Fall 2022

Coastal Stormwater Pond Pollutants and the Potential for Development of Antimicrobial Resistance in Vibrio and Enterococcus Bacteria

Cassandra L. Horton

Coastal Stormwater Pond Pollutants and the Potential for Development of Antimicrobial Resistance in *Vibrio* and *Enterococcus* Bacteria

by

Cassandra L. Horton

Bachelor of Science
Clemson University, 2012

Submitted in Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy in

Environmental Health Sciences

Norman J. Arnold School of Public Health

University of South Carolina

2022

Accepted by:

Geoffrey I. Scott, Major Professor

Marie E. DeLorenzo, Committee Member

Dwayne E. Porter, Committee Member

Alan W. Decho, Committee Member

Cheryl L. Addy, Interim Vice Provost and Dean of the Graduate School

Dedication

This dissertation is dedicated to all the people who loved and supported me while I chased my PhD, including:

My family, for never doubting for a moment how much I could achieve;

My friends, for refusing to let me become a hermit (no matter how hard I might have tried);

My pets, Poe, Pepper, and Allie, for somehow always knowing when I need a fluffy hug; and

My Leo, for somehow being both a rock and a life preserver at the same time. I love you.

Thanks, y'all. I couldn't have done it without you.

Acknowledgements

I would like to express my deepest gratitude to my committee, especially Drs. Scott and DeLorenzo, for motivating me through a pandemic and helping to keeping my head on straight.

I would also like to thank the staff of NCCOS Charleston, especially Dr. Paul Pennington and Katie Chung. Paul - you taught me more about statistics in a handful of video chats than I learned in an entire semester of Biostats. Katie - your knowledge of all things lab-related are unparalleled.

Finally, I want to thank the NIEHS Center for Oceans and Human Health, for the security of a fully-funded PhD program.

Abstract

The Southeastern coastal plain is the most rapidly urbanizing region in the United States. Associated landscape changes which increase imperviousness lead to hydrological cycle alterations, increasing runoff of nonpoint source (NPS) pollution. Urban and agricultural NPS runoff is generally discharged into stormwater ponds, which sequester chemical contaminants, nutrients, and microbes to reduce loading into coastal ecosystems. Studies of these ponds have indicated elevated levels of trace metals, antimicrobial compounds, and bacterial contamination (SCSGC 2018).

Interactions of aquatic pathogens *Vibrio vulnificus* and *Enterococcus faecium* with trace metals (arsenic, copper, zinc) and clinically relevant antimicrobials (triclosan, ciprofloxacin, oxytetracycline) commonly found in coastal ponds and estuaries were examined to determine how they may affect growth and impact antimicrobial resistance. Both species have significant environmental and public health significance in terms of water quality, seafood safety and contact recreation. Experimental data indicate that binary mixtures of environmentally relevant concentrations of some antimicrobials and metals inhibit growth in *V. vulnificus* and *E. faecium*. A mixture of copper and oxytetracycline is of note due to a biostimulatory effect at levels a dose 60% lower than the copper Effects Range Low (ERL) sediment quality guideline and at the Probable No-Effect Concentration (PNEC) of oxytetracycline. This dose combination also resulted in susceptibility changes to three clinically relevant antibiotics of different classes.

Table of Contents

Dedication iii

Acknowledgements iv

Abstract v

List of Tables viii

List of Figures xiii

List of Abbreviations xix

Chapter 1: Background and Significance 1

- Runoff Pollution and Urbanization 1
- Antimicrobials as Contaminants of Emerging Concern 4
- Aquatic Pathogens 5
- Rise of Antimicrobial Resistance 10
- Antimicrobial Mechanisms of Action 13
- Summary 28

Chapter 2: Aims and Approach 31

- Problem Statement 31
- Experimental Design 31
- Specific Goals 33
- Hypotheses 33

Chapter 3: *Vibrio vulnificus* 35

- Materials 35
- Methods 37
- Results 48
- Discussion 100

Chapter 4: *Enterococcus faecium* 111

Materials..... 111
Methods..... 112
Results..... 124
Discussion 176

Chapter 5: Conclusions and Future Work.....180

Summary 180
Future Work 181

References.....187

List of Tables

Table 1.1: Examples of cross-resistance between heavy metals and antibiotics (Baker-Austin et al. 2006). 14

Table 3.1: Nominal concentrations of antimicrobials upon which range finding assay exposures were based (Bayer Pharmaceuticals 2004; Agwuh and MacGowan 2006; Rodricks et al. 2010; Nietch et al. 2013; Bengtsson-Palme and Larsson 2016). 39

Table 3.2: Nominal concentrations of metals upon which range finding assay exposures were based (Aarestrup and Hasman 2004; EPA 2004; Fulladosa et al. 2005; Rebelo et al. 2012). 40

Table 3.3: Sediment Quality Guidelines for Arsenic, Copper, and Zinc (Long 1995). 41

Table 3.4: Minimum Effective Concentration (MEC) of each toxicant used as a constant in binary exposure experiments, as determined in Protocol 1 experiments. 44

Table 3.5: *V. vulnificus* Inhibition Zone Breakpoints (CLSI 2016). 47

Table 3.6: ANOVA Assumptions Legend, as applied to results presented in tables that follow. 50

Table 3.7: ANOVA statistical analysis of results from the PNEC range of antimicrobials. 52

Table 3.8: ANOVA statistical analysis of results from the clinical range of antimicrobials. 54

Table 3.9: ANOVA statistical analysis of results from the CCC range of metals. 57

Table 3.10: ANOVA statistical analysis of results from the EC20 range of metals. 59

Table 3.11: ANOVA statistical analysis of results from the MIC range of metals. 61

Table 3.12: ANOVA statistical analysis of results from the mid-range of copper concentrations. 64

Table 3.13: ANOVA statistical analysis of results from the Binary Group 1 Mixtures of MEC As with PNEC ranges of TCS, OTC, or CIP. 66

Table 3.14: ANOVA statistical analysis of results from the Binary Group 2 Mixtures of MEC Cu with PNEC ranges of TCS, OTC, or CIP. 69

Table 3.15: ANOVA statistical analysis of results from the Binary Group 3 Mixtures of MEC Zn with PNEC ranges of TCS, OTC, or CIP. 72

Table 3.16: ANOVA statistical analysis of results from the Binary Group 4 Mixtures of MEC TCS with CCC range of As, Cu, or Zn. 74

Table 3.17: ANOVA statistical analysis of results from the Binary Group 5 Mixtures of MEC OTC with CCC range of As, Cu, or Zn. 76

Table 3.18: ANOVA statistical analysis of results from the Binary Group 6 Mixtures of MEC CIP with CCC range of As, Cu, or Zn. 79

Table 3.19(a): AMR breakpoints for *V. vulnificus* exposure to ciprofloxacin (CIP), cefotaxime (CTX), and oxytetracycline (OTC) (CLSI 2016). 81

Table 3.19(b): *V. vulnificus* post-exposure Zones of Inhibition with susceptibility designations. 82

Table 3.20: ANOVA statistical analysis of the results for Kirby-Bauer Low Dose Exposures to CCC doses of As, Cu, and Zn and PNEC doses of TCS, OTC, or CIP. 85

Table 3.21: ANOVA statistical analysis of results for the Kirby-Bauer High Dose Exposures to MEC doses of As, Cu, Zn, TCS, OTC, or CIP. 87

Table 3.22: ANOVA statistical analysis of results for Kirby-Bauer Assay Binary Group 1 Mixtures of MEC As with PNEC ranges of TCS, OTC, or CIP. 89

Table 3.23: ANOVA statistical analysis of results for Kirby-Bauer Assay Binary Group 2 Mixtures of MEC Cu with PNEC ranges of TCS, OTC, or CIP. 92

Table 3.24: ANOVA statistical analysis of results for Kirby-Bauer Assay Binary Group 3 Mixtures of MEC Zn with PNEC ranges of TCS, OTC, or CIP. 95

Table 3.25: ANOVA statistical analysis of results for the Kirby-Bauer Assay Binary Group 4 Mixtures of MEC TCS with CCC of As, Cu, or Zn. 97

Table 3.26: ANOVA statistical analysis of results for the Kirby-Bauer Assay Binary Group 5 Mixtures of MEC OTC with CCC range of As, Cu, or Zn. 99

Table 3.27: ANOVA statistical analysis of results for Kirby-Bauer Assay Binary Group 6 Mixtures of MEC CIP with CCC range of As, Cu, or Zn. 102

Table 3.28: A comparison of the two binary mixture groups combining copper and oxytetracycline at different concentrations. 103

Table 3.29: A comparison of the two binary mixture groups combining zinc and triclosan at different concentrations. 105

Table 3.30: A comparison of the two binary mixture groups combining copper and triclosan at different concentrations. 106

Table 4.1: Nominal antimicrobial concentrations upon which range finding assay exposures were based (Bayer Pharmaceuticals 2004; Agwuh and MacGowan 2006; Rodricks et al. 2010; Nietch et al. 2013; Bengtsson-Palme and Larsson 2016). 114

Table 4.2: Nominal metals concentrations upon which range finding assay exposures were based (Aarestrup and Hasman 2004; EPA 2004; Fulladosa et al. 2005; Rebelo et al. 2012). 115

Table 4.3: Sediment Quality Guidelines for Arsenic, Copper, and Zinc (Long 1995).....117

Table 4.4: Minimum Effective Concentration (MEC) of each toxicant used as a constant in binary exposure experiments, as determined in Protocol 1 experiments. 120

Table 4.5: *E. faecium* Inhibition Zone Breakpoints (CLSI 2017). 123

Table 4.6: ANOVA Assumptions Legend, as applied in results presented in tables that follow. 125

Table 4.7: ANOVA statistical analysis of results from the PNEC range of antimicrobials.....128

Table 4.8: ANOVA statistical analysis of results from the clinical range of antimicrobials. 131

Table 4.9: ANOVA statistical analysis of results from a mid-range of (a) triclosan or (b) ciprofloxacin. 133

Table 4.10: ANOVA statistical analysis of results from the CCC range of metals. 135

Table 4.11: ANOVA statistical analysis of results from the EC20 range of metals. 137

Table 4.12: ANOVA statistical analysis of results from the MIC range of metals.....140

Table 4.13: ANOVA statistical analysis of results from the mid-range of copper concentrations. 142

Table 4.14: ANOVA statistical analysis of results from the Binary Group 1 Mixtures of MEC As with a PNEC range of TCS, OTC, or CIP. 145

Table 4.15: ANOVA statistical analysis of results from the Binary Group 2 Mixtures of MEC Cu with PNEC ranges of TCS, OTC, or CIP.....147

Table 4.16: ANOVA statistical analysis of results from the Binary Group 3 Mixtures of MEC zinc with a PNEC range of TCS, OTC, or CIP. 150

Table 4.17: ANOVA statistical analysis of results from the Binary Group 4 Mixtures of MEC TCS with a CCC range of As, Cu, or Zn. 152

Table 4.18: ANOVA statistical analysis of results from the Binary Group 5 Mixtures of MEC OTC with a CCC range of As, Cu, or Zn. 155

Table 4.19: ANOVA statistical analysis of results from the Binary Group 6 Mixtures of MEC CIP with a CCC range of As, Cu, or Zn. 157

Table 4.20(a): AMR breakpoints for *E. faecium* exposure to ciprofloxacin (CIP), vancomycin (VAN), and oxytetracycline (OTC). 159

Table 4.20(b): Post-exposure Zones of Inhibition with susceptibility designations. 160

Table 4.21: ANOVA statistical analysis of the results for Kirby-Bauer Low Dose Exposures to CCC doses of As, Cu, and Zn and PNEC doses of TCS, OTC, or CIP. 163

Table 4.22: ANOVA statistical analysis of results for the Kirby-Bauer High Dose Exposures to MEC doses of As, Cu, Zn, TCS, OTC, or CIP. 165

Table 4.23: ANOVA statistical analysis of results for Kirby-Bauer Binary Group 1 Mixtures of MEC As with PNEC ranges of TCS, OTC, or CIP. 168

Table 4.24: ANOVA statistical analysis of results for Kirby-Bauer Binary Group 3 Mixtures of MEC Zn with PNEC ranges of TCS, OTC, or CIP. 171

Table 4.25: ANOVA statistical analysis of results for the Kirby-Bauer Binary Group 4 Mixtures of MEC TCS with CCC range of As, Cu, or Zn. 173

Table 4.26: ANOVA statistical analysis of results for the Kirby-Bauer Binary Group 5 Mixtures of MEC OTC with CCC range of As, Cu, or Zn. 175

Table 4.27: ANOVA statistical analysis of results for Kirby-Bauer Binary Group 6 Mixtures of MEC CIP with CCC range of As, Cu, or Zn. 178

List of Figures

Figure 1.1: Resistance response for V. vulnificus (Baker-Austin et al. 2008, 2009) from the Atlantic coast in South Carolina and Georgia. 9

Figure 1.1: Resistance response for V. vulnificus (Baker-Austin et al. 2008, 2009) from the Atlantic coast in South Carolina and Georgia.. 9

Figure 1.2: Chemical structure of oxytetracycline (PubChem 2011 Dec 26).....15

Figure 1.3: Chemical structure of ciprofloxacin (PubChem 2005 Mar 25). 17

Figure 1.4: Chemical structure of cefotaxime [PubChem]. 19

Figure 1.5: Chemical structure of vancomycin (PubChem 2005 Jun 24).....20

Figure 1.6: Chemical structure of triclosan (PubChem 2005b Mar 25). 22

Figure 1.7: Chemical structure of roxarsone (PubChem 2005c Mar 25).....24

Figure 1.8: Chemical structure of sodium arsenate (PubChem 2013 May 27. 25

Figure 1.9: Chemical structure of copper sulfate (PubChem 2004 Sep 16). 27

Figure 1.10: Chemical structure of zinc sulfate (PubChem 2004b Sep 16. 29

Figure 3.1: Kirby-Bauer procedure, adapted from Hudzicki 2009. 45

Figure 3.2: Inhibition of *V. vulnificus* following exposure to the PNEC range of antimicrobials. 51

Figure 3.3: Inhibition of *V. vulnificus* following exposure to the clinical range of antimicrobials. 53

Figure 3.4: Inhibition of *V. vulnificus* following exposure to the CCC range of metals. 55

Figure 3.5: Inhibition of *V. vulnificus* following exposure to EC20 range of metals. 58

Figure 3.6: Inhibition of *V. vulnificus* following exposure to MIC range of metals. 60

Figure 3.7: Inhibition of *V. vulnificus* following exposure to a range of copper concentrations falling between the CCC and EC20 concentration ranges. 63

Figure 3.8: Inhibition of *V. vulnificus* following exposure to the Binary Group 1 mixture of the As MEC with a PNEC range of (a) TCS, (b) OTC, or (c) CIP. 65

Figure 3.9: Inhibition of *V. vulnificus* following exposure to the Binary Group 2 mixture of the Cu MEC with a PNEC range of (a) TCS, (b) OTC, or (c) CIP. 68

Figure 3.10: Inhibition of *V. vulnificus* following exposure to the Binary Group 3 mixture of the Zn MEC with a PNEC range of (a) TCS, (b) OTC, or (c) CIP. 70

Figure 3.11: Inhibition of *V. vulnificus* following exposure to the Binary Group 4 mixture of the TCS MEC with a CCC range of (a) As, (b) Cu, or (c) Zn. 73

Figure 3.12: Inhibition of *V. vulnificus* following exposure to the Binary Group 5 mixture of the OTC MEC with a CCC range of (a) As, (b) Cu, or (c) Zn. 75

Figure 3.13: Inhibition of *V. vulnificus* following exposure to the Binary Group 6 mixture of the CIP MEC with a CCC range of (a) As, (b) Cu, or (c) Zn. 78

Figure 3.14: *V. vulnificus* change in susceptibility to (a) ciprofloxacin (-0.7 - 7.17%), (b) cefotaxime (5.12 - 9.83%), and (c) oxytetracycline (1.89-8.74%) following 24-hour acclimation to the EPA Criterion Continuous Concentration of arsenic, copper, or

zinc, or the Probable No Effects Concentration of triclosan, oxytetracycline, or ciprofloxacin. 84

Figure 3.15: *V. vulnificus* change in susceptibility to (a) ciprofloxacin, (b) cefotaxime, and (c) oxytetracycline following 24-hour acclimation to MEC of arsenic, copper, zinc, triclosan, oxytetracycline, or ciprofloxacin. 86

Figure 3.16: *V. vulnificus* change in susceptibility to (a) ciprofloxacin (1.98 - 4.00%), (b) cefotaxime (-9.58 - 1.56%), and (c) oxytetracycline (-7.84 - -3.01%) following 24-hour acclimation to the Binary Group 1 mixture of the As MEC with the PNEC of triclosan, oxytetracycline, or ciprofloxacin. 88

Figure 3.17: *V. vulnificus* change in susceptibility to (a) ciprofloxacin (1.54 - 3.4%), (b) cefotaxime (-1.20 - 7.13%), and (c) oxytetracycline (-3.822 - -0.41%) following 24-hour acclimation to the Binary Group 2 mixture of the Cu MEC with the PNEC of triclosan, oxytetracycline, or ciprofloxacin. 91

Figure 3.18: *V. vulnificus* change in susceptibility to (a) ciprofloxacin (-0.92 - 5.62%), (b) cefotaxime (-1.21 - 0.02%), and (c) oxytetracycline (-10.82 - 5.70%) following 24-hour acclimation to the Binary Group 3 mixture of the Zn MEC with the PNEC of TCS, OTC, or CIP.....94

Figure 3.19: *V. vulnificus* change in susceptibility to (a) ciprofloxacin (-2.01 - 1.38%), (b) cefotaxime (-6.35 - 0.8%), and (c) oxytetracycline (0.26 - 7.25%) following 24-hour acclimation to the Binary Group 4 mixture of the TCS MEC with the CCC of arsenic, copper, or zinc.....96

Figure 3.20: *V. vulnificus* change in susceptibility to (a) ciprofloxacin (0.17 - 9.91%), (b) cefotaxime (3.96 - 11.60%), and (c) oxytetracycline (4.06 - 5.99%) following 24-hour acclimation to the Binary Group 5 mixture of the OTC MEC with the CCC of As, Cu, or Zn.....98

Figure 3.21: *V. vulnificus* change in susceptibility to (a) ciprofloxacin, (b) cefotaxime, and (c) oxytetracycline following 24-hour acclimation to the

Binary Group 6 mixture of the CIP MEC with the CCC of As, Cu, or Zn. 101

Figure 3.22: Comparison of antagonistic and synergistic relationship across exposure types. 108

Figure 4.1: Kirby-Bauer procedure, adapted from Hudzicki 2009. 121

Figure 4.2: Inhibition of *E. faecium* following exposure to the PNEC range of antimicrobials. 127

Figure 4.3: Inhibition of E. faecium following exposure to the clinical range of antimicrobials. 130

Figure 4.4: Inhibition of *E. faecium* following exposure to a range of triclosan or ciprofloxacin concentrations falling between the CCC and EC20 concentration ranges. 132

Figure 4.5: Inhibition of *E. faecium* following exposure to the CCC range of metals. 134

Figure 4.6: Inhibition of E. faecium following exposure to the EC20 range of metals. 136

Figure 4.7: Inhibition of *E. faecium* following exposure to the MIC range of metals. 139

Figure 4.8: Inhibition of *E. faecium* following exposure to a range of copper concentrations falling between the CCC and EC20 concentration ranges. 141

Figure 4.9: Inhibition of *E. faecium* following exposure to the Binary Group 1 Mixtures of the As MEC with a PNEC range of (a) TCS, (b) OTC, or (c) CIP. 144

Figure 4.11: Inhibition of *E. faecium* following exposure to the Binary Group 3 Mixtures of the Zn MEC with a PNEC range of (a) TCS, (b) OTC, or (c) CIP. 149

Figure 4.12: Inhibition of *E. faecium* following exposure to the Binary Group 4 Mixtures of the TCS MEC with a CCC range of (a) As, (b) Cu, or (c) Zn. 151

Figure 4.13: Inhibition of *E. faecium* following exposure to the Binary Group 5 Mixtures of the OTC MEC with a CCC range of (a) As, (b) Cu, or (c) Zn. 154

Figure 4.14: Inhibition of *E. faecium* following exposure to the Binary Group 6 Mixtures of the CIP MEC with a CCC range of (a) As, (b) Cu, or (c) Zn. 156

Figure 4.15: *E. faecium* change in susceptibility to (a) ciprofloxacin (-0.64% - 7.58%), (b) vancomycin (1.02% - 4.95%), and (c) oxytetracycline (0.78% - 4.59%) following 24-hour acclimation to the EPA Criterion Continuous Concentration of As, Cu, or Zn, or the Probable No Effects Concentration of TCS, OTC, or CIP.....162

Figure 4.16: *E. faecium* change in susceptibility to (a) ciprofloxacin (1.25% - 19.69%), (b) vancomycin (0.18% - 14.57%), and (c) oxytetracycline (-0.87 – 16.67%) following 24-hour acclimation to the MEC of As, Zn, TCS, OTC, or CIP. 164

Figure 4.17: *E. faecium* change in susceptibility to (a) ciprofloxacin (-4.65 – 3.66%), (b) vancomycin (-3.73% - 0.22%), and (c) oxytetracycline (-1.92% - 4.79%) following 24-hour acclimation to the Binary Group 1 Mixtures of the As MEC with the PNEC of TCS, OTC, or CIP.....167

Figure 4.18: *E. faecium* change in susceptibility to (a) ciprofloxacin (2.08% - 6.09%), (b) vancomycin (2.99% - 5.63%), and (c) oxytetracycline (3.00% - 5.03%) following 24-hour acclimation to the Binary Group 3 mixture of the Zn MEC with the PNEC of TCS, OTC, or CIP.....170

Figure 4.19: *E. faecium* change in susceptibility to (a) ciprofloxacin (1.60% - 5.02%), (b) vancomycin (-1.68% - 1.53%), and (c) oxytetracycline (4.06% - 5.14%) following 24-hour acclimation to the Binary Group 4 mixture of the TCS MEC with the CCC of As, Cu, or Zn.....172

Figure 4.20: *E. faecium* change in susceptibility to (a) ciprofloxacin (4.19% - 6.87%), (b) vancomycin (0.87% - 5.22%), and (c) oxytetracycline (0.22% - 2.88%) following 24-hour acclimation to the Binary

Group 5 mixture of the OTC MEC with the CCC of As, Cu, or Zn.....174

Figure 4.21: *E. faecium* change in susceptibility to (a) ciprofloxacin (0.68% - 4.58%), (b) vancomycin (0.76% - 3.72%), and (c) oxytetracycline (-0.64% - 3.37%) following 24-hour acclimation to the Binary Group 6 mixture of the CIP MEC with the CCC of As, Cu, or Zn.....177

Figure 5.1: Summary of findings and potential future implications.....182

List of Abbreviations

%I.....Percent Inhibition

µg.....Microgram

µL..... Microliter

AMR.....Antimicrobial Resistance

ANOVA.....Analysis of Variance

As..... Arsenic

ATCC.....American Type Culture Collection

°C.....Degrees Celsius

CAFO(s).....Confined Animal Feeding Operation(s)

CCC.....Criterion Continuous Concentration

Cd.....Cadmium

CDC.....Centers for Disease Control and Prevention

CFU.....Colony-Forming Units

CIP..... Ciprofloxacin

CLSI..... Clinical and Laboratory Standards Institute

COT..... Chlortetracycline, Oxytetracycline, and Tetracycline

Cr..... Chromium

CTX.....Cefotaxime

Cu.....Copper

DNA ..... Deoxyribonucleic Acid

EC20 ..... 20% Effect Concentration

Efm ..... *Enterococcus faecium*

EPA ..... Environmental Protection Agency

ERL ..... Effects Range Low

ERM ..... Effects Range Median

FDA ..... Food and Drug Administration

GI ..... Gastrointestinal

HABs ..... Harmful Algal Bloom(s)

Hg ..... Mercury

MEC ..... Median Effect Concentration

MIC ..... Minimum Inhibitory Concentration

mL ..... Milliliter

mm ..... millimeters

NHS ..... National Health Service

Ni ..... Nickel

nm ..... Nanometers

NOAA ..... National Oceanic and Atmospheric Administration

NPS ..... Nonpoint Source

OD_{608} ..... Optical Density at 608 nanometers

OTC ..... Oxytetracycline

Pb ..... Lead

PNEC ..... Probable No-Effect Concentration

ppb.....Parts per billion

TCS ..... Triclosan

tRNA.....Transfer Ribonucleic Acid

USGS ..... United States Geographic Survey

VAN..... Vancomycin

VRE..... Vancomycin-Resistant *Enterococcus*

Vv.....*Vibrio vulnificus*

WHO.....World Health Organization

WWTP(s) .....Wastewater Treatment Plant(s)

Zn ..... Zinc

ZOI..... Zone of Inhibition

Chapter 1: Background and Significance

Runoff Pollution and Urbanization

The coastal zone of South Carolina, comprising Horry, Georgetown, Charleston, Berkeley, Dorchester, Colleton, Beaufort, and Jasper counties, is currently experiencing extremely high rates of population growth and urbanization: The population of this region is expected to exceed 1.5 million by 2030 (SCSGC 2018). Population data from the 2020 census indicate that the Charleston Tri-County (Charleston, Berkeley, and Dorchester counties) area alone experienced a 20.32% growth in population over the past ten years, compared to 10.65% for the state of South Carolina and 7.35% overall growth across the United States (Bureau 2020). This indicates that the Charleston metro area alone is growing at a rate nearly twice that of South Carolina overall, and nearly three times that of the United States as a whole.

Urbanization results in significant landscape ecology changes which increase imperviousness and cause alterations in the hydrological cycle, increasing runoff of nonpoint source (NPS) pollution including heightened levels of nutrients, microbes, and chemical contaminants. These contaminants may include legacy pollutants such as trace metals (Scott et al. 2006; Baalousha et al. 2015) in addition to contaminants of emerging concern (CECs), such as pharmaceuticals and personal care products (PPCPs) (Uyaguari et al. 2013; Maruya et al. 2014; Scott 2017; Apeti et al. 2018).

Associated hardscaping has caused an increase in the flashiness – the likelihood of a body of running water to flood during rainstorms, as defined by the American

Meteorological Society (AMS 2012) – of Southeastern urban drainage systems, and when coupled with more extreme weather associated with climate change has led to the construction of an extensive network of surface impoundments for managing stormwater runoff. Increasing sea level rise may result in more frequent flooding of these stormwater ponds, adding to their ecological complexity and management. Within the state of South Carolina alone there are more than 21,500 retention/detention ponds along the coastal zone, which are increasing at a rate of approximately 4% per year (SCSGC 2018).

Stormwater ponds collect runoff from a variety of land uses, including agricultural, recreational, residential, and industrial areas. Agricultural ponds collect runoff from farms, which carries pesticides and fertilizers from crop application and manure from livestock operations. Additionally, lagoons are used in aquaculture and confined animal feeding operations (CAFOs) to contain rainwater that washes away fecal bacteria, metals, antimicrobial products, and other pharmaceutical and chemical contaminants used as feed additives for tens of thousands of fish and livestock annually (Bradford et al. 2008; Kitiyodom et al. 2010; Landers et al. 2012). Stormwater ponds in recreational areas may be affected by fertilizer and pesticide runoff from sports fields and golf courses as well as increased petroleum hydrocarbons emissions from automobiles along roadways and in parking lots. Likewise, fertilizer and pesticides from home yard care, increased petroleum hydrocarbons from roadways, and bacteria from pet and wildlife waste inevitably wash into the stormwater ponds scattered throughout residential urban and suburban communities to mitigate flooding and surface discharges.

Ideally, detained stormwater is remediated of contaminants by a combination of physical settlement, microbial remediation, photodegradation, and dilution by precipitation

(Booth et al. 2002; Vezzaro et al. 2011; Borne et al. 2014) before discharging into adjacent natural waters. Assessment of pollutant levels in SC stormwater ponds indicates that such remediation is, to a large extent, successful, as sediment concentrations of several trace metals (Cd, Cu, and Zn) and petroleum hydrocarbons (fluoranthene, phenanthrene, and pyrene) were higher in ponds than in adjoining tidal creeks (SCSGC 2018). As most ponds are very effective at pollutant retention, greatly reducing pollution in adjoining surface waters, many pollutants have been measured in stormwater ponds, including pesticides, polycyclic aromatic hydrocarbons, and trace metals.

Levels of trace metals (As, Cu, and Zn) exceeding sediment quality guidelines (Long and Morgan Lee G 1991; Macdonald et al. 1996) have been measured in coastal stormwater ponds, often considerably higher than those measured in downstream estuaries into which they drain (Baalousha et al. 2015). In theory, stormwater impoundments would also serve as protected environments for local flora and fauna. However, high nutrient and chemical contaminant loads alter the growth of eukaryotic and prokaryotic microbes, which in turn impact water quality (SCSGC 2018). This is evidenced by harmful algal blooms and high levels of fecal coliform and *Vibrio* bacteria that have been measured in stormwater ponds. The high levels of trace metals and bacteria in ponds pose a significant risk for inducement of highly antibiotic resistant pathogens; several studies (Baker-Austin et al. 2006; Stepanauskas et al. 2006; Seiler and Berendonk 2012; Xu et al. 2017) have found significant statistical associations between trace metals concentrations in surface waters and sediments and increased rates of antimicrobial resistance (AMR).

Antimicrobials as Contaminants of Emerging Concern

There are many anthropogenic sources of antibiotic resistance which end up in the environment. Agricultural runoff, particularly from poultry and swine farms, comprises a major source of antibiotic inputs into aquatic ecosystems. An estimated 61% of U.S. antibiotics sold annually are for agricultural purposes (FDA 2014). Approximately 88% of pigs raised in the United States are fed antibiotics daily for disease prevention and growth promotion, most commonly tetracyclines, aminoglycosides, and sulfonamides (Landers et al. 2012; FDA 2014). These antibiotics are then excreted from the animals and washed into adjacent catchment lagoons and water systems (Bradford et al. 2008).

Recreational areas are also of concern; golf courses are of particular interest due to their land application of biosolids from wastewater treatment plants (WWTPs) as fertilizer and spray irrigation of treated wastewater (National Research Council 2002; Stacey et al. 2019), which may still contain trace amounts of pharmaceutical contaminants (Edwards et al. 2009). WWTPs are yet another potential source. Their pre- and post-treatment containment ponds as well as final effluent can contain pharmaceutical products generally unaffected by the treatment process, including antimicrobials like triclosan (Cooper et al. 2008; Uyaguari et al. 2011; Hedgespeth et al. 2012).

