	0.08365	Means Significantly Different
(2) to (3)	0.0866667	0.08365	Means Significantly Different
(2) to (4)	0.2566667	0.08365	Means Significantly Different
(3) to (4)	0.17	0.08365	Means Significantly Different

Table A3. ANOVA and Tukey's test for manual augmentation of thymidine via cell lysing.

	Post-Sonication Filtrate			
	(1)_TRB	(2)_TRB in 0.01% PSF	(3)_TRB in 0.1% PSF	(4)_TRB in 1% PSF
Trial 1	10%	10%	18%	22%
Trial 2	10%	13%	21%	23%
Trial 3	11%	11%	16%	24%
Trial 4	13%	18%	32%	39%
Trial 5	14%	17%	29%	42%
Trial 6	13%	16%	30%	38%
Trial 7	13%	12%	22%	24%
Trial 8	11%	16%	22%	29%
Trial 9	14%	12%	21%	29%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1)_TRB	9	1.081111	0.1201234	0.000302045
(2)_TRB in 0.01% SM	9	1.246131	0.138459	0.000853068
(3)_TRB in 0.1% SM	9	2.104993	0.2338881	0.003122229
(4)_TRB in 1% SM	9	2.686527	0.298503	0.005729452

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.1889859	3	0.0629953	25.18101038	1.48E-08	2.90112
Within Groups	0.0800544	32	0.0025017			
Total	0.2690402	35				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.0183356	0.110889	Means Not Significantly Different
(1) to (3)	0.1137647	0.110889	Means Significantly Different
(1) to (4)	0.1783796	0.110889	Means Significantly Different
(2) to (3)	0.0954291	0.110889	Means Not Significantly Different
(2) to (4)	0.160044	0.110889	Means Significantly Different
(3) to (4)	0.0646149	0.110889	Means Not Significantly Different

المنارة للاستشارات
www.manaraa.com

Table A4. ANOVA and Tukey's test to evaluate the effects of varying MLSS filtrate volumes (from 7-day SRT) on apparent TMP resistance among bacteria from the MLSS operated with a 2-day SRT.

MLSS Filtrate and 2-Day SRT				
	(1)_TRB	*(2)_TRB in 0.01% FM*	*(3)_TRB in 0.1% FM*	*(4)_TRB in 1% FM*
Trial 1	8%	21%	33%	47%
Trial 2	8%	25%	28%	51%
Trial 3	8%	18%	42%	54%
Trial 4	11%	19%	27%	41%
Trial 5	10%	24%	30%	45%
Trial 6	13%	25%	28%	41%
Trial 7	8%	21%	26%	38%
Trial 8	7%	14%	30%	43%
Trial 9	4%	17%	28%	36%

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
(1)_TRB	9	0.77	0.0855556	0.000652778
(2)_TRB in 0.01% FM	9	1.84	0.2044444	0.001452778
(3)_TRB in 0.1% FM	9	2.72	0.3022222	0.002369444
(4)_TRB in 1% FM	9	3.96	0.44	0.003475

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.6091639	3	0.2030546	102.1658514	1.79E-16	2.90112
Within Groups	0.0636	32	0.0019875			
Total	0.6727639	35				

Tukey Test

Comparison	*Absolute Difference*	*Critical Range*	*Results*
(1) to (2)	0.1188889	0.098838	Means Significantly Different
(1) to (3)	0.2166667	0.098838	Means Significantly Different
(1) to (4)	0.3544444	0.098838	Means Significantly Different
(2) to (3)	0.0977778	0.098838	Means Not Significantly Different
(2) to (4)	0.2355556	0.098838	Means Significantly Different
(3) to (4)	0.1377778	0.098838	Means Significantly Different

Table A5. ANOVA and Tukey's test to evaluate the effects of varying MLSS filtrate volumes (from 7-day SRT) on apparent TMP resistance among bacteria from the MLSS operated with a 7-day SRT.

MLSS Filtrate and 7-Day SRT				
	(1)_TRB	*(2)_TRB in 0.01% FM*	*(3)_TRB in 0.1% FM*	*(4)_TRB in 1% FM*
Trial 1	10%	23%	35%	55%
Trial 2	13%	25%	32%	49%
Trial 3	12%	31%	45%	47%
Trial 4	12%	17%	34%	46%
Trial 5	12%	24%	29%	43%
Trial 6	15%	27%	32%	38%
Trial 7	9%	18%	33%	43%
Trial 8	11%	18%	33%	41%
Trial 9	11%	23%	27%	37%

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
(1)_TRB	9	1.05	0.1166667	0.0003
(2)_TRB in 0.01% FM	9	2.06	0.2288889	0.002136111
(3)_TRB in 0.1% FM	9	3	0.3333333	0.002525
(4)_TRB in 1% FM	9	3.99	0.4433333	0.003175

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.5293	3	0.1764333	86.74086719	1.87E-15	2.90112
Within Groups	0.0650889	32	0.002034			
Total	0.5943889	35				

Tukey Test

Comparison	*Absolute Difference*	*Critical Range*	*Results*
(1) to (2)	0.1122222	0.099988	Means Significantly Different
(1) to (3)	0.2166667	0.099988	Means Significantly Different
(1) to (4)	0.3266667	0.099988	Means Significantly Different
(2) to (3)	0.1044444	0.099988	Means Significantly Different
(2) to (4)	0.2144444	0.099988	Means Significantly Different
(3) to (4)	0.11	0.099988	Means Significantly Different

www.manaraa.com

Table A6. ANOVA and Tukey's test to evaluate the effects of varying MLSS filtrate volumes (from 7-day SRT) on apparent TMP resistance among bacteria from the MLSS operated with a 20-day SRT.

	MLSS Filtrate and 20-Day SRT			
	(1)_TRB	(2)_TRB in 0.01% FM	(3)_TRB in 0.1% FM	(4)_TRB in 1% FM
Trial 1	14%	35%	43%	50%
Trial 2	15%	35%	46%	56%
Trial 3	17%	33%	48%	52%
Trial 4	15%	25%	39%	49%
Trial 5	15%	27%	36%	43%
Trial 6	18%	22%	30%	42%
Trial 7	11%	20%	39%	48%
Trial 8	13%	25%	35%	43%
Trial 9	11%	26%	30%	42%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1)_TRB	9	1.29	0.1433333	0.000575
(2)_TRB in 0.01% FM	9	2.48	0.2755556	0.003052778
(3)_TRB in 0.1% FM	9	3.46	0.3844444	0.004127778
(4)_TRB in 1% FM	9	4.25	0.4722222	0.002519444

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.5445556	3	0.1815185	70.66414346	3.31E-14	2.90112
Within Groups	0.0822	32	0.0025688			
Total	0.6267556	35				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.1322222	0.112365	Means Significantly Different
(1) to (3)	0.2411111	0.112365	Means Significantly Different
(1) to (4)	0.3288889	0.112365	Means Significantly Different
(2) to (3)	0.1088889	0.112365	Means Not Significantly Different
(2) to (4)	0.1966667	0.112365	Means Significantly Different
(3) to (4)	0.0877778	0.112365	Means Not Significantly Different

Table A7. ANOVA and Tukey's test to evaluate the effects of varying SRT on apparent TMP resistance.

	SRT Data		
	(1)_MH+TMP(2day)	(2)_MH+TMP(7day)	(3)_MH+TMP(20day)
Trial 1	8%	10%	14%
Trial 2	8%	13%	15%
Trial 3	8%	12%	17%
Trial 4	11%	12%	15%
Trial 5	10%	12%	15%
Trial 6	13%	15%	18%
Trial 7	8%	9%	11%
Trial 8	7%	11%	13%
Trial 9	4%	11%	11%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1)_MH+TMP(2day)	9	0.77	0.08555556	0.00065278
(2)_MH+TMP(7day)	9	1.05	0.11666667	0.0003
(3)_MH+TMP(20day)	9	1.29	0.14333333	0.000575

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.01505185	2	0.00752593	14.7781818	6.5585E-05	3.40282611
Within Groups	0.01222222	24	0.00050926			
Total	0.02727407	26				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.03111111	0.04599213	Means Not Significantly Different
(1) to (3)	0.05777778	0.04599213	Means Significantly Different
(2) to (3)	0.02666667	0.04599213	Means Not Significantly Different

المنارة للاستشارات
www.manaraa.com

Table A8. ANOVA and Tukey's test to evaluate the effects of varying SRT on multi-drug resistance across three separate sample events. Separate ANOVAs were performed for the individual sample events due to the effect of temperature on relative resistance.

TMP/SMX First Event

	(1)_SRT 2 Days (CFU/ 100 µL)	(2)_SRT 7 Days A (CFU/ 100 µL)	(3)_SRT 7 Days B (CFU/ 100 µL)	(4)_SRT 20 Days (CFU/ 100 µL)
Trial 1	19%	25%	22%	31%
Trial 2	18%	27%	24%	27%
Trial 3	19%	26%	21%	33%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1)_SRT 2 Days	3	0.5625	0.1875	6.7E-05
(2)_SRT 7 Days A	3	0.78228	0.26076	5.5E-05
(3)_SRT 7 Days B	3	0.666667	0.222222	0.00019
(4)_SRT 20 Days	3	0.906393	0.302131	0.000665

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.021971	3	0.007324	29.98032	0.000106	4.066181
Within Groups	0.001954	8	0.000244			
Total	0.023926	11				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.07326	0.047104	Means Significantly Different
(1) to (3)	0.034722	0.047104	Means Not Significantly Different
(1) to (4)	0.114631	0.047104	Means Significantly Different
(2) to (3)	0.038538	0.047104	Means Not Significantly Different
(2) to (4)	0.041371	0.047104	Means Not Significantly Different
(3) to (4)	0.079909	0.047104	Means Significantly Different

TMP/SMX Second Event

	(1)_SRT 2 Days (CFU/ 100 µL)	(2)_SRT 7 Days A (CFU/ 100 µL)	(3)_SRT 7 Days B (CFU/ 100 µL)	(4)_SRT 20 Days (CFU/ 100 µL)
Trial 1	2%	14%	12%	14%
Trial 2	3%	12%	11%	14%
Trial 3	3%	13%	10%	15%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1)_SRT 2 Days	3	0.076842	0.025614	7.29E-06
(2)_SRT 7 Days A	3	0.382632	0.127544	0.000139
(3)_SRT 7 Days B	3	0.328302	0.109434	5.7E-05
(4)_SRT 20 Days	3	0.437647	0.145882	5.12E-05

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.025405	3	0.008468	133.2376	3.62E-07	4.066181
Within Groups	0.000508	8	6.36E-05			
Total	0.025913	11				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.10193	0.024027	Means Significantly Different
(1) to (3)	0.08382	0.024027	Means Significantly Different
(1) to (4)	0.120268	0.024027	Means Significantly Different
(2) to (3)	0.01811	0.024027	Means Not Significantly Different
(2) to (4)	0.018338	0.024027	Means Not Significantly Different
(3) to (4)	0.036448	0.024027	Means Significantly Different

TMP/SMX Third Event

	(1)_SRT 2 Days (CFU/ 100 µL)	(2)_SRT 7 Days A (CFU/ 100 µL)	(3)_SRT 7 Days B (CFU/ 100 µL)	(4)_SRT 20 Days (CFU/ 100 µL)
Trial 1	2%	5%	5%	9%
Trial 2	2%	5%	7%	8%
Trial 3	2%	11%	8%	8%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1)_SRT 2 Days	3	0.063054	0.021018	7E-06
(2)_SRT 7 Days A	3	0.204478	0.068159	0.001074
(3)_SRT 7 Days B	3	0.202537	0.067512	0.000135
(4)_SRT 20 Days	3	0.245745	0.081915	2.63E-05