Once trace amounts of antimicrobials reach natural aquatic ecosystems, they are not generally a point of concern from a toxicological standpoint. Despite the frequency of detection, the levels at which they are present in the environment are so low they are not considered to be a toxicological threat to aquatic organisms (Kolpin et al. 2002). However, studies have not traditionally included microbes as organisms of interest when compiling risk assessments. With that in mind, it must be noted that the harmful effect of antibiotics

in the environment tends to be opposite that of other chemical contaminants. While most chemicals are more harmful at higher concentrations, low concentrations of antibiotics serve as a sub-lethal dose to the natural bacterial components of aquatic ecosystems (Carey and McNamara 2014; DeLorenzo et al. 2016; Scott et al. 2016). This, in turn, fosters selection of resistant bacteria and propagation of resistance genes through a variety of horizontal gene transfer mechanisms (Aminov 2010; McDaniel et al. 2010). For example, a study of the gut microbiota of the grass shrimp *Palaemonetes pugio* showed a significant change in resistance profiles after exposure to oxytetracycline in sub-lethal doses (Uyaguari et al. 2009). Another study indicated that acclimation to levels of triclosan found in a variety of natural and man-made environments increases antimicrobial resistance (AMR) in several human pathogens (Carey and McNamara 2014).

Aquatic Pathogens

Enterococci are Gram-positive coccus-shaped bacteria ubiquitous to the gastrointestinal tract (GI) of most animals. The opportunistic pathogens *E. faecium* and *E. faecalis* are among the most common causes of GI illness; vancomycin-resistant *E. faecium* (VRE), in fact, is one of the most concerning sources of nosocomial infection worldwide (Willems et al. 2005). Enterococcal illness can be contracted from contact recreation, ingestion of contaminated water, or contact with improperly sanitized hospital equipment. Outside of clinical settings, enterococci are the standard indicator bacteria for fecal contamination of recreational waters in coastal ecosystems (US EPA; Byappanahalli et al. 2012; Boehm and Sassoubre 2014). This is due to their near-ubiquitous presence in fecal samples as well as a well-defined correlation between enterococcal presence and human health impacts. Due to these associations, it has been suggested that *E. faecium* carrying

the *esp* surface protein can be used as a human-specific indicator of human fecal contamination, though *E. faecium* in other mammals may also express *esp* (Boehm and Sassoubre 2014).

Environmental sources of enterococci include runoff from agricultural areas, sewage overflow during flood events, and pet or wildlife waste (Bradford et al. 2008; Boehm and Sassoubre 2014). These same sources may also introduce enterococcal bacteria to stormwater ponds. Concentrations of fecal enterococci up to 13.13×10^5 colony forming units (CFU) per 100 milliliters (mL) have been measured in agricultural lagoons, and a 2014 report described levels of fecal enterococci in urban stormwater systems up to 2.4×10^3 CFU/mL (Clary et al. 2014).

E. faecium is an opportunistic pathogen; that is, they will generally only infect hosts with immune systems that are compromised in some way. Several virulence genes that have been noted to enhance disease-causing abilities of *E. faecium* include *gelE*, a gelatinase which hydrolyzes gelatin and collagen and exacerbates endocarditis (Vankerckhoven et al. 2004; Van Wamel et al. 2007; Al-Talib et al. 2015); aggregation substance *as1*, which both enables conjugative transfer of plasmids by clumping bacterial cells together and increases adherence to internal host cells (Vankerckhoven et al. 2004); and *esp*, the enterococcal surface protein. *Esp* is extremely important in cell adherence and biofilm formation (Vankerckhoven et al. 2004; Al-Talib et al. 2015) and increases conjugation frequency. Notably, expression of *esp* is increased when cultured at physiological temperature (37°C) compared to an approximated ambient temperature of 21°C, as well as in anaerobic conditions like those found in the gastrointestinal tract (Vankerckhoven et al. 2004; Lund et al. 2006; Van Wamel et al. 2007; Al-Talib et al. 2015).

Vibrio vulnificus is a gram-negative, halophilic, rod-shaped saltwater bacterium. An opportunistic pathogen, *V. vulnificus* is often found in warm coastal and estuarine waters. It can be transmitted by ingestion of contaminated seafood, such as oysters, causing severe gastrointestinal illness. *V. vulnificus* can also enter the body directly through broken skin, leading to necrotizing fasciitis and septicemia (Jones and Oliver 2009; CDC 2019b). *Vibrio* bacteria are the main source of human illness and death from shellfish (Jones and Oliver 2009) and *Vibrio* also have immense ability to develop resistance to a broad range of antibiotic agents. It is estimated that *V. vulnificus* accounts for about a third of the total seafood-borne illness costs in the U.S. (Ralston et al. 2011) and has a 51% mortality rate (Jones and Oliver 2009).

Seafood like shrimp and oysters are of particular concern. Resistant bacteria can not only be present in seafood sold in stores but are pathogenic to the shrimp themselves as well (Kitiyodom et al. 2010; Dash et al. 2017). *Vibrios*is can affect both larval and adult shrimp, causing decreases in growth rate, lethargy, and discolored tissue. Infections can cause widespread mortality in mariculture systems (Dash et al. 2017). Oysters, meanwhile, ingest *Vibrio* bacteria while filter feeding, sometimes accumulating the bacteria to over 100 times the concentration in the water from which they were harvested (Froelich and Noble 2016). Consumption of raw or undercooked oysters can therefore be a significant source of *Vibrio* illness.

There are two main established subtypes of *V. vulnificus,* differentiated by the virulence correlated gene *vcg* and categorized based on specific sequence variations which are generally correlated with environmental (*vcgE*) or clinical (*vcgC*) isolation (Jones and Oliver 2009). Both subtypes, however, can possess genes which cause illness in humans

(Bier et al. 2013). Genes which confer increased ability for cell-to-cell contact—including flagellar structural proteins like *flgC* and *flgE*, and pilus formation and structural proteins (*pilA, pilD,* and *pilF*)—are vital for cytotoxicity in wound infections (Jones and Oliver 2009; Roig et al. 2010; Yamazaki et al. 2019). *PilF* has been used to determine potential pathogenicity of *V. vulnificus* (Roig et al. 2010; Baker-Austin et al. 2012). Capsular polysaccharide (*cps*) is also essential in evading the host defenses. Additionally, toxin-producing genes like *vvhA* enhance virulence, but are not necessary to induce lethality (Jones and Oliver 2009). A recent study from the University of South Carolian (Correa Velez and Norman 2021) found that exposure to WWTP effluent resulted in increased upregulation of genes involved in biofilm formation and downregulation of genes associated with motility in *V. vulnificus*; increased biofilm formation is associated with increased antibiotic resistance in *Vibrios*.

Isolates of *V. vulnificus* from coastal Georgia and South Carolina were noted to express resistance to an enormous range of antibiotics. Of 151 *V. vulnificus* isolates, 45% were resistant to three or more of eleven tested classes of antibiotics, indicating widespread multidrug resistance (**Figure 1.1**). This impressive ability to display resistance to so many different types of antimicrobials is especially concerning when the pathogenicity of the bacteria is considered. Not only does *V. vulnificus* cause serious and deadly foodborne illness, but it is associated with serious wound infections after exposure to affected water, quickly leading to necrotizing fasciitis, septicemia, and death. Skin infections tend to be acquired by swimming or wading in warm, coastal waters (Koh et al. 2017; CDC 2019b) Once these infections reach the bloodstream, the considerable virulence of this pathogen

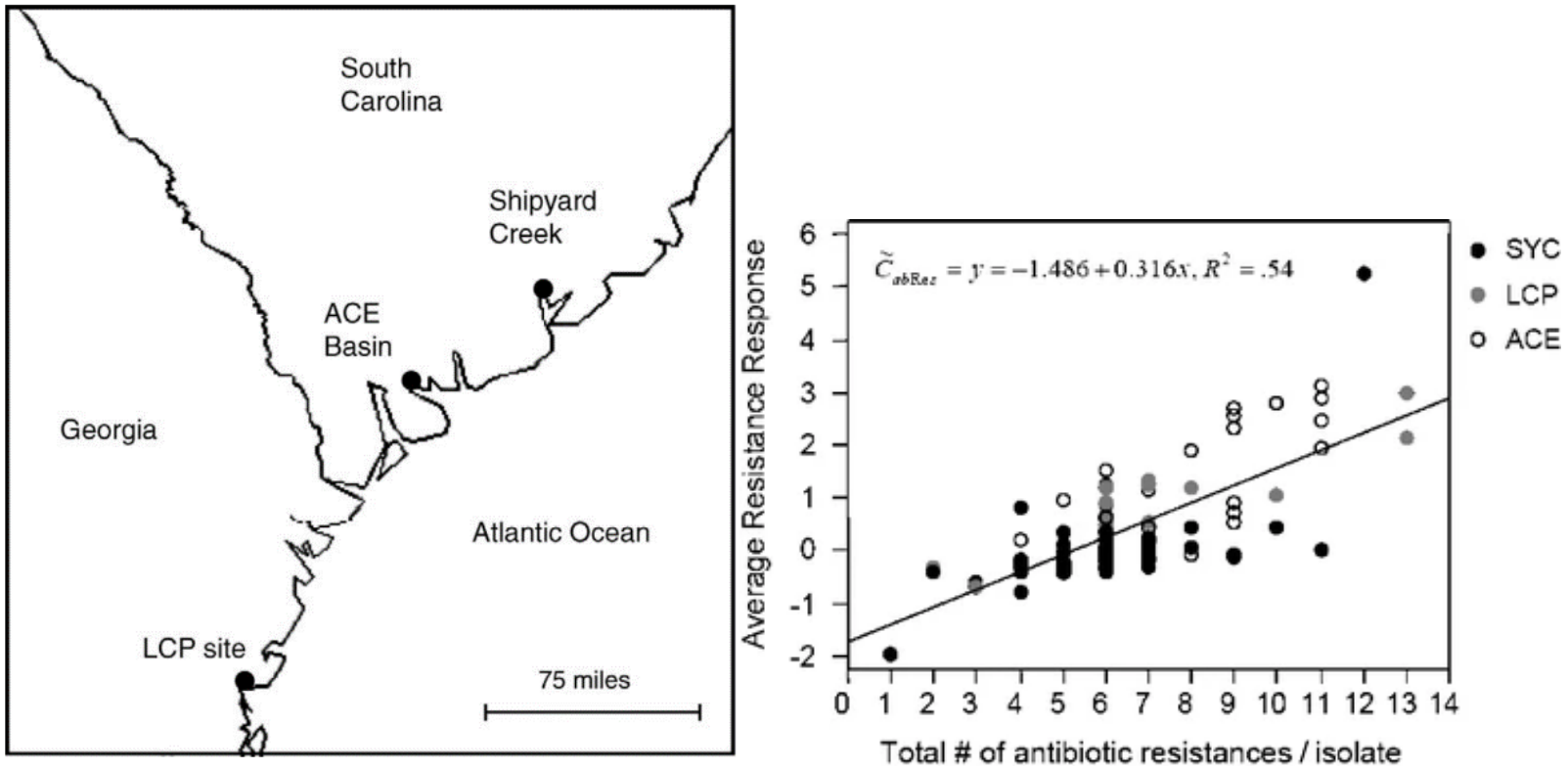


Figure 1.1: Resistance response for *V. vulnificus* (Baker-Austin et al. 2008, 2009) from the Atlantic coast in South Carolina and Georgia.

becomes especially apparent: If *V. vulnificus* septicemia is not diagnosed and treated within 72 hours of onset, the mortality rate jumps to 100% (Baker-Austin et al. 2009). As climate change expands the geographic and temporal range of the pathogens due to increased salinity (Jones et al. 2008; Deeb et al. 2018) and temperature (Muhling et al. 2017), cases of *Vibrio* infections are on the rise (King et al. 2019).

With this increasing frequency of cases, news organizations have sensationalized the microbe as a "flesh-eating bacterium" (Woosten Jr. 2016; Loria 2018; Baer 2019; O'Kane 2019; Shamard 2019). Though awareness of these illnesses is vital in getting infected people treated in a timely manner, such alarmism could potentially affect mariculture, the seafood industry, and tourism economies which rely on seafood and contact recreation activities. The more researchers and medical professionals understand about these bacteria and how they interact with humans within coastal environments, the better we can alert the public and prepare these industries to deal with this rising infectious disease threat.

Rise of Antimicrobial Resistance

Following Alexander Fleming's discovery of penicillin in 1928, fewer than twenty years passed before the first cases of penicillin-resistant infections were noted in a clinical setting (Ventola 2015). Since then, the phenomenon has accelerated at an unprecedented rate, hurried along by human activities. The Centers for Disease Control and Prevention (CDC) estimate that there are 2.8 million illnesses and 35,000 deaths each year from drug-resistant bacteria in the U.S. alone (CDC 2019a), with an estimated health care cost of $21-34 billion (WHO 2014).

A more worrisome number is the future predictions. A report from the United Kingdom's National Health Service (NHS) estimates that by 2050, there will be ten million deaths attributable to antimicrobial resistance globally each year, compared with approximately 700,000 deaths observed today (O 'Neill 2016). In 2014, the World Health Organization (WHO) warned that if antimicrobial resistance continued to trend upwards, "A post-antibiotic era – in which common infections and minor injuries can kill--is a very real possibility for the 21st century," (WHO 2014). Despite preventive measures helping to decrease the number of annual deaths from AMR infections, a similar report from the CDC in 2019 insists that it is already here (CDC 2019a).

A global epidemiological study from Murray et al. at the University of Washington paints a bleak portrait of the current burden imposed by AMR infections. The predictive models utilized in this study estimate 1.27 million deaths in 2019 to have been directly attributable to antimicrobial-resistant bacteria. By these estimates, AMR is, "a leading cause of death around the world, with the highest burdens in low-resource settings" (Murray et al. 2022).

A truly post-antibiotic era will have far-reaching consequences beyond untreatable foodborne and skin infections: Our entire modern medical system hinges on antibiotic use. Common, easily treated childhood illnesses like strep throat will be life-threatening again. Young adults having their wisdom teeth removed will have more to worry about than the anesthesia, as the infection risk will jump dramatically. Surgeries which already carry high infection risk—from orthopedic implants and pacemakers to organ transplants and cardiac bypasses—will become too risky to perform as readily as they are now. In short, increasing antimicrobial resistance is, arguably, the greatest public health threat facing current

generations. In order to combat it, closer study is required of often-overlooked environmental causes and mechanisms that may cause AMR.

As concerning as the role of pharmaceuticals in the environment is, antimicrobial contamination is not the only stressor in aquatic environments noted to induce bacterial AMR. There is a growing body of evidence indicating that metals contamination plays a role in bacteria developing multidrug resistance, even if those bacteria are in areas in which antimicrobial contamination has never been detected (Baker-Austin et al. 2006; Stepanauskas et al. 2006; Seiler and Berendonk 2012; Xu et al. 2017). A number of different bacterial species have been found to develop high levels of AMR, including pathogens (e.g., *Vibrio* species), indicator bacteria (e.g., *Escherichia coli* and *Enterococcus* species), and bacterial fauna in microbial loop communities (e.g., *Pseudomonas* species). Co-resistance mechanisms for metals and antimicrobial resistance have also been noted (**Table 1.1**). However, most of this research has been performed with field studies, with which come a myriad of potential confounding factors. Additional laboratory studies are needed to definitively link bacterial exposure to metals with development of antimicrobial resistance.

As indicated in **Table 1.1**, bacterial exposure to trace metals may cause cross resistance with these five different classes of antibiotics. This is a significant finding as trace metal pollution is highly pervasive in coastal regions of the US, including Superfund Sites (Cr, Hg, and Zn), stormwater ponds (Cd, Cu, and Zn) and coastal tidal creeks (As, Cd, Cr, Cu, Hg, Ni, Pb, and Zn). Potentially toxic levels of these trace metals have been reported by the National Oceanic and Atmospheric Administration (NOAA) throughout SC and the southeastern U.S. at pervasively high levels (e.g., 8.7-85% prevalence in SC sites (Sanger

et al. 1999). Of additional concern are the distributions of low-level trace metals in coastal waters which may induce antimicrobial resistance

Antimicrobial Mechanisms of Action

Antibiotics are chemical substances either synthesized or produced by living organisms, such as bacteria and fungi, which kill or prevent growth of bacterial pathogens. They can be either bacteriostatic (preventing growth) or bactericidal (killing the bacteria outright). There are several classes of antibiotics with varying mechanisms of action, including disruption of cell wall synthesis, DNA synthesis inhibition, and prevention of ribosomal translation. What follows is a discussion of several major classes of antibiotics used in this study.

(a) Tetracyclines

Tetracyclines are a class of bacteriostatic antibiotics which inhibit bacterial growth by preventing protein synthesis. More specifically, they competitively bind to the 30S ribosomal subunit, effectively impeding translation of tRNA into proteins. They are generally considered to be broad-spectrum antibiotics, but are frequently found to be ineffective against enteric, gram-negative bacilli and staphylococci (Papich 2016).

Oxytetracycline (**Figure 1.2**) is a common treatment for many human illnesses but is also often used in agricultural and veterinary practices. As such, it is often found in the soil of large-scale farming operations like CAFOs and washes into catchment lagoons with stormwater runoff (Bradford et al. 2008). It can also be directly introduced to waterways through mariculture and aquaculture practices (Thurman 2003; Burridge et al. 2010).

Table 1.1: Examples of cross-resistance between heavy metals and antibiotics (Baker-Austin et al. 2006)**.**

Resistance mechanism	Metal ions	Antibiotics
Reduction in permeability[b]	As, Cu, Zn, Mn, Co, Ag	Cip, Tet, Chlor, ß-lactams
Drug and metal alteration[c]	As, Hg	ß-lactams, Chlor
Drug and metal efflux[d]	Cu, Co, Zn, Cd, Ni, As	Tet, Chlor, ß-lactams
Alteration of cellular target(s)[e]	Hg, Zn, Cu	Cip, ß-lactams, Trim, Rif
Drug and metal sequestration[f]	Zn, Cd, Cu	CouA

[a]Abbreviations: Chlor, chloramphenicol; Cip, ciprofloxacin; CouA, coumermycin A; Rif, rifampicin; Tet, tetracycline; Trim, trimethoprim.
[b]Includes reduction of membrane permeability to metals and antibiotics.
[c]Includes drug and metal inactivation and modification.
[d]Includes rapid efflux of the metal and antibiotic.
[e]Includes alteration of a cellular component to lower its sensitivity to the toxic metal and antibiotic.
[f]Includes drug and metal sequestration.

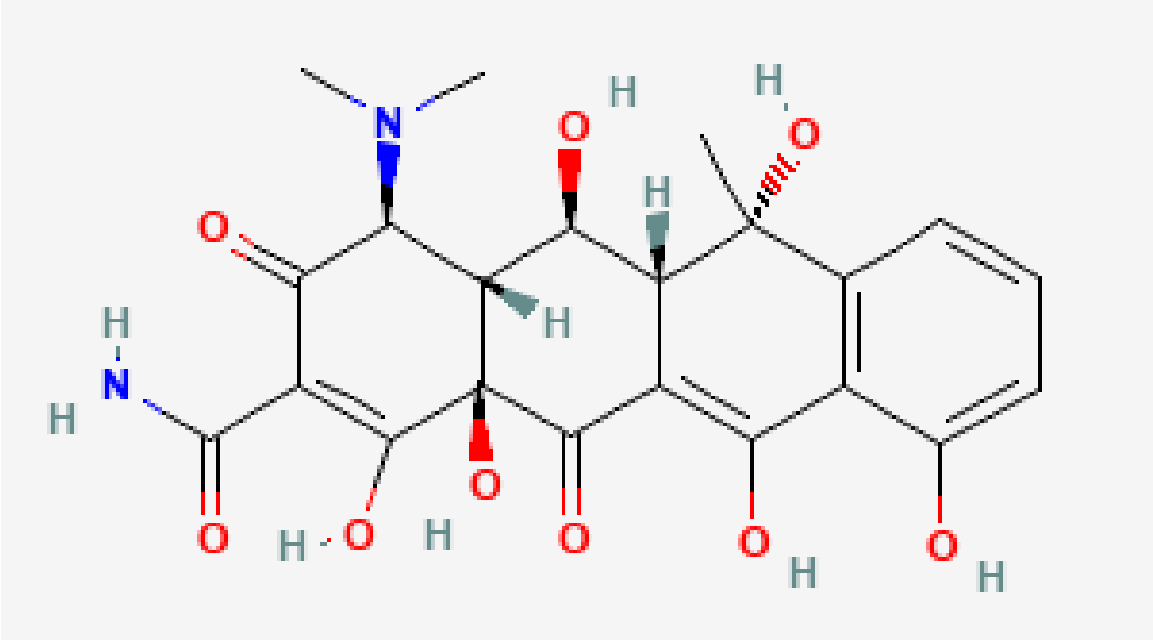


Figure 1.2: Chemical structure of Oxytetracycline (PubChem 2011 Dec 26).

(b) Fluoroquinolones

Fluoroquinolones are broad-spectrum antibiotics which exhibit bactericidal properties in both gram-negative and gram-positive bacteria. They are highly effective in preventing DNA replication, transcription, and repair by inhibiting the activity of DNA topoisomerases. Fluoroquinolones are often used as alternative treatment for bacteria resistant to cell wall inhibiting antibiotics such as penicillins, as the mechanisms of action are so vastly different (Bayer Pharmaceuticals 2004; Silva et al. 2011).

The fluoroquinolone, ciprofloxacin (**Figure 1.3**), is of special interest in antimicrobial resistance studies due to its wide-ranging effectiveness. It has long been included on the WHO's Essential Medicines List and is on their Watch Group of antimicrobials with a higher potential for development of antimicrobial resistance (WHO 2021). It is especially relevant to this study in that it is found in sublethal concentrations in a growing number of aquatic systems worldwide (Kelly and Brooks 2018). Kolpin et al. (2002) reported maximum surface water concentrations of 30 ng/L in watersheds across the US.

(c) Cephalosporins

Cephalosporins are bactericidal β-lactam antibiotics arranged into five generations, with efficacy against aerobic Gram-positive and Gram-negative microbes varying depending on the generation (Merck/Werth 2020). During peptidoglycan synthesis, β-lactam antibiotics replace a key component in peptidoglycan with an unstable β-lactam ring. As these altered peptidoglycans are incorporated into the cell wall, they cause overall instability and eventual collapse of the cell. The class in general is very commonly used in clinical

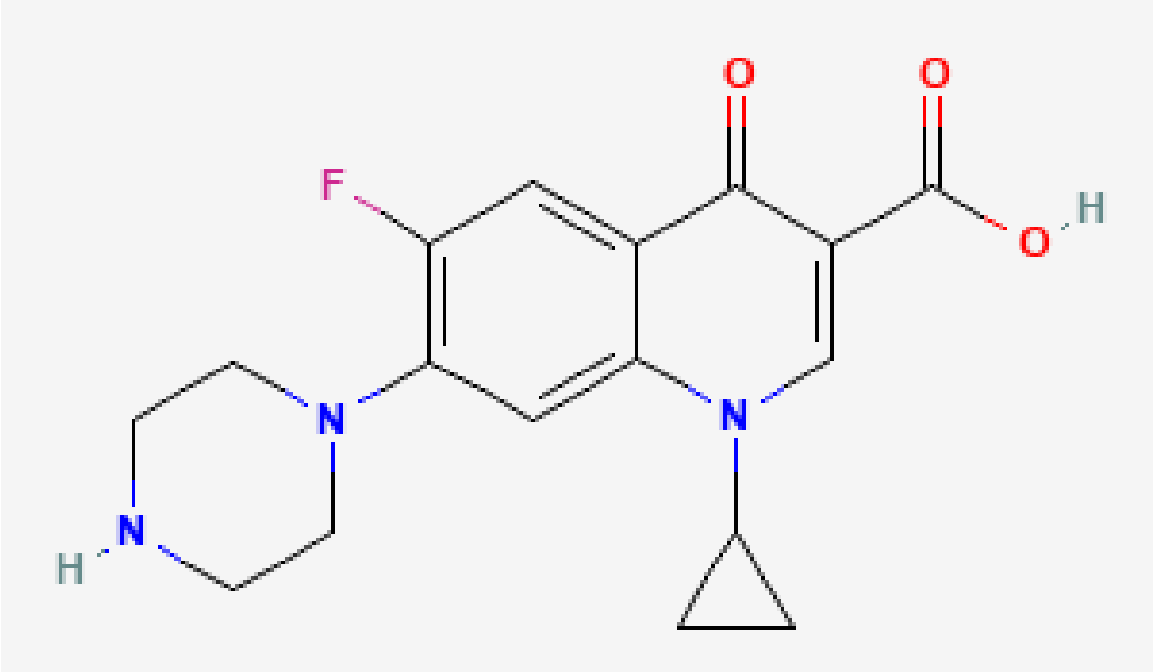


Figure 1.3: Chemical structure of ciprofloxacin (PubChem 2005a Mar 25).

settings, due to wide-ranging effectiveness and high patient tolerance (Chaudhry et al. 2019).

Cefotaxime (**Figure 1.4**) is a third generation, extended-spectrum cephalosporin with widespread uses, from severe rhinosinusitis and pneumonia to bacteremia and bacterial meningitis (Chaudhry et al. 2019). Third generation cephalosporins are often used as first-line therapy against necrotizing fasciitis, a condition often associated with *Vibrio vulnificus* infections, especially in conjunction with a fluoroquinolone like doxycycline (CDC 2019b).

(d) Glycopeptides

Glycopeptide antibiotics are bactericidal, preventing construction of the cell wall by inhibiting peptidoglycan synthesis. Contrary to β-lactams, however, they achieve this by competitively binding cell wall proteins and preventing addition of new subunits to the peptidoglycan layer. Glycopeptides are narrow spectrum and only indicated for use in Gram-positive infections, especially enterococcal infections that exhibit resistance to a wide range of other antimicrobials. As such, glycopeptides are, "drug[s] of last resort for treatment of life-threatening infections caused by Gram-positive bacteria" (Donadio and Sosio 2009 Jan 1).

In recent years, vancomycin (**Figure 1.5**) resistance has come to the forefront of clinical use of glycopeptides. Vancomycin-resistant Enterococcus (VRE) is considered by the CDC as a serious concern in nosocomial infections in the U.S., accounting for an estimated 54,000 cases in hospitalized patients, 5,400 deaths, and $539M in attributable

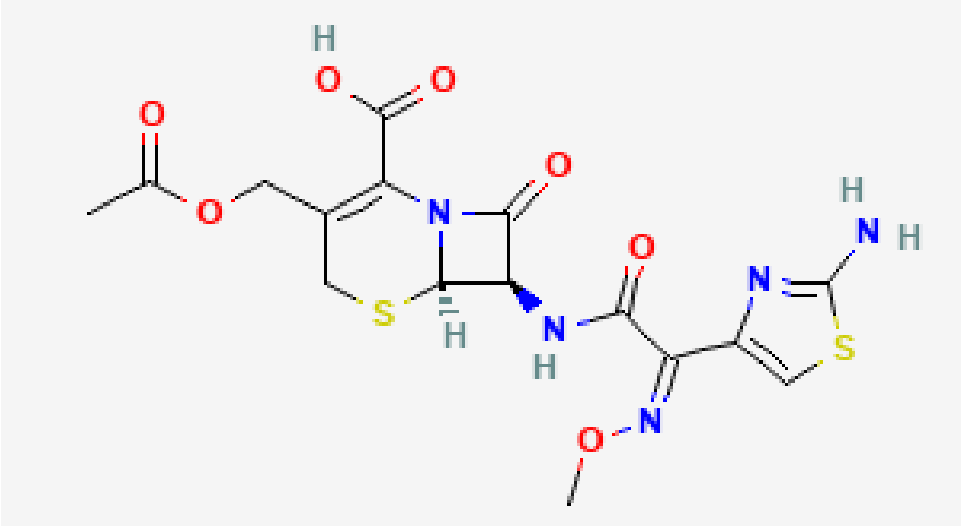


Figure 1.4: Chemical structure of cefotaxime (PubChem 2005 Aug 1).

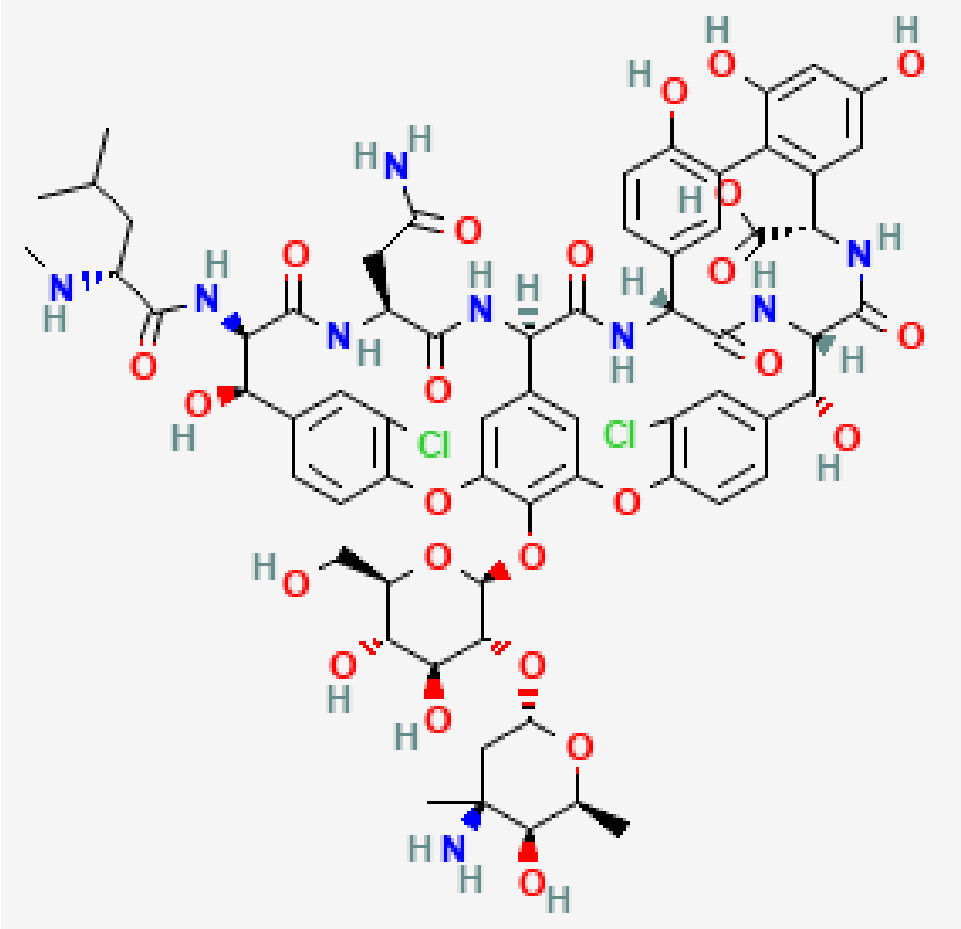


Figure 1.5: Chemical structure of vancomycin (PubChem 2005 Jun 24).

healthcare costs in 2017 (CDC 2019a). A major concern with VRE is that, given the broad distribution of enterococci in healthcare settings, resistance to vancomycin – a drug of last resort – may be conferred to other multidrug-resistant bacterial pathogens.

(e) Biocides

Biocides are generally synthetic in origin and are readily used for surface disinfection in many settings, including healthcare and household uses. Contrary to antibiotics, instead of only targeting bacteria, biocides are effective against microbes such as fungi and viruses as well. There are a great variety of effective biocides, each with their own specific mechanism of action, and all of which may be contributing significantly to the increase in antimicrobial resistant infections (Jones and Joshi 2021).

Triclosan (**Figure 1.6**) is one of the most important biocides used in healthcare today. It features heavily in hand washes and surface disinfectants in hospitals, and until 2017 was approved for household products such as hand soap and toothpaste (FDA 2017). The 2017 ban on domestic triclosan use came in response to increasing evidence of a relationship between sublethal triclosan exposure and multidrug resistance in bacterial pathogens. Beyond the excessive use of triclosan in products outside of healthcare settings, triclosan is often found in WWTP effluent from populated areas, particularly in effluent affected by large medical communities (Diamond et al. 2011). Once in the aquatic ecosystem, triclosan is highly persistent in sediments and may expose aquatic bacteria to constant sublethal doses. *Vibrio* bacteria may be especially vulnerable to this exposure, as sediments tend to be seasonal reservoirs of *Vibrio* species in marine environments (Chase et al. 2015).

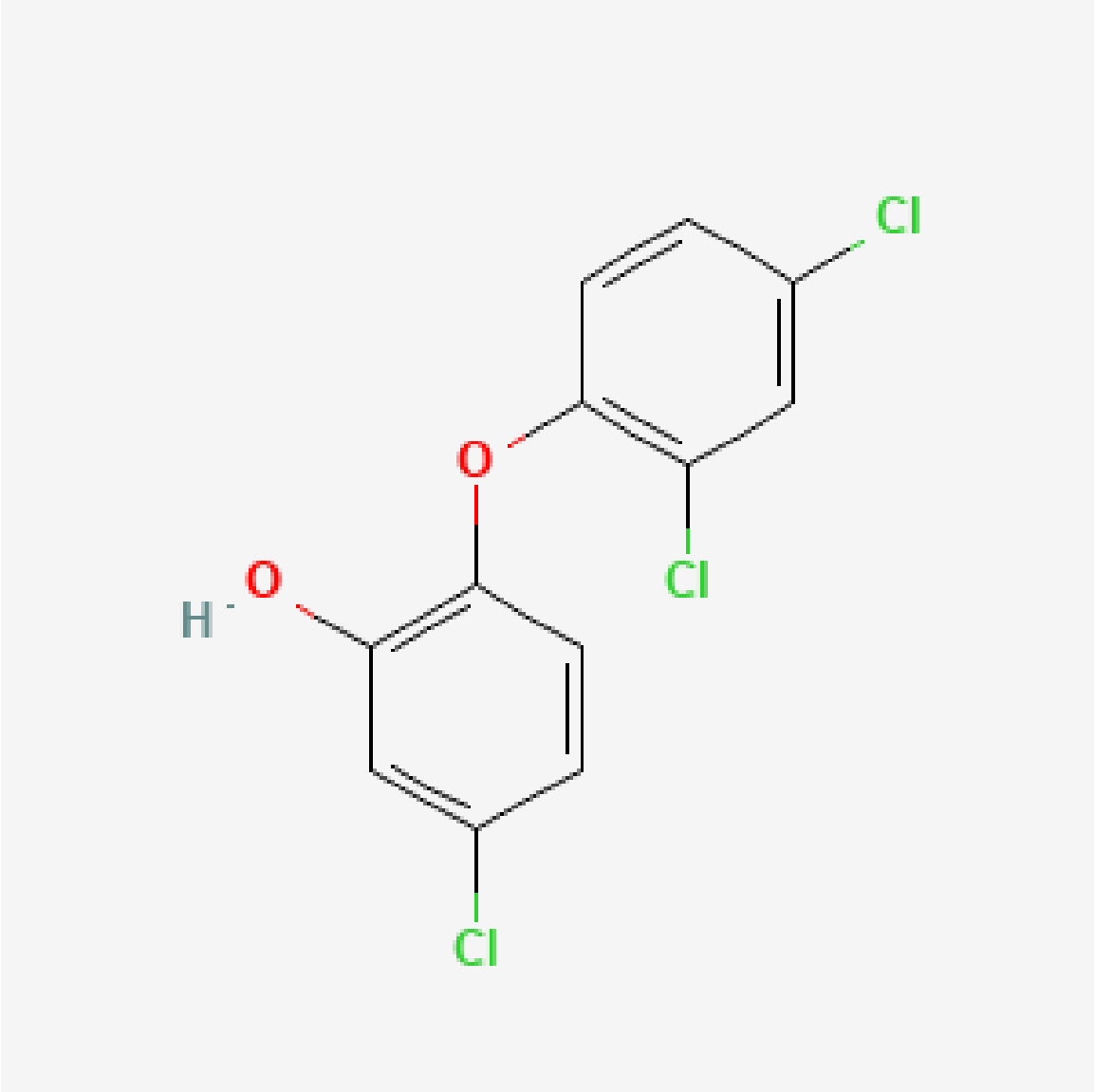


Figure 1.6: Chemical structure of triclosan (PubChem 2005b Mar 25).

(f) Arsenicals

Arsenic has long been known to possess antimicrobial properties. In 1908, the Nobel Prize was awarded to Paul Erlich for his work on an arsenic-based treatment for syphilis (Satter 2022). In 1944, shortly after the discovery of penicillin as an antibiotic, organic arsenic compounds were found to have antimicrobial activity nearly to the level of penicillin and contemporary mercury-based antimicrobials
(Albert et al. 1944). Despite a long-established use of arsenic as an antimicrobial, however, the mechanism of action for its antimicrobial activity in prokaryotes is not well understood.

The first arsenic-containing product to be approved by the Food and Drug Administration for use in animal drug products was 3-Nitro, or roxarsone (**Figure 1.7**), in 1944 (U.S. FDA 2021 Apr 30). It remained a common additive in animal feed until its ban in 2013 (Dunham 2013); while it was actively available as animal feed, livestock byproducts like poultry litter were often used as fertilizers in food crops (Garbarino et al. 2003; Rutherford et al. 2003). A study from the U.S. Geological Survey (USGS) in 2003 examining the "Environmental Fate of Roxarsone in Poultry Litter," found that while stable in dry litter, roxarsone degrades to arsenate (**Figure 1.8**) in approximately 30 days if water is added and the mixture allowed to compost (Garbarino et al. 2003). This degradation time decreased as the amount of water added increased. A related study of soils from agricultural fields indicated that long-term application of poultry litter strongly correlated with elevated levels of arsenic in the surrounding soils (Rutherford et al. 2003). Arsenate is highly persistent in the environment, as it binds strongly to soils and sediments (Panagiotaras and Nikolopoulos 2015).

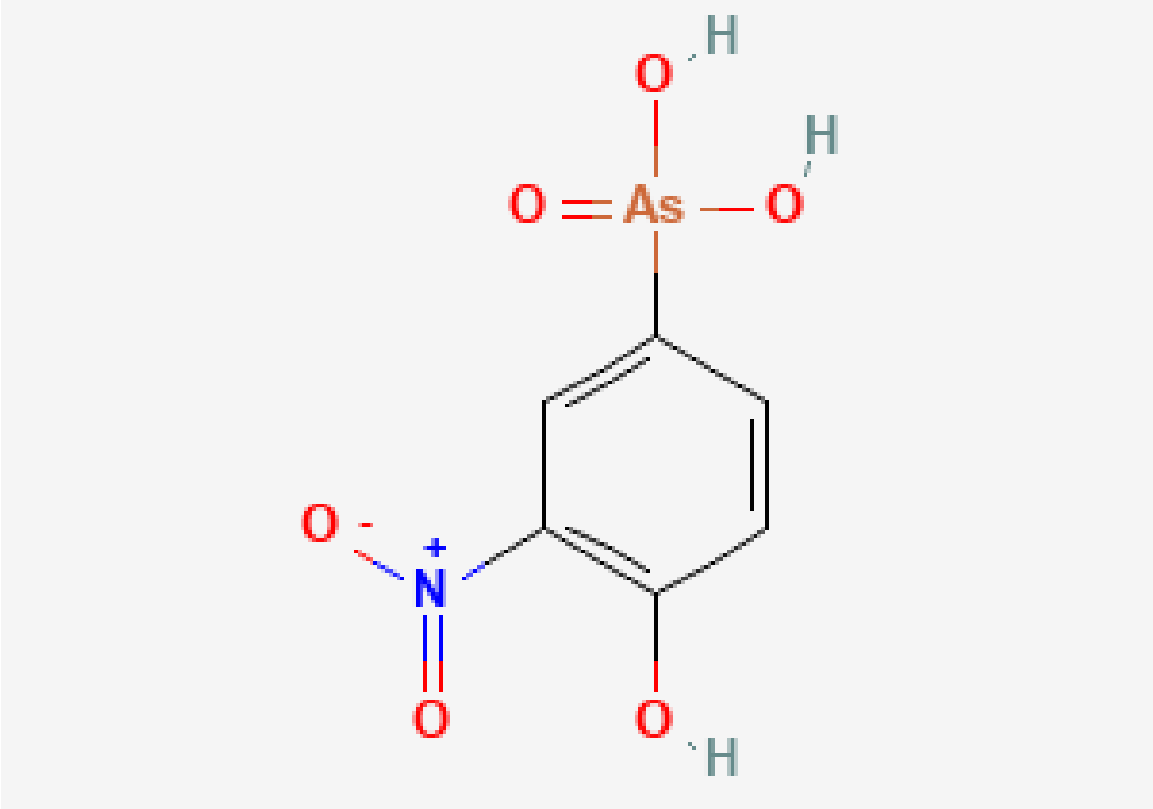


Figure 1.7: Chemical structure of roxarsone (PubChem 2005c Mar 25).

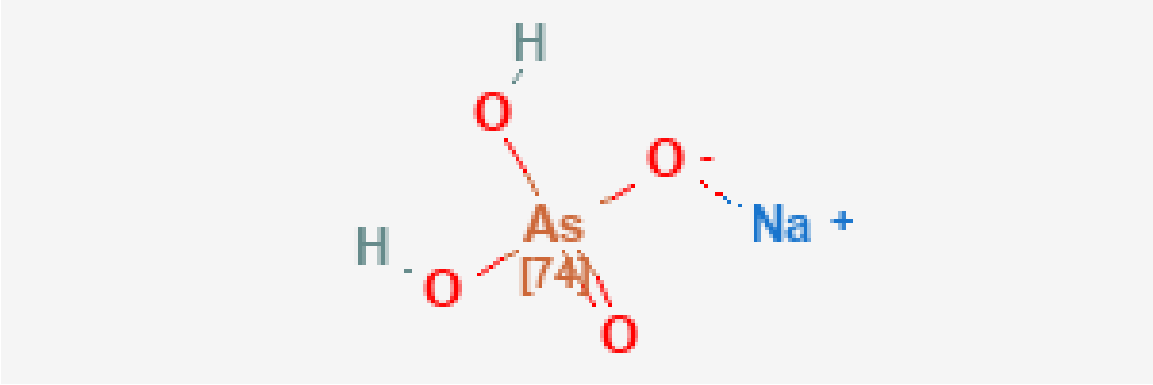


Figure 1.8: Chemical structure of sodium arsenate (PubChem 2013 May 27).

(g) Copper

Copper surfaces and nanoparticles are popular emerging potential solutions for multidrug resistance in nosocomial infections (Benhalima et al. 2019; Montero et al. 2019; Ermini and Voliani 2021). Though the antimicrobial activity of copper surfaces is well established, the specific mechanism of action is not, although studies suggest involvement of dissolved copper ions (Grass et al. 2011) or membrane damage (Santo et al. 2011). Additionally, studies indicate that copper ions and nanoparticles may induce oxidative stress in bacterial cells due to the production of reactive oxygen species (Applerot et al. 2012; Ermini and Voliani 2021). Copper is also frequently used as a feed additive in fish farms (Burridge et al. 2010) and often found in the sediments of aquaculture operations (Seiler and Berendonk 2012).

In addition to potential use in healthcare settings (Benhalima et al. 2019), copper sulfate (**Figure 1.9**) has been long recommended for prevention and remediation of algal blooms in lakes and stormwater ponds, especially in residential neighborhoods and recreational settings like golf courses (SC DNR 2020). In this context, copper sulfate is applied directly to the aquatic environment, where it will quickly be diluted by rainfall and tidal changes.

(h) Zinc

During the search for alternatives to antibiotic use on multidrug resistant pathogens, zinc oxide – especially in nanoparticle form – has been posed as a possible way to get around the multitude of resistance genes exhibited by the bacteria (Sirelkhatim et al. 2015). The antimicrobial activity of zinc ions is thought to be related to reactive oxygen species causing oxidative stress or direct disruption of the bacterial cell wall (Pasquet et al. 2014).

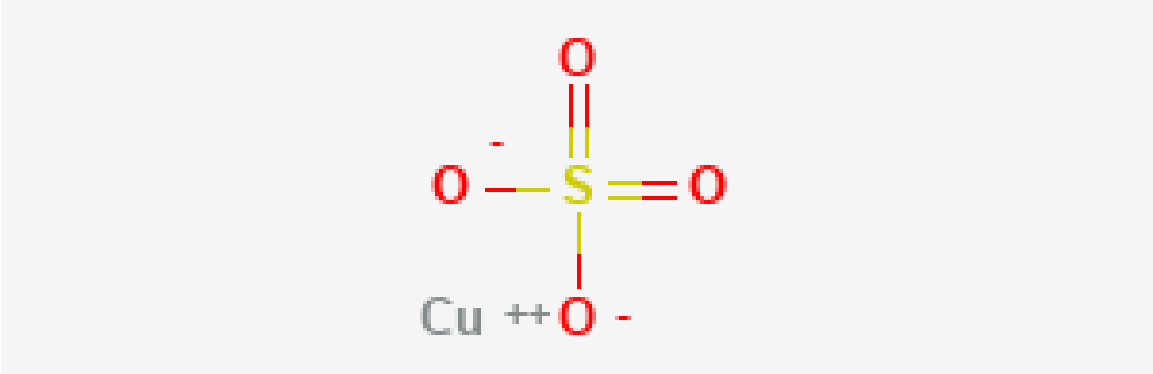


Figure 1.9: Chemical structure of copper sulfate (PubChem 2004a Sep 16).

Like copper, aquaculture sediments are often enriched with zinc, likely due to runoff from terrestrial agriculture operations (Seiler and Berendonk 2012) as well as its use as a feed additive (Burridge et al. 2010).

The antimicrobial activity of zinc sulfate (**Figure 1.10**) against a wide range of microbes is well established (Abdalkader and Al-Saedi). Outside of antimicrobial uses, zinc sulfate is a common additive to fertilizers in agricultural practices (UMN-Extension 2016). As with copper sulfate, agricultural use often results in zinc sulfate being carried away with stormwater runoff, to be sequestered in catchment ponds and lagoons.

Summary

Antimicrobial resistance is among the most pressing of modern public health concerns. Bacteria such as *Enterococci* and *Vibrios* are pathogens which may greatly affect healthcare as well as coastal mariculture and tourism industries. Meanwhile, a wide variety of human activities may introduce contaminants like heavy metals and antimicrobial compounds to the waterways in which such pathogens are ubiquitous.

While some studies have examined the abilities of individual chemical contaminants to affect the AMR of *Vibrios* and other microbes (e.g., Uyaguari et al, 2009), very few studies have examined the effects of chemical mixtures on antimicrobial resistance. This issue is further complicated by the abilities of microbes to adapt to a changing environment: Often, AMR may be increased at lower doses of chemical exposure to antibiotics, altering the prevailing and conventional risk assessment norm that, "the higher the dose, the greater the effect," (Scott et al. 2016). This conundrum is a major impediment to better understanding AMR and how to manage it more effectively from an environmental perspective.

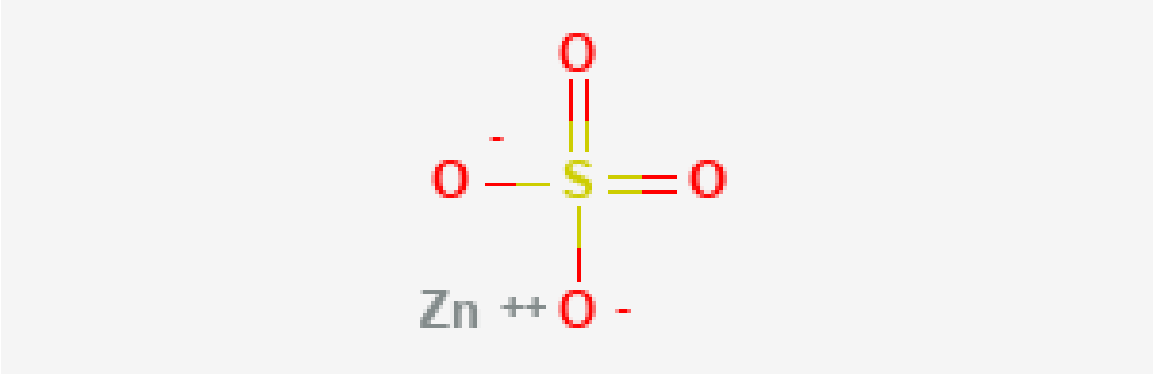


Figure 1.10: Chemical structure of zinc sulfate (PubChem 2004b Sep 16).

This study represents an effort to better understand the interactions of aquatic microbes to legacy pollutants (e.g., trace metals) and CECs (e.g., pharmaceutical products like antimicrobials). How these interactions affect the progression of AMR from a public health outlook will be explored and is the focus of this research. The importance of dose dependency and individual contaminant versus mixture exposures will be examined in both Gram-negative (e.g., *Vibrio* species) and Gram-positive microbes (e.g., *Enterococcus* species). Both microbes are important indicator bacteria, having environmental and public health significance in terms of water quality, seafood safety and wound infections. Their abilities to develop AMR in a changing coastal environment pose significant public health threats to individuals with underlying health issues including diabetes, autoimmune diseases, obesity, and liver and kidney disease, especially in children and senior citizens.

Chapter 2: Aims and Approach

Problem Statement

The short-term goal of this study is to determine whether the presence of trace metals and clinically relevant antimicrobial products found in coastal waters exerts a significant effect on the growth and development of antimicrobial resistance (AMR) in the aquatic pathogenic bacteria, *Vibrio vulnificus* and *Enterococcus faecium*. Both species were selected due to their impact on healthcare, including the problems presented by multidrug-resistant strains of each, and their ubiquitous presence in coastal waterways. Long-term, this project serves to establish a baseline for predicting the growth of these pathogens in the presence of metal and antimicrobial toxicants. This research will establish the foundational relationship between trace metals and antimicrobial products in enhancing AMR.

Experimental Design

The study consists of a series of high-throughput analyses of bacterial growth under a variety of exposure conditions to metals and antimicrobials, followed by phenotypic resistance profiles. The antimicrobials which will be used (triclosan [TCS], oxytetracycline [OTC], and ciprofloxacin [CIP]) are all frequently found in coastal aquatic systems (Kolpin et al. 2002; Thurman 2003; Hedgespeth et al. 2012; Kelly and Brooks 2018) and are also important in clinical settings. Triclosan is frequently used for decontaminating skin and surfaces in hospital settings, and until 2017 was allowed by the Food and Drug Administration (FDA) for use in household soaps and antiseptics (FDA 2017). Due to this

widespread use, TCS is commonly found in both natural aquatic environments and man-made water systems (i.e., WWTP holding ponds) (Carey and McNamara 2014). Ciprofloxacin is included on the WHO's list of essential medicines (WHO 2019). Although oxytetracycline is no longer used in clinical settings, it is in the same class as doxycycline, another antibiotic on the WHO's essential medicines list and a component of the first-line treatment against *Vibrio* septicemia (WHO 2019; CDC 2019b). OTC is used frequently as a feed additive in CAFOs (Bradford et al. 2008) and aquaculture (Uyaguari et al. 2009).

Additional antibiotics selected for building resistance profiles are applicable for each of these microbial organisms of interest. Cefotaxime (CTX) is commonly used in conjunction with doxycycline, a tetracycline antibiotic, as the first-line treatment against *Vibrio* septicemia (CDC 2019b). Vancomycin (VAN), meanwhile, is relevant in that vancomycin-resistant *Enterococci* are among the most important nosocomial infections (Willems et al. 2005; Al-Talib et al. 2015; Ventola 2015) and are considered a serious public health threat by the CDC (CDC 2019a).

Meanwhile, the metals selected (arsenic [As], copper [Cu], and zinc [Zn]) have been found to have a high potential for increasing antibiotic resistance and exhibit co-resistance mechanisms with several classes of antibiotics (Baker-Austin et al. 2006; Seiler and Berendonk 2012; Xu et al. 2017). Elevated levels of As, Cu, and Zn have also been measured in agricultural, industrial, and residential stormwater ponds (Bradford et al. 2008; Cooper et al. 2008), while Cu and Zn are frequent contaminants of aquaculture sediments (Burridge et al. 2010; Seiler and Berendonk 2012).

In this study, a varied range of concentrations of each metal and antimicrobial

was selected rather than simply using the minimal inhibitory concentration (MIC) as do a large contingent of toxicology studies investigating antimicrobial properties of various compounds. Specifically, the probable no effects concentration (PNEC) and other sub-lethal levels of the toxicants were tested, as these levels are both more environmentally relevant as well as more likely to induce changes in gene expression related to bacterial survival in the presence of those toxicants (Aminov 2010; McDaniel et al. 2010; Carey and McNamara 2014; DeLorenzo et al. 2016).

Specific Goals

Goal 1: Determine baseline growth curves and resistance for *V. vulnificus* and *E. faecium* exposure to three individual antimicrobials (CIP, OTC, TCS) and three individual trace metals (As, Cu, Zn) as compared to control.

Goal 2: Compare growth effects of nine binary antimicrobial + metal mixtures to control and individual exposures of *V. vulnificus* and *E. faecium*.

Goal 3: Compare phenotypic changes in resistance profiles for nine binary antimicrobial + metal mixtures to control and individual exposures of *V. vulnificus* and *E. faecium.*

Hypotheses

This research specifically tested the hypothesis that exposure to varying concentrations of trace metal and antimicrobial agents, both individually and in mixture, will adversely affect the survival, growth and development of *Vibrio* and *Enterococcus* bacteria. Specific sub-hypotheses tested included:

H_0: Antimicrobial products and trace metals will not significantly alter microbial growth patterns.

Ha: Antimicrobial products and trace metals will significantly alter microbial growth patterns.

H_0: Co-exposure of metals and antimicrobials will have no significant effect on either the growth or the development of antimicrobial resistance for either test organism as compared to exposure to individual toxicants.

Ha_1: Co-exposure of metals and antimicrobials is expected to alter the microbial growth patterns compared to the individual compound growth patterns.

Ha_2: Co-exposure of metals and antimicrobials is expected to alter the development of antimicrobial resistance compared to either class of contaminant alone.

Chapter 3: Vibrio vulnificus

Materials

(a) Microbial Organisms

V. vulnificus (ATCC 27562) was grown in Marine Agar/Broth 2216 (BD Difco). Kirby-Bauer assays were performed on Mueller-Hinton Agar (BD Difco). All cultures were grown at 30°C, while the Kirby-Bauer agar plates were incubated at 35°C, per the protocol instructions (Hudzicki 2009).

A clinical lab strain from American Type Culture Company (ATCC) was selected rather than using an environmental isolate. As the strain was clinically isolated, it is known to cause disease without the need for genotyping and therefore clearly relevant to the public health application of this experiment. Lab-kept strains are also further ecologically removed from environmental stressors which may influence initial growth.

(b) Antimicrobials

Analytical grade antimicrobial compounds were obtained as follows: Triclosan (TCS) (Irgasan, Fluka, 97.0 to 103.0%), oxytetracycline dihydrate (OTC) (Sigma LifeSciences, 94.5 to 102.0%), and ciprofloxacin hydrochloride monohydrate (CIP) (Alfa Aesar, 98.0 to 102.0%).Antimicrobial stocks were created in acetone (TCS and OTC) or deionized water (CIP) and kept in opaque containers in the dark at 4°C. CIP stocks were remade on a biweekly basis to avoid degradation (Eghianruwa 2014).

Antimicrobial diffusion discs were used at the following concentrations: CIP 5µg, Cefotaxime (CTX) 30µg, and OTC 30µg (BD BBL™ SensiDiscs™). These are 6-mm filter-paper discs impregnated with a standard concentration of antimicrobials, as listed.

(c) Metals

Analytical grade metal compounds were obtained as follows: Sodium hydrogen arsenate heptahydrate (As) (Alfa Aesar**,** ≥97.5 to ≤102.5%), cupric sulfate pentahydrate (Cu) (Sigma CellCulture, ≥98%), and zinc sulfate heptahydrate (Zn) (Sigma CellCulture, ≥99.0%). Metals stocks were made up in deionized water, wrapped in aluminum foil, and kept at 4℃.

(d) Toxicity Tests with Antibiotics, Biocides, and Trace Metals

V. vulnificus (ATCC 27562) bacteria were exposed for 24 hours to each antimicrobial agent (TCS, OTC, and CIP), and trace metal (As, Cu, and Zn), individually and in binary mixtures and % survival and growth of the bacterial cultures were measured spectrophotometrically using optical density at a wavelength of 608 nm.

(e) Statistical Analysis

Data from the range finding and binary exposure experiments were blanked against the growth medium using MARS Data Analysis software before exporting to Microsoft Excel, from which these data were reformatted and transferred to SAS®. All statistical analyses were performed using SAS® University Edition software, replaced in August 2021 by the manufacturer with SAS® OnDemand for Academics.

(f) Instruments and Software

- Spectrophotometer: BMG LabTech NOVOstar Microplate Reader running MARS Data Analysis Software

- Data Visualization: Microsoft Excel and SigmaPlot v. 12.5
- Statistical Analysis: SAS® University Edition (later SAS® OnDemand for Academics)

Methods

(a) Protocol 1: Single Exposure Experiments

The first phase of the study was a range-finding project, which doubled as single-exposure assays of *V. vulnificus* (Vv) to antimicrobial products (triclosan [TCS], oxytetracycline [OTC], or ciprofloxacin [CIP]) and/or trace metals (arsenic [As], copper [Cu], or zinc [Zn]). Glycerol stocks of the microbes were plated on agar and grown overnight at 30°C. After 24 hours, an isolated colony was selected at random and inoculated into broth media. This was again incubated overnight at 30°C with orbital shaking to discourage biofilm formation.

After 24 hours, the overnight suspension was diluted to an optical density of approximately 0.02 at 608 nm ($OD_{608} \approx 0.02$). The diluted suspension was divided into individual conical tubes. One tube was spiked with the toxicant, after which serial dilutions were performed to achieve the desired exposure concentrations (**Table 3.1, Table 3.2**).

Environmental concentrations of antimicrobials (e.g., low dose) were selected based on published Probable No-Effects Level (PNEC) (**Table 3.1**) and correspond to levels measured in coastal waters impacted by human activity. Triclosan, for example, was measured in wastewater effluent discharging into Charleston Harbor, Charleston, SC, at a concentration of 0.3 ppb (Hedgespeth et al. 2012). Oxytetracycline has been found in effluent from mariculture operations at levels measuring up to 2.3 ppb (Thurman 2003), and a U.S. Geological Survey study on streams susceptible to contamination by human sources measured levels of ciprofloxacin up to 0.03 ppb (Kolpin et al. 2002). Clinical levels

of ciprofloxacin and oxytetracycline were selected from the maximum serum concentration of an adult oral dose (**Table 3.1**). Clinical triclosan levels were

based on the concentration found in antimicrobial hand soap (**Table 3.1**).

Environmental metals concentrations were based on the Environmental Protection Agency's (EPA's) Criterion Continuous Concentration (CCC) for saltwater exposure (**Table 3.2**). Levels selected to ensure a toxicological response in the microbes were chosen using the published 20th-percentile effect concentration (EC20) values for *Vibrio fischerii* (**Table 3.2**) as well as published minimum inhibitory concentrations (MIC) for *Enterococcus* species (**Table 3.2**). The *Enterococcus* MIC range encompasses the effects range median (ERM) sediment quality guideline (Long and Morgan,1990)for both copper and zinc, while the *Vibrio* EC20 range roughly corresponds to the arsenic Effects Range Low (ERL) sediment quality guideline (**Table 3.3**). These are similar to concentrations measured in sites like agricultural lagoons and commercial stormwater ponds (Bradford et al. 2008; Baalousha et al. 2015), where copper and zinc were both measured in excess of the ERM and arsenic in excess of the ERL.

For those antimicrobials whose stocks were made in acetone due to solubility limits (TCS and OTC), an equivalent amount of acetone was added to all samples containing lower concentrations of the antimicrobial so that all samples contained 0.3% acetone, and a carrier control was also tested. An early pilot test indicated no significant difference in growth patterns of *V. vulnificus* between a diluent control and a carrier control containing 0.3% acetone, thus the 0.3% acetone levels assured optimum antimicrobial agent dissolution into solution without affecting survival and growth.

Table 3.1: Nominal concentrations of antimicrobials upon which range finding assay exposures were based (Bayer Pharmaceuticals 2004; Agwuh and MacGowan 2006; Rodricks et al. 2010; Nietch et al. 2013; Bengtsson-Palme and Larsson 2016).

Antimicrobial	PNEC (ppb)	Clinical (ppb)
Triclosan	0.5	4.50E+06
Oxytetracycline	0.5	4.00E+03
Ciprofloxacin	6.40E-02	5.40E+03

Table 3.2: Nominal concentrations of metals upon which range finding assay exposures were based (Aarestrup and Hasman 2004; EPA 2004; Fulladosa et al. 2005; Rebelo et al. 2012).

Metal	CCC (ppb)	*Vibrio* EC20 (ppb)	*Enterococcus* MIC (ppb)
Arsenic	36	2.54E+03	5.98E+05
Copper	3.1	60	9.73E+05
Zinc	81	460	2.48E+05

Table 3.3: Sediment Quality Guidelines for Arsenic, Copper, and Zinc (Long 1995).

Metal	ERL (ppb)	ERM (ppb)
Arsenic	8.20E+03	7.00E+04
Copper	3.40E+04	2.70E+05
Zinc	1.50E+05	4.10E+05

Diluted and spiked bacterial suspensions were added in triplicate to a 96-well plate at 200 μL per well, along with media blanks, a control containing only bacteria, and a carrier control (acetone or water). This plate was read in a NOVOstar Microplate Reader (BMG LabTech), with a pre-programmed protocol which takes OD_{608} absorption measurements every 15 minutes for 24 hours, maintained at 30°C with dual orbital shaking for 0.2 seconds before each read. The growth rate of *Vibrio vulnificus* is approximately 3.05 generations per hour at optimal conditions of 30°C and 20% salinity, meaning the culture doubles in density every 19.7 minutes (Chase and Harwood 2011), so taking measurements every 15 minutes ensures that these data will capture logarithmic phase growth. Meanwhile, plate shaking prevents bacterial settling and biofilm formation, which can interfere with optical density readings.

MARS data analysis software was then used to compile and blank these data using the media control wells before exporting to SAS for analysis. This process was repeated three times, for a total of four replicates in triplicate within each exposure condition. In total, nine data points were obtained for each concentration of antimicrobial agent or trace metal tested. The blanked data was exported into Microsoft Excel to obtain these data in a format readable by most computers without the proprietary MARS software. From there, it was organized and compiled into SAS OnDemand for Academics, where the statistical analysis proceeded as described below in Section 2(d).