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.006367	3	0.002122	6.829547	0.013469	4.066181
Within Groups	0.002486	8	0.000311			
Total	0.008853	11				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.047141	0.053129	Means Not Significantly Different
(1) to (3)	0.046495	0.053129	Means Not Significantly Different
(1) to (4)	0.060897	0.053129	Means Significantly Different
(2) to (3)	0.000647	0.053129	Means Not Significantly Different
(2) to (4)	0.013756	0.053129	Means Not Significantly Different
(3) to (4)	0.014409	0.053129	Means Not Significantly Different

www.manaraa.com

8.0 APPENDIX B

المنارة للاستشارات
www.manaraa.com

Table B1. Summary of methods for water quality parameters

Measurement	Sampling and Measurement Method	Analysis Method	Sample Container/ Quantity of Sample	Preservation/ Storage	Hold Time
pH	Orion Model 720A pH meter	Standard Method 4500-H B	20 mL glass vials/10 mL	None	Immediate analysis
MLSS	0.45-µm glass fiber filters, 25-mL baking crucibles, 105ºC oven, analytical balance	Standard Methods 2540 D	50 mL centrifuge tube/10 mL	Refrigeration/ Store @ 4±2°C	7 d
MLVSS	0.45-µm glass fiber filters, 25-mL baking crucibles, 550ºC oven, analytical balance	Standard Methods 2540 D,E	50 mL centrifuge tube/10 mL	Refrigeration/ Store @ 4±2°C	7 d
NH_3	Hach DR/5000 spectrophotometer, Salicylate Method	Hach Method 10031	150 mL amber glass bottle/100 µL	HCl addition to pH<2 / Store @ 4±2°C	28 d
NO_3	Hach DR/5000 spectrophotometer, Cadmium Reduction Method	Hach Method 8039	150 mL amber glass bottle/10 mL	Filter / Store @ 4±2°C	48 h
NO_2	Hach DR/5000 spectrophotometer, Diazotization Method	Hach Method 8507	150 mL amber glass bottle/10 mL	Filter / Store @ 4±2°C	48 h
DO	O_2 electrode probe	Standard Method 4500-O G	40 mL glass vials/20 mL	None	Immediate analysis
Soluble COD (sCOD)	Hach DR/5000 spectrophotometer, Reactor Digestion Method	U.S. EPA method 410.4, Hach Method 8000	20 mL glass vials/2 mL	H_2SO_4 addition to pH<2 / Store @ 4±2°C	28 d
Spread Plates	Spread plate on select nutrient media agars	Described in main text	50 mL conical tube/100 µL per plate	None	8 h
TOrCs	LC-MS/MS, API 4000 triple-quadrupole mass spectrometer	Trenholm et al. (2006); Vanderford and Snyder (2006)	500 mL pre-cleaned amber bottle	1 g/L NaN_3 and 50 mg/L Ascorbic Acid / Store @ 4±2°C	28 days

www.manaraa.com

9.0 APPENDIX C

المنارة للاستشارات
www.manaraa.com

Table C1. ANOVA and Tukey's test to evaluate the effects of varying SRT on SMX/TMP resistance.

SMX/TMP Resistant Bacteria First Event

	(1) PE	(2) 2-Day SRT	(3) 7-Day SRT A	(4) 7-Day SRT B	(5) 20-Day SRT
Trial 1	1%	19%	25%	22%	31%
Trial 2	1%	18%	27%	24%	27%
Trial 3	1%	19%	26%	21%	33%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1)_PE	3	0.021149	0.00704981	4.53605E-07
(2)_2-Day SRT	3	0.5625	0.1875	6.69643E-05
(3)_7-Day SRT A	3	0.782243	0.26074768	5.50266E-05
(4)_7-Day SRT B	3	0.666667	0.22222222	0.00018967
(5)_20-Day SRT	3	0.908383	0.30212766	0.00068548

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.155752	4	0.03893801	199.1561904	1.54E-09	3.47805
Within Groups	0.0019551	10	0.00019551			
Total	0.1577072	14				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.1804502	0.037539	Means Significantly Different
(1) to (3)	0.2536979	0.037539	Means Significantly Different
(1) to (4)	0.2151724	0.037539	Means Significantly Different
(1) to (5)	0.2950779	0.037539	Means Significantly Different
(2) to (3)	0.0732477	0.037539	Means Significantly Different
(2) to (4)	0.0347222	0.037539	No Significantly Different
(2) to (5)	0.3021277	0.037539	Means Significantly Different
(3) to (4)	0.0385254	0.037539	Means Significantly Different
(3) to (5)	0.04138	0.037539	Means Significantly Different
(4) to (5)	0.0799054	0.037539	Means Significantly Different

SMX/TMP Resistant Bacteria Second Event

	(1) PE	(2) 2-Day SRT	(3) 7-Day SRT A	(4) 7-Day SRT B	(5) 20-Day SRT
Trial 1	1.5%	2.3%	14.0%	11.7%	14.2%
Trial 2	2.2%	2.6%	11.7%	10.9%	14.1%
Trial 3	1.7%	2.8%	12.6%	10.2%	15.4%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1)_PE	3	0.0534	0.0178	0.00001164
(2)_2-Day SRT	3	0.076842	0.02561404	7.29455E-06
(3)_7-Day SRT A	3	0.382635	0.12754491	0.00013876
(4)_7-Day SRT B	3	0.328302	0.10943396	5.64598E-05
(5)_20-Day SRT	3	0.437647	0.14588235	5.12111E-05

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.0424679	4	0.01061697	199.6647964	1.72E-09	3.47805
Within Groups	0.0005317	10	5.31742E-05			
Total	0.0429996	14				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.0078140	0.019577	No Significantly Different
(1) to (3)	0.1097449	0.019577	Means Significantly Different
(1) to (4)	0.091634	0.019577	Means Significantly Different
(1) to (5)	0.1280824	0.019577	Means Significantly Different
(2) to (3)	0.1019309	0.019577	Means Significantly Different
(2) to (4)	0.0838199	0.019577	Means Significantly Different
(2) to (5)	0.1458824	0.019577	Means Significantly Different
(3) to (4)	0.0181109	0.019577	No Significantly Different
(3) to (5)	0.0183374	0.019577	No Significantly Different
(4) to (5)	0.0364484	0.019577	Means Significantly Different

SMX/TMP Resistant Bacteria Third Event

	(1) PE	(2) 2-Day SRT	(3) 7-Day SRT A	(4) 7-Day SRT B	(5) 20-Day SRT
Trial 1	1.1%	1.8%	5.1%	5.5%	8.8%
Trial 2	1.1%	2.2%	4.8%	7.0%	7.9%
Trial 3	1.2%	2.3%	10.8%	7.8%	8.0%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1)_PE	3	0.034636	0.01154545	1.21488E-06
(2)_2-Day SRT	3	0.063054	0.02101796	7.00276E-06
(3)_7-Day SRT A	3	0.204478	0.0681592	0.00115448
(4)_7-Day SRT B	3	0.202538	0.06751269	0.00013528
(5)_20-Day SRT	3	0.245745	0.08191489	2.63128E-05

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.0119212	4	0.00298029	11.9588968	0.000789	3.47805
Within Groups	0.0024885	10	0.00024885			
Total	0.0144097	14				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.0094725	0.042351	No Significantly Different
(1) to (3)	0.0566137	0.042351	Means Significantly Different
(1) to (4)	0.0559672	0.042351	Means Significantly Different
(1) to (5)	0.0703694	0.042351	Means Significantly Different
(2) to (3)	0.0471412	0.042351	Means Significantly Different
(2) to (4)	0.0464947	0.042351	Means Significantly Different
(2) to (5)	0.0819149	0.042351	Means Significantly Different
(3) to (4)	0.0006465	0.042351	No Significantly Different
(3) to (5)	0.0137557	0.042351	No Significantly Different
(4) to (5)	0.0144022	0.042351	No Significantly Different

المنارة للاستشارات
www.manaraa.com

Table C2. ANOVA and Tukey's test to evaluate the effects of varying SRT on AMP resistance.

AMP Resistant Bacteria: First Event

	(1) PE	(2) 2-Day SRT	(3) 7-Day SRT A	(4) 7-Day SRT B	(5) 20-Day SRT
Trial 1	1%	17%	18%	28%	34%
Trial 2	1%	18%	20%	27%	32%
Trial 3	1%	19%	19%	26%	35%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1) PE	3	0.024943	0.0083142	5.08275E-06
(2) 2-Day SRT	3	0.544643	0.1815476	0.000103104
(3) 7-Day SRT A	3	0.576168	0.1920561	0.000112019
(4) 7-Day SRT B	3	0.813725	0.2712418	3.58836E-05
(5) 20-Day SRT	3	1.005319	0.3351064	0.000285197

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.1813459	4	0.0453365	422.7307094	4.15E-11	3.47805
Within Groups	0.0010725	10	0.0001072			
Total	0.1824184	14				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.1732334	0.027803	Means Significantly Different
(1) to (3)	0.1837419	0.027803	Means Significantly Different
(1) to (4)	0.2629277	0.027803	Means Significantly Different
(1) to (5)	0.3267922	0.027803	Means Significantly Different
(2) to (3)	0.0105085	0.027803	Means Significantly Different
(2) to (4)	0.0896942	0.027803	Means Significantly Different
(2) to (5)	0.3351064	0.027803	Means Significantly Different
(3) to (4)	0.0791858	0.027803	Means Significantly Different
(3) to (5)	0.1430503	0.027803	Means Significantly Different
(4) to (5)	0.0638646	0.027803	Means Significantly Different

AMP Resistant Bacteria: Second Event

	(1) PE	(2) 2-Day SRT	(3) 7-Day SRT A	(4) 7-Day SRT B	(5) 20-Day SRT
Trial 1	2%	2%	4%	3%	10%
Trial 2	1%	3%	5%	4%	10%
Trial 3	1%	2%	5%	4%	11%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1) PE	3	0.042	0.014	0.00000572
(2) 2-Day SRT	3	0.068421	0.022807	1.58818E-05
(3) 7-Day SRT A	3	0.143713	0.0479042	2.04382E-05
(4) 7-Day SRT B	3	0.109434	0.036478	2.25466E-05
(5) 20-Day SRT	3	0.315294	0.105098	5.22953E-06

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.0154381	4	0.0038595	293.2051617	2.57E-10	3.47805
Within Groups	0.0001316	10	1.316E-05			
Total	0.0155697	14				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.008807	0.00974	No Significantly Different
(1) to (3)	0.0339042	0.00974	Means Significantly Different
(1) to (4)	0.022478	0.00974	Means Significantly Different
(1) to (5)	0.091098	0.00974	Means Significantly Different
(2) to (3)	0.0250972	0.00974	Means Significantly Different
(2) to (4)	0.013671	0.00974	Means Significantly Different
(2) to (5)	0.105098	0.00974	Means Significantly Different
(3) to (4)	*0.0114262*	0.00974	*Means Significantly Different*
(3) to (5)	0.0571938	0.00974	Means Significantly Different
(4) to (5)	0.068620	0.00974	Means Significantly Different

AMP Resistant Bacteria: Third Event

	(1) PE	(2) 2-Day SRT	(3) 7-Day SRT A	(4) 7-Day SRT B	(5) 20-Day SRT
Trial 1	0%	1%	1%	1%	4%
Trial 2	0%	1%	1%	2%	3%
Trial 3	0%	1%	1%	2%	3%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1) PE	3	0.011182	0.0037273	5.2231E-07
(2) 2-Day SRT	3	0.026228	0.0087425	5.2376E-06
(3) 7-Day SRT A	3	0.04194	0.0139801	6.0938E-07
(4) 7-Day SRT B	3	0.046751	0.0155838	1.0281E-06
(5) 20-Day SRT	3	0.10133	0.0337766	5.9416E-06

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.0013601	4	0.00034	173.9825078	3.58E-09	3.47805
Within Groups	2.268E-05	10	2.268E-06			
Total	0.0013828	14				

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.0050152	0.004043	Means Significantly Different
(1) to (3)	0.0102528	0.004043	Means Significantly Different
(1) to (4)	0.0118565	0.004043	Means Significantly Different
(1) to (5)	0.0300493	0.004043	Means Significantly Different
(2) to (3)	0.0052376	0.004043	Means Significantly Different
(2) to (4)	0.0068412	0.004043	Means Significantly Different
(2) to (5)	0.0237766	0.004043	Means Significantly Different
(3) to (4)	0.0016037	0.004043	No Significantly Different
(3) to (5)	0.0197965	0.004043	Means Significantly Different
(4) to (5)	0.0181929	0.004043	Means Significantly Different

المنارة للاستشارات
www.manaraa.com

Table C3. ANOVA and Tukey's test to evaluate the effects of varying SRT on TC resistance.