(b) Protocol 2: Binary Exposure Experiments

Binary exposure experiments were conducted to examine the potential interactive toxicity (e.g., joint toxicity) between two individual compounds, between different classes of chemicals (e.g., antimicrobials and metals). Using data obtained during the first group

of experiments, the same general protocol was used to obtain binary exposure data. This time, instead of spiking the diluted bacterial suspensions with a single toxicant, this set of experiments used one of two conditions. The first was a single concentration of antimicrobial (TCS, OTC, or CIP) which was shown to have a sublethal toxicological effect – the Minimum Effective Concentration, or MEC (**Table 3.4**) – on bacterial growth in combination with a range of metal (As, Cu, or Zn) concentrations at environmentally-relevant levels based on the CCC. A second set of experiments was performed using the MEC of trace metals (As, Cu, or Zn) in combination with a range of environmentally relevant antimicrobial (TCS, OTC, or CIP) levels based on the PNEC. Culture, dilution, microbial measurements, and statistical analysis methods remained the same as for the single-exposure experiments, culminating in eight individual replicates. In total, twenty-four individual data points were obtained for each treatment group.

(c) Protocol 3: Phenotypic Analysis for Antimicrobial Resistance

To determine a phenotypic resistance profile of *V. vulnificus*, a set of Kirby-Bauer assays (Hudzicki 2009) were performed using a modified inoculum preparation. First, a frozen glycerol stock of *V. vulnificus* was streaked on agar plates and incubated overnight in order to obtain isolated colonies. A single colony was then inoculated into broth medium and incubated overnight at 30°C. This overnight culture was split into a series of tubes containing broth spiked with treatment groups corresponding to those from the binary exposure experiments. To prepare the inocula, these acclimated cultures were diluted in sterile phosphate-buffered saline until they corresponded with the 0.5 McFarland Standard, and the assay proceeded using the standard protocol (**Figure 3.1**). Antimicrobial challenge discs included OTC and CIP, in addition to clinically important cefotaxime to assess effects

Table 3.4: Minimum Effective Concentration (MEC) of each toxicant used as a constant in binary exposure experiments, as determined in Protocol 1 experiments.

Treatment	Minimum Effective Concentration (ppb)	Percent Inhibition (%)
Arsenic	510	7.28
Copper	1.39E+04	14.84
Zinc	1.15E+04	-1.07
Triclosan	3.60E+04	6.56
Oxytetracycline	500	4.71
Ciprofloxacin	338	24.41

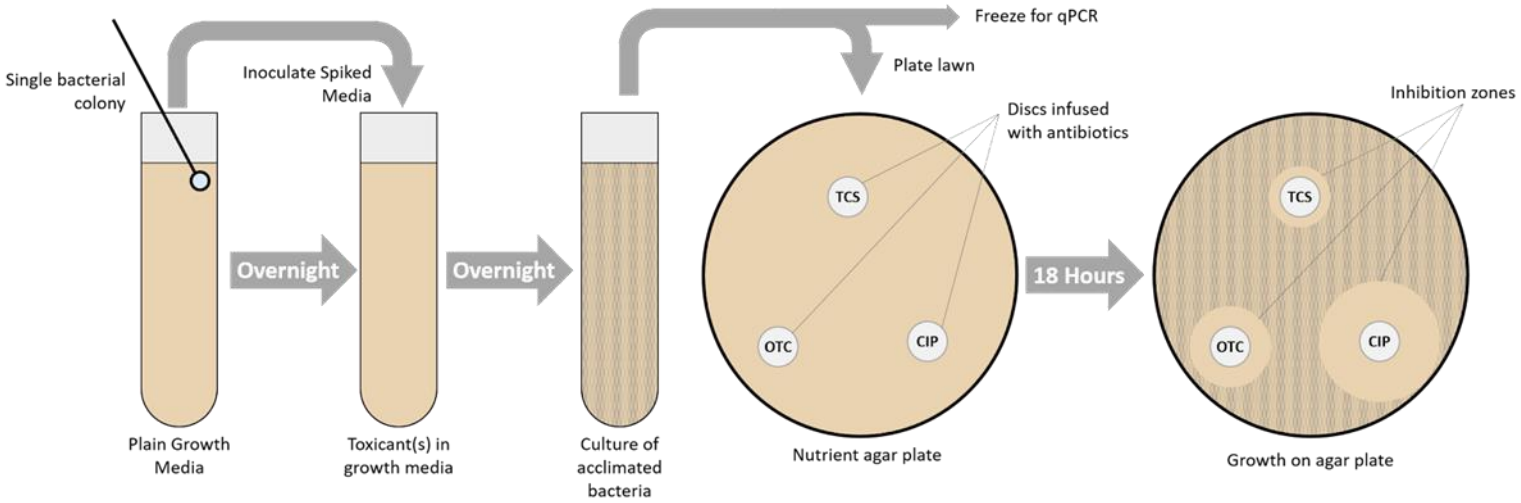


Figure 3.1: Kirby-Bauer procedure, adapted from Hudzicki 2009.

of the cephalosporins. Three discs of each antimicrobial plus three blank controls were placed in triplicate in each of three replicated experiments. Additionally, several 0.5 mL aliquots of acclimated bacterial culture from each exposure criterion (antimicrobial alone, metal alone, combination antimicrobial + metal, or control) were added to cryovials containing 0.5 mL 50% glycerol solution, resulting in a final concentration of 25% glycerol, and frozen at -80°C to save for future studies. The inoculated plates were incubated at 30°C for 18 hours, then the Zones of Inhibition (ZOI) were measured (Hudzicki 2009; CLSI 2016) using a digital caliper, recorded, and analyzed using a nested ANOVA with Dunnett's test. Resistance levels (Susceptible, Intermediate, or Resistant) were determined using breakpoint guidelines published by the Clinical and Laboratory Standards Institute (CLSI) (CLSI 2016) (**Table 3.5**). On Figure 3.1, please note that the Inhibition Zones and disc placement are for demonstration only and do not represent actual results. A smaller diameter inhibition zone is indicative of more resistant strains while larger diameter inhibition zones indicate sensitive isolates.

(d) Statistical Analysis

As each experimental stage consisted of four to eight replicated experiments in which each exposure group was examined in triplicate, a one-way nested Analysis of Variance (ANOVA) with post-hoc Dunnett's test was performed to account for these triplicate subgroups when comparing treatment results to the control. Studentized Residual and Cook's Distance statistics were used to identify outliers. Those data points for which both the $Studentized\ Residual > |3|$ and $Cook's\ Distance > 4/N$ were then removed from the ANOVA.

Table 3.5: *V. vulnificus* Inhibition Zone Breakpoints (CLSI 2016).

Antimicrobial Challenge	Breakpoints Zone of Inhibition (ZOI) [mm]		
Legend	▲ Susceptible	– Intermediate	▼ Resistant
Ciprofloxacin (5 µg)	≥21	16-20	≤15
Cefotaxime (30 µg)	≥15	12-14	≤11
(Oxy)tetracycline (30 µg)	≥26	23-25	≤22

Results

Percent inhibition (% I) was calculated using the mean OD_{608} and the following formula:

$$\%I = [(\frac{A - B)}{A}] * 100$$

where A = Control OD_{608} at stationary phase and B = Treatment OD_{608} at stationary phase.

For each figure below in Chapter 3, an asterisk (*) indicates exposures which were significantly different from the controls (Dunnett's test $p \leq 0.05$). Negative inhibition indicates growth exceeding that of the control. For both the Shapiro-Wilk test for normality and Levene's test for homogeneity of variance, these data sets were classified as "*meets assumptions*" if $p \geq 0.05$, "*minor departure from assumptions*" if $p\ 0.05 \geq 0.02$, "*moderate departure from assumptions*" if $0.02 \geq 0.005$, and "*fails to meet assumptions*" if $p < 0.005$ (Zar 1999; Pennington 2022). A data set met assumptions for computed power if $p \geq 0.8$, and "*failed to meet assumptions*" if $p < 0.8$.

(a) Single Exposure Experiments

The first set of experiments involved exposure of *V. vulnificus* individually to triclosan, oxytetracycline, and ciprofloxacin. The second set was nearly identical, using arsenic, copper, and zinc instead of antimicrobials as the exposure agents. These assays were set up as a range-finding pilot studies, as they served to both determine the exposure range at which binary exposure experiments would be run as well as to determine a baseline growth curve against which to compare the binary exposure study. "Difference in growth" is defined as change in OD_{608} from t=0 until the onset of stationary phase. For *V. vulnificus*, this metric occurred at approximately t=24 hours. Difference in growth was used instead of growth rate because pilot experiments of these exposures reached log phase growth at

nearly the same time as control for most experiments. Thus, the difference in total growth observed was used instead. For all of the figures below, a dagger (†) denotes published PNEC value for antimicrobials or CCC value for metals, asterisk (*) indicates statistical significance (Dunnett's test $p \leq 0.05$), positive (+) inhibition values were indicative of reduced growth, and negative (-) suggest growth stimulation.

In the Probable No-Effects level range, nested ANOVA results indicated that, for all of the antimicrobials tested, there were only slight differences in growth when compared to control (**Figure 3.2)**. Though *Vibrio vulnificus* showed a response to all three antimicrobials, it was only a slight difference and was not different enough from controls to be considered statistically significant (**Table 3.7**). Interestingly, treated *V. vulnificus* slightly outgrew the control under several low dose exposures: 0.25 ppb OTC (7.52%), 0.5 ppb OTC (3.51%), 1.00 ppb OTC (0.91%), and 2.00 ppb TCS (0.81%).

In the clinical exposure range (**Figure 3.3**), significant growth inhibition was observed in *V. vulnificus* for all exposures of CIP and the highest TCS concentration. CIP doses of 0.675, 1.35, 2.7, and 5.4 ppm all exceeded 95% inhibition, while 0.338 ppm CIP had 24.41% inhibition. TCS exposure experienced 93.95% inhibition at 4500 ppm, 77.12% at 900 ppm, and 29.75% at 180 ppm. While there was a slight, defined dose response to OTC, none of the exposure levels experienced statistically significant inhibition (**Table 3.8**).

Results for *V. vulnificus* exposure to Criterion Continuous Concentration ranges of two of the three metals (As and Zn) expressed similar variation between exposures as to the PNEC antimicrobials (**Figure 3.4**). Arsenic treatment at a level exceeding the CCC by 800% (144 ppb) caused growth inhibition of 6.2%, with the corresponding concentration of zinc (324

Table 3.6: ANOVA Assumptions Legend, as applied to results presented in tables that follow.

Meets Assumptions	Minor Departure from Assumptions	Moderate Departure from Assumptions	Fails to Meet Assumptions
			

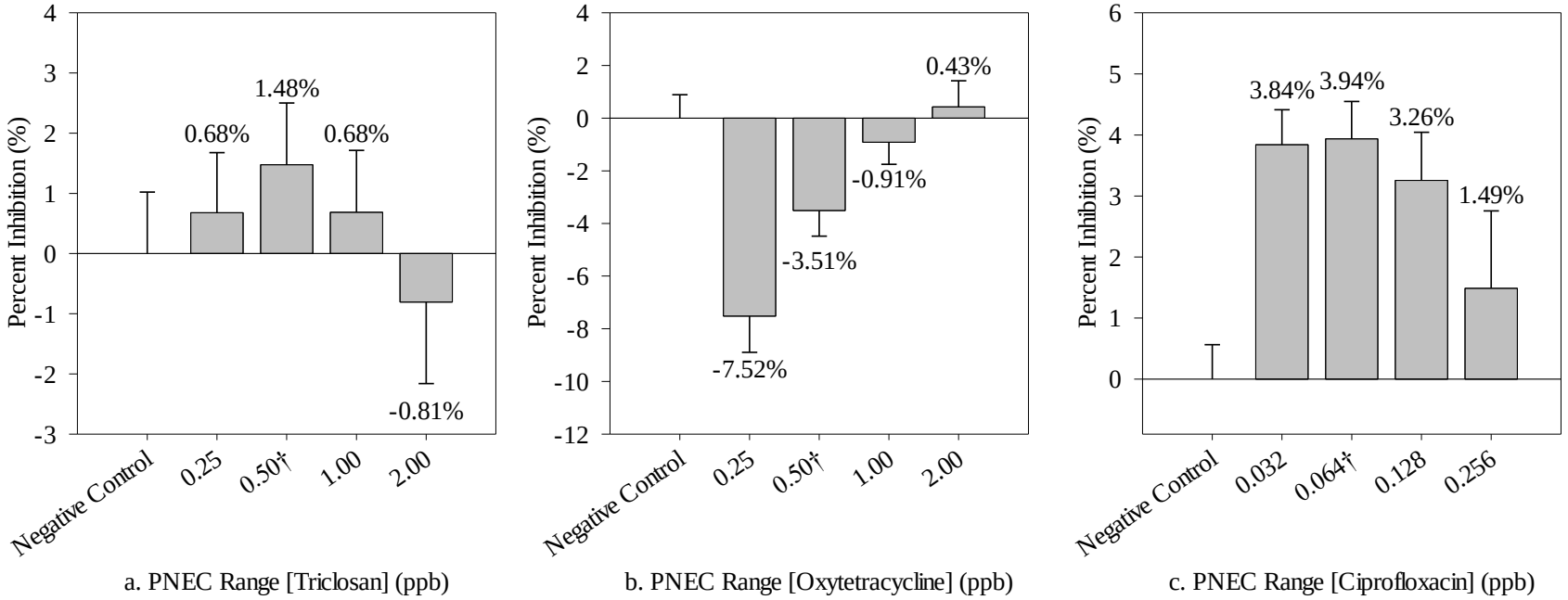


Figure 3.2: Inhibition of *V. vulnificus* following exposure to the PNEC range of antimicrobials.

Table 3.7: ANOVA statistical analysis of results from the PNEC range of antimicrobials.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control p ≤ 0.05	Shapiro-Wilk: Normality p ≥ 0.05	Levene's Test: Homogeneity of Variance p ≥ 0.05	Computed Power p ≥ 0.8
PNEC [Ciprofloxacin] (ppb)	Negative Control	8		✓ 0.0589	✓ 0.5135	✓ 0.999
	0.032	9	✗ 0.2599			
	0.064†	9	✗ 0.2437			
	0.128	9	✗ 0.3803			
	0.256	9	✗ 0.8728			
PNEC [Oxytetracycline] (ppb)	Negative Control	7		✓ 0.2466	✓ 0.1697	✓ 0.999
	0.25	9	✗ 0.0972			
	0.50†	9	✗ 0.654			
	1	9	✗ 0.999			
	2	8	✗ 0.9994			
PNEC [Triclosan] (ppb)	Negative Control	9		✓ 0.1162	✓ 0.1857	✗ 0.763
	0.25	9	✗ 0.9975			
	0.50†	9	✗ 0.9576			
	1	9	✗ 0.9974			
	2	9	✗ 0.9951			

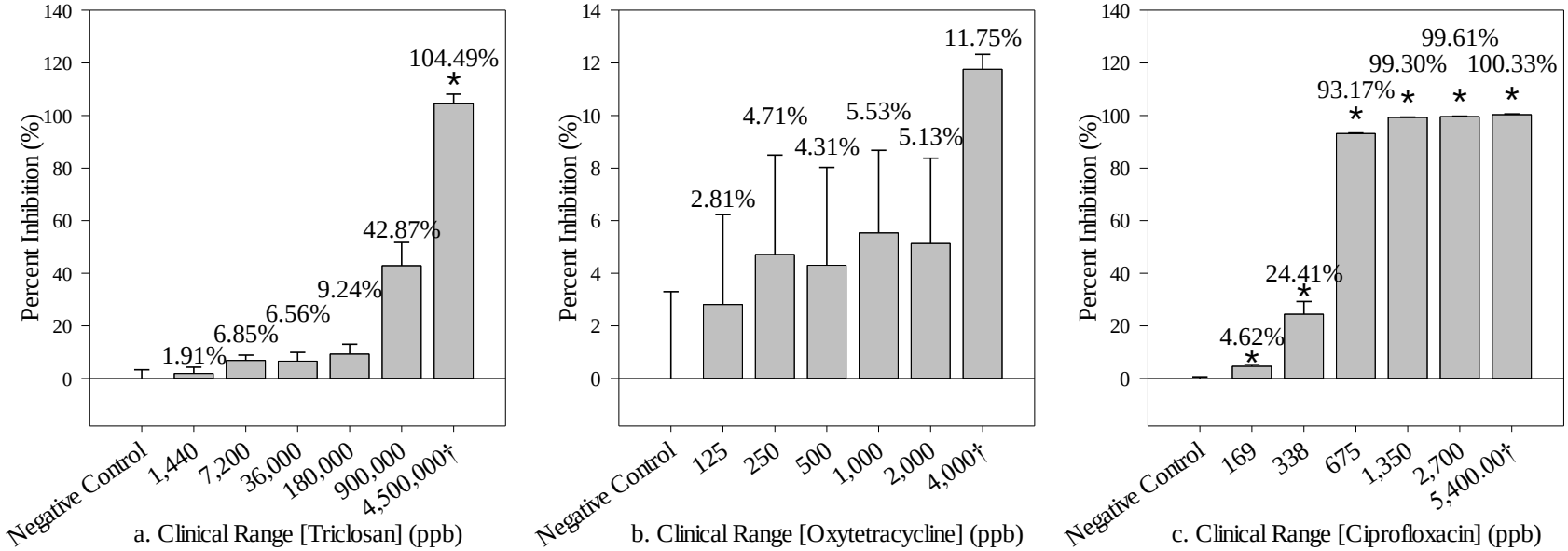


Figure 3.3: Inhibition of *V. vulnificus* following exposure to the clinical range of antimicrobials.

Table 3.8: ANOVA statistical analysis of results from the clinical range of antimicrobials.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control p ≤ 0.05	Shapiro-Wilk: Normality p ≥ 0.05	Levene's Test: Homogeneity of Variance p ≥ 0.05	Computed Power p ≥ 0.8
Clinical [Ciprofloxacin] (ppb)	Negative Control	6		✓ 0.0759	! 0.0186	✓ 0.999
	169	6	✗ 0.6741			
	338	4	✓ 2.60E-03			
	675	6	✓ 1.00E-04			
	1,350	6	✓ 1.00E-04			
	2,700	6	✓ 1.00E-04			
	5,400.00†	6	✓ 1.00E-04			
Clinical [Oxytetracycline] (ppb)	Negative Control	6		✓ 0.243	✓ 0.104	✓ 0.999
	125	6	✗ 0.9996			
	250	6	✗ 0.9939			
	500	6	✗ 0.9961			
	1,000	6	✗ 0.9867			
	2,000	6	✗ 0.9907			
	4,000†	3	✗ 0.877			
Clinical [Triclosan] (ppb)	Negative Control	6		✓ 0.819	✓ 0.0599	✓ 0.999
	1,440	6	✗ 1			
	7,200	6	✗ 0.9809			
	36,000	6	✗ 0.9844			
	180,000	6	✗ 0.9323			
	900,000	5	✗ 0.0785			
	4,500,000	6	✓ 3.00E-04			

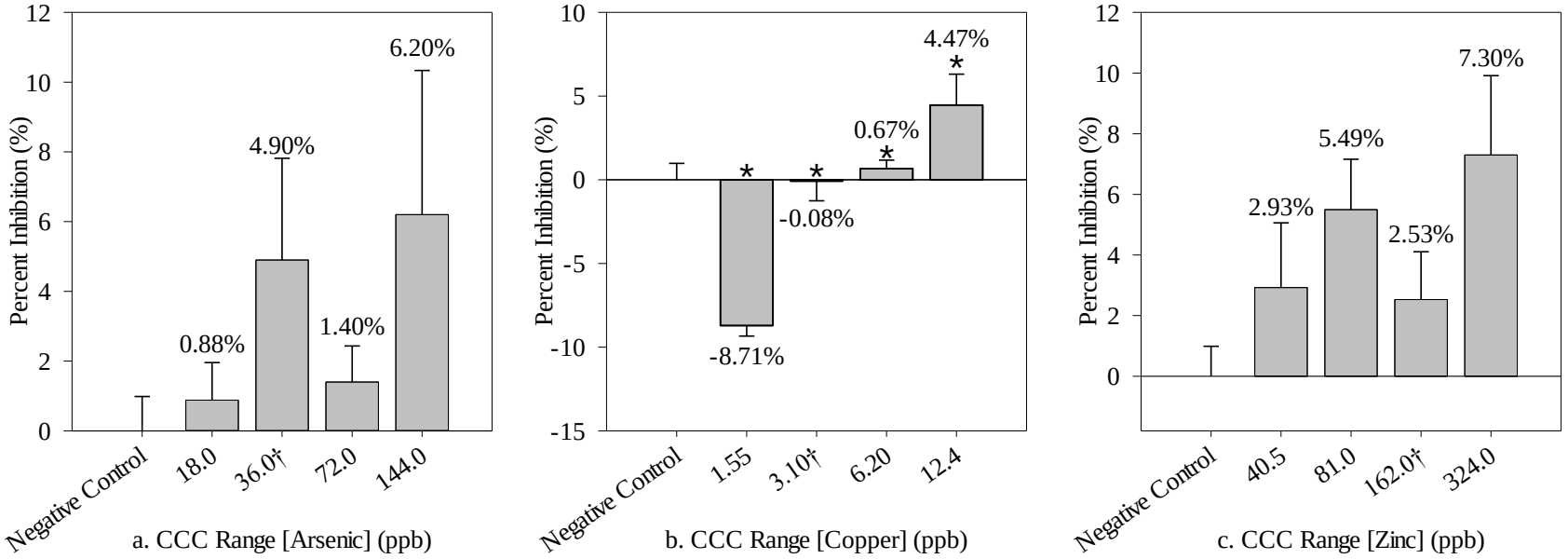


Figure 3.4: Inhibition of *V. vulnificus* following exposure to the CCC range of metals.

ppb) reaching 7.3% inhibition. None of the arsenic or zinc treatments achieved statistical significance. Conversely, all four of the copper concentrations showed significant changes in growth inhibition as compared to control (**Table 3.9**). The highest copper concentration in this range, 12.4 ppb Cu, reached nearly 4.5% inhibition. Also note that at the lowest- tested concentration of copper (1.55 ppb), growth exceeded the control by a significant 8.7%.Results of exposures at the range based on *V. fischerii* EC20 values (**Figure 3.5**), *V. vulnificus* displayed a distinct and significant dose-response curve when exposed to arsenic, ranging from approximately 7.3% at 0.51 ppb As to 47.6% inhibition at 63.3 ppb As. Again, at two concentrations of copper (0.012 ppb and 0.060 ppb), *V. vulnificus* outgrew the control by 4.7% and 2.5%, respectively, but only the lowest dose was significantly different from the controls (**Table 3.10**). At higher Cu doses of 0.5 ppb and 1.5 ppb, the bacteria only experienced up to 1% inhibition and were not significantly different from the controls. *V. vulnificus* exposures to Zn did not result in statistically significant inhibition or biostimulation, although the 11,500 ppb Zn slightly outgrew the control by approximately 1%, while it was inhibited by up to 1.8% at the three lower concentrations.

At the highest-tested concentration range -- based on the published *Enterococcus* minimum inhibitory concentrations (MIC) for each metal -- *V. vulnificus* growth was significantly inhibited compared to controls (**Figure 3.6, Table 3.11**). Both copper and zinc exposures exceeded 98% inhibition for all concentrations, and arsenic exposure ranged from ~65-93% inhibition.

As a secondary goal of the single-exposure study was to determine a sublethal yet effective concentration (between 10% and 20% inhibition) of the toxicants to use in a series of binary exposure experiments, a fourth range of copper concentrations was tested, at concentrations

Table 3.9: ANOVA statistical analysis of results from the CCC range of metals.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control p ≤ 0.05	Shapiro-Wilk: Normality p ≥ 0.05	Levene's Test: Homogeneity of Variance p ≥ 0.05	Computed Power p ≥ 0.8
CCC [Arsenic] (ppb)	Negative Control	9		✓ 0.0674	✓ 0.2061	✓ 0.987
	18	9	✗ 0.9995			
	36.0†	8	✗ 0.6761			
	72	9	✗ 0.997			
	144	8	✗ 0.7858			
CCC [Copper] (ppb)	Negative Control	9		✓ 0.9105	✓ 0.1328	✓ 0.999
	1.55	7	✓ 0.0189			
	3.10†	9	✗ 1			
	6.2	8	✗ 0.9954			
	12.4	8	✗ 0.1615			
CCC [Zinc] (ppb)	Negative Control	9		✓ 0.9289	! 0.045	✓ 0.997
	40.5	7	✗ 0.8954			
	81	9	✗ 0.5822			
	162.0†	9	✗ 0.9447			
	324	9	✗ 0.3559			

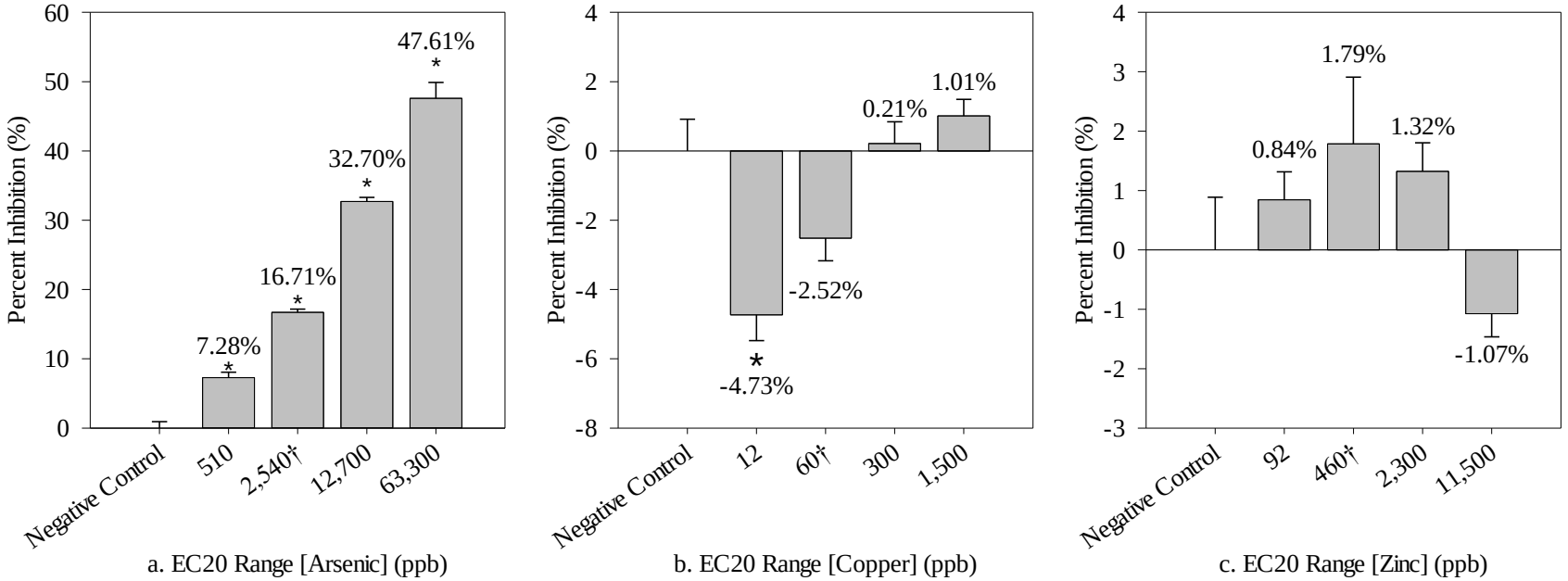


Figure 3.5: Inhibition of *V. vulnificus* following exposure to EC20 range of metals.

Table 3.10: ANOVA statistical analysis of results from the EC20 range of metals.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control p ≤ 0.05	Shapiro-Wilk: Normality p ≥ 0.05	Levene's Test: Homogeneity of Variance p ≥ 0.05	Computed Power p ≥ 0.8
EC20 [Arsenic] (ppb)	Negative Control	9		✓ 0.3551	! 0.0472	✓ 0.999
	510	9	✓ 0.0127			
	2,540†	9	✓ 1.00E-04			
	12,700	9	✓ 1.00E-04			
	63,300	6	✓ 1.00E-04			
EC20 [Copper] (ppb)	Negative Control	9		✓ 0.066	! 0.0478	✓ 0.999
	12	9	✓ 0.0137			
	60†	8	✗ 0.2742			
	300	9	✗ 0.9994			
	1,500	9	✗ 0.8462			
EC20 [Zinc] (ppb)	Negative Control	7		✓ 0.1294	✓ 0.0545	✓ 0.966
	92	9	✗ 0.9413			
	460†	9	✗ 0.6547			
	2,300	9	✗ 0.8143			
	11,500	9	✗ 0.943			

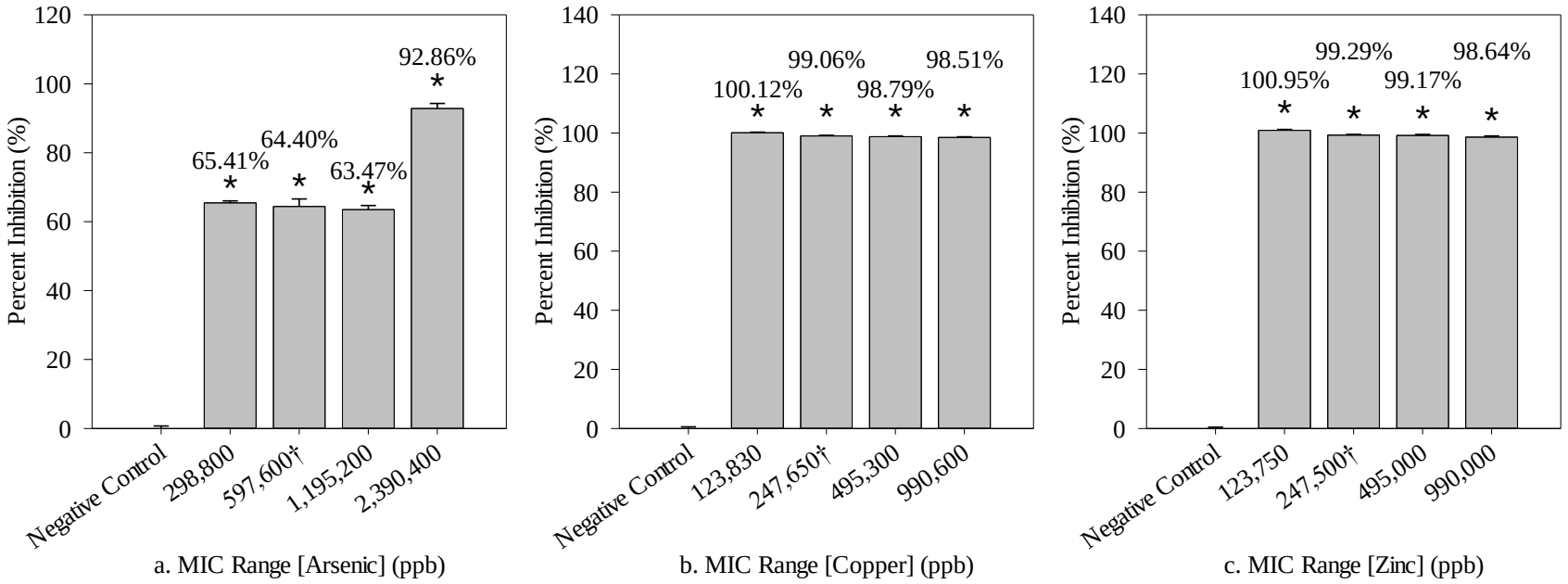


Figure 3.6: Inhibition of *V. vulnificus* following exposure to MIC range of metals.

Table 3.11: ANOVA statistical analysis of results from the MIC range of metals.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control p ≤ 0.05	Shapiro-Wilk: Normality p ≥ 0.05	Levene's Test: Homogeneity of Variance p ≥ 0.05	Computed Power p ≥ 0.8
MIC [Arsenic] (ppb)	Negative Control	9		✓ 0.83	✓ 0.0869	✓ 0.999
	298,800	9	✓ 1.00E-04			
	597,600†	7	✓ 1.00E-04			
	1,195,200	9	✓ 1.00E-04			
	2,390,400	9	✓ 1.00E-04			
MIC [Copper] (ppb)	Negative Control	3		✓ 0.5856	✓ 0.2676	✓ 0.999
	123,830	9	✓ 1.00E-04			
	247,650†	9	✓ 1.00E-04			
	495,300	9	✓ 1.00E-04			
	990,600	7	✓ 1.00E-04			
MIC [Zinc] (ppb)	Negative Control	7		✓ 0.8722	! 0.0462	✓ 0.999
	123,750	9	✓ 1.00E-04			
	247,500†	9	✓ 1.00E-04			
	495,000	9	✓ 1.00E-04			
	990,000	8	✓ 1.00E-04			

ranging between the CCC and EC20 exposure doses. This range of Cu exposures achieved that goal, as all doses were significantly inhibited compared to controls and the 13,890 ppb Cu resulted in a 14.84% inhibition (**Figure 3.7, Table 3.12**).

(b) Binary Exposure Experiments

Following the conclusion of the single-compound exposure experiments, concentrations of the six toxicants which caused a sublethal toxicological effect at environmentally relevant levels were identified. These concentrations will be referred to as the minimum effective concentration (MEC). The MEC values for each compound (trace metals and antimicrobials) were used as constants and secondary controls in a series of experiments examining binary exposures of *V. vulnificus* to the sublethal toxicant in combination with the PNEC range of antimicrobials or CCC range of metals.

For all of the figures below, a dagger (†) denotes published PNEC value for antimicrobials or CCC value for metals, asterisk (*) indicates statistical significance (Dunnett's test $p \leq 0.05$), positive (+) inhibition values were indicative of reduced growth, and negative (-) suggest growth stimulation compared to the negative control.

The Binary Group 1 mixture consisted of 510 ppb As in combination with the PNEC ranges of TCS, OTC, or CIP (**Figure 3.8**). Most of the binary treatments were very similar to the arsenic control, with some notable exceptions. The combination of 510 ppb arsenic with 2.0 ppb triclosan, instead of causing approximately 2% inhibition like arsenic alone, induced growth stimulation of 2.5%, while the 0.256 ppb CIP caused similar stimulation of 1.8%. The 510 ppb As with PNEC for OTC caused 5.6% growth stimulation, which was the only combination significantly different from the control (**Table 3.13**).

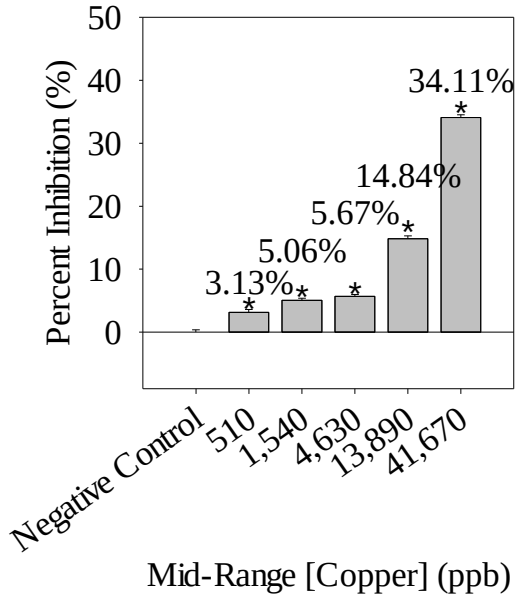
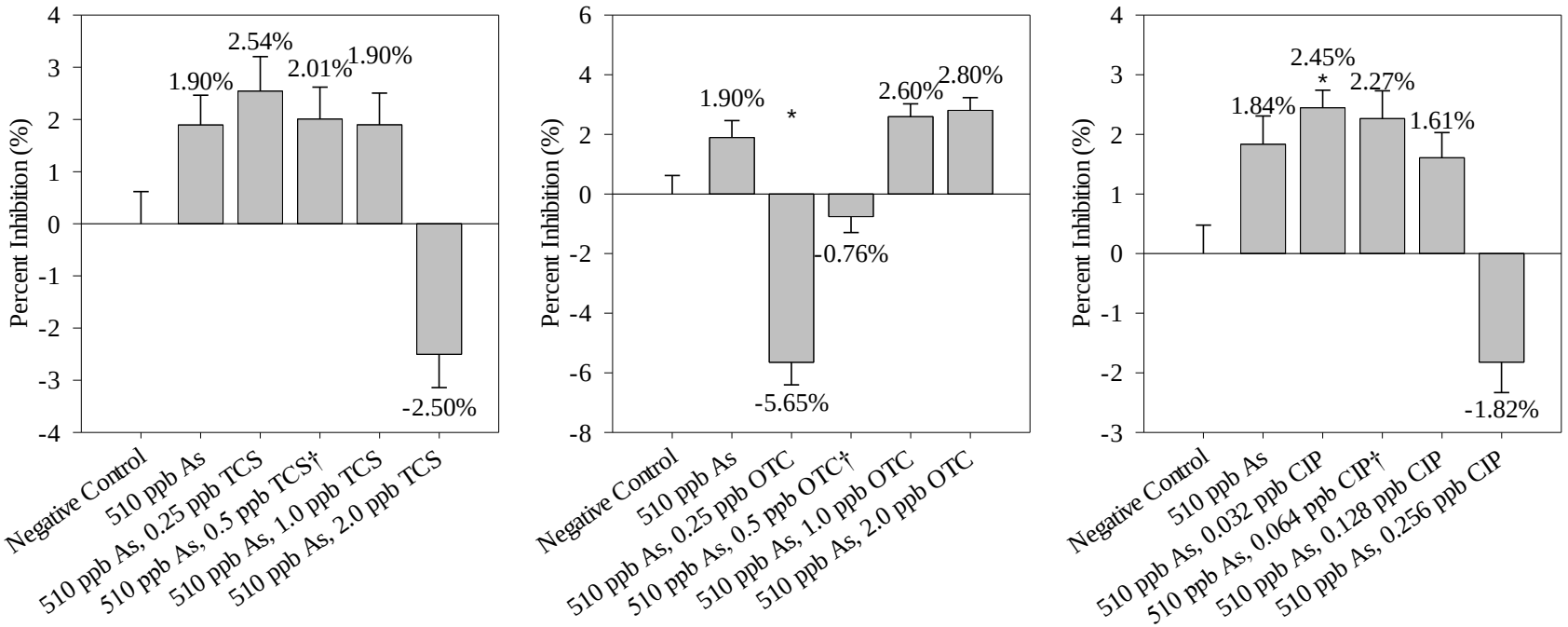


Figure 3.7: Inhibition of *V. vulnificus* following exposure to a range of copper concentrations falling between the CCC and EC20 concentration ranges.

Table 3.12: ANOVA statistical analysis of results from the mid-range of copper concentrations.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control p ≤ 0.05	Shapiro-Wilk: Normality p ≥ 0.05	Levene's Test: Homogeneity of Variance p ≥ 0.05	Computed Power p ≥ 0.8
Midrange [Copper] (ppb)	Negative Control	9		✓ 0.6117	✓ 0.0747	✓ 0.999
	510	8	✓ 0.0105			
	1,540	9	✓ 2.00E-04			
	4,630	9	✓ 1.00E-04			
	13,890	7	✓ 1.00E-04			
	41,670	9	✓ 1.00E-04			



a. Binary Group 1.1

b. Binary Group 1.2

c. Binary Group 1.3

Figure 3.8: Inhibition of *V. vulnificus* following exposure to the Binary Group 1 mixture of the As MEC with a PNEC range of (a) TCS, (b) OTC, or (c) CIP.

Table 3.13: ANOVA statistical analysis of results from the Binary Group 1 Mixtures of MEC As with PNEC ranges of TCS, OTC, or CIP.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control p ≤ 0.05	Shapiro-Wilk: Normality p ≥ 0.05	Levene's Test: Homogeneity of Variance p ≥ 0.05	Computed Power p ≥ 0.8
Binary 1.1: 510 ppb As, PNEC [TCS] (ppb)	Negative Control	12		✓ 0.6321	! 0.0376	✓ 0.999
	510 ppb As	12	✗ 0.3952			
	510 ppb As, 0.25 ppb TCS	12	✗ 0.1651			
	510 ppb As, 0.5 ppb TCS†	12	✗ 0.3443			
	510 ppb As, 1.0 ppb TCS	12	✗ 0.3944			
	510 ppb As, 2.0 ppb TCS	12	✗ 0.1756			
Binary 1.2: 510 ppb As, PNEC [OTC] (ppb)	Negative Control	12		✓ 0.6321	! 0.0376	✓ 0.999
	510 ppb As	12	✗ 0.3246			
	510 ppb As, 0.25 ppb OTC	12	✓ 3.00E-04			
	510 ppb As, 0.5 ppb OTC†	12	✗ 0.9317			
	510 ppb As, 1.0 ppb OTC	12	✗ 0.1092			
	510 ppb As, 2.0 ppb OTC	12	✗ 0.0763			
Binary 1.3: 510 ppb As, PNEC [CIP] (ppb)	Negative Control	11		✓ 0.1157	✓ 0.0772	✓ 0.999
	510 ppb As	11	✗ 0.1851			
	510 ppb As, 0.032 ppb CIP	12	✓ 0.0532			
	510 ppb As, 0.064 ppb CIP†	12	✗ 0.0777			
	510 ppb As, 0.128 ppb CIP	12	✗ 0.2709			
	510 ppb As, 0.256 ppb CIP	12	✗ 0.263			

In contrast to Binary Group 1, the copper group did not experience any growth stimulation when combined with PNEC-level antimicrobials, and all treatment groups were statistically significant for increased growth inhibition compared to control (**Table 3.14**). The copper MEC control exposure (13,890 ppb Cu) exceeded the growth inhibition of all of the TCS treatment groups by approximately 1%, while in the MEC copper + CIP groups all exceeded the MEC copper control growth inhibition by between 0.3% and 1.2%. For both groups, growth inhibition in the binary exposures were either all significantly ($p \leq 0.05$) different from the negative control but were not significantly different from the MEC copper control. The copper + OTC group also mostly exceeded the copper control, by between 0.12% and 1.5%. A notable exception is 13,890 ppb Cu in combination with 0.25 ppb OTC. This treatment group experienced 23-.63% lower growth inhibition than 13,890 ppb Cu exposure alone, although this value was not statistically significant (**Figure 3.9, Table 3.14**).

Following co-exposure to 11,500 ppb MEC Zn exposure and TCS, there were only negligible reductions in growth inhibition for three of the four TCS PNEC concentrations tested (**Figure 3.10**). At the TCS concentration representing approximately 800 times the PNEC value, however, biostimulation was observed as the bacteria outgrew the MEC zinc exposure by 6.25%. Additionally, MEC Zn in combination with the PNEC of oxytetracycline experienced growth stimulation of 11.3% as compared to the MEC Zn exposure alone. Additionally, the combination of ciprofloxacin representing an 800% exceedance of the PNEC in combination with zinc experienced 6.3% growth stimulation,

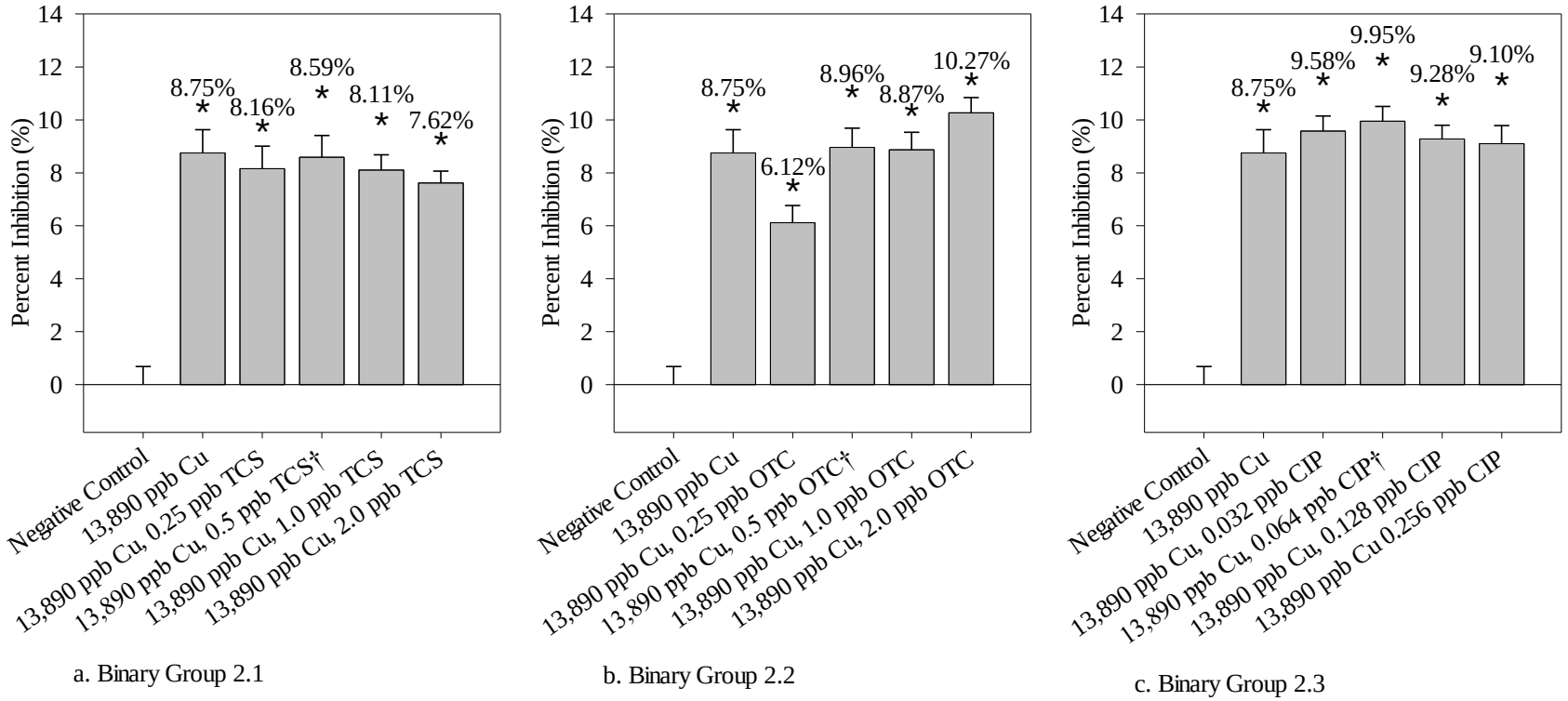


Figure 3.9: Inhibition of *V. vulnificus* following exposure to the Binary Group 2 mixture of the Cu MEC with a PNEC range of (a) TCS, (b) OTC, or (c) CIP.

Table 3.14: ANOVA statistical analysis of results from the Binary Group 2 Mixtures of MEC Cu with PNEC ranges of TCS, OTC, or CIP.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control p ≤ 0.05	Shapiro-Wilk: Normality p ≥ 0.05	Levene's Test: Homogeneity of Variance p ≥ 0.05	Computed Power p ≥ 0.8
Binary 2.1: 13,890 ppb Cu, PNEC [TCS] (ppb)	Negative Control	11		✓ 0.9381	! 0.0222	✓ 0.999
	13,890 ppb Cu	12	✓ 4.00E-04			
	13,890 ppb Cu, 0.25 ppb TCS	12	✓ 8.00E-04			
	13,890 ppb Cu, 0.5 ppb TCS†	11	✓ 5.00E-04			
	13,890 ppb Cu, 1.0 ppb TCS	12	✓ 9.00E-04			
	13,890 ppb Cu, 2.0 ppb TCS	12	✓ 2.20E-03			
Binary 2.2: 13,890 ppb Cu, PNEC [OTC] (ppb)	Negative Control	11		✓ 0.9202	! 0.0164	✓ 0.999
	13,890 ppb Cu	12	✓ 3.00E-04			
	13,890 ppb Cu, 0.25 ppb OTC	10	✓ 7.30E-03			
	13,890 ppb Cu, 0.5 ppb OTC†	12	✓ 2.00E-04			
	13,890 ppb Cu, 1.0 ppb OTC	11	✓ 3.00E-04			
	13,890 ppb Cu, 2.0 ppb OTC	12	✓ 1.00E-04			
Binary 2.3: 13,890 ppb Cu, PNEC [CIP] (ppb)	Negative Control	11		✓ 0.6088	! 0.0206	✓ 0.999
	13,890 ppb Cu	12	✓ 1.00E-04			
	13,890 ppb Cu, 0.032 ppb CIP	11	✓ 1.00E-04			
	13,890 ppb Cu, 0.064 ppb CIP†	12	✓ 1.00E-04			
	13,890 ppb Cu, 0.128 ppb CIP	12	✓ 1.00E-04			
	13,890 ppb Cu 0.256 ppb CIP	10	✓ 1.00E-04			

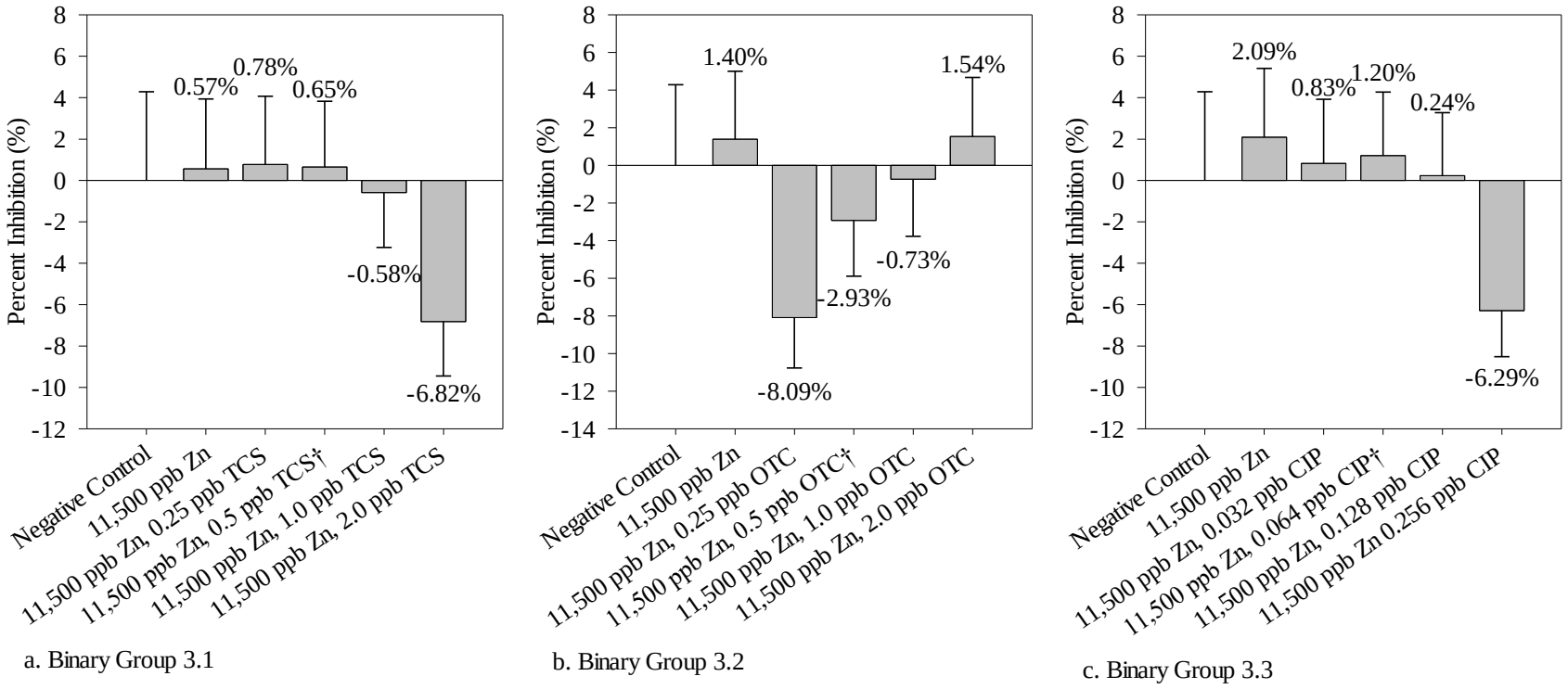


Figure 3.10: Inhibition of *V. vulnificus* following exposure to the Binary Group 3 mixture of the Zn MEC with a PNEC range of (a) TCS, (b) OTC, or (c) CIP.

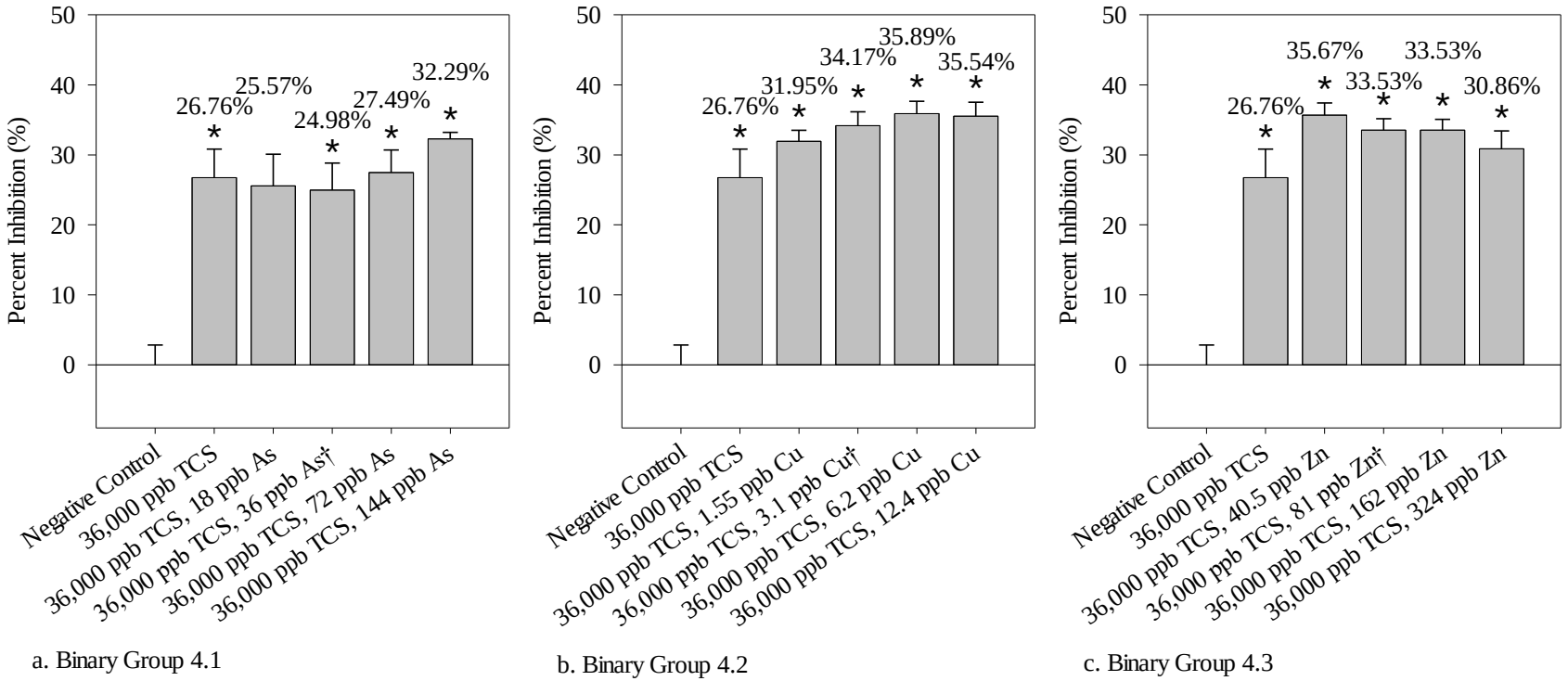
outgrowing the MEC Zn exposure by 8.4%. However, statistical analysis (**Table 3.15**) indicated that none of the PNEC TCS, OTC, or CIP and MEC Zn mixtures were significantly different from the negative controls or individual MEC Zn exposure alone (**Table 3.15**).

Exposure to binary mixtures of MEC TCS and all three CCC-range trace metal concentrations experienced significant growth inhibition (**Table 3.16**). Co-exposure to arsenic (18-172 ppb) and triclosan (36,000 ppb) resulted in slightly less inhibition in the two lowest arsenic exposures than the TCS MEC exposure alone, but the highest arsenic concentration tested (144 ppb) outgrew the TCS MEC exposure by 5.5% (**Figure 3.11**). The three highest arsenic and MEC TCS exposure group mixtures had significantly ($p \leq 0.05$) reduced growth compared to the negative control but were not significantly different from the TCS MEC exposure. Similarly, all of the co-exposures of TCS with Cu and TCS with Zn outgrew the TCS MEC by up to 9%, had significantly ($p \leq 0.05$) reduced growth compared to the negative control, and were not significantly different from the TCS MEC exposure group.

The majority of co-exposures to OTC at the MEC concentration and CCC-level metals resulted in the binary mixtures having only slightly increased growth inhibition (< 3.72%) when compared to the OTC MEC exposure and were not significantly different ($p \leq 0.05$) from the OTC MEC exposure or negative control alone (**Table 3.17**). Each metal, however, had one concentration which, in combination with oxytetracycline, had biostimulated growth and outgrew the OTC MEC exposure group alone (**Figure 3.12**). For example, the 144 ppb arsenic with oxytetracycline experienced 2.1% growth stimulation, outgrowing the triclosan control by nearly 3%; the 1.5 ppb copper experienced

Table 3.15: ANOVA statistical analysis of results from the Binary Group 3 Mixtures of MEC Zn with PNEC ranges of TCS, OTC, or CIP.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control p ≤ 0.05	Shapiro-Wilk: Normality p ≥ 0.05	Levene's Test: Homogeneity of Variance p ≥ 0.05	Computed Power p ≥ 0.8
Binary 3.1: 11,500 ppb Zn, PNEC [TCS] (ppb)	Negative Control	10		! 0.0238	! 0.0104	✓ 0.999
	11,500 ppb Zn	11	✗ 0.9995			
	11,500 ppb Zn, 0.25 ppb TCS	11	✗ 0.9993			
	11,500 ppb Zn, 0.5 ppb TCS†	11	✗ 0.9994			
	11,500 ppb Zn, 1.0 ppb TCS	12	✗ 1			
	11,500 ppb Zn, 2.0 ppb TCS	12	✗ 0.8954			
Binary 3.2: 11,500 ppb Zn, PNEC [OTC] (ppb)	Negative Control	10		✓ 0.902	✓ 0.0603	✓ 0.999
	11,500 ppb Zn	10	✗ 0.9999			
	11,500 ppb Zn, 0.25 ppb OTC	11	✗ 0.7899			
	11,500 ppb Zn, 0.5 ppb OTC†	11	✗ 0.9997			
	11,500 ppb Zn, 1.0 ppb OTC	11	✗ 1			
	11,500 ppb Zn, 2.0 ppb OTC	11	✗ 0.9977			
Binary 3.3: 11,500 ppb Zn, PNEC [CIP] (ppb)	Negative Control	10		✓ 0.1704	✓ 0.0669	✓ 0.999
	11,500 ppb Zn	10	✗ 0.9987			
	11,500 ppb Zn, 0.032 ppb CIP	11	✗ 0.1651			
	11,500 ppb Zn, 0.064 ppb CIP†	11	✗ 0.3443			
	11,500 ppb Zn, 0.128 ppb CIP	11	✗ 0.3944			
	11,500 ppb Zn 0.256 ppb CIP	12	✗ 0.1756			



a. Binary Group 4.1

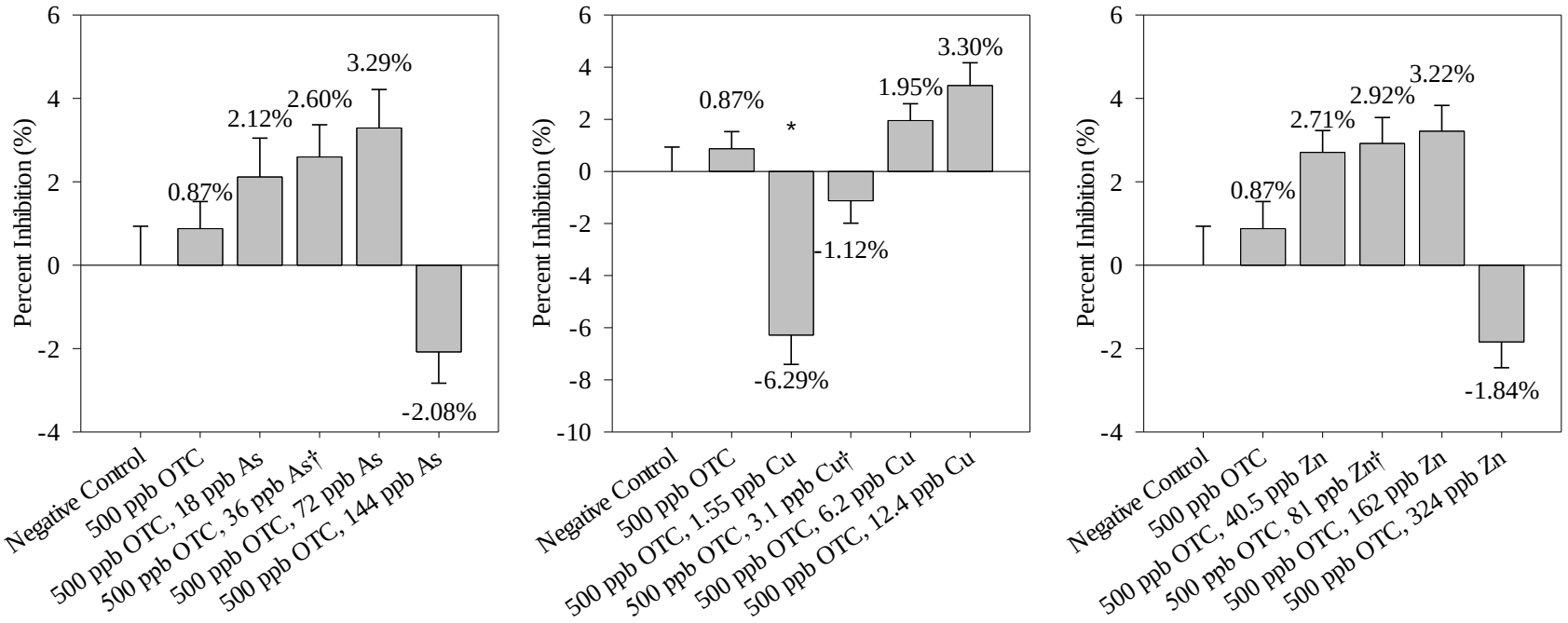
b. Binary Group 4.2

c. Binary Group 4.3

Figure 3.11: Inhibition of *V. vulnificus* following exposure to the Binary Group 4 mixture of the TCS MEC with a CCC range of (a) As, (b) Cu, or (c) Zn.