TC Resistant Bacteria, First Event	(1) PE	(2) 2-Day SRT	(3) 7-Day SRT A	(4) 7-Day SRT B	(5) 20-Day SRT
Trial 1	1.2%	6.7%	9.5%	8.9%	10.0%
Trial 2	1.4%	7.4%	10.3%	8.9%	10.4%
Trial 3	1.2%	7.2%	9.5%	7.9%	10.4%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1)_PE	3	0.03725	0.0124183	9.35538E-07
(2)_2-Day SRT	3	0.21282	0.0709402	1.53408E-05
(3)_7-Day SRT A	3	0.29545	0.0984848	2.75482E-05
(4)_7-Day SRT B	3	0.25714	0.0857143	3.82653E-05
(5)_20-Day SRT	3	0.3075	0.1025	4.6875E-06

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.016052	4	0.004013	231.2232385	8.3E-10	3.47805
Within Groups	0.000174	10	1.7356E-05			
Total	0.016226	14				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.058522	0.01118	Means Significantly Different
(1) to (3)	0.086067	0.01118	Means Significantly Different
(1) to (4)	0.073296	0.01118	Means Significantly Different
(1) to (5)	0.090082	0.01118	Means Significantly Different
(2) to (3)	0.027545	0.01118	Means Significantly Different
(2) to (4)	0.014774	0.01118	Means Significantly Different
(2) to (5)	0.1025	0.01118	Means Significantly Different
(3) to (4)	0.012771	0.01118	Means Significantly Different
(3) to (5)	0.004015	0.01118	No Significantly Different
(4) to (5)	0.016786	0.01118	Means Significantly Different

TC Resistant Bacteria, Second Event	(1) PE	(2) 2-Day SRT	(3) 7-Day SRT A	(4) 7-Day SRT B	(5) 20-Day SRT
Trial 1	0.8%	0.9%	1.3%	0.9%	5.7%
Trial 2	0.8%	1.2%	1.5%	1.2%	4.8%
Trial 3	1.0%	1.1%	1.3%	1.1%	4.1%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1)_PE	3	0.02698	0.0089855	1.5753E-06
(2)_2-Day SRT	3	0.03195	0.0106494	3.84551E-06
(3)_7-Day SRT A	3	0.04276	0.0142529	9.90884E-07
(4)_7-Day SRT B	3	0.03196	0.0106542	2.20107E-06
(5)_20-Day SRT	3	0.13985	0.0466167	8.09365E-05

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.003065	4	0.0007662	77.31480048	1.8E-07	3.47805
Within Groups	9.91E-05	10	9.91E-06			
Total	0.003164	14				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.001664	0.00845	No Significantly Different
(1) to (3)	0.005267	0.00845	No Significantly Different
(1) to (4)	0.001669	0.00845	No Significantly Different
(1) to (5)	0.037625	0.00845	Means Significantly Different
(2) to (3)	0.003604	0.00845	No Significantly Different
(2) to (4)	4.85E-06	0.00845	No Significantly Different
(2) to (5)	0.04661	0.00845	Means Significantly Different
(3) to (4)	0.003599	0.00845	No Significantly Different
(3) to (5)	0.032357	0.00845	Means Significantly Different
(4) to (5)	0.035956	0.00845	Means Significantly Different

TC Resistant Bacteria, Third Event	(1) PE	(2) 2-Day SRT	(3) 7-Day SRT A	(4) 7-Day SRT B	(5) 20-Day SRT
Trial 1	0.1%	0.3%	0.3%	0.4%	3.2%
Trial 2	0.1%	0.4%	0.4%	0.5%	2.0%
Trial 3	0.1%	0.4%	0.5%	0.5%	2.1%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1)_PE	3	0.003	0.001	2.36688E-08
(2)_2-Day SRT	3	0.01039	0.0034637	1.77897E-07
(3)_7-Day SRT A	3	0.01328	0.0044269	3.5621E-07
(4)_7-Day SRT B	3	0.01477	0.0049225	2.74923E-07
(5)_20-Day SRT	3	0.07324	0.0244147	4.46304E-05

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.001082	4	0.0002705	29.7464996	1.6E-05	3.47805
Within Groups	9.09E-05	10	9.093E-06			
Total	0.001173	14				

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.002464	0.0081	No Significantly Different
(1) to (3)	0.003427	0.0081	No Significantly Different
(1) to (4)	0.003922	0.0081	No Significantly Different
(1) to (5)	0.023415	0.0081	Means Significantly Different
(2) to (3)	0.000963	0.0081	No Significantly Different
(2) to (4)	0.001459	0.0081	No Significantly Different
(2) to (5)	0.024415	0.0081	Means Significantly Different
(3) to (4)	0.000496	0.0081	No Significantly Different
(3) to (5)	0.019988	0.0081	Means Significantly Different
(4) to (5)	0.019492	0.0081	Means Significantly Different

المنارة للاستشارات
www.manaraa.com

Table C4. ANOVA and Tukey's test to evaluate the effects of varying SRT on VA resistance.

VA Resistant Bacteria First Event

	(1) PE	(2) 2-Day SRT	(3) 7-Day SRT A	(4) 7-Day SRT B	(5) 20-Day SRT
Trial 1	1%	9%	16%	11%	15%
Trial 2	1%	10%	17%	14%	15%
Trial 3	1%	10%	15%	12%	15%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1) PE	3	0.020392	0.0067974	8.97091E-08
(2) 2-Day SRT	3	0.291795	0.097265	2.37563E-05
(3) 7-Day SRT A	3	0.481818	0.1606061	0.000192837
(4) 7-Day SRT B	3	0.367857	0.122619	0.000131803
(5) 20-Day SRT	3	0.45	0.15	1.09375E-05

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.0453026	4	0.0113256	157.5527681	3.51E-09	3.47805
Within Groups	0.0007188	10	7.188E-05			
Total	0.0460214	14				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.0904676	0.022762	Means Significantly Different
(1) to (3)	0.1538087	0.022762	Means Significantly Different
(1) to (4)	0.1158217	0.022762	Means Significantly Different
(1) to (5)	0.1432026	0.022762	Means Significantly Different
(2) to (3)	0.0633411	0.022762	Means Significantly Different
(2) to (4)	0.0253541	0.022762	Means Significantly Different
(2) to (5)	0.15	0.022762	Means Significantly Different
(3) to (4)	0.037987	0.022762	Means Significantly Different
(3) to (5)	0.0106061	0.022762	No Significantly Different
(4) to (5)	0.027381	0.022762	Means Significantly Different

VA Resistant Bacteria Second Event

	(1) PE	(2) 2-Day SRT	(3) 7-Day SRT A	(4) 7-Day SRT B	(5) 20-Day SRT
Trial 1	2%	15%	18%	17%	28%
Trial 2	2%	17%	18%	16%	25%
Trial 3	2%	12%	22%	20%	24%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1) PE	3	0.065217	0.0217391	7.3724E-06
(2) 2-Day SRT	3	0.444156	0.1480519	0.000548467
(3) 7-Day SRT A	3	0.575862	0.191954	0.000479588
(4) 7-Day SRT B	3	0.53271	0.1775701	0.000364224
(5) 20-Day SRT	3	0.767797	0.2559322	0.000415828

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.0893612	4	0.0223403	61.59511898	5.24E-07	3.47805
Within Groups	0.003627	10	0.0003627			
Total	0.0929881	14				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.1263128	0.051129	Means Significantly Different
(1) to (3)	0.1702149	0.051129	Means Significantly Different
(1) to (4)	0.155831	0.051129	Means Significantly Different
(1) to (5)	0.2341931	0.051129	Means Significantly Different
(2) to (3)	0.0439021	0.051129	No Significantly Different
(2) to (4)	0.0295181	0.051129	No Significantly Different
(2) to (5)	0.2559322	0.051129	Means Significantly Different
(3) to (4)	0.0143839	0.051129	No Significantly Different
(3) to (5)	0.0639782	0.051129	Means Significantly Different
(4) to (5)	0.0783621	0.051129	Means Significantly Different

VA Resistant Bacteria Third Event

	(1) PE	(2) 2-Day SRT	(3) 7-Day SRT A	(4) 7-Day SRT B	(5) 20-Day SRT
Trial 1	1%	13%	15%	16%	26%
Trial 2	2%	16%	14%	13%	22%
Trial 3	2%	15%	20%	21%	21%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1) PE	3	0.047949	0.0159829	1.88557E-06
(2) 2-Day SRT	3	0.435754	0.1452514	0.000271711
(3) 7-Day SRT A	3	0.498024	0.1660079	0.000984237
(4) 7-Day SRT B	3	0.5	0.1666667	0.001667568
(5) 20-Day SRT	3	0.692308	0.2307692	0.00070469

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.0747378	4	0.0186844	25.0470325	3.41E-05	3.47805
Within Groups	0.0074597	10	0.000746			
Total	0.0821975	14				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.1292685	0.073325	Means Significantly Different
(1) to (3)	0.150025	0.073325	Means Significantly Different
(1) to (4)	0.1506838	0.073325	Means Significantly Different
(1) to (5)	0.2147863	0.073325	Means Significantly Different
(2) to (3)	0.0207565	0.073325	No Significantly Different
(2) to (4)	0.0214153	0.073325	No Significantly Different
(2) to (5)	0.2307692	0.073325	Means Significantly Different
(3) to (4)	0.0006588	0.073325	No Significantly Different
(3) to (5)	0.0647613	0.073325	No Significantly Different
(4) to (5)	0.0641026	0.073325	No Significantly Different

المنارة للاستشارات
www.manaraa.com

Table C5. ANOVA and Tukey's test to evaluate the effects of varying influent antibiotic concentrations on VA resistance.

VA Resistant Bacteria: First Event

	(1) PE	(2) 1x	(3) 10x A	(4) 10x B	(5) 100x
Trial 1	7%	10%	13%	16%	15%
Trial 2	7%	10%	13%	14%	18%
Trial 3	7%	8%	14%	14%	22%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1) PE	3	0.216457	0.0721524	1.70164E-05
(2) 1x	3	0.281899	0.0939629	0.000133042
(3) 10x A	3	0.403606	0.1345353	2.01109E-05
(4) 10x B	3	0.438869	0.1462896	9.24793E-05
(5) 100x	3	0.556239	0.1854196	0.001102512

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.0238201	4	0.005955	21.8446775	6.27E-05	3.47805
Within Groups	0.0027261	10	0.0002726			
Total	0.0265462	14				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.0218105	0.044326	No Significantly Different
(1) to (3)	0.0623831	0.044326	Means Significantly Different
(1) to (4)	0.0741372	0.044326	Means Significantly Different
(1) to (5)	0.1132672	0.044326	Means Significantly Different
(2) to (3)	0.0405726	0.044326	No Significantly Different
(2) to (4)	0.0523267	0.044326	Means Significantly Different
(2) to (5)	0.1854196	0.044326	Means Significantly Different
(3) to (4)	0.0117541	0.044326	No Significantly Different
(3) to (5)	0.0508841	0.044326	Means Significantly Different
(4) to (5)	0.03913	0.044326	No Significantly Different

VA Resistant Bacteria: Second Event

	(1) PE	(2) 1x	(3) 10x A	(4) 10x B	(5) 100x
Trial 1	11%	17%	25%	25%	29%
Trial 2	10%	12%	22%	23%	30%
Trial 3	11%	13%	24%	23%	30%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1) PE	3	0.320126	0.1067086	7.58286E-06
(2) 1x	3	0.417392	0.1391308	0.000595118
(3) 10x A	3	0.718166	0.2393887	0.000237539
(4) 10x B	3	0.708026	0.2360085	0.000151046
(5) 100x	3	0.880657	0.2935522	8.24359E-05

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.0718941	4	0.0179735	83.69764607	1.2E-07	3.47805
Within Groups	0.0021474	10	0.0002147			
Total	0.0740416	14				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.032422	0.039342	No Significantly Different
(1) to (3)	0.13268	0.039342	Means Significantly Different
(1) to (4)	0.1292999	0.039342	Means Significantly Different
(1) to (5)	0.1868436	0.039342	Means Significantly Different
(2) to (3)	0.100258	0.039342	Means Significantly Different
(2) to (4)	0.0968779	0.039342	Means Significantly Different
(2) to (5)	0.2935522	0.039342	Means Significantly Different
(3) to (4)	0.0033801	0.039342	No Significantly Different
(3) to (5)	0.0541635	0.039342	Means Significantly Different
(4) to (5)	0.0575437	0.039342	Means Significantly Different