Table 3.16: ANOVA statistical analysis of results from the Binary Group 4 Mixtures of MEC TCS with CCC range of As, Cu, or Zn.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control p ≤ 0.05	Shapiro-Wilk: Normality p ≥ 0.05	Levene's Test: Homogeneity of Variance p ≥ 0.05	Computed Power p ≥ 0.8
Binary 4.1: 36,000 ppb TCS, CCC [As] (ppb)	Negative Control	10		✓ 0.0912	! 0.0426	✓ 0.999
	36,000 ppb TCS	11	✓ 0.0225			
	36,000 ppb TCS, 18 ppb As	9	✗ 0.0603			
	36,000 ppb TCS, 36 ppb As†	11	✓ 0.0372			
	36,000 ppb TCS, 72 ppb As	12	✓ 0.0158			
	36,000 ppb TCS, 144 ppb As	12	✓ 4.70E-03			
Binary 4.2: 36,000 ppb TCS, CCC [Cu] (ppb)	Negative Control	10		✓ 0.493	! 0.0225	✓ 0.999
	36,000 ppb TCS	11	✓ 2.60E-03			
	36,000 ppb TCS, 1.55 ppb Cu	12	✓ 4.00E-04			
	36,000 ppb TCS, 3.1 ppb Cu†	12	✓ 2.00E-04			
	36,000 ppb TCS, 6.2 ppb Cu	12	✓ 1.00E-04			
	36,000 ppb TCS, 12.4 ppb Cu	12	✓ 1.00E-04			
Binary 4.3: 36,000 ppb TCS, CCC [Zn] (ppb)	Negative Control	10		✓ 0.6998	✓ 0.068	✓ 0.999
	36,000 ppb TCS	11	✓ 2.40E-03			
	36,000 ppb TCS, 40.5 ppb Zn	12	✓ 1.00E-03			
	36,000 ppb TCS, 81 ppb Zn†	12	✓ 2.00E-04			
	36,000 ppb TCS, 162 ppb Zn	12	✓ 2.00E-04			
	36,000 ppb TCS, 324 ppb Zn	11	✓ 7.00E-04			



a. Binary Group 5.1

b. Binary Group 5.2

c. Binary Group 5.3

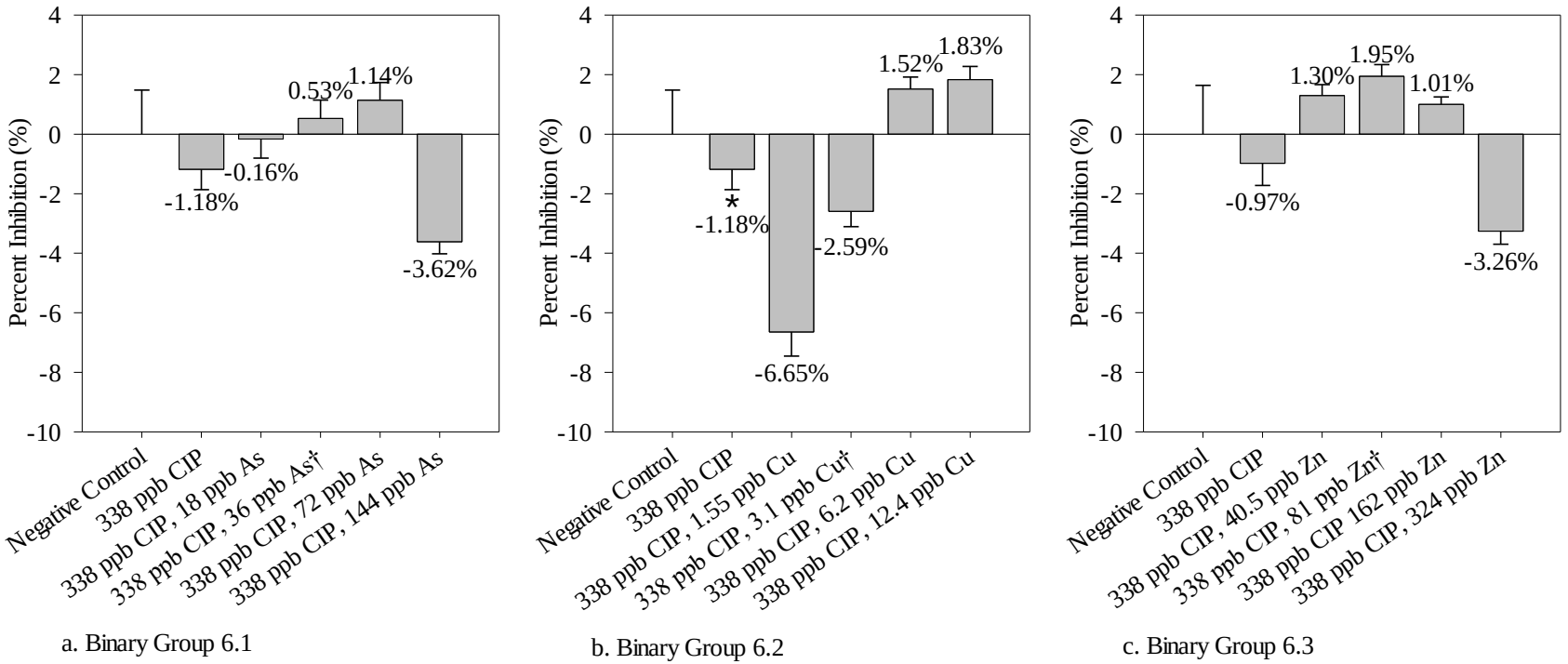
Figure 3.12: Inhibition of *V. vulnificus* following exposure to the Binary Group 5 mixture of the OTC MEC with a CCC range of (a) As, (b) Cu, or (c) Zn.

Table 3.17: ANOVA statistical analysis of results from the Binary Group 5 Mixtures of MEC OTC with CCC range of As, Cu, or Zn.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control p ≤ 0.05	Shapiro-Wilk: Normality p ≥ 0.05	Levene's Test: Homogeneity of Variance p ≥ 0.05	Computed Power p ≥ 0.8
Binary 5.1: 500 ppb OTC, CCC [As] (ppb)	Negative Control	10		! 0.0217	✓ 0.2034	✓ 0.999
	500 ppb OTC	12	✗ 1			
	500 ppb OTC, 18 ppb As	12	✗ 0.9106			
	500 ppb OTC, 36 ppb As†	12	✗ 0.7859			
	500 ppb OTC, 72 ppb As	12	✗ 0.566			
	500 ppb OTC, 144 ppb As	12	✗ 0.5467			
Binary 5.2: 500 ppb OTC, CCC [Cu] (ppb)	Negative Control	10		✓ 0.0987	! 0.0356	✓ 0.999
	500 ppb OTC	12	✗ 1			
	500 ppb OTC, 1.55 ppb Cu	11	✓ 0.0236			
	500 ppb OTC, 3.1 ppb Cu†	11	✗ 0.7554			
	500 ppb OTC, 6.2 ppb Cu	12	✗ 0.9371			
	500 ppb OTC, 12.4 ppb Cu	12	✗ 0.5477			
Binary 5.3: 500 ppb OTC, CCC [Zn] (ppb)	Negative Control	10		✓ 0.4016	! 0.0231	✓ 0.999
	500 ppb OTC	12	✗ 0.9999			
	500 ppb OTC, 40.5 ppb Zn	12	✗ 0.5376			
	500 ppb OTC, 81 ppb Zn†	12	✗ 0.4539			
	500 ppb OTC, 162 ppb Zn	12	✗ 0.35			
	500 ppb OTC, 324 ppb Zn	12	✗ 0.3844			

6.3% growth stimulation and outgrew the OTC MEC exposure by 5.4%; and the 324 ppb zinc experienced 1.8% growth stimulation, outgrowing the OTC MEC by 2.7%. Statistical analysis revealed that none of these OTC and CCC trace metals mixtures causing biostimulated growth were statistically different ($p \leq 0.05$) from the negative controls or the OTC MEC exposure alone (**Table 3.17**).

The ciprofloxacin-based binary exposure group was different from the rest of the binary mixture groups in that the CIP MEC exposure group caused much greater stimulation as opposed to growth inhibition. More importantly, biostimulation was observed across a broad range of exposures in each of the three trace metals tested and was not seen in the single-exposure experiments. Growth inhibition ranged from 0.5% - 11.95% across all three metals and varied with exposure levels for each trace metal. Regardless, slight differences were seen between the CIP MEC exposure and co-exposures to CIP and metals. Growth inhibition was observed in co-exposure to CIP and As at 36 ppb and 72 ppb, up to 1.4% inhibition and a 2.3% change from the CIP MEC exposure group. Additionally, ciprofloxacin with 1.55 ppb copper experienced an additional 5.5% stimulation compared to growth in the CIP MEC exposure group, while at 12.4 Cu, 1.8% growth inhibition was observed. Finally, exposure to both CIP and Zn experienced growth inhibition up to 1.9% at 81 ppb zinc, and 3.3% stimulation at 324 ppb Zn exposure. None of these slight increases in biostimulation or growth inhibition in the Binary Group 6 metals were significantly ($p \leq 0.05$) different when compared to the negative control or the CIP MEC exposure group (**Figure 3.13; Table 3.18**).



a. Binary Group 6.1

b. Binary Group 6.2

c. Binary Group 6.3

Figure 3.13: Inhibition of *V. vulnificus* following exposure to the Binary Group 6 mixture of the CIP MEC with a CCC range of (a) As, (b) Cu, or (c) Zn.

Table 3.18: ANOVA statistical analysis of results from the Binary Group 6 Mixtures of MEC CIP with CCC range of As, Cu, or Zn.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control p ≤ 0.05	Shapiro-Wilk: Normality p ≥ 0.05	Levene's Test: Homogeneity of Variance p ≥ 0.05	Computed Power p ≥ 0.8
Binary 6.1: 338 ppb CIP, CCC [As] (ppb)	Negative Control	11		✓ 0.2833	! 0.0185	✓ 0.999
	338 ppb CIP	12	✗ 0.7297			
	338 ppb CIP, 18 ppb As	12	✗ 0.9812			
	338 ppb CIP, 36 ppb As†	12	✗ 1			
	338 ppb CIP, 72 ppb As	12	✗ 0.9994			
	338 ppb CIP, 144 ppb As	12	✗ 0.0989			
Binary 6.2: 338 ppb CIP, CCC [Cu] (ppb)	Negative Control	11		✓ 0.8003	! 0.0467	✓ 0.999
	338 ppb CIP	12	✗ 0.7345			
	338 ppb CIP, 1.55 ppb Cu	12	✓ 3.20E-03			
	338 ppb CIP, 3.1 ppb Cu†	12	✗ 0.2716			
	338 ppb CIP, 6.2 ppb Cu	12	✗ 0.9889			
	338 ppb CIP, 12.4 ppb Cu	12	✗ 0.9557			
Binary 6.3: 338 ppb CIP, CCC [Zn] (ppb)	Negative Control	10		✓ 0.8161	✓ 0.0541	✓ 0.999
	338 ppb CIP	10	✗ 0.9117			
	338 ppb CIP, 40.5 ppb Zn	12	✗ 0.9523			
	338 ppb CIP, 81 ppb Zn†	12	✗ 0.7792			
	338 ppb CIP 162 ppb Zn	12	✗ 0.9865			
	338 ppb CIP, 324 ppb Zn	12	✗ 0.2316			

(c) Kirby-Bauer Assays

For all of the figures below, an asterisk (*) indicates statistical significance (Dunnett's test $p \leq 0.05$). Positive (+) susceptibility values indicate that the antimicrobial challenge was more toxic to the exposure group than the negative control, and negative (-) susceptibility values indicate that the antimicrobial challenge was less toxic for the exposure group than the negative control. No more than two data points (~22%) were removed as outliers from any given treatment group, and most had no data points removed.

When compared against the CLSI breakpoints for inhibition zones, all of the treatment groups were categorized as susceptible to both ciprofloxacin and cefotaxime (**Table 3.19b**). The negative control and all Low Dose Control treatments except ciprofloxacin were of intermediate susceptibility to oxytetracycline, as were the High Dose Controls for zinc, triclosan, and oxytetracycline. Binary groups of intermediate susceptibility to oxytetracycline included: 510 ppb As with 0.5 ppb OTC, 510 ppb As with 0.064 ppb CIP, 11,500 ppb Zn with 0.5 ppb OTC and 11,500 ppb Zn with 0.064 ppb CIP, and 36,000 ppb TCS with 36 ppb As. There was only one treatment group with a small enough ZOI to be considered resistant, which was 11,500 ppb Zn with 0.5 ppb TCS. All other control and exposure groups were susceptible to oxytetracycline.

Controls for each of the concentrations used in binary mixture for the Kirby-Bauer assays were assessed alongside the binary mixture groups. The Low Dose group consisted of exposures to the published CCC for arsenic, copper, and zinc, and the published PNEC values of triclosan, oxytetracycline, and ciprofloxacin. Each treatment had at least slight non-statistically significant changes in susceptibility to both ciprofloxacin and oxytetracycline from the negative control. Only 6.2 ppb Cu resulted in a decrease in

Table 3.19(a): AMR breakpoints for *V. vulnificus* exposure to ciprofloxacin (CIP), cefotaxime (CTX), and oxytetracycline (OTC) (CLSI 2016).

Antimicrobial Challenge	Breakpoints		
Legend	▲ Susceptible	– Intermediate	▼ Resistant
Ciprofloxacin (5 µg)	≥21	16-20	≤15
Cefotaxime (30 µg)	≥15	12-14	≤11
(Oxy)tetracycline (30 µg)	≥26	23-25	≤22

Table 3.19(b): *V. vulnificus* post-exposure Zones of Inhibition with susceptibility designations.

Average Zone of Inhibition (mm)				
Treatment Group	**Treatment**	**CIP Challenge**	**CTX Challenge**	**OTC Challenge**
Negative Control	None	23.7	24.0	24.0
Low Controls	36 ppb As	25.3	25.0	24.8
	6.2 ppb Cu	24.8	24.8	24.9
	81 ppb Zn	25.3	25.3	25.1
	0.5 ppb TCS	24.8	24.8	24.9
	0.5 ppb OTC	25.1	25.1	24.2
	0.064 ppb CIP	25.9	25.9	26.1
High Controls	510 ppb As	29.2	27.6	26.9
	13,890 ppb Cu	30.4	29.0	27.4
	11,500 ppb Zn	29.4	25.7	24.5
	36,000 ppb TCS	31.4	29.1	25.9
	500 ppb OTC	29.2	26.3	25.4
	338 ppb CIP	31.3	28.3	26.7
Binary Group 1	510 ppb As, 0.5 ppb TCS	29.7	25.0	26.1
	510 ppb As, 0.5 ppb OTC	30.0	26.2	25.2
	510 ppb As,0.064 ppb CIP	30.3	26.5	24.6
Binary Group 2	13,890 ppb Cu, 0.5 ppb TCS	31.1	30.9	26.3
	13,890 ppb Cu, 0.5 ppb OTC	30.8	28.5	27.1
	13,890 ppb Cu, 0.064 ppb CIP	31.4	29.4	27.3
Binary Group 3	11,500 ppb Zn, 0.5 ppb TCS	29.7	26.4	21.9
	11,500 ppb Zn, 0.5 ppb OTC	29.6	26.3	25.9
	11,500 ppb Zn, 0.064 ppb CIP	31.1	26.0	25.9
Binary Group 4	36,000 ppb TCS, 36 ppb As	30.5	27.3	26.0
	36,000 ppb TCS, 3.1 ppb Cu	31.4	29.4	27.7
	36,000 ppb TCS, 81 ppb Zn	31.9	28.8	27.8
Binary Group 5	500 ppb OTC, 36 ppb As	30.1	27.4	26.8
	500 ppb OTC, 3.1 ppb Cu	30.7	27.6	26.5
	500 ppb OTC, 81 ppb Zn	32.4	29.4	27.0
Binary Group 6	338 ppb CIP, 36 ppb As	28.2	28.2	27.3
	338 ppb CIP, 3.1 ppb Cu	32.3	32.4	29.2
	338 ppb CIP, 81 ppb Zn	28.0	28.0	27.9

susceptibility, though not enough to qualify it as "resistant." The largest susceptibility changes were seen in those cultures acclimated to 0.064 ppb CIP, with a 7.17% increase in susceptibility to ciprofloxacin and 8.74% increase in susceptibility to oxytetracycline. None of these changes in susceptibility were significantly ($p \leq 0.05$) different in comparison with the negative control (**Figure 3.14; Table 3.20**).

The High Dose Group consisted of the toxicant concentrations used as constants in each binary treatment group. Changes in susceptibility to ciprofloxacin, cefotaxime, and oxytetracycline were varied, ranging from 1.27% increase in susceptibility to oxytetracycline after acclimation to 11,500 ppb Zn and 23.4% increase in susceptibility to cefotaxime following acclimation to 36 ppm triclosan. None induced a decrease in susceptibility, which would indicate increased resistance. None of these changes in susceptibility were significantly ($p \leq 0.05$) different in comparison to the negative control (**Figure 3.15; Table 3.21**).

Binary Group 1 consisted of a constant 0.51 ppb arsenic exposure in combination with three treatment groups which combined 0.51 ppb arsenic with either 0.5 ppb TCS, 0.5 ppb OTC, or 0.064 ppb CIP. In tandem with the arsenic, all three antimicrobials slightly increased *V. vulnificus* susceptibility to ciprofloxacin as compared to arsenic exposure alone, while all decreased susceptibility to oxytetracycline. The effect on cefotaxime susceptibility was mixed - co-exposure to triclosan and oxytetracycline slightly decreased susceptibility compared to the arsenic control, while co-exposure to ciprofloxacin slightly increased susceptibility. None of these changes in susceptibility were significantly ($p \leq 0.05$) different in comparison to the negative control (**Figure 3.16; Table 3.22**).

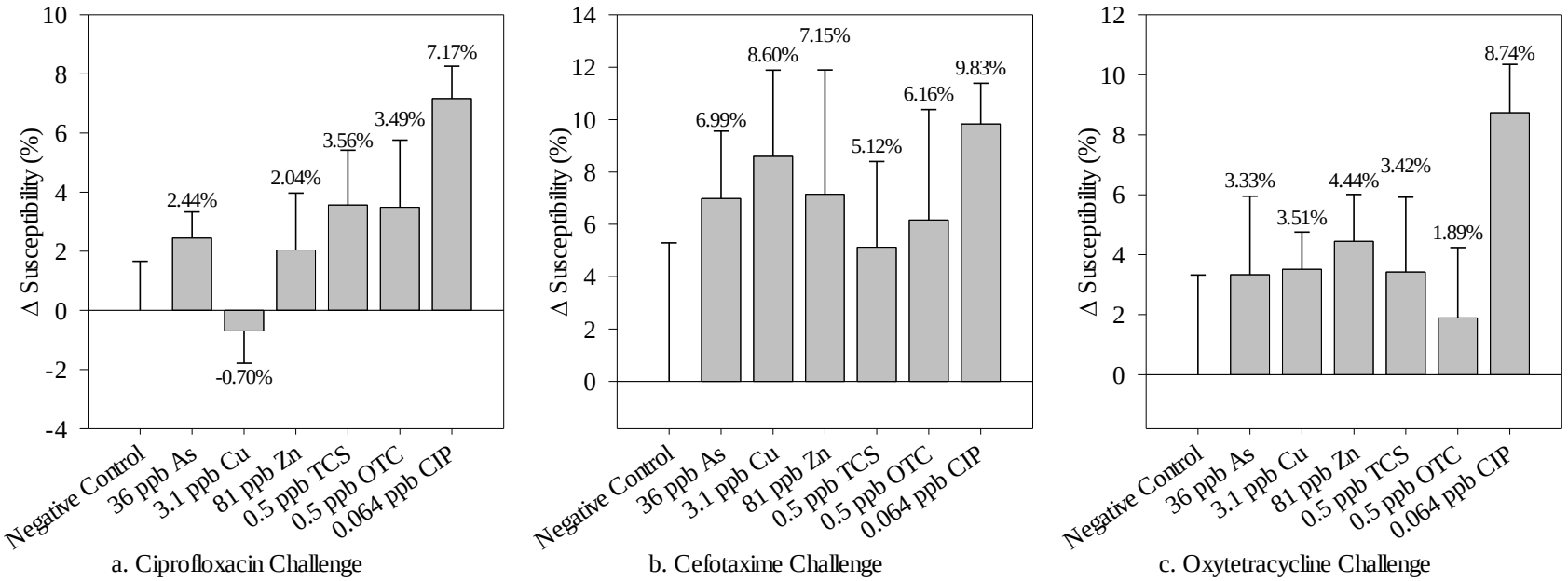


Figure 3.14: *V. vulnificus* change in susceptibility to (a) ciprofloxacin (-0.7 - 7.17%), (b) cefotaxime (5.12 - 9.83%), and (c) oxytetracycline (1.89-8.74%) following 24-hour acclimation to the EPA Criterion Continuous Concentration of arsenic, copper, or zinc, or the Probable No Effects Concentration of triclosan, oxytetracycline, or ciprofloxacin.

Table 3.20: ANOVA statistical analysis of the results for Kirby-Bauer Low Dose Exposures to CCC doses of As, Cu, and Zn and PNEC doses of TCS, OTC, or CIP.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control p ≤ 0.05	Shapiro-Wilk: Normality p ≥ 0.05	Levene's Test: Homogeneity of Variance p ≥ 0.05	Computed Power p ≥ 0.8
Low Doses: CIP Challenge	Negative Control	9		✓ 0.0661	! 0.0425	✓ 0.999
	36 ppb As	9	✗ 0.9742			
	6.2 ppb Cu	9	✗ 1			
	81 ppb Zn	8	✗ 0.9888			
	0.5 ppb TCS	9	✗ 0.8782			
	0.5 ppb OTC	9	✗ 0.8875			
	0.064 ppb CIP	9	✗ 0.3371			
Low Doses: CTX Challenge	Negative Control	8		✓ 0.4607	! 0.0296	✓ 0.998
	36 ppb As	8	✗ 0.997			
	6.2 ppb Cu	7	✗ 0.9996			
	81 ppb Zn	9	✗ 0.9847			
	0.5 ppb TCS	9	✗ 0.9986			
	0.5 ppb OTC	9	✗ 0.9944			
	0.064 ppb CIP	9	✗ 0.9134			
Low Doses: OTC Challenge	Negative Control	9		✓ 0.2231	! 0.01	✓ 0.999
	36 ppb As	9	✗ 0.9841			
	6.2 ppb Cu	9	✗ 0.9794			
	81 ppb Zn	9	✗ 0.9422			
	0.5 ppb TCS	9	✗ 0.9818			
	0.5 ppb OTC	8	✗ 1			
	0.064 ppb CIP	9	✗ 0.5342			

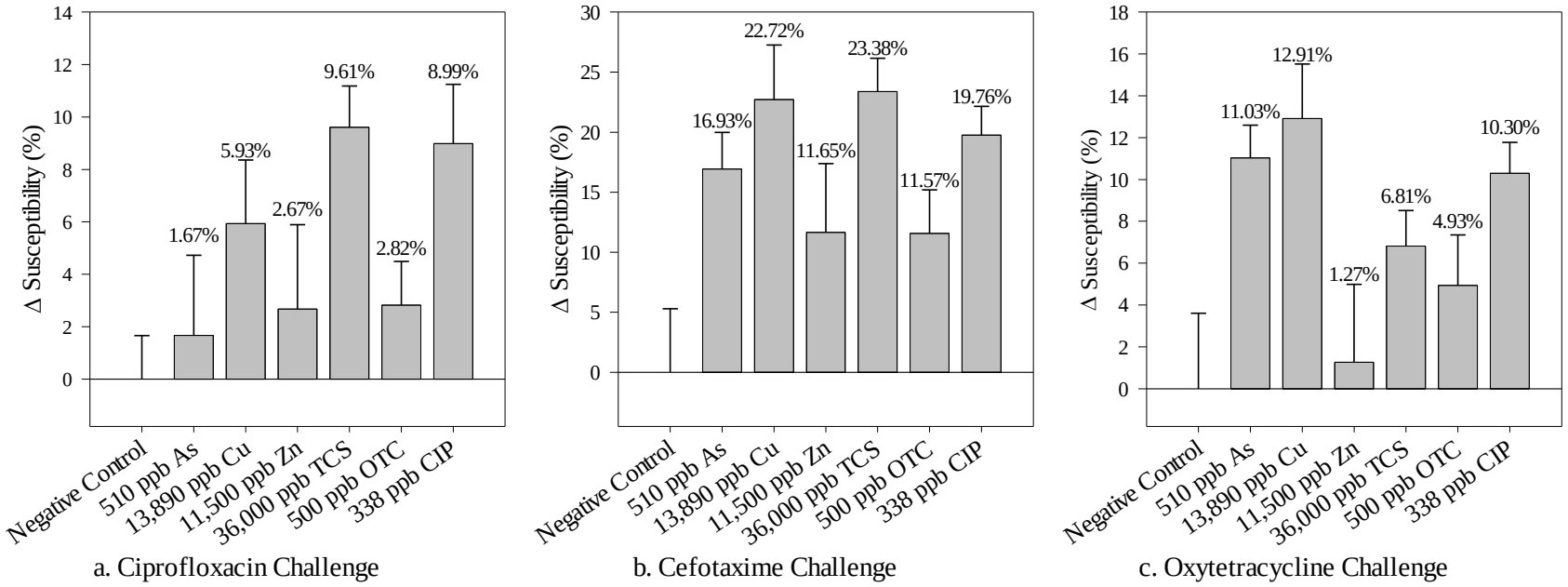


Figure 3.15: *V. vulnificus* change in susceptibility to (a) ciprofloxacin, (b) cefotaxime, and (c) oxytetracycline following 24-hour acclimation to MEC of arsenic, copper, zinc, triclosan, oxytetracycline, or ciprofloxacin.

Table 3.21: ANOVA statistical analysis of results for the Kirby-Bauer High Dose Exposures to MEC doses of As, Cu, Zn, TCS, OTC, or CIP.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control $p \leq 0.05$	Shapiro-Wilk: Normality $p \geq 0.05$	Levene's Test: Homogeneity of Variance $p \geq 0.05$	Computed Power $p \geq 0.8$
High Doses: CIP Challenge	Negative Control	9		✓ 0.7356	✓ 0.07	✓ 0.999
	510 ppb As	9	✗ 0.9997			
	13,890 ppb Cu		✗ 0.8631			
	11,500 ppb Zn		✗ 0.9962			
	36,000 ppb TCS		✗ 0.5041			
	500 ppb OTC	9	✗ 0.9911			
	338 ppb CIP	9	✗ 0.5654			
High Doses: CTX Challenge	Negative Control	8		✓ 0.8707	! 0.0151	✓ 0.999
	510 ppb As	9	✗ 0.5645			
	13,890 ppb Cu	9	✗ 0.2789			
	11,500 ppb Zn	8	✗ 0.9662			
	36,000 ppb TCS	9	✗ 0.2546			
	500 ppb OTC	9	✗ 0.8681			
	338 ppb CIP	9	✗ 0.4099			
High Doses: OTC Challenge	Negative Control	8		✓ 0.8986	✓ 0.1052	✓ 0.999
	510 ppb As	9	✗ 0.3492			
	13,890 ppb Cu	9	✗ 0.236			
	11,500 ppb Zn	9	✗ 0.9968			
	36,000 ppb TCS	9	✗ 0.7066			
	500 ppb OTC	9	✗ 0.8639			
	338 ppb CIP	9	✗ 0.4025			

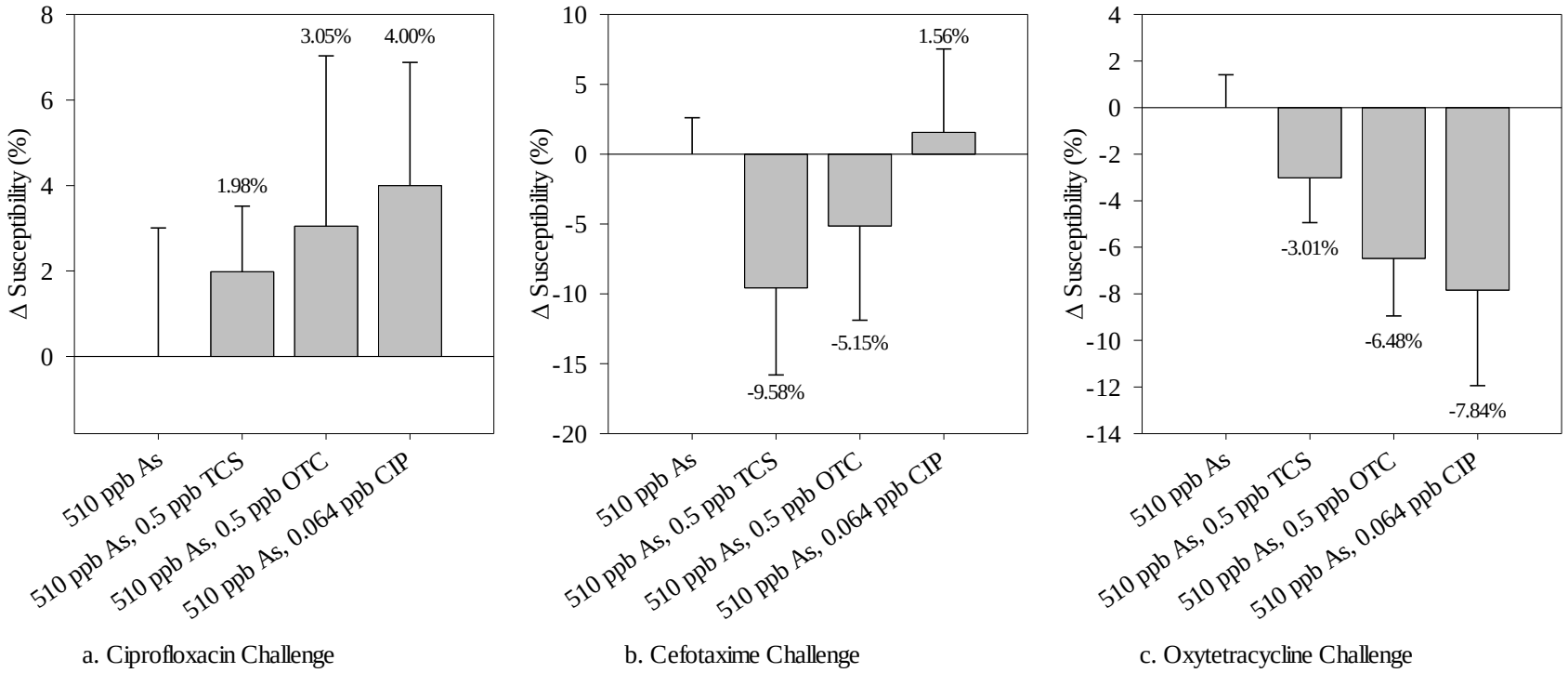


Figure 3.16: *V. vulnificus* change in susceptibility to (a) ciprofloxacin (1.98 - 4.00%), (b) cefotaxime (-9.58 - 1.56%), and (c) oxytetracycline (-7.84 - -3.01%) following 24-hour acclimation to the Binary Group 1 mixture of the As MEC with the PNEC of triclosan, oxytetracycline, or ciprofloxacin.

Table 3.22: ANOVA statistical analysis of results for Kirby-Bauer Assay Binary Group 1 Mixtures of MEC As with PNEC ranges of TCS, OTC, or CIP.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control $p \leq 0.05$	Shapiro-Wilk: Normality $p \geq 0.05$	Levene's Test: Homogeneity of Variance $p \geq 0.05$	Computed Power $p \geq 0.8$
Binary Group 1: CIP Challenge	510 ppbAs	9		✓ 0.7841	✓ 0.0847	✓ 0.989
	510 ppb As, 0.5 ppb TCS†	9	✗ 0.99			
	510 ppb As, 0.5 ppb OTC†	9	✗ 0.9662			
	510 ppb As, 0.064 ppb CIP†	9	✗ 0.9301			
Binary Group 1 CTX Challenge	510 ppbAs	9		✓ 0.4075	✓ 0.0942	✓ 0.989
	510 ppb As, 0.5 ppb TCS†	9	✗ 0.8619			
	510 ppb As, 0.5 ppb OTC†	9	✗ 0.9725			
	510 ppb As, 0.064 ppb CIP†	7	✗ 0.991			
Binary Group 1 OTC Challenge	510 ppbAs	9		✓ 0.8403	✓ 0.0971	✓ 0.999
	510 ppb As, 0.5 ppb TCS†	9	✗ 0.9448			
	510 ppb As, 0.5 ppb OTC†	9	✗ 0.6771			
	510 ppb As, 0.064 ppb CIP†	8	✗ 0.45			

When compared back to the antimicrobial Low Dose groups, these reactions indicate that co-exposure to arsenic and antimicrobials reduces susceptibility to both ciprofloxacin and oxytetracycline when compared to the antimicrobials alone. Notably, co-exposure of arsenic and ciprofloxacin increased susceptibility of *V. vulnificus* to oxytetracycline by a total of 16.58% when compared to ciprofloxacin alone. This is not, however, enough of a reduction in susceptibility to consider it to be resistant.

Binary Group 2 is a constant treatment of copper (13,890 ppb) with each of the three PNEC antimicrobials. Similar to Binary Group 1 results, all three antimicrobial treatments slightly increased susceptibility to ciprofloxacin when compared to the copper exposure alone, and all three decreased susceptibility to oxytetracycline. Cefotaxime susceptibility slightly increased compared to the copper exposure alone after exposure to triclosan and ciprofloxacin mixtures with copper, but slightly decreased following acclimation to oxytetracycline along with the copper. None of these changes in susceptibility were significantly ($p \leq 0.05$) different in comparison to the negative control (**Figure 3.17; Table 3.23**).

Co-exposure of *V. vulnificus* to zinc and triclosan resulted in very little change in susceptibility to either ciprofloxacin or cefotaxime but induced nearly an 11% decrease in susceptibility to oxytetracycline. Likewise, a <1% change in susceptibility to either ciprofloxacin or cefotaxime was observed following exposure to zinc with oxytetracycline, but the susceptibility to oxytetracycline increased by 5.7%. Exposure to zinc in conjunction with ciprofloxacin resulted in a 5.6% increase in susceptibility to ciprofloxacin, 1.3% decrease in susceptibility to cefotaxime, and 5.4% increase in susceptibility to

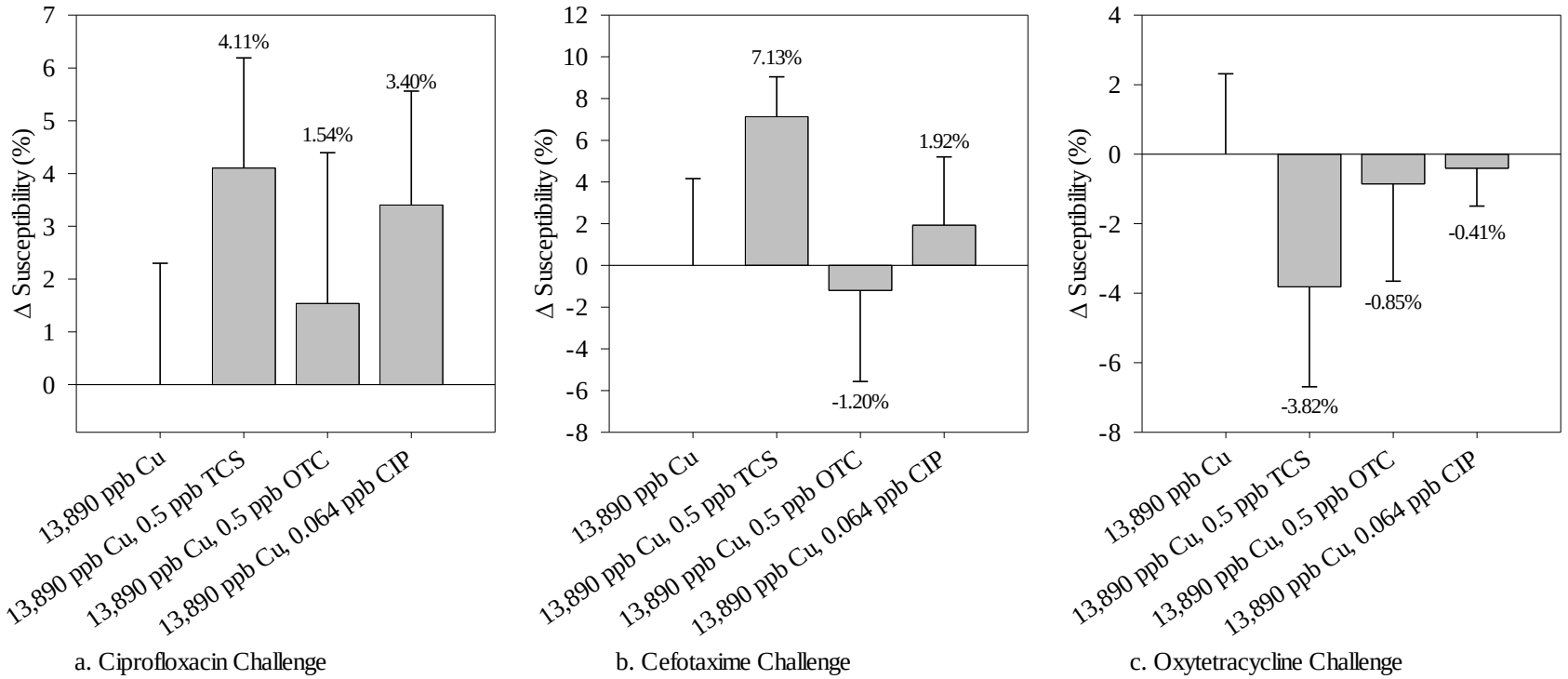


Figure 3.17: *V. vulnificus* change in susceptibility to (a) ciprofloxacin (1.54 - 3.4%), (b) cefotaxime (-1.20 - 7.13%), and (c) oxytetracycline (-3.822 - -0.41%) following 24-hour acclimation to the Binary Group 2 mixture of the Cu MEC with the PNEC of triclosan, oxytetracycline, or ciprofloxacin.

Table 3.23: ANOVA statistical analysis of results for Kirby-Bauer Assay Binary Group 2 Mixtures of MEC Cu with PNEC ranges of TCS, OTC, or CIP.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control p ≤ 0.05	Shapiro-Wilk: Normality p ≥ 0.05	Levene's Test: Homogeneity of Variance p ≥ 0.05	Computed Power p ≥ 0.8
Binary Group 2: CIP Challenge	13,890 ppb Cu	9		✓ 0.2689	✓ 0.2294	✓ 0.9
	13,890 ppb Cu, 0.5 ppb TCS†	8	✗ 0.9035			
	13,890 ppb Cu, 0.5 ppb OTC†	9	✗ 0.9897			
	13,890 ppb Cu, 0.064 ppb CIP†	9	✗ 0.9088			
Binary Group 2: CTX Challenge	13,890 ppb Cu	8		✓ 0.3497	! 0.0382	✓ 0.999
	13,890 ppb Cu, 0.5 ppb TCS†	9	✗ 0.8874			
	13,890 ppb Cu, 0.5 ppb OTC†	9	✗ 0.986			
	13,890 ppb Cu, 0.064 ppb CIP†	9	✗ 0.9999			
Binary Group 2: OTC Challenge	13,890 ppb Cu	9		✓ 0.3008	✓ 0.1558	✓ 0.983
	13,890 ppb Cu, 0.5 ppb TCS†	9	✗ 0.8878			
	13,890 ppb Cu, 0.5 ppb OTC†	9	✗ 0.9983			
	13,890 ppb Cu, 0.064 ppb CIP†	9	✗ 0.9998			

oxytetracycline. None of these changes in susceptibility were significantly ($p \leq 0.05$) different in comparison to the negative control (**Figure 3.18; Table 3.24**).

Binary Group 4 combined exposure to triclosan with exposure to CCC-level metals. A reduction in susceptibility to both ciprofloxacin and cefotaxime was observed following exposure to triclosan with arsenic, and very little increase in susceptibility to oxytetracycline. A <1.5% change in susceptibility to ciprofloxacin and cefotaxime was seen following co-exposures to triclosan with either copper or zinc, but both copper and zinc individually caused a slight increase in susceptibility to oxytetracycline of approximately 7% (**Figure 3.19; Table 3.25**). None of these changes in susceptibility were significantly ($p \leq 0.05$) different in comparison to the negative control.

Increases in susceptibility to all three antimicrobial challenges was observed following co-exposure of oxytetracycline with each of the three metals. The largest changes in each challenge were in the oxytetracycline and zinc treatment group, leading to a 9.9% increase in susceptibility to ciprofloxacin, an 11.6% increase in susceptibility to cefotaxime, and a 6% increase in susceptibility to oxytetracycline. None of these slight changes in susceptibility were significantly ($p \leq 0.05$) different in comparison to the negative control (**Figure 3.20; Table 3.26**).

Approximately 10% decrease in susceptibility to ciprofloxacin was observed in the treatment groups combining ciprofloxacin with arsenic and with zinc, though ciprofloxacin with copper led to a 3.3% increase in susceptibility. Neither arsenic nor zinc considerably affected susceptibility to cefotaxime, while copper increased cefotaxime by 15.2% when compared to the ciprofloxacin control. Oxytetracycline susceptibility was increased by approximately 2% when copper was added to the ciprofloxacin, 4.2% when zinc was

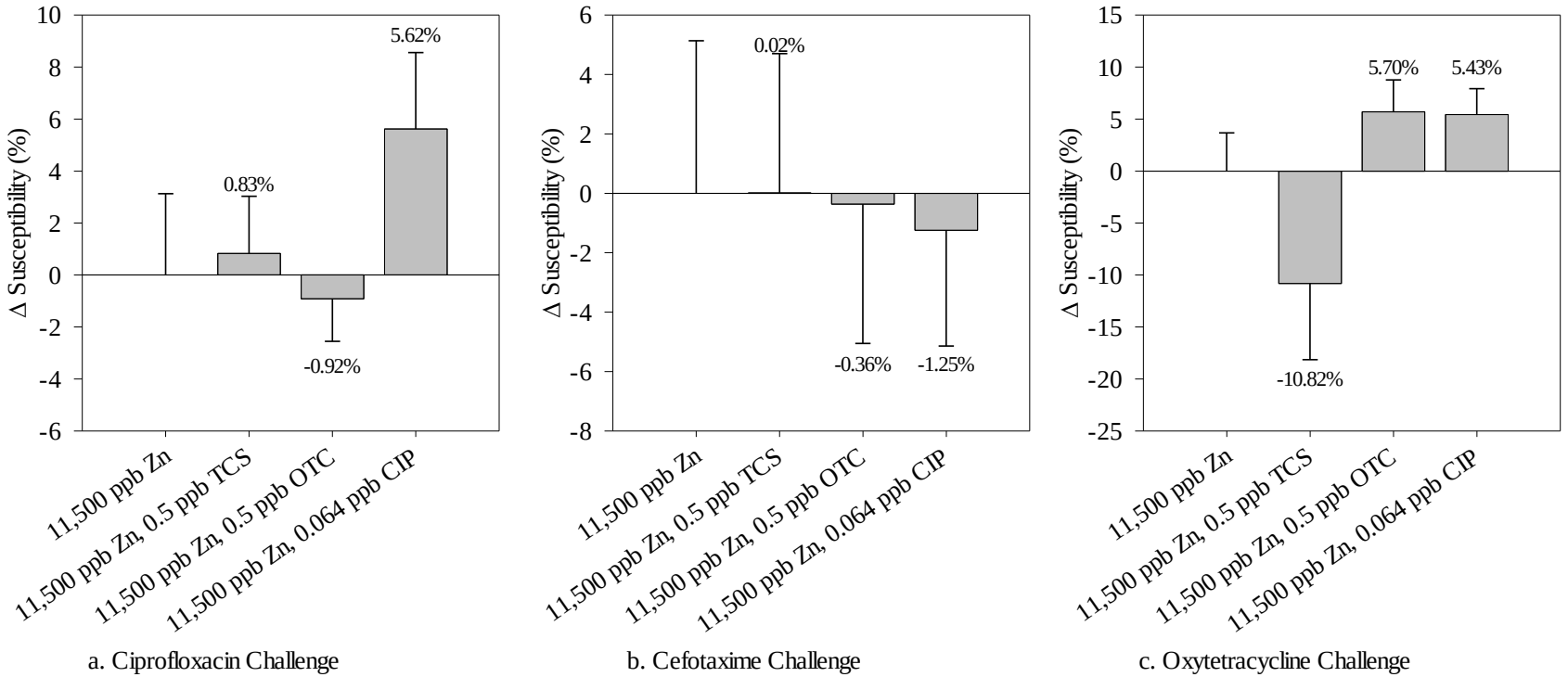


Figure 3.18: *V. vulnificus* change in susceptibility to (a) ciprofloxacin (-0.92 - 5.62%), (b) cefotaxime (-1.21 - 0.02%), and (c) oxytetracycline (-10.82 - 5.70%) following 24-hour acclimation to the Binary Group 3 mixture of the Zn MEC with the PNEC of TCS, OTC, or CIP.

Table 3.24: ANOVA statistical analysis of results for Kirby-Bauer Assay Binary Group 3 Mixtures of MEC Zn with PNEC ranges of TCS, OTC, or CIP.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control $p \leq 0.05$	Shapiro-Wilk: Normality $p \geq 0.05$	Levene's Test: Homogeneity of Variance $p \geq 0.05$	Computed Power $p \geq 0.8$
Binary Group 3: CIP Challenge	11,500 ppb Zn	9		✓ 0.2165	✓ 0.0662	✓ 0.999
	11,500 ppb Zn, 0.5 ppb TCS†	9	✗ 0.9988			
	11,500 ppb Zn, 0.5 ppb OTC†	8	✗ 1			
	11,500 ppb Zn, 0.064 ppb CIP†	9	✗ 0.771			
Binary Group 3: CTX Challenge	11,500 ppb Zn	8		✓ 0.9741	✓ 0.1021	✗ 0.444
	0.5 ppb TCS†	9	✗ 0.9948			
	11,500 ppb Zn, 0.5 ppb OTC†	9	✗ 0.9969			
	11,500 ppb Zn, 0.064 ppb CIP†	9	✗ 0.9994			
Binary Group 3: OTC Challenge	11,500 ppb Zn	9		✓ 0.346	✓ 0.0895	✓ 0.999
	11,500 ppb Zn, 0.5 ppb TCS†	9	✗ 0.7379			
	11,500 ppb Zn, 0.5 ppb OTC†	9	✗ 0.942			
	11,500 ppb Zn, 0.064 ppb CIP†	9	✗ 0.9491			

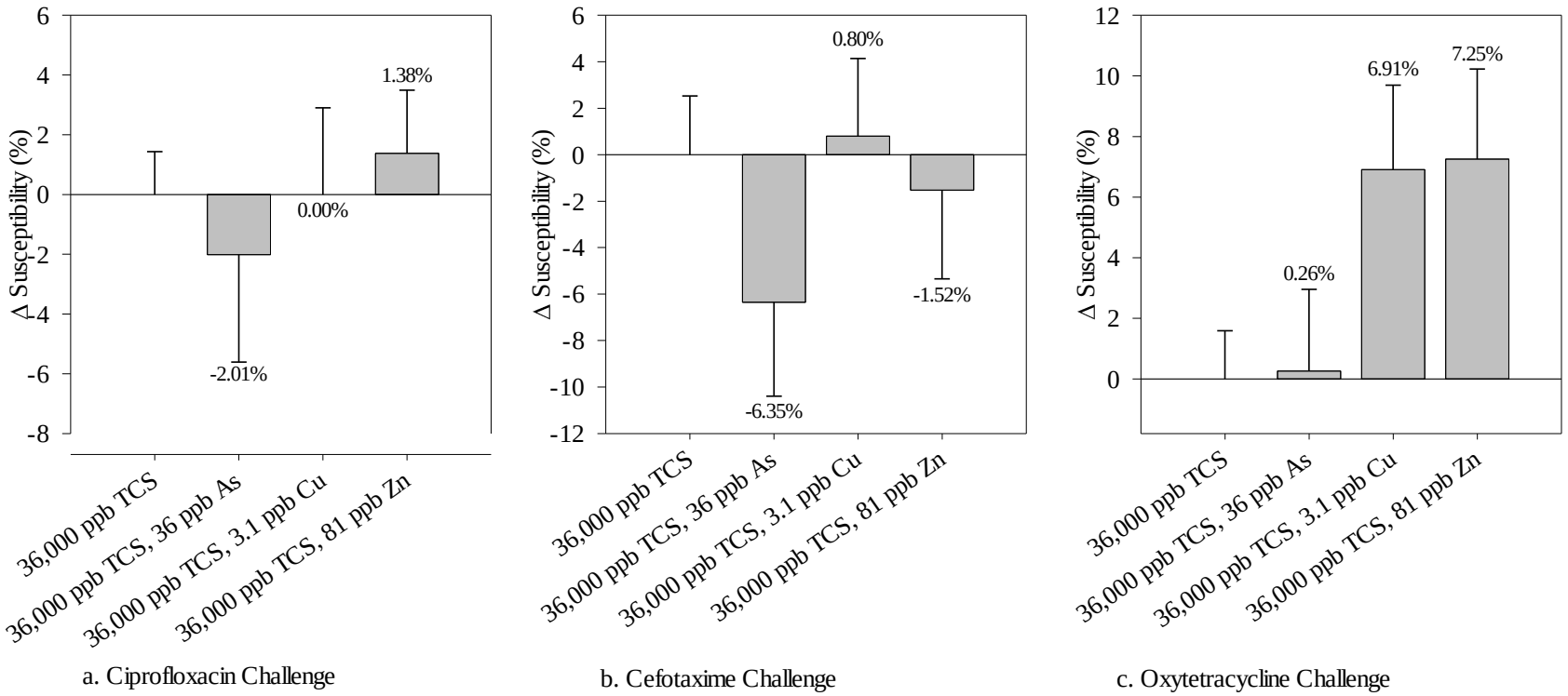


Figure 3.19: *V. vulnificus* change in susceptibility to (a) ciprofloxacin (-2.01 - 1.38%), (b) cefotaxime (-6.35 - 0.8%), and (c) oxytetracycline (0.26 - 7.25%) following 24-hour acclimation to the Binary Group 4 mixture of the TCS MEC with the CCC of As, Cu, or Zn.

Table 3.25: ANOVA statistical analysis of results for the Kirby-Bauer Assay Binary Group 4 Mixtures of MEC TCS with CCC of As, Cu, or Zn.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control p ≤ 0.05	Shapiro-Wilk: Normality p ≥ 0.05	Levene's Test: Homogeneity of Variance p ≥ 0.05	Computed Power p ≥ 0.8
Binary Group 4: CIP Challenge	36,000 ppb TCS	9		✓ 0.2208	! 0.0213	✓ 0.999
	36,000 ppb TCS, 36 ppb As†	7	✗ 0.9661			
	36,000 ppb TCS, 3.1 ppb Cu†	9	✗ 1			
	36,000 ppb TCS, 81 ppb Zn†	9	✗ 0.9936			
Binary Group 4: CTX Challenge	36,000 ppb TCS	8		✓ 0.885	✓ 0.0649	✓ 0.999
	36,000 ppb TCS, 36 ppb As†	9	✗ 0.9065			
	36,000 ppb TCS, 3.1 ppb Cu†	9	✗ 0.9952			
	36,000 ppb TCS, 81 ppb Zn†	9	✗ 0.9999			
Binary Group 4: OTC Challenge	36,000 ppb TCS	9		✓ 0.8984	✓ 0.1422	✓ 0.999
	36,000 ppb TCS, 36 ppb As†	9	✗ 1			
	36,000 ppb TCS, 3.1 ppb Cu†	9	✗ 0.6598			
	36,000 ppb TCS, 81 ppb Zn†	9	✗ 0.6294			

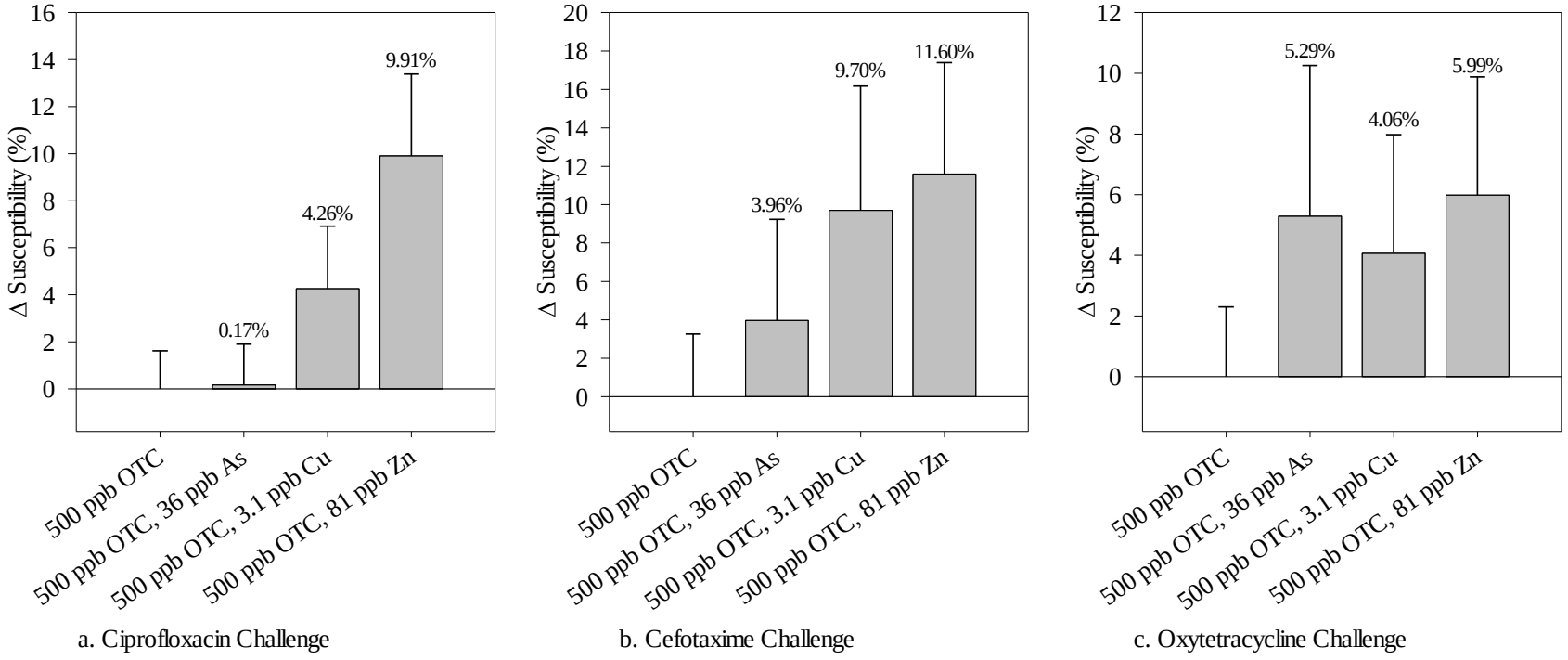


Figure 3.20: *V. vulnificus* change in susceptibility to (a) ciprofloxacin (0.17 - 9.91%), (b) cefotaxime (3.96 - 11.60%), and (c) oxytetracycline (4.06 - 5.99%) following 24-hour acclimation to the Binary Group 5 mixture of the OTC MEC with the CCC of As, Cu, or Zn.

Table 3.26: ANOVA statistical analysis of results for the Kirby-Bauer Assay Binary Group 5 Mixtures of MEC OTC with CCC range of As, Cu, or Zn.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control p ≤ 0.05	Shapiro-Wilk: Normality p ≥ 0.05	Levene's Test: Homogeneity of Variance p ≥ 0.05	Computed Power p ≥ 0.8
Binary Group 5: CIP Challenge	500 ppb OTC	8		✓ 0.1135	! 0.0409	✓ 0.999
	500 ppb OTC, 36 ppb As†	8	✗ 0.9998			
	500 ppb OTC, 3.1 ppb Cu†	9	✗ 0.9021			
	500 ppb OTC, 81 ppb Zn†	9	✗ 0.4349			
Binary Group 5: CTX Challenge	500 ppb OTC	9		✓ 0.0576	✓ 0.3974	✓ 0.999
	500 ppb OTC, 36 ppb As†	9	✗ 0.9838			
	500 ppb OTC, 3.1 ppb Cu†	7	✗ 0.9804			
	500 ppb OTC, 81 ppb Zn†	9	✗ 0.7511			
Binary Group 5: OTC Challenge	500 ppb OTC	9		✓ 0.8977	✓ 0.1391	✓ 0.999
	500 ppb OTC, 36 ppb As†	9	✗ 0.9288			
	500 ppb OTC, 3.1 ppb Cu†	9	✗ 0.9647			
	500 ppb OTC, 81 ppb Zn†	9	✗ 0.9025			

introduced, and 8.2% after co-exposure to ciprofloxacin and copper. None of these slight changes in susceptibility were significantly ($p \leq 0.05$) different in comparison to the negative control (**Figure 3.21; Table 3.27**).

Discussion

(a) Key Points

Several binary mixture groups stood out as deserving of closer study. For example, Binary Group 2.2 (13,890 ppb Cu and 0.5 ppb OTC) exhibited a synergistic relationship - that is, bacterial growth inhibition in mixture was greater than the sum of the inhibition for both compounds individually, meaning the compounds amplify the effects of each other. In contrast, Binary Group 5.2, which contained the same compounds in different concentrations (500 ppb OTC and 3.1 ppb Cu) were antagonistic, having less toxic effect in combination than the sum of each alone, and ultimately resulted in 6.3% biostimulation as compared to the negative control (**Table 3.28**). These divergent results of two similar mixture treatment groups exemplify the importance of examining different concentrations of test compounds in microbial toxicology. That is, dose is very important in determining whether a compound is antagonistic or synergistic. OTC has been found in mariculture effluents at concentrations up to 2.3 ppb (Thurman 2003; Bradford et al. 2008) and will be quickly diluted during rainfall events and as it moves from catchments into larger waterways, while copper in the form of copper sulfate is a common algicide in stormwater ponds. It is also used to treat toxic cyanobacteria in drinking water plants to eliminate potential toxins along with taste and odor problems. In a risk assessment for OTC, Uyaguari et al. (2009) found that shrimp mariculture pond sediments and effluent may pose significant risk for an increased potential for antibiotic resistance. Results further

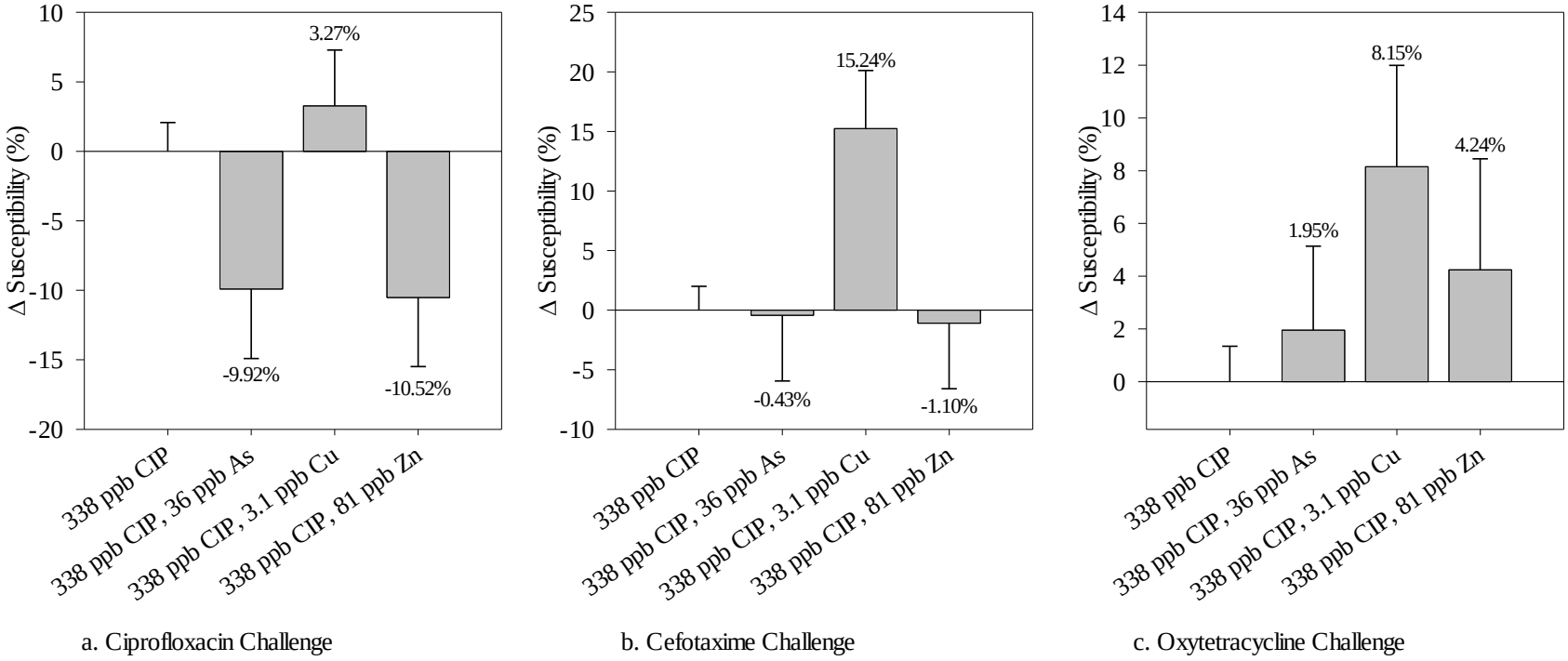


Figure 3.21: *V. vulnificus* change in susceptibility to (a) ciprofloxacin, (b) cefotaxime, and (c) oxytetracycline following 24-hour acclimation to the Binary Group 6 mixture of the CIP MEC with the CCC of As, Cu, or Zn.