VA Resistant Bacteria: Third Event

	(1) PE	(2) 1x	(3) 10x A	(4) 10x B	(5) 100x
Trial 1	14%	22%	25%	19%	32%
Trial 2	15%	19%	29%	20%	36%
Trial 3	14%	19%	29%	31%	30%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1) PE	3	0.434334	0.144778	7.40751E-05
(2) 1x	3	0.597332	0.1991106	0.000496944
(3) 10x A	3	0.834446	0.2781486	0.000706679
(4) 10x B	3	0.691747	0.2305825	0.005145717
(5) 100x	3	0.971177	0.3237256	0.000975408

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.0575438	4	0.0143859	9.735462056	0.001773	3.47805
Within Groups	0.0147768	10	0.0014777			
Total	0.0723206	14				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.0543326	0.103201	No Significantly Different
(1) to (3)	0.1333706	0.103201	Means Significantly Different
(1) to (4)	0.0858045	0.103201	No Significantly Different
(1) to (5)	0.1789477	0.103201	Means Significantly Different
(2) to (3)	0.079038	0.103201	No Significantly Different
(2) to (4)	0.0314719	0.103201	No Significantly Different
(2) to (5)	0.1237256	0.103201	Means Significantly Different
(3) to (4)	0.0475661	0.103201	No Significantly Different
(3) to (5)	0.045577	0.103201	No Significantly Different
(4) to (5)	0.0931432	0.103201	No Significantly Different

المنارة للاستشارات
www.manaraa.com

Table C6. ANOVA and Tukey's test to evaluate the effects of varying influent antibiotic concentrations on SMX/TMP resistance.

SMX/TMP Resistant Bacteria: First Event

	(1) PE	(2) 1x	(3) 10x A	(4) 10x B	(5) 100x
Trial 1	3%	4%	8%	9%	11%
Trial 2	3%	4%	11%	9%	11%
Trial 3	3%	4%	7%	9%	8%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1)_PE	3	0.085071	0.028357	8.34725E-06
(2)_1x	3	0.119077	0.0396923	8.05578E-06
(3)_10x A	3	0.256949	0.0856498	0.000687502
(4)_10x B	3	0.272818	0.0909388	9.92384E-06
(5)_100x	3	0.29552	0.0985068	0.000372105

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.0124178	4	0.0031045	14.3621...	0.000356	3.47805
Within Groups	0.0021319	10	0.0002132			
Total	0.0145497	14				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.0113354	0.039199	No Significantly Different
(1) to (3)	0.0572928	0.039199	Means Significantly Different
(1) to (4)	0.0625818	0.039199	Means Significantly Different
(1) to (5)	0.0701498	0.039199	Means Significantly Different
(2) to (3)	0.0459574	0.039199	Means Significantly Different
(2) to (4)	0.0512465	0.039199	Means Significantly Different
(2) to (5)	0.0588068	0.039199	Means Significantly Different
(3) to (4)	0.005289	0.039199	No Significantly Different
(3) to (5)	0.012857	0.039199	No Significantly Different
(4) to (5)	0.007568	0.039199	No Significantly Different

SMX/TMP Resistant Bacteria: Second Event

	(1) PE	(2) 1x	(3) 10x A	(4) 10x B	(5) 100x
Trial 1	1.9%	5.5%	7.8%	8.4%	11.8%
Trial 2	2.0%	4.8%	7.7%	8.0%	10.3%
Trial 3	2.1%	4.0%	6.8%	7.0%	12.0%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1)_PE	3	0.059316	0.019772	7.94078E-07
(2)_1x	3	0.120828	0.0402759	3.31053E-05
(3)_10x A	3	0.223479	0.074493	3.24515E-05
(4)_10x B	3	0.23415	0.0780501	4.75912E-05
(5)_100x	3	0.341291	0.1137637	8.57444E-05

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.0158844	4	0.0039711	99.43376023	5.22E-08	3.47805
Within Groups	0.0003994	10	3.994E-05			
Total	0.0162838	14				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.0205039	0.016968	Means Significantly Different
(1) to (3)	0.054721	0.016968	Means Significantly Different
(1) to (4)	0.058278	0.016968	Means Significantly Different
(1) to (5)	0.0939919	0.016968	Means Significantly Different
(2) to (3)	0.0342171	0.016968	Means Significantly Different
(2) to (4)	0.0377742	0.016968	Means Significantly Different
(2) to (5)	0.1137637	0.016968	Means Significantly Different
(3) to (4)	0.003557	0.016968	No Significantly Different
(3) to (5)	0.0392707	0.016968	Means Significantly Different
(4) to (5)	0.0357137	0.016968	Means Significantly Different

SMX/TMP Resistant Bacteria: Third Event

	(1) PE	(2) 1x	(3) 10x A	(4) 10x B	(5) 100x
Trial 1	4.4%	9.1%	13.7%	11.9%	16.1%
Trial 2	5.2%	9.3%	14.6%	11.6%	15.1%
Trial 3	6.1%	9.8%	13.2%	12.5%	15.2%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1)_PE	3	0.156721	0.0522402	6.58085E-05
(2)_1x	3	0.283534	0.0945113	1.46808E-05
(3)_10x A	3	0.41516	0.1383868	5.43766E-05
(4)_10x B	3	0.360077	0.1200258	2.01603E-05
(5)_100x	3	0.464028	0.1546759	3.24133E-05

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.0193769	4	0.0048442	129.2212538	1.45E-08	3.47805
Within Groups	0.0003749	10	3.749E-05			
Total	0.0197518	14				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.0422713	0.016438	Means Significantly Different
(1) to (3)	0.0861466	0.016438	Means Significantly Different
(1) to (4)	0.0677856	0.016438	Means Significantly Different
(1) to (5)	0.1024356	0.016438	Means Significantly Different
(2) to (3)	0.0438755	0.016438	Means Significantly Different
(2) to (4)	0.0255145	0.016438	Means Significantly Different
(2) to (5)	0.1546759	0.016438	Means Significantly Different
(3) to (4)	0.018361	0.016438	Means Significantly Different
(3) to (5)	0.016289	0.016438	No Significantly Different
(4) to (5)	0.03465	0.016438	Means Significantly Different

المنارة للاستشارات
www.manaraa.com

Table C7. ANOVA and Tukey's test to evaluate the effects of varying influent antibiotic concentrations on TC resistance.

TC Resistant Bacteria First Event

	(1) PE	(2) 1x	(3) 10x A	(4) 10x B	(5) 100x
Trial 1	1.1%	2.1%	3.6%	3.8%	4.6%
Trial 2	1.4%	2.1%	3.1%	3.0%	4.2%
Trial 3	1.2%	1.7%	3.6%	3.4%	4.5%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1) PE	3	0.037449	0.012483	2.43092E-06
(2) 1x	3	0.060015	0.020005	5.58131E-06
(3) 10x A	3	0.103144	0.034381	6.70363E-06
(4) 10x B	3	0.100247	0.033416	1.05284E-05
(5) 100x	3	0.133196	0.044399	3.36567E-06

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.0019178	4	0.0004795	83.73290234	1.22E-07	3.47805
Within Groups	5.7282E-05	10	5.7282E-06			
Total	0.0019751	14				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.0075219	0.006424	Means Significantly Different
(1) to (3)	0.0218982	0.006424	Means Significantly Different
(1) to (4)	0.0209325	0.006424	Means Significantly Different
(1) to (5)	0.0319155	0.006424	Means Significantly Different
(2) to (3)	0.0143763	0.006424	Means Significantly Different
(2) to (4)	0.0134106	0.006424	Means Significantly Different
(2) to (5)	0.0443985	0.006424	Means Significantly Different
(3) to (4)	0.0009657	0.006424	No Significantly Different
(3) to (5)	0.0100173	0.006424	Means Significantly Different
(4) to (5)	0.010983	0.006424	Means Significantly Different

TC Resistant Bacteria Second Event

	(1) PE	(2) 1x	(3) 10x A	(4) 10x B	(5) 100x
Trial 1	1.7%	7.6%	5.9%	11.3%	11.8%
Trial 2	2.4%	8.3%	8.9%	5.6%	14.2%
Trial 3	1.5%	3.8%	6.7%	8.9%	8.6%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1) PE	3	0.055559	0.018519	2.27488E-05
(2) 1x	3	0.197313	0.065771	0.000595118
(3) 10x A	3	0.214708	0.071569	0.000237535
(4) 10x B	3	0.257464	0.085821	0.000798386
(5) 100x	3	0.345972	0.115324	0.000762551

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.014896	4	0.003724	7.705949509	0.004211	3.47805
Within Groups	0.0048326	10	0.0004833			
Total	0.0197287	14				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.0472512	0.059018	No Significantly Different
(1) to (3)	0.0530501	0.059018	No Significantly Different
(1) to (4)	0.0673016	0.059018	Means Significantly Different
(1) to (5)	0.0968044	0.059018	Means Significantly Different
(2) to (3)	0.005799	0.059018	No Significantly Different
(2) to (4)	0.0200504	0.059018	No Significantly Different
(2) to (5)	0.1153241	0.059018	Means Significantly Different
(3) to (4)	0.0142515	0.059018	No Significantly Different
(3) to (5)	0.0437543	0.059018	No Significantly Different
(4) to (5)	0.0295028	0.059018	No Significantly Different

TC Resistant Bacteria Third Event

	(1) PE	(2) 1x	(3) 10x A	(4) 10x B	(5) 100x
Trial 1	0.8%	8.4%	18.8%	13.4%	22.3%
Trial 2	0.9%	7.2%	9.4%	11.2%	21.5%
Trial 3	0.8%	8.4%	11.8%	17.9%	25.6%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1) PE	3	0.022414	0.0074713	1.09161E-06
(2) 1x	3	0.240375	0.080125	4.81501E-05
(3) 10x A	3	0.399594	0.133198	0.002384207
(4) 10x B	3	0.423974	0.141325	0.001161646
(5) 100x	3	0.695158	0.231719	0.000452868

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.0819547	4	0.0204887	25.24377852	3.3E-05	3.47805
Within Groups	0.0081163	10	0.0008116			
Total	0.090071	14				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.0726537	0.076484	No Significantly Different
(1) to (3)	0.12558	0.076484	Means Significantly Different
(1) to (4)	0.1338538	0.076484	Means Significantly Different
(1) to (5)	0.2242473	0.076484	Means Significantly Different
(2) to (3)	0.0530729	0.076484	No Significantly Different
(2) to (4)	0.0611998	0.076484	No Significantly Different
(2) to (5)	0.2317194	0.076484	Means Significantly Different
(3) to (4)	0.0081268	0.076484	No Significantly Different
(3) to (5)	0.0985215	0.076484	Means Significantly Different
(4) to (5)	0.0903947	0.076484	Means Significantly Different

المنارة للاستشارات
www.manaraa.com

Table C8. ANOVA and Tukey's test to evaluate the effects of varying influent antibiotic concentrations on AMP resistance.

AMP Resistant Bacteria, First Event

	(1) PE	(2) 1x	(3) 10x A	(4) 10x B	(5) 100x
Trial 1	1%	3%	15%	10%	15%
Trial 2	1%	3%	17%	12%	14%
Trial 3	1%	3%	15%	11%	15%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1) PE	3	0.028023	0.00934112	5.84308E-07
(2) 1x	3	0.086894	0.02896468	3.74019E-06
(3) 10x A	3	0.475629	0.15854317	0.000209217
(4) 10x B	3	0.32738	0.10912656	0.000119088
(5) 100x	3	0.443281	0.14776018	7.78825E-05

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.0558858	4	0.01397145	170.1718178	3.77E-09	3.47805
Within Groups	0.000821	10	8.21022E-05			
Total	0.0567068	14				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.0196236	0.024326	No Significantly Different
(1) to (3)	0.149202	0.024326	Means Significantly Different
(1) to (4)	0.0997854	0.024326	Means Significantly Different
(1) to (5)	0.1384191	0.024326	Means Significantly Different
(2) to (3)	0.1295785	0.024326	Means Significantly Different
(2) to (4)	0.0801619	0.024326	Means Significantly Different
(2) to (5)	0.1477602	0.024326	Means Significantly Different
(3) to (4)	0.0494166	0.024326	Means Significantly Different
(3) to (5)	0.010783	0.024326	No Significantly Different
(4) to (5)	0.0386336	0.024326	Means Significantly Different

AMP Resistant Bacteria, Second Event

	(1) PE	(2) 1x	(3) 10x A	(4) 10x B	(5) 100x
Trial 1	8%	10%	16%	12%	23%
Trial 2	9%	13%	13%	15%	24%
Trial 3	6%	9%	16%	19%	20%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1) PE	3	0.234792	0.07826404	0.000160852
(2) 1x	3	0.316453	0.10548442	0.000048278
(3) 10x A	3	0.444344	0.14811475	0.00022773
(4) 10x B	3	0.461021	0.15367374	0.001373762
(5) 100x	3	0.687874	0.22929126	0.000758296

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.0395533	4	0.00988832	16.46182122	0.000213	3.47805
Within Groups	0.0060068	10	0.00060068			
Total	0.0455601	14				

Tukey Test

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.0272204	0.065798	No Significantly Different
(1) to (3)	0.0698507	0.065798	Means Significantly Different
(1) to (4)	0.0754097	0.065798	Means Significantly Different
(1) to (5)	0.1510272	0.065798	Means Significantly Different
(2) to (3)	0.0426303	0.065798	No Significantly Different
(2) to (4)	0.0481893	0.065798	No Significantly Different
(2) to (5)	0.2292913	0.065798	Means Significantly Different
(3) to (4)	*0.055589*	0.065798	No Significantly Different
(3) to (5)	0.0811765	0.065798	Means Significantly Different
(4) to (5)	0.0756175	0.065798	Means Significantly Different

AMP Resistant Bacteria, Third Event

	(1) PE	(2) 1x	(3) 10x A	(4) 10x B	(5) 100x
Trial 1	3%	6%	19%	16%	25%
Trial 2	3%	9%	14%	14%	27%
Trial 3	2%	8%	15%	15%	27%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
(1) PE	3	0.087547	0.02918247	2.45322E-05
(2) 1x	3	0.224784	0.07492803	9.84154E-05
(3) 10x A	3	0.466653	0.15555107	0.000347837
(4) 10x B	3	0.44828	0.1494198	0.000126002
(5) 100x	3	0.784596	0.26153208	0.000145338

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.0943284	4	0.02358209	158.8823205	5.28E-09	3.47805
Within Groups	0.0014842	10	0.00014842			
Total	0.0958126	14				

Comparison	Absolute Difference	Critical Range	Results
(1) to (2)	0.0457456	0.032707	Means Significantly Different
(1) to (3)	0.1263686	0.032707	Means Significantly Different
(1) to (4)	0.1202374	0.032707	Means Significantly Different
(1) to (5)	0.2323496	0.032707	Means Significantly Different
(2) to (3)	0.080623	0.032707	Means Significantly Different
(2) to (4)	0.0744919	0.032707	Means Significantly Different
(2) to (5)	0.2615321	0.032707	Means Significantly Different
(3) to (4)	0.0061312	0.032707	No Significantly Different
(3) to (5)	0.105981	0.032707	Means Significantly Different
(4) to (5)	0.1121122	0.032707	Means Significantly Different

www.manaraa.com

10.0 REFERENCES

Abdel-el-haleem, D., 2003. Minireview Acinetobacter : environmental and biotechnological applications. African J. Biotechnol. 2, 71–74. doi:10.4314/ajb.v2i4.14828

Ahmed, Z., Cho, J., Lim, B.-R., Song, K.-G., Ahn, K.-H., 2007. Effects of sludge retention time on membrane fouling and microbial community structure in a membrane bioreactor. J. Memb. Sci. 287, 211–218. doi:10.1016/j.memsci.2006.10.036

Alcaide, E., Garay, E. 1984. R. Plasmid transfer in salmonella spp. isolated from wastewater and sewage-contaminated surface waters. Appl. Environ. Microbiol. 48, 435-438.

Alonso, A., Sanchez, P., Martinez, J, L. 2001. Environmental selection of antibiotic resistance genes. Environmental Microbiology, Vol. 3., Issue 1., 1-9.

Amador, P.P., Fernandes, R.M., Prudêncio, M.C., Barreto, M.P., Duarte, I.M., 2015. Antibiotic resistance in wastewater: Occurrence and fate of Enterobacteriaceae producers of Class A and Class C β-lactamases. J. Environ. Sci. Health. A. Tox. Hazard. Subst. Environ. Eng. 50, 26–39. doi:10.1080/10934529.2015.964602

Amann, R.I., Ludwig, W., Schleifer, K.H. 1995. Phylogenetic Identification and In Situ Detection of Individual Microbial Cells without Cultivation. Microbiol. Rev.59. 143-170.

Aminov, R.I., Garrigues-Jeanjean, N., Mackie, R.I., 2001. Molecular ecology of tetracycline resistance: Development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. Appl. Environ. Microbiol. 67, 22–32. doi:10.1128/AEM.67.1.22-32.2001

Amyes, S.G.B., Smith, J.T. 1974. Trimethoprim-sensitivity testing and thymineless mutants. Journal of Medical Microbiology 7, 143-153.

المنارة للاستشارات
www.manaraa.com

Anderl, Jeff N., Michael J. Franklin, and Philip S. Stewart. 2000. Role of antibiotic penetration limitation in klebsiella pneumoniae biofilm resistance to ampicillin and ciprofloxacin. Antimicrobial Agents and Chemotherapy 44 (7): 1818-24.

Ang, W.S., Elimelech, M., 2007. Protein (BSA) fouling of reverse osmosis membranes: Implications for wastewater reclamation. J. Memb. Sci. 296, 83–92. doi:10.1016/j.memsci.2007.03.018

Antibiotic resistance: how serious is the problem, and what can be done? Clinical Chemistry, 1182-1186. Antimicrob. Chemother. 43(1), 1-4.

Anwar, H., J. L. Strap, and J. W. Costerton. 1992. Kinetic interaction of biofilm cells of staphylococcus aureus with cephalexin and tobramycin in a chemostat system. Antimicrobial Agents and Chemotherapy 36 (4): 890-3.

Auerbach, E.A., Seyfried, E.E., McMahon, K.D., 2007. Tetracycline resistance genes in activated sludge wastewater treatment plants. Water Res. 41, 1143–1151. doi:10.1016/j.watres.2006.11.045

Baquero, F., Martínez, J.L., Cantón, R., 2008. Antibiotics and antibiotic resistance in water environments. Curr. Opin. Biotechnol. doi:10.1016/j.copbio.2008.05.006

Beales, N., 2004. Adaptation of Microorganisms to Cold Temperatures, Weak Acid Preservatives, Low pH, and Osmotic Stress: A Review. Compr. Rev. Food Sci. Food Saf. 3, 1–20. doi:10.1111/j.1541-4337.2004.tb00057.x

Blair, J.M.A., Webber, M.A., Baylay, A.J., Ogbolu, D.O., Piddock, L.J.V. 2015. Molecular mechanisms of antibiotic resistance. Nature. 13, 42-51.

Bushby, S.R.M., Hitchings, G.H. 1968. Trimethoprim, A Sulphonamide Potentiator. British Journal of Pharmacology and chemotherapy. 33 (1), 72-90.

المنارة للاستشارات
www.manaraa.com

Canales, A., Pareilleux, A., Rols, J.L., Goma, G., Huyard, A., 1994. Decreased sludge production strategy for domestic wastewater treatment, in: Water Science and Technology. pp. 97–106.

Chadha, Tejpreet. 2014. Bacterial biofilms: Survival mechanisms and antibiotic resistance. Journal of Bacteriology & Parasitology 5 (3): 1.

Chen, H., Zhang, M., 2013. Effects of advanced treatment systems on the removal of antibiotic resistance genes in wastewater treatment plants from Hangzhou, China. Environ. Sci. Technol. 55, 8157–8153. doi:10.1016/j.envint.2013.01.019

Chen, S. H. Aitken, M.D. 1999. Salicylate stimulates the degradation of high-molecular weight polycyclic aromatic hydrocarbons by pseudomonas saccharophila P15. Environmental Science and Technology 33 (3): 435.

Chen, W., Westerhoff, P., Leenheer, J.A., Booksh, K., 2003. Fluorescence Excitation-Emission Matrix Regional Integration to Quantify Spectra for Dissolved Organic Matter. Environ. Sci. Technol. 37, 5701–5710. doi:10.1021/es034354c

Christian, E., Batista, J.R., Gerrity, D., 2016. Use of COD, TOC, and Fluorescence Spectroscopy to Estimate BOD in Wastewater. Water Environ. Res. doi:10.2175/106143016X14504669768976

Clara, M., Gans, O., Windhofer, G., Krenn, U., Hartl, W., Braun, K., Scharf, S., Scheffknecht, C., 2011. Occurrence of polycyclic musks in wastewater and receiving water bodies and fate during wastewater treatment. Chemosphere 82, 1116–1123. doi:10.1016/j.chemosphere.2010.11.041

Clara, M., Strenn, B., Gans, O., Martinez, E., Kreuzinger, N., Kroiss, H., 2005. Removal of selected pharmaceuticals, fragrances and endocrine disrupting compounds in a membrane

المنارة للاستشارات
www.manaraa.com

bioreactor and conventional wastewater treatment plants. Water Res. 39, 4797–4807.

CLSI. 2012. Performance standards for antimicrobial susceptibility testing. Twenty-fifth information supplement. Clinical Laboratory and Standards Institute. M100-S25.

Cochran, W. L., G. A. McFeters, and P. S. Stewart. 2000. Reduced susceptibility of thin pseudomonas aeruginosa biofilms to hydrogen peroxide and monochloramine. Journal of Applied Microbiology 88 (1): 22-30.

Collado, L., Levican, A., Perez, J., Figueras, M.J., 2011. Arcobacter defluvii sp. nov., isolated from sewage samples. Int. J. Syst. Evol. Microbiol. 61, 2155–2161. doi:10.1099/ijs.0.025668-0

Colomer-Lluch, M., Imamovic, L., Jofre, J., Muniesa, M. 2011. Antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples. PLos One. 6: e17549.

Costa, P.M., VAz-Pires, P., Bernardo, F. 2006. Antimicrobial resistance in Enterococcus spp. Isolated in inflow, effluent and sludge from municipal sewage water treatment plants. Water Res, 1735-1740.

Coutu, S., Rossi, L., Barry, D.A., Rudaz, S., Vernaz, N., 2013. Temporal Variability of Antibiotics Fluxes in Wastewater and Contribution from Hospitals. PLoS One 8. doi:10.1371/journal.pone.0053592

Dancer, S.J., Shears, P., Platt, D.J. 1997. Isolation and characterization of coliforms from glacial ice and water in Canada's high arctic. J. Appl. Microbiol. 82, 597-609.

Davies, J, Davies, D. 2010. Origins and Evolution of antibiotic Resistance. Microbiology and Molecular Biology Reviews, 417-433.

de Beer, D., P. Stoodley, F. Roe, and Z. Lewandowski. 1994. Effects of biofilm structures on oxygen distribution and mass transport. Biotechnology and Bioengineering 43 (11): 1131-8.

www.manaraa.com

Dodd, M.C. 2012. Potential impacts of disinfection processes on elimination and deactivation of antibiotic resistance gene during water and wastewater treatment. J. Environ. Monit. 14, 1754.

Espigares, E., Bueno, A., Espigares, M., Gálvez, R., 2006. Isolation of Salmonella serotypes in wastewater and effluent: Effect of treatment and potential risk. Int. J. Hyg. Environ. Health 209, 103–107.

Fahrenfeld, N., Ma, Y., O'Brien, M., Pruden, A. 2013. Reclaimed water as a reservoir of antibiotic resistance genes: Distribution system and irrigation implications.

Farias, E.L., Howe, K.J., Thomson, B.M., 2014. Effect of membrane bioreactor solids retention time on reverse osmosis membrane fouling for wastewater reuse. Water Res. 49, 53–61. doi:10.1016/j.watres.2013.11.006

Gao, P., Munir, M., Xagoraraki, I., 2012. Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. Sci. Total Environ. 421–422, 173–183. doi:10.1016/j.scitotenv.2012.01.061

Gerrity, D. Holady, J.C., Mawhinney, D.B., Quinones, O., Trenholm, R.A., Snyder, S.A. 2013. The effects of solids retention time in full-scale activated sludge basins on trace organic contaminant concentrations. Water Env. Res. 85(8), 715-724.

Gerrity, D., Owens-Bennett, E., Venezia, T., Stanford, B.D., Plumlee, M.H., Debroux, J., Trussell, R.S., 2014. Applicability of Ozone and Biological Activated Carbon for Potable Reuse. Ozone Sci. Eng. 36, 123–137. doi:10.1080/01919512.2013.866886

Gerrity, D., Pecson, B., Shane Trussell, R., Rhodes Trussell, R., 2013. Potable reuse treatment trains throughout the world. J. Water Supply Res. Technol. - AQUA 62, 321–338.

المنارة للاستشارات
www.manaraa.com

doi:10.2166/aqua.2013.041

Gerrity, D., Pisarenko, A.N., Marti, E., Trenholm, R.A., Gerringer, F., Reungoat, J., Dickenson, E., 2015. Nitrosamines in pilot-scale and full-scale wastewater treatment plants with ozonation. Water Res. 72, 251–261. doi:10.1016/j.watres.2014.06.025

Gerrity, D., Trenholm, R.A., Snyder, S.A., 2011. Temporal variability of pharmaceuticals and illicit drugs in wastewater and the effects of a major sporting event. Water Res. 45, 5399–5411. doi:10.1016/j.watres.2011.07.020

Gonzalez-Martinez, A., Rodriguez-Sanchez, A., Lotti, T., Garcia-Ruiz, M.J., Osorio, F., Gonzalez-Lopez, J., van Loosdrecht, M.C., 2016. Comparison of bacterial communities of conventional and A-stage activated sludge systems. Sci Rep 6, 18786. doi:10.1038/srep18786

Graham, D.W., Olivares-Rieumont, S., Knapp, C.W., Lima, L., Werner, D., Bowen, E., 2011. Antibiotic resistance gene abundances associated with waste discharges to the Almendares river near Havana, Cuba. Environ. Sci. Technol. 45, 418–424. doi:10.1021/es102473z

Green, H.C., Dick, L.K., Gilpin, B., Samadpour, M., Field, K.G. 2011. Genetic Markers for Rapid PCR-Based Identification of Gull, Canada Goose, Duck, and Chicken Fecal Contamination in Water. Appl. Environ. Microbiol. 78, 503-510.

Gros, M., Rodríguez-Mozaz, S. & Barceló, D. 2013, "Rapid analysis of multiclass antibiotic residues and some of their metabolites in hospital, urban wastewater and river water by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry", Journal of Chromatography A, vol. 1292, pp. 173-188.

Gullberg. E., Albrecht, L.M., Karlsson, C., Sandegren, L., Andersson, D.I. 2014. Selection of a

المنارة للاستشارات

www.manaraa.com

multidrug resistance plasmid by sublethal levels of antibiotics and heavy metals. mBio 5(5):e01918-14.

Guo, M.T., Yuan, Q. Bin, Yang, J., 2015. Insights into the amplification of bacterial resistance to erythromycin in activated sludge. Chemosphere 136, 79–85.

Hall, R.M., Stokes, H.W. 1993. Integrons: Novel DNA elements which capture genes by site-specific recombination. Genetica. Vol. 90. Issue 2, 115-132.

Head, M.A., Oleszkiewicz, J.A., 2004. Bioaugmentation for nitrification at cold temperatures. Water Res. 38, 523–530. doi:10.1016/j.watres.2003.11.003

Henderson, R.K., Baker, A., Murphy, K.R., Hambly, A., Stuetz, R.M., Khan, S.J., 2009. Fluorescence as a potential monitoring tool for recycled water systems: A review. Water Res. 43, 863-881.

Heylen, K., Lebbe, L., de Vos, P., 2008. Acidovorax caeni sp. nov., a denitrifying species with genetically diverse isolates from activated sludge. Int. J. Syst. Evol. Microbiol. 58, 73–77. doi:10.1099/ijs.0.65387-0

Hirsch, R., Ternes, T., Haberer, K., Kratz, K.L., 1999. Occurrence of antibiotics in the aquatic environment. Sci. Total Environ. 225, 109–118. doi:10.1016/S0048-9697(98)00337-4

Hoshino, T., Terahara, T., Tsuneda, S., Hirata, A., Inamori, Y., 2005. Molecular analysis of microbial population transition associated with the start of denitrification in a wastewater treatment process. J. Appl. Microbiol. 99, 1165–1175. doi:10.1111/j.1365-2672.2005.02698.x

Hoyle, B. D., J. Alcantara, and J. W. Costerton. 1992. Pseudomonas aeruginosa biofilm as a diffusion barrier to piperacillin. Antimicrobial Agents and Chemotherapy 36 (9): 2054-6.

المنارة للاستشارات
www.manaraa.com

Huang, J.-J., Hu, H.-Y., Lu, S.-Q., Li, Y., Tang, F., Lu, Y., Wei, B., 2012. Monitoring and evaluation of antibiotic-resistant bacteria at a municipal wastewater treatment plant in China. Environ. Int. 42, 31–36. doi:10.1016/j.envint.2011.03.001

Huang, M-H., Zhang, W., Liu, C., Hu, H-Y. 2014. Fate of trace tetracycline with resistant bacteria and resistance genes in an improved AAO wastewater treatment plant. Process Safety and Environmental Protection. http://dx.doi.org/10.1016/j.psep.2014.04.004

Hugenholtz, P., 2002. Exploring prokaryotic diversity in the genomic era. Genome Biol. 3, REVIEWS0003. doi:10.1186/gb-2002-3-2-reviews0003

Huovinen, P. 2001. Resistance to trimethoprim-sulfamethoxazole. Clin. Infect. Dis. 32, 1608-1614.

Igbinosa, I.H., Igumbor, E.U., Aghdasi, F., Tom, M., Okoh, A.I., 2012. Emerging Aeromonas Species Infections and Their Significance in Public Health. Sci. World J. 2012, 1–13. doi:10.1100/2012/625023

Jarusutthirak, C., Amy, G., 2001. Membrane filtration of wastewater effluents for reuse: Effluent organic matter rejection and fouling, in: Water Science and Technology. pp. 225–232.

Jarusutthirak, C., Amy, G., Croué, J.P., 2002. Fouling characteristics of wastewater effluent organic matter (EfOM) isolates on NF and UF membranes. Desalination 145, 247–255. doi:10.1016/S0011-9164(02)00419-8

Kim, S., Eichhorn, P., Jensen, J.N., Weber, A.S., Aga, D.S., 2005. Removal of antibiotics in wastewater: Effect of hydraulic and solid retention times on the fate of tetracycline in the activated sludge process. Environ. Sci. Technol. 39, 5816–5823. doi:10.1021/es050006u

Kim, S., Park, H., Chandran, K., 2010. Propensity of activated sludge to amplify or attenuate

المنارة للاستشارات
www.manaraa.com

tetracycline resistance genes and tetracycline resistant bacteria: A mathematical modeling approach. Chemosphere 78, 1071–1077.

Kim, S.D., Cho, J., Kim, I.S., Vanderford, B.J., Synder, S.A., 2007. Occurrence and removal of pharmaceuticals and endocrine disruptors in South Korean surface, drinking, and wastewaters. Water Research. Vol. 41. Issue 5, 1013-1021.

Kim, Sungpyo, Peter Eichhorn, James N. Jensen, A. Scott Weber, and Diana S. Aga. 2005. Removal of antibiotics in wastewater: Effect of hydraulic and solid retention times on the fate of tetracycline in the activated sludge process. Environmental Science & Technology 39 (15): 5816-23.

Kniemeyer, O., Probian, C., Rosselló-Mora, R., Harder, J., 1999. Anaerobic mineralization of quaternary carbon atoms: Isolation of denitrifying bacteria on dimethylmalonate. Appl. Environ. Microbiol. 65, 3319–3324.

Koditschek, L.K., Guyre, P. 1974. Antimicrobial-resistant coliforms in New York Bight. Marine Pollution Bulletin. Vol. 5. Issue 5, 71-74.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: a national reconnaissance. Environ. Sci. Technol. 36(6), 1202-1211.

Kolpin, D.W., Kolpin, D.W., Furlong, E.T., Furlong, E.T., Meyer, M.T., Meyer, M.T., 2002. Environ. Sci. Technol. 2002, 36, 1202-1211. Environ. Sci. Technol. 36, 1202–1211. doi:10.1021/es011055j

Kristiansson, E., Fick, J., Janzon, A., Grabic, R., Rutgersson, C., Weijdegard, B., Soderstorm, H., Larsson, D.G.J. 2011. Pyrosequencing of antibiotic-contaminated river sediments reveals high levels of resistance and gene transfer elements. PLoS ONE 6(2): e17038.

المنارة للاستشارات
www.manaraa.com

Lapworth, D.J., Baran, N., Stuart, M.E., Ward, R.S., 2012. Emerging organic contaminants in groundwater: A review of sources, fate and occurrence. Environ. Pollut. doi:10.1016/j.envpol.2011.12.034

Lee, S., Ang, W.S., Elimelech, M., 2006. Fouling of reverse osmosis membranes by hydrophilic organic matter: Implications for water reuse. Desalination 187, 313–321. doi:10.1016/j.desal.2005.04.090

Lee, Y., Gerrity, D., Lee, M., Bogeat, A.E., Salhi, E., Gamage, S., Trenholm, R.A., Wert, E.C., Snyder, S.A., Von Gunten, U., 2013. Prediction of micropollutant elimination during ozonation of municipal wastewater effluents: Use of kinetic and water specific information. Environ. Sci. Technol. 47, 5872–5881. doi:10.1021/es400781r

Lee, Y., Gerrity, D., Lee, M., Gamage, S., Pisarenko, A., Trenholm, R.A., Canonica, S., Snyder, S.A., Von Gunten, U., 2016. Organic Contaminant Abatement in Reclaimed Water by UV/H2O2 and a Combined Process Consisting of O3/H2O2 Followed by UV/H2O2: Prediction of Abatement Efficiency, Energy Consumption, and Byproduct Formation. Environ. Sci. Technol. 50, 3809–3819. doi:10.1021/acs.est.5b04904

Leu, S., Chan, L., Stenstrom, M. K., 2012. Toward long solids retention time of activated sludge processes: benefits in energy saving, effluent quality, and stability. Water Research 84 (1) : 42–53.

Li, B., Wu, G., 2014. Effects of sludge retention times on nutrient removal and nitrous oxide emission in biological nutrient removal processes. Int. J. Environ. Res. Public Health 11, 3553–3569. doi:10.3390/ijerph110403553

Li, B., Zhang, T., 2010. Biodegradation and adsorption of antibiotics in the activated sludge

المنارة للاستشارات
www.manaraa.com

process. Environ. Sci. Technol. 44, 3468–3473.

Li, B., Zhang, T., 2011. Mass flows and removal of antibiotics in two municipal wastewater treatment plants. Chemosphere 83, 1284–1289. doi:10.1016/j.chemosphere.2011.03.002

Lu, X., Zhang, X.X., Wang, Z., Huang, K., Wang, Y., Liang, W., Tan, Y., Liu, B., Tang, J., 2015. Bacterial pathogens and community composition in advanced sewage treatment systems revealed by metagenomics analysis based on high-throughput sequencing. PLoS One 10. doi:10.1371/journal.pone.0125549

Luo, Y., Guo, W., Ngo, H.H., Nghiem, L.D., Hai, F.I., Zhang, J., Liang, S., Wang, X.C., 2014. A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. Sci. Total Environ. doi:10.1016/j.scitotenv.2013.12.065

Luo, Y., Yang, F., Mathieu, J., Mao, D., Wang, Q., Alvarez, P.J.J. 2014. Proliferation of Multidrug-resistant new delhi metallo-β-lactamse Genes in Municipal Wastewater Treatment Plants in Northern China. Environ. Sci. Technol. 1, 26-30.

Martínez, J.L., 2008. Antibiotics and antibiotic resistance genes in natural environments. Science 321, 365–7. doi:10.1126/science.1159483

McAdam, A.J., Hooper, C.D., DeMaria, A., Limbago, B.M., O'Brien, T.F., McCaughey, B. 2010.

McKinney, C.W., Pruden, A, 2012. Ultraviolet Disinfection of Antibiotic Resistant Bacteria and Their Antibiotic Resistance Genes in Water and Wastewater. Environ. Sci. Technol. 46, 13393–13400. doi:10.1021/es303652q

McLellan, S.L., Huse, S.M., Mueller-Spitz, S.R., Andreishcheva, E.N., Sogin, M.L., 2010.

المنارة للاستشارات
www.manaraa.com

Diversity and population structure of sewage-derived microorganisms in wastewater treatment plant influent. Environ. Microbiol. 12, 378–392. doi:10.1111/j.1462-2920.2009.02075.x

McMahon, M.A.S., Xu, J., Moore, J.E., Blair, I.S., McDowell, D.A., 2007. Environmental stress and antibiotic resistance in food-related pathogens. Appl. Environ. Microbiol. 73, 211–217. doi:10.1128/AEM.00578-06

Melcer, H., Klecka, G., 2011. Treatment of wastewaters containing bisphenol A: state of the science review. Water Environ. Res. 83, 650–66. doi:doi:10.2175/106143010X12851009156925

Messi, P., Guerrieri, E., de Niederhausern, S., Sabia, C., Bondi, M. 2006. Vancomycin-resistant enterococci (VRE) in meat and environmental samples. Int. J. Food Microbiol. 107, 218-222.

Metcalf and Eddy, Wastewater Engineerings Treatment and Resource Recovery, New York: McGraw-Hill, 2014.

Miner, G. 2007, "Wastewater Bacteria", American Water Works Association. Journal, vol. 99, no. 7, pp. 127.

Muniesa, M., Garcia, A., Miro, E., Mirelis, B., Prats, G., Jofre, J., Navarro, F. 2004. Bacteriophages and Diffusion of β-lactamase Genes. Emerg. Infect. Dis. 10(6), 1134-1137.

Nagulapally, S.R., Ahmad, A., Henry, A., Marchin, G.L., Zurek, L., Bhandari, A., 2009. Occurrence of ciprofloxacin-, trimethoprim-sulfamethoxazole-, and vancomycin-resistant bacteria in a municipal wastewater treatment plant. Water Environ. Res. 81, 82–90. doi:10.2175/106143008X304596

المنارة للاستشارات
www.manaraa.com

Nandi, S., Maurer, J. J., Hofacre, C., Summers, A. O. 2004. Gram-positive bacteria are a major reservoir of Class 1 antibiotic resistance integrons in poultry litter. Proceedings of the National Academy of Science of the United States of America. 101(18), 7118-7122.

Neidhardt, F. C. 1931- (Frederick Carl), Ingraham, J.L. & Schaechter, M. 1990, Physiology of the bacterial cell: a molecular approach, Sinauer Associates.

Novo, A., Manaia, C.M. 2010. Factors influencing antibiotic resistance burden in municipal wastewater treatment plants. Appl. Microbiol. Biotechnol. 87(3), 1157-1166.

Nzila, Alexis. 2013. Update on the cometabolism of organic pollutants by bacteria. Environmental Pollution (Barking, Essex : 1987) 178 : 474-82.

Oggioni, M.R., Furi, L., Coelho, J.R., Maillard, J.Y., Martinez, J.L., 2013. Recent advances in the potential interconnection between antimicrobial resistance to biocides and antibiotics. Expert Rev. Anti. Infect. Ther. 11, 363-366.

Oliver, J.D., Dagher, M., Linden, K. 2005. Induction of Escherichia coli and Salmonella typhimurium into the viable but nonculturable state following chlorination of wastewater. J Water Health. 3(3), 249-257.

Oppenheimer, J., Eaton, A., Badruzzaman, M., Haghani, A.W., Jacangelo, J.G. 2011. Occurrence and suitability of sucralose as an indicator compound of wastewater loading to surface waters in urbanized regions. Water Res. 45, 4019-4027.

Oppenheimer, J., Stephenson, R., Burbano, A., Liu, L., 2007. Characterizing the passage of personal care products through wastewater treatment processes. Water Environ. Res. 79, 2564–2577. doi:10.2175/106143007X184573

OUYANG, K., LIU, J., 2009. Effect of sludge retention time on sludge characteristics and membrane fouling of membrane bioreactor. J. Environ. Sci. 21, 1329–1335.

المنارة للاستشارات
www.manaraa.com

doi:10.1016/S1001-0742(08)62422-5

Pal, A., Gin, K.Y.H., Lin, A.Y.C., Reinhard, M., 2010. Impacts of emerging organic contaminants on freshwater resources: Review of recent occurrences, sources, fate and effects. Sci. Total Environ. doi:10.1016/j.scitotenv.2010.09.026

Parsley, L.C., Consuegra, E.J., Kakirde, K.S., Land, A.M., Harper, W.F., Liles, M.R. 2010. Identification of diverse antimicrobial resistance determinants carried on bacterial, plasmid, or viral metagenomes from an activated sludge microbial assemblage. Appl. Environ. Microbial. 76 (11), 3753-3757.

Pei, R.T., Kim, S.C., Carlson, K.H., Pruden, A. 2006. Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). Water Res. 40, 2427-2435.

Pruden, A., Arabi, M., Storteboom, H.N., 2012. Correlation between upstream human activities and riverine antibiotic resistance genes. Environ. Sci. Technol., 46, 11541-11549.

Pruden, A., Pei, R., Storteboom, H., Carlson, K.H., 2006. Antibiotic resistance genes as emerging contaminants: Studies in northern Colorado. Environ. Sci. Technol. 40, 7445–7450. doi:10.1021/es060413l

Qiu, P., Guo, X., Zhang, Y., Chen, X. & Wang, N., 2016. Occurrence, fate, and risk assessment of vancomycin in two typical pharmaceutical wastewater treatment plants in Eastern China. Environmental Science and Pollution Research. 23, 16513-23.

Radjenovic, J., Petrovic, M., Barcelo, D. 2009. Fate and distribution of pharmaceuticals in wastewater and sewage sludge of the conventional activated sludge (CAS) and advanced membrane bioreactor (MBR) treatment. Water Res. 43(3), 831-841.

Research and Testing Laboratory, 2014. Data analysis methodology. Lubbock, TX.

المنارة للاستشارات
www.manaraa.com

http://www.researchandtesting.com/docs/Data_Analysis_Methodology.pdf.

Ritalahti, K.M., Amos, B.K., Sung, Y., Wu, O., Koenigsberg, S.S., Lofflen, F. E. 2006. Quantitative PCR targeting 16S rRNA and reductive dehalogenase genes simultaneously monitors multiple Dehalococcoides strains. Appl. Environ. Microbiol. 72, 2765-2774.

Roberts, M.C. 2005. Update on acquired tetracycline resistance genes. FEMS Microbiol. Lett. 245, 195-203.

Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sànchez-Melsió, A., Borrego, C.M., Barceló, D., Balcázar, J.L., 2015. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. Water Res. 69, 234–242. doi:10.1016/j.watres.2014.11.021

Roh, H., Chu, K.H., 2011. Effects of solids retention time on the performance of bioreactors bioaugmented with a 17??-estradiol-utilizing bacterium, Sphingomonas strain KC8. Chemosphere 84, 227–233. doi:10.1016/j.chemosphere.2011.04.029

Rowe-Magnus, D. A., Mazel, D. 2002. The role of Integrons in antibiotic resistance gene capture. International Journal of Medical Microbiology. Vol. 292, Issue 2, 115-125.

Saikaly, P.E., Stroot, P.G., Oerther, D.B. 2005. Use of 16S rRNA Gene Terminal Restriction Fragment Analysis to Assess the Impact of Solids Retention Time on the Bacterial Diversity of Activated Sludge. Appl. Microbiol. 71, 5814-5822.

Salveson, A., T. Rauch-Williams, E. Dickenson, J. Drewes, D. Drury, D. McAvoy and S. Snyder. 2012. Trace Organic Compound Indicator Removal During Conventional Wastewater Treatment, Water Environment Research Foundation, Stock No. CEC4R08, Co-published by IWA Publishing, Alexandria, VA.

Salyers, A.A., Amabile-Cuevas, C.F. 1997. Why are antibiotic resistance gene so resistant to

المنارة للاستشارات

www.manaraa.com

elimination?. Antimicrob Agents Chemother. Vol. 41. Issue 11, 2321-2325.

Samaras, V.G., Stasinakis, A.S., Mamais, D., Thomaidis, N.S., Lekkas, T.D., 2013. Fate of selected pharmaceuticals and synthetic endocrine disrupting compounds during wastewater treatment and sludge anaerobic digestion. J. Hazard. Mater. 244–245, 259–267. doi:10.1016/j.jhazmat.2012.11.039

Sawyer, Clair N., and Perry L. McCarty. 2003. Chemistry for environmental engineering and science. 5th ed. McGraw-Hill.

Schwartz, T., Kohnen, W., Jansen, B., Obst, U., 2003. Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. FEMS Microbiol. Ecol. 43, 325–335. doi:10.1016/S0168-6496(02)00444-0

Seiler, C., Berendonk, T.U. 2012. Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. Front. Microbio. 3, 1-10.

Sharp, J.O., Sales, C.M., Alvarez-Cohen, L., 2010. Functional characterization of propane-enhanced N-nitrosodimethylamine degradation by two actinomycetales. Biotechnol. Bioeng. 107, 924–932. doi:10.1002/bit.22899

Shi, P., Jia, S., Zhang, X.X., Zhang, T., Cheng, S., Li, A. 2013. Metagenomic insights into chlorination effects on microbial antibiotic resistance in drinking water. Water Res. 47, 111-120.

Shih, Pei-Ching, and Ching-Tsan Huang. 2002. Effects of quorum-sensing deficiency on pseudomonas aeruginosa biofilm formation and antibiotic resistance. The Journal of Antimicrobial Chemotherapy 49 (2): 309-14.

Shoemaker, N.B, Vlamakis, H., Hayes, K. Salyers, A.A. 2001. Evidence for extensive resistance gene transfer among bacteriodes spp. and among bacteriodes and other genera in the human

للاستشارات المنارة
www.manaraa.com

colon. Appl. Microbiol. 67, 561-568.

Silver, S., Phung, L, T. 1996. Bacterial heavy metal resistance: new surprises. Annual review of Microbiology. Vol. 50, 753-789.

Skold, O. 2001. Resistance to trimethoprim and sulfonamides. Vet. Res. 32, 261-273.

Smith, M. 2009. IDSA: MRSA linked to necrotizing fasciitis. MedPage Today. www.medpagetoday.com/MeetingCoverage/IDSA/4278. Accessed: April 30, 2015.

Stanford, B.D., Pisarenko, A.N., Holbrook, R.D., Snyder, S. a., 2011. Preozonation Effects on the Reduction of Reverse Osmosis Membrane Fouling in Water Reuse. Ozone Sci. Eng. 33, 379–388. doi:10.1080/01919512.2011.607385

Stewart, P. S. 1998. A review of experimental measurements of effective diffusive permeabilities and effective diffusion coefficients in biofilms. Biotechnology and Bioengineering 59 (3): 261-72

Stewart, Philip S., and J. William Costerton. 2001. Antibiotic resistance of bacteria in biofilms. The Lancet 358 (9276): 135-8.

Struthers, K.J., Westran, R.P. 2003. Clinical bacteriology. ASM Press. p. 49-65.

Su, H.-C., Ying, G.-G., He, L.-Y., Liu, Y.-S., Zhang, R.-Q., Tao, R., 2014. Antibiotic resistance, plasmid-mediated quinolone resistance (PMQR) genes and ampC gene in two typical municipal wastewater treatment plants. Environ. Sci. Process. Impacts 16, 324–32.

Suarez, S., Lema, J.M., Omil, F., 2010. Removal of Pharmaceutical and Personal Care Products (PPCPs) under nitrifying and denitrifying conditions. Water Res. 44, 3214–3224. doi:10.1016/j.watres.2010.02.040

Sulakvelidze, A. 2005. Phage therapy: an attractive option for dealing with antibiotic-resistant bacterial infections. 10(12), 807-809.

المنارة للاستشارات
www.manaraa.com

Suller, M.T., Russell, a D., 2000. Triclosan and antibiotic resistance in Staphylococcus aureus. J. Antimicrob. Chemother. 46, 11–18. doi:10.1093/jac/46.1.11

Summers, R.M., Mohanty, S.K., Gopishetty, S., Subramanian, M., 2015. Genetic characterization of caffeine degradation by bacteria and its potential applications. Microb. Biotechnol. doi:10.1111/1751-7915.12262

Tai, CS, KS Singh, and SR Grant. 2006. Post-treatment of anaerobic bioreactor effluent using jet loop reactor (JLR). Water Quality Research Journal of Canada 41 (3): 316-24.

TamTam, F., Mercier, F., Le Bot, B., Eurin, J., Tuc Dinh, Q., Clement, M., Chevreuil, M. 2008. Occurrence and fate of antibiotics in the Seine River in various hydrological conditions. Science of the Total Environment. Vol. 393, Issue 1, 84-95.

Taylor, Paul J. 2005. Matrix effects: The achilles heel of quantitative high-performance liquid chromatography–electrospray–tandem mass spectrometry. Clinical Biochemistry 38 (4): 328-34.

Towner, K.J., 2009. Acinetobacter: an old friend, but a new enemy. J. Hosp. Infect. doi:10.1016/j.jhin.2009.03.032

Trevors, J.T. 2010. Suitable microscopic entropy for the origin of microbial life: microbiological methods are challenges. J Microbiol Methods. 83(3), 341-344.

Trussell, R.S., Adham, S., Gagliardo, P., Merlo, R., Trussell, R.R., 2000. WERF: application of membrane bioreactor (MBR) technology for wastewater treatment. WEFTEC, Anaheim, CA.

Ueki, A., Abe, K., Kaku, N., Watanabe, K., Ueki, K., 2008. Bacteroides propionicifaciens sp. nov., isolated from rice-straw residue in a methanogenic reactor treating waste from cattle

المنارة للاستشارات

www.manaraa.com

farms. Int. J. Syst. Evol. Microbiol. 58, 346–352. doi:10.1099/ijs.0.65486-0

Van den Broeck, R., Van Dierdonck, J., Nijskens, P., Dotremont, C., Krzeminski, P., van der Graaf, J.H.J.M., van Lier, J.B., Van Impe, J.F.M., Smets, I.Y., 2012. The influence of solids retention time on activated sludge bioflocculation and membrane fouling in a membrane bioreactor (MBR). J. Memb. Sci. 401–402, 48–55. doi:10.1016/j.memsci.2012.01.028

Vanderford, B.J., Snyder, S.A., 2006. Analysis of pharmaceuticals in water by isotope dilution liquid chromatography/tandem mass spectrometry. Environ. Sci. Technol. 40, 7312–7320. doi:10.1021/es0613198

Volkmann, H., Schwartz, T., Bischoff, P., Kirchen, S., Obst, U. 2004. Detection of clinically relevant antibiotic-resistance genes in municipal wastewater using real-time PCR (TaqMan). J. Microbiol. Methods. 56, 277-286.

Wang, H.H., Schaffner, D.W., 2011. Antibiotic resistance: How much do we know and where do we go from here? Appl. Environ. Microbiol. doi:10.1128/AEM.06565-11

Wang, J., Mao, D., Mu, Q., Luo, Y., 2015. Fate and proliferation of typical antibiotic resistance genes in five full-scale pharmaceutical wastewater treatment plants. Sci. Total Environ. 526, 366–373. doi:10.1016/j.scitotenv.2015.05.046

Wang, L., Li, Y., He, G., 2014. Degradation of typical N-nitrosodimethylamine (NDMA) precursors and its formation potential in anoxic-aerobic (AO) activated sludge system. J. Environ. Sci. Heal. Part A 49, 1727–1739. doi:10.1080/10934529.2014.951263

Wang, X. chun, Shen, J. min, Chen, Z. lin, Zhao, X., Xu, H., 2016. Removal of pharmaceuticals from synthetic wastewater in an aerobic granular sludge membrane bioreactor and determination of the bioreactor microbial diversity. Appl. Microbiol. Biotechnol. 100,

المنارة للاستشارات
www.manaraa.com

8213–8223. doi:10.1007/s00253-016-7577-6

Watkinson, A.J., Murby, E.J., Kolpin, D.W., Costanzo, S.D., 2009. The occurrence of antibiotics in an urban watershed: From wastewater to drinking water. Sci. Total Environ. 407, 2711–2723. doi:10.1016/j.scitotenv.2008.11.059

WHO. 2011. Antimicrobial resistance: no action today, no cure tomorrow. World Health Organization. http://www.who.int/world-health-day/2011/en/index.html. Accessed: April 30, 2015.

Wojcieszyńska, D., Domaradzka, D., Hupert-Kocurek, K., Guzik, U., 2014. Bacterial degradation of naproxen - Undisclosed pollutant in the environment. J. Environ. Manage. 145, 157–161. doi:10.1016/j.jenvman.2014.06.023

Wu, S., Zhang, L., Chen, J., 2012. Paracetamol in the environment and its degradation by microorganisms. Appl. Microbiol. Biotechnol. doi:10.1007/s00253-012-4414-4

Xu, W., Zhang, G., Li, X., Zou, X., Li, P., Hu, Z., Li, J. 2007. Ocurrence and elimination of antibiotics at four sewage treatment plants in the Pearl River Delta (PRD), South China. Water Research. Vol. 41. Issue 19, 4526-4534.

Yang, B., Ying, G.G., Zhao, J.L., Liu, S., Zhou, L.J., Chen, F., 2012. Removal of selected endocrine disrupting chemicals (EDCs) and pharmaceuticals and personal care products (PPCPs) during ferrate(VI) treatment of secondary wastewater effluents. Water Res. 46, 2194–2204. doi:10.1016/j.watres.2012.01.047

Yong, D., Toleman, W.A., Giske, C.G., Cho, H.S., Sundman, K., Lee, K., Walsh, T.R. 2009. Characterization of a new metallic-beta-lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumonia

المنارة للاستشارات
www.manaraa.com

sequence type 14 from India. Antimicrob. Agents. Chemother. Vol. 53. Issue 12, 5046-5054.

Zhang, S., Han, B., Gu, J., Wang, C., Wang, P., Ma, Y., Cao, J., He, Z., 2015. Fate of antibiotic resistant cultivable heterotrophic bacteria and antibiotic resistance genes in wastewater treatment processes. Chemosphere 135, 138–145. doi:10.1016/j.chemosphere.2015.04.001

Zhang, T., Fang, H.H.P. 2006. Applications of real-time polymerase chain reaction for quantification of microorganisms in environmental samples. Appl. Microbiol. Biotechnol. 70, 281-289.

Zhang, T., Shao, M.-F., Ye, L., 2012. 454 Pyrosequencing Reveals Bacterial Diversity of Activated Sludge From 14 Sewage Treatment Plants. ISME J. 6, 1137–47. doi:10.1038/ismej.2011.188

Zhang, Tian C., and Paul L. Bishop. 1996. Evaluation of substrate and pH effects in a nitrifying biofilm. Water Environment Research 68 (7): 1107-15.

Zhang, Y., Marrs, C.F., Simon, C., Xi, C. 2009. Wastewater treatment contribute to selective increase of antibiotic resistance among Acinetobacter spp. Total. Environ. 407, 3702-3706.

Zhao, Y., Song, L., Ong, S.L., 2010. Fouling of RO membranes by effluent organic matter (EfOM): Relating major components of EfOM to their characteristic fouling behaviors. J. Memb. Sci. 349, 75–82. doi:10.1016/j.memsci.2009.11.024

المنارة للاستشارات
www.manaraa.com

CURRICULUM VITAE

Majid Neyestani, Ph.D., E.I.T

Email:
neyestan@unlv.nevada.edu
Phone: (702)600-9930

EDUCATION:

Doctor of Philosophy, Civil and Environmental Engineering, University of Nevada, Las Vegas, United States

- Graduation: Fall 2016

Master of Science, Civil and Environmental Engineering, Isfahan University of Technology, Iran

- Graduation: Spring 2012

Bachelor of Science, Civil Engineering, University of Qom, Iran

- Graduation: Spring 2010

PROFESSIONAL EXPERIENCE:

Civil Engineer, Greeley and Hansen, (November 2016 – Present)

Graduate Intern, MWH Global, (May 2016 – August 2016)

- Developed mass balances on waste streams for wastewater treatment design
- Designed iron and manganese removal systems
- Assisted in design of disinfection by product (DBP) removal systems
- Assisted in design of arsenic removal systems from groundwater
- Assisted in design of reverse osmosis (RO) system
- Evaluated chlorine decay rate in storage tanks in wastewater treatment plants
- Assisted in preparation of technical memoranda and basis of design reports (BODRs)

Research Assistant, University of Nevada, Las Vegas, (August 2013 – December 2016)

- Designed and developed laboratory-scale sequencing batch reactors
- Designed experiments to detect antibiotics and trace organic compounds in biological treatment systems
- Designed activated sludge systems to reduce COD and ammonia
- Performed culture-based and molecular-based techniques to detect and quantify the fate of antibiotic resistance in wastewater treatment plants
- Analyzed data on biological activated sludge pilot-study
- Performed batch testing, data collection, sample analysis, data compilation, and results analysis
- Performed DNA extraction and microbial community analysis in activated sludge reactors
- Analyzed the effect of solids retention time (SRT) on antibiotics, antibiotic

المنارة للاستشارات
www.manaraa.com

resistance, microbial community structure, and effluent water quality in biological wastewater treatment
- Designed packed-tower air stripping system for VOCs removal

Graduate Intern, MWH Global, (May 2015 – August 2015)
- Prepared preliminary designs for solids thickening and for solids dewatering process
- Assisted in the preparation of thickening and dewatering technology evaluation and cost estimation
- Designed biological nutrient removal systems: sizing anaerobic, anoxic and aerobic tanks
- Performed solids mass balances on wastewater treatment plants
- Prepared process flow diagrams for different wastewater treatment projects
- Prepared technical memoranda and basis of design reports (BODRs)

Teacher Assistant, University of Nevada, Las Vegas, 2012-2013
- Graded assignments, quizzes, and exams
- Taught water quality analysis, water and wastewater treatment processes, and Statics
- Provided mentorship to undergraduate students
- Lectured the water quality testing concepts in environmental engineering lab
- Assisted undergraduate students in performing laboratory experiments

PROFESSIONAL PRESENTATION:

- Neyestani, M., Dickenson, E., McLain, J., Rock, C., Gerrity, D., 2016 "*Occurrence and proliferation of antibiotics and antibiotic resistance during wastewater treatment*", WEFTEC 2016, New Orleans, Louisiana
- Neyestani, M., Gerrity, D., 2015 "*Fate of antibiotic resistant bacteria in wastewater treatment process*", UNCOWR/NIWR/CUAHSI Annual Conference, Las Vegas, Nevada
- Neyestani, M., Gerrity, D., 2016 "*Microbial community and antibiotic resistance in biological wastewater treatment*", 55th Annual ASM AZ/NV Branch Meeting, Phoenix, Arizona

HONORS AND AWARDS:

- Doctoral Graduate Research Assistantship, 2015-present
- Graduate Research Scholarship, 2013-2014
- Graduate Assistantship, 2012-2013
- UNLV Access Grant Award, 2013-Present
- College of Engineering Undergraduate Student Award, 2009

ACTIVITIES:

- Vice President, UNLV Student Chapter of the American Water Works Association, 2014 - 2015
- National Society of Leadership and Success, Member, 2014 - Present
- American Society of Civil Engineers, Member, 2012 - Present
- Water Environment Federation, Member, 2016 - Present

COMPUTER SKILLS:

Microsoft Office Suite, AutoCAD, Civil3D, Primavera P6, Groundwater Vistas, ArcGIS

المنارة للاستشارات
www.manaraa.com