Table 3.27: ANOVA statistical analysis of results for Kirby-Bauer Assay Binary Group 6 Mixtures of MEC CIP with CCC range of As, Cu, or Zn.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control p ≤ 0.05	Shapiro-Wilk: Normality p ≥ 0.05	Levene's Test: Homogeneity of Variance p ≥ 0.05	Computed Power p ≥ 0.8
Binary Group 6: CIP Challenge	338 ppb CIP	9		✓ 0.4242	✓ 0.1445	✓ 0.999
	338 ppb CIP, 36 ppb As†	9	✗ 0.7388			
	338 ppb CIP, 3.1 ppb Cu†	9	✗ 0.9842			
	338 ppb CIP, 81 ppb Zn†	9	✗ 0.7067			
Binary Group 6: CTX Challenge	338 ppb CIP	9		✓ 0.4242	✓ 0.1445	✓ 0.999
	36 ppb As†	9	✗ 1			
	338 ppb CIP, 3.1 ppb Cu†	8	✗ 0.5793			
	338 ppb CIP, 81 ppb Zn†	9	✗ 0.9995			
Binary Group 6: OTC Challenge	338 ppb CIP	9		✓ 0.6008	! 0.0448	✓ 0.999
	338 ppb CIP, 36 ppb As†	9	✗ 0.9925			
	338 ppb CIP, 3.1 ppb Cu†	8	✗ 0.702			
	338 ppb CIP, 81 ppb Zn†	9	✗ 0.9345			

Table 3.28: A comparison of the two binary mixture groups combining copper and oxytetracycline at different concentrations.

Treatment Group	Treatment	%I Alone	%I in Mixture	Toxicological Relationship	Susceptibility to OTC
Binary Group 2.2	13,890 ppb Cu	3.2	6	SYNERGISTIC*	S
	0.5 ppb OTC	-3.5113			
Binary Group 5.2	3.1 ppb Cu	-0.0798	-6.3	ANTAGONISTIC	S
	500 ppb OTC	4.3			

support these findings. The combination of copper and oxytetracycline led to considerable changes in susceptibility to all three antimicrobial challenges, including a slight decrease in susceptibility to both cefotaxime and oxytetracycline when in the configuration of Binary Group 2.2 (**Figures 3.17 and 3.20**), which uses concentrations of both toxicants which may be found in environmental settings.

Another mixture of environmental relevance is zinc and triclosan (Binary Groups 3.1 and 4.3) (**Table 3.29**). At 36,000 ppb triclosan and 162 ppb Zn, these two compounds are synergistic and susceptible to oxytetracycline. However, at 11,500 ppb Zn and 0.5 ppb TCS, these compounds are antagonistic and induce slight resistance to oxytetracycline. Given the prevalence of both these compounds at similar levels in stormwater ponds and estuarine systems, the interactions of these two toxicants should be closely monitored and further studied. In terms of synergism, it appears high doses of each trace metals is a major driver producing synergisms (enhanced toxicity) while it appears high doses of each antimicrobial appeared to be a driver that reduced toxicity and produce antagonism

Triclosan in combination with copper also exhibits opposite toxicological relationships when the concentrations change, corresponding to varied reactions to the susceptibility tests (**Table 3.30**). More specifically, the antagonistic relationship corresponds to a slight increase in susceptibility to ciprofloxacin and a slight decrease in susceptibility to cefotaxime and oxytetracycline. Meanwhile, the synergistic relationship corresponds to no change in susceptibility to ciprofloxacin and slight increases in susceptibility to cefotaxime and oxytetracycline. These results suggest that antagonistic and synergistic responses correlate with opposite (increase versus decrease) susceptibility

Table 3.29: A comparison of the two binary mixture groups combining zinc and triclosan at different concentrations.

Treatment Group	Treatment	%I Alone	%I in Mixture	Toxicological Relationship	Susceptibility to OTC
Binary Group 3.1	11,500 ppb Zn	0.5658	0.6531	ANTAGONISTIC	R
	0.5ppb TCS	1.4756			
Binary Group 4.3	162 ppb Zn	2.5283	33.5273	SYNERGISTIC*	S
	36,000 ppb TCS	26.7593			

Table 3.30: A comparison of the two binary mixture groups combining copper and triclosan at different concentrations.

Treatment Group	Treatment	%I Alone	%I in Mixture	Toxicological Relationship	Susceptibility to OTC
Binary Group 2.1	13,890 ppb Cu	8.7534	8.5932	ANTAGONISTIC*	S
	0.5ppb TCS	1.4756			
Binary Group 4.2	3.1 ppb Cu	-0.0798	34.1681	SYNERGISTIC*	S
	36,000 ppb TCS	26.7593			

changes, but that whether those changes are positive or negative is dependent on the specific antimicrobial challenge itself.

Of a total of eighteen treatment groups, thirteen displayed antagonistic relationships while only five were synergistic. Of those five, three included the oxytetracycline PNEC and the other two were the triclosan MEC (combined with CCC copper or zinc). When the exposure groups are distilled down to the two overarching exposure types, the breakdown remains similar: The MEC of a metal combined with the PNEC of an antimicrobial resulted in three synergistic and six antagonistic relationships, while the MEC of an antimicrobial combined with the CCC of a metal included seven antagonistic relationships and two synergistic (**Figure 3.22**). These similar results emphasize the importance of toxic equivalency in looking at these two classes of compounds. Another factor may be the similarities or dissimilarities in the mechanisms of action between trace metals and antimicrobials tested.

(b) Confounding Factors

There are several potential confounding factors which may affect the statistical results in these data. As evidenced by some considerable variability between replicates in the single and binary exposure experiments, minute changes in treatment dilutions or inoculation density, likely stemming from material loss during pipetting, may exert an effect on overall responses to exposure. Smudges or minor scratches on polystyrene 96-well plates may change the optical density recorded by the spectrophotometer, as can settling of dead bacterial cells.

The Kirby-Bauer assay also has several points in the protocol which may introduce variability within and between replicates. Minor differences in the depth of Muller-Hinton

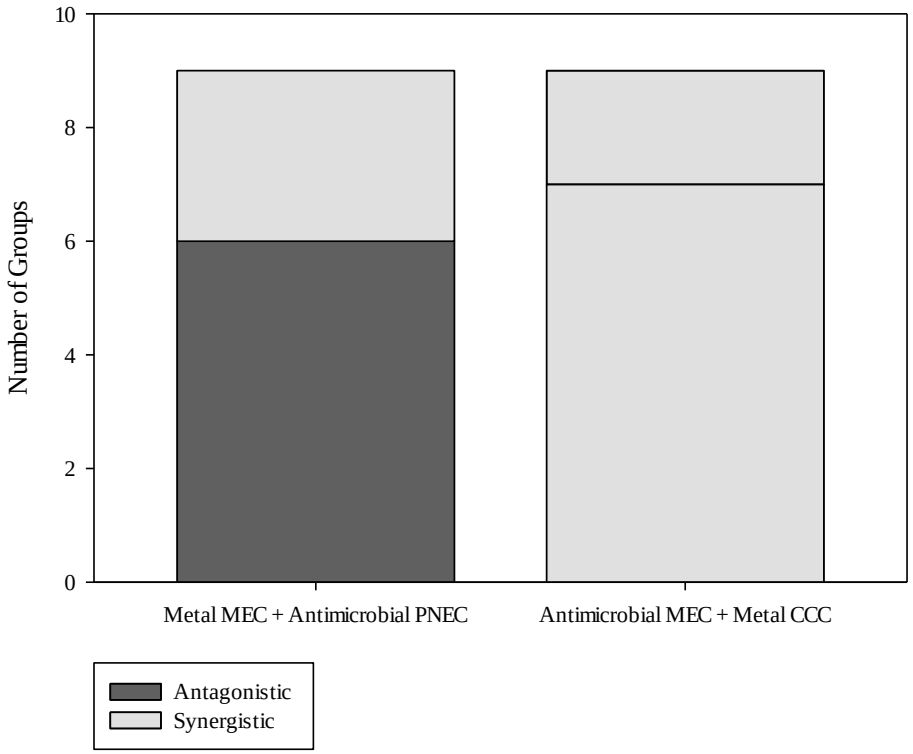


Figure 3.22: Comparison of antagonistic and synergistic relationship across exposure types.

agar from plate to plate will affect the diffusion of antibiotics through the media. Additionally, if the antimicrobial filter discs are not pressed onto the plate with the same pressure throughout, the antibiotic diffusion will again be affected. As with the prior set of experiments, slight changes in treatment dilutions or inoculation density may affect both the acclimation of the bacteria to the toxicants as well as the growth on the plate and interactions with the antimicrobial discs. Additionally, blurry margins on some inhibition zones made measurement difficult. If the protocol in this study is used for future work, the author recommends using a 150 mm susceptibility disc dispenser for more consistent application of discs and therefore more even diffusion of antimicrobials.

In two tests – the Triclosan PNEC-range single exposure treatment group and the cefotaxime challenge to the Zinc group in the antimicrobial resistance assay – the data set exhibited low computed power. This, again, is likely due to high variation within and between replicates.

Despite these limitations, these data are robust and clearly stand as a new major set of findings on AMR and the importance of two major pollutant classes – trace metals and antimicrobials – which often co-occur within the environment. These results clearly show that modeling potential toxicological interactions is difficult as the findings clearly show the importance of dose and mixture ratios within the environmental setting.

(c) Conclusions

The most significant takeaway from this series of experiments is that the effect of co-exposure to antimicrobials and trace metals is both distinctly dose-dependent and compound dependent. Both high and low doses are important as is the specific mixture composition of trace metals and antibiotics/antimicrobial agents. Many mixture treatment

groups had an antagonistic relationship between the two toxicants when in one dose configuration yet a synergistic relationship when the exposure concentrations of each toxicant changed. Specifically, the combinations of oxytetracycline with copper and zinc should be the subject of more investigations due to the high prevalence of these compounds in wastewater treatment plants, stormwater ponds, estuaries, aquaculture, and aquatic ecosystems and the varied responses to antimicrobial susceptibility challenges which were observed.

From an AMR perspective, it is still unclear which toxicological interaction - antagonism or synergism is more important in enhancing AMR. Future genetic sequencing research should be focused on mixture combinations which are antagonistic or synergistic: This may help build models that can better predict AMR and better identify mixture combinations in the environment that may pose the greatest risk to the public. New research also needs to focus on risk factors as to whether increased AMR risk exists only in a select high risk exposure group or is more broadly distributed among the general public. This additional information may provide insight into modeling mixture effects on AMR and understanding their importance on ecosystem and human health.

Chapter 4: Enterococcus faecium

Materials

(a) Microbial Organisms

E. faecium (ATCC 6569) was grown in Brain-Heart Infusion Agar/Broth (BD Difco). Kirby-Bauer assays were performed on Mueller-Hinton Agar (BD Difco). All cultures were grown at 30°C, while the Mueller-Hinton Agar plates for the Kirby-Bauer assays were incubated at 35°C, per the protocol instructions (Hudzicki 2009).

A clinical lab strain from American Type Culture Company (ATCC) was selected rather than using an environmental isolate. As the strain was clinically isolated, it is known to cause disease without the need for genotyping and therefore clearly relevant to the public health application of this experiment. In addition, laboratory strains are also further removed from environmental stressors which may influence initial growth.

(b) Antimicrobials

Analytical grade antimicrobial compounds were obtained as follows: Triclosan (TCS) (Irgasan, Fluka, 97.0 to 103.0%), oxytetracycline dihydrate (OTC) (Sigma LifeSciences, 94.5 to 102.0%), and ciprofloxacin hydrochloride monohydrate (CIP) (Alfa Aesar, 98.0 to 102.0%).

Antimicrobial stocks were created in acetone (TCS and OTC) or deionized water (CIP) and kept in opaque containers in the dark inside a refrigerator at 4°C. CIP stocks were remade on a biweekly basis to avoid degradation (Eghianruwa 2014).

Antimicrobial diffusion discs were obtained at the following concentrations: CIP 5µg, OTC 30µg, and Vancomycin (VAN) 30µg (BD BBL™ SensiDiscs™). These are 6-mm filter-paper discs impregnated with the listed concentrations of antimicrobials.

(c) Metals

Analytical grade metal compounds were obtained as follows: Sodium hydrogen arsenate heptahydrate (As) (Alfa Aesar**,** ≥97.5 to ≤102.5%), cupric sulfate pentahydrate (Cu) (Sigma CellCulture, ≥98%), and zinc sulfate heptahydrate (Zn) (Sigma CellCulture, ≥99.0%). Metals stocks were made up in deionized water in conical tubes, wrapped in aluminum foil, and kept at 4°C.

(d) Toxicity Tests with Antibiotics, Biocides, and Trace Metals

E. faecium (ATCC 6569) bacteria were exposed for 24 hours to each antimicrobial agent (TCS, OTC, and CIP), and trace metal (As, Cu, and Zn), individually and in binary mixtures, and percent survival and growth using the optical density of the bacterial cultures.

(e) Statistical Analysis

Data from the range finding and binary exposure experiments were blanked against the growth medium using MARS Data Analysis software, before exporting to Microsoft Excel, from which these data were reformatted and transferred to SAS®. All statistical analyses were performed using SAS® University Edition software, replaced in August 2021 by the manufacturer with SAS® OnDemand for Academics.

Methods

(a) Protocol 1: Single Exposure Experiments

The first phase of the study was a range-finding project, which doubled as single-exposure assays of *E. faecium* (Efm) to antimicrobial products (triclosan [TCS],

oxytetracycline [OTC], or ciprofloxacin [CIP]) and/or trace metals (arsenic [As], copper [Cu], or zinc [Zn]). Glycerol stocks of the microbes were plated on agar and grown overnight at 30°C. After 24 hours, an isolated colony was selected at random and inoculated into broth media. This was again incubated overnight at 30°C with orbital shaking to discourage biofilm formation.

After 24 hours, the overnight suspension was diluted to an optical density of approximately 0.02 at 608 nm ($OD_{608} \approx 0.02$). The diluted suspension was divided into individual conical tubes. One tube was spiked with the toxicant, after which serial dilutions were performed to achieve the desired exposure concentrations (**Table 4.1, Table 4.2**).

Environmental concentrations of antimicrobials (e.g., low dose) were selected based on published Probable No-Effects Level (PNEC) (**Table 4.1**) and correspond to levels measured in coastal waters impacted by human activity. Triclosan, for example, was measured in wastewater effluent discharging into Charleston Harbor, Charleston, SC, at a concentration of 0.3 ppb (Hedgespeth et al. 2012). Oxytetracycline has been found in effluent from mariculture operations at reported levels of up to 2.3 ppb (Thurman 2003), and a U.S. Geological Survey study on streams across the US susceptible to contamination by human sources measured levels of ciprofloxacin up to 0.03 ppb (Kolpin et al. 2002). Clinical levels (e.g., high dose) of ciprofloxacin and oxytetracycline were selected from the maximum serum concentration of an adult oral dose (**Table 4.1**). Clinical triclosan levels were based on the concentration found in antimicrobial hand soap (**Table 4.1**).

Environmental metals concentrations were based on the Environmental Protection Agency's (EPA's) Criterion Continuous Concentration (CCC) for saltwater exposure (**Table 4.2**). Levels selected to ensure a toxicological response in the microbes were chosen

Table 4.1: Nominal antimicrobial concentrations upon which range finding assay exposures were based (Bayer Pharmaceuticals 2004; Agwuh and MacGowan 2006; Rodricks et al. 2010; Nietch et al. 2013; Bengtsson-Palme and Larsson 2016)**.**

Antimicrobial	PNEC (ppb)	Clinical (ppb)
Triclosan	0.5	4.50E+06
Oxytetracycline	0.5	4.00E+03
Ciprofloxacin	6.40E-02	5.40E+03

Table 4.2: Nominal metals concentrations upon which range finding assay exposures were based (Aarestrup and Hasman 2004; EPA 2004; Fulladosa et al. 2005; Rebelo et al. 2012).

Metal	CCC (ppb)	*Vibrio* EC20 (ppb)	*Enterococcus* MIC (ppb)
Arsenic	36	2.54E+03	5.98E+05
Copper	3.1	60	9.73E+05
Zinc	81	460	2.48E+05

using the published 20th-percentile effect concentration (EC20) values for *Vibrio fischerii* (**Table 4.2**) as well as published minimum inhibitory concentrations (MIC) for *Enterococcus* species (**Table 4.2**). The *Enterococcus* MIC range encompassed the effects range median (ERM) concentration in sediments for both copper and zinc, while the *Vibrio* EC20 range roughly corresponds to the arsenic Effects Range Low (ERL) in sediments (**Table 4.3**). These are similar to concentrations measured in sites like agricultural lagoons and commercial stormwater ponds (Bradford et al. 2008; Baalousha et al. 2015), where copper and zinc concentrations in sediments were both measured in excess of the ERM and arsenic in excess of the ERL.

For those antimicrobials whose stocks were made in acetone due to solubility limits (TCS and OTC), an equivalent amount of acetone was be added to all samples containing lower concentrations of the antimicrobial so that all samples contain 0.3% acetone, and a carrier control was added. An early pilot test indicated no significant difference in growth patterns of *E. faecium* between a diluent control and a test group containing 0.3% acetone, thus the 0.3% acetone levels assured optimum antimicrobial agent dissolution into solution without affecting survival and growth.

Diluted and spiked bacterial suspensions were added in triplicate to a 96-well plate at 200 µL per well, along with media blanks, a control containing only bacteria, and a carrier control (acetone or water). This plate was read in a NOVOstar Microplate Reader (BMG LabTech), with a pre-programmed protocol which takes OD_{608} absorption measurements every 15 minutes for 24 hours, maintained at 30°C with dual-orbital shaking for 0.2 seconds before each read to prevent settling and biofilm formation. MARS data analysis software was then used to compile and blank the data against the media control

Table 4.3: Sediment Quality Guidelines for Arsenic, Copper, and Zinc (Long 1995)**.**

Metal	ERL (ppb)	ERM (ppb)
Arsenic	8.20E+03	7.00E+04
Copper	3.40E+04	2.70E+05
Zinc	1.50E+05	4.10E+05

wells before exporting to SAS for analysis. This process was repeated twice, for a total of three replicates in triplicate within each exposure condition. In total, nine replicate measurements were obtained for each concentration of antimicrobial agent or trace metal tested.

The generation rate of *Enterococcus faecium* is approximately 2 generations per hour at an optimal temperature of 37°C in an aerobic environment, meaning the culture doubles in density every 30 minutes (Morandi et al. 2005). Taking measurements every 15 minutes ensures that the data will capture logarithmic phase growth. Meanwhile, plate shaking prevents bacterial settling and biofilm formation, which can interfere with optical density readings.

The blanked data was exported into Microsoft Excel to obtain the data in a format readable by most computers without the proprietary MARS software. From there, it was organized and compiled into SAS OnDemand for Academics, where the statistical analysis proceeded as described below.

(b) Protocol 2: Binary Mixture Exposure Experiments

Binary mixture exposure experiments were conducted to examine the potential interactive toxicity (e.g., joint toxicity) between two individual compounds, between different classes of chemicals (e.g., antimicrobials and metals). Using data obtained during the initial single compound toxicity tests, the same general protocol was used to obtain binary mixture exposure data. This time, instead of spiking the diluted bacterial suspensions with a single toxicant, this set of experiments used one of two conditions. The first was a single concentration of antimicrobial (TCS, OTC, or CIP) which was shown to have a sublethal toxicological effect – the Minimum Effective Concentration, or MEC –

on bacterial growth in combination with a range of metal (As, Cu, or Zn) concentrations at environmentally-relevant levels based on the CCC (**Table 4.4**). A second set of experiments was performed using the MEC of each trace metal (As, Cu, or Zn) in combination with a range of environmentally relevant antimicrobial (TCS, OTC, or CIP) levels based on the PNEC. Culture, dilution, microbial measurements, and statistical analysis methods remained the same as for the single-exposure experiments, culminating in eight individual replicates with each treatment plated in triplicate. In total, twenty-four individual data points were obtained for each treatment group.

(c) Protocol 3: Phenotypic Analysis for Antimicrobial Resistance

To determine a phenotypic resistance profile of *E. faecium*, a set of Kirby-Bauer assays (Hudzicki 2009) were performed using a modified inoculum preparation. First, a frozen glycerol stock of *E. faecium* was streaked on agar plates and incubated overnight in order to obtain isolated colonies. A single colony was then inoculated into broth medium and incubated overnight at 30°C. This overnight culture was split into a series of tubes containing broth spiked with treatment groups corresponding to those from the binary exposure experiments. To prepare the inocula, these acclimated cultures were diluted in sterile phosphate-buffered saline until they corresponded with the 0.5 McFarland Standard, and the assay proceeded using the standard protocol (**Figure 4.1**). Antimicrobial challenge discs included OTC and CIP, in addition to clinically important vancomycin to assess effect on this clinically important glycopeptide. Three discs of VAN and two to three of CIP and OTC, plus three blank controls, were placed for three pseudoreplicates in each of three replicated experiments. The disparity in number of discs added to the plates resulted from a miscalculation when ordering supplies - there were insufficient CIP and OTC discs to

Table 4.4: Minimum Effective Concentration (MEC) of each toxicant used as a constant in binary exposure experiments, as determined in Protocol 1 experiments.

Treatment	Minimum Effective Concentration (ppb)	Percent Inhibition (%)
Arsenic	2.39E+06	3.22
Copper	1.24E+05	18.38
Zinc	1.39E+04	3.00
Triclosan	230	6.93
Oxytetracycline	250	24.49
Ciprofloxacin	75	22.55

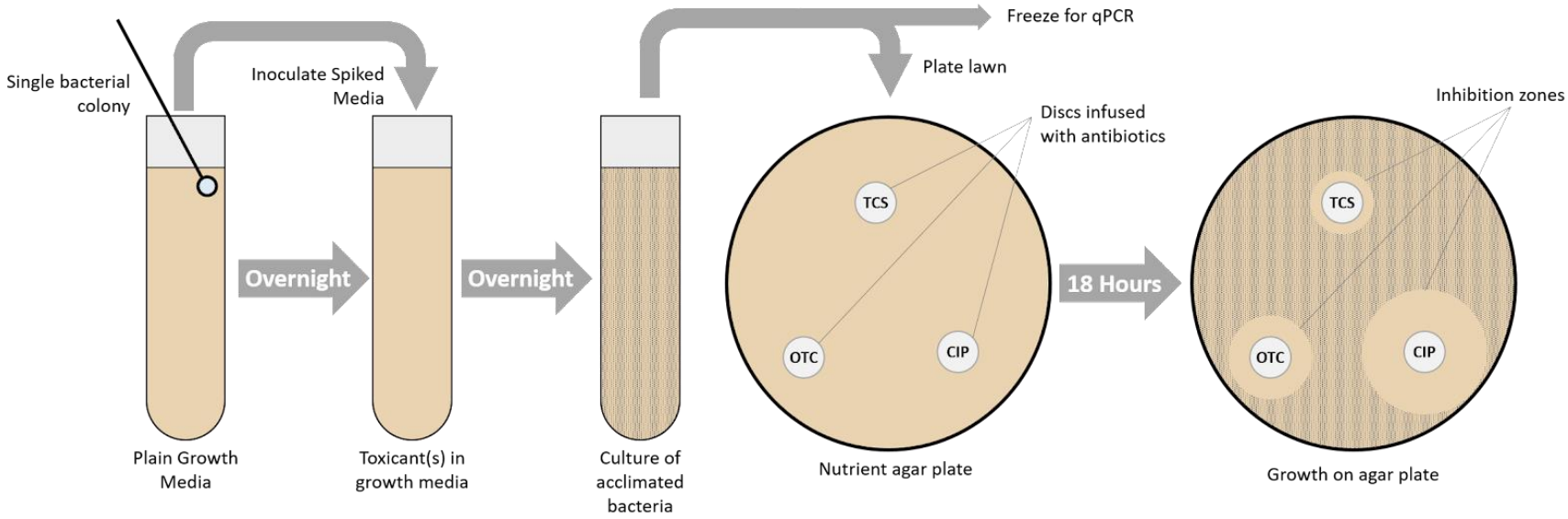


Figure 4.1: Kirby-Bauer procedure, adapted from Hudzicki 2009.

place three on all plates. Additionally, several 0.5 mL aliquots of acclimated bacterial culture from each exposure criterion (antimicrobial alone, metal alone, combination antimicrobial + metal, or control) were added to cryovials containing 0.5 mL 50% glycerol solution, resulting in a final concentration of 25% glycerol, and frozen at -80°C to save for future studies. Inoculated plates were incubated at 30°C for 24 hours, then Zones of inhibition (ZOI) were measured (Hudzicki 2009; CLSI 2017) using a digital caliper, recorded, and analyzed using a nested ANOVA with Dunnett's test. Resistance levels (Susceptible, Intermediate, or Resistant) were determined using breakpoint guidelines published by the Clinical and Laboratory Standards Institute (CLSI) (CLSI 2017) (**Table 4.5).** On Figure 4.1, please note that the Inhibition Zones and disc placement are for demonstration only and do not represent actual results. A smaller diameter inhibition zone is indicative of more resistant strains while larger diameter inhibition zones indicate sensitive isolates.

(d) Statistical Analysis

As each stage consisted of four replicated experiments in which each exposure group was examined in triplicate, a one-way nested Analysis of Variance (ANOVA) with *post-hoc* Dunnett's test was performed to account for these triplicate subgroups when comparing treatment results to the control. Studentized Residual and Cook's Distance statistics were used to identify outliers. Those data points for which both the $Studentized\ Residual > |3|$ and $Cook's\ Distance > 4/N$, were then removed from the ANOVA.

Table 4.5: *E. faecium* Inhibition Zone Breakpoints (CLSI 2017).

Antimicrobial Challenge	Breakpoints Zone of Inhibition (ZOI) [mm]		
Legend	▲ Susceptible	– Intermediate	▼ Resistant
Ciprofloxacin (5 µg)	> 21	16-20	<15
Vancomycin (30 µg)	>17	15-16	<14
(Oxy)tetracycline (30 µg)	>19	15-18	<14

Results

Percent inhibition (% I) was calculated using the mean OD_{608} and the following formula:

$$\%I = [(A - B)/A\,] * 100$$

where A = Control OD_{608} at stationary phase and B = Treatment OD_{608} at stationary phase.

For each figure below, an asterisk (*) indicates exposures which were significantly different from the controls (Dunnett's test $p \leq 0.05$). Negative inhibition indicates biostimulatory growth exceeding that of the control. For both the Shapiro-Wilk test for normality and Levene's test for homogeneity of variance, the data set was classified as "*meets assumptions*" if $p \geq 0.05$, "*minor departure from assumptions*" if p $0.05 \geq 0.02$, "*moderate departure from assumptions*" if $0.02 \geq 0.005$, and "*fails to meet assumptions*" if $p < 0.005$ (Zar 1999; Pennington 2022). A data set met assumptions for computed power if $p \geq 0.8$, and "*failed to meet assumptions*" if $p < 0.8$.

(a) Single Exposure Experiments

The first set of experiments involved exposure of *E. faecium* individually to triclosan, oxytetracycline, and ciprofloxacin. The second set was nearly identical, using arsenic, copper, and zinc instead of antimicrobials as the exposure agents. These assays were set up as a range-finding pilot studies, serving to both determine the range at which binary exposure experiments would be run as well as to determine a baseline growth curve against which to compare the binary exposure study. "Difference in growth" is defined as change in OD_{608} from t=0 until the onset of stationary phase. For *E. faecium*, this metric occurred at approximately t=24 hours. Difference in growth was used instead of growth rate because pilot experiments of these exposures reached log phase growth at nearly the

Table 4.6: ANOVA Assumptions Legend, as applied in results presented in tables that follow.

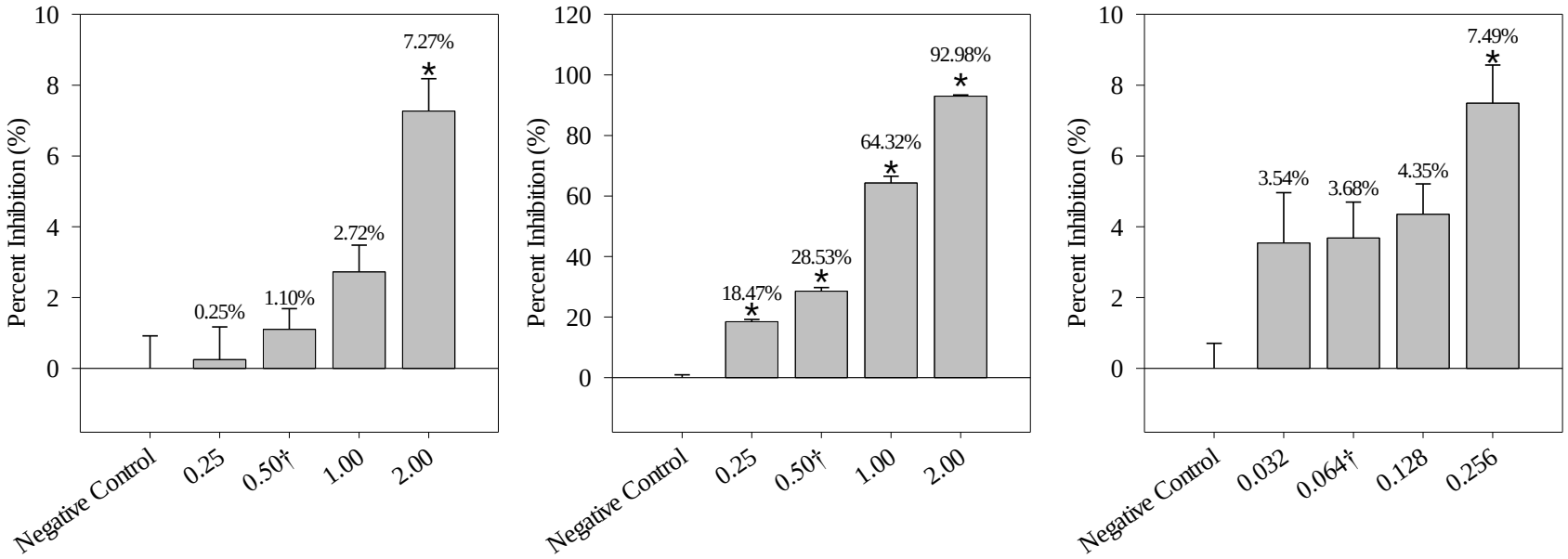
Meets Assumptions	Minor Departure from Assumptions	Moderate Departure from Assumptions	Fails to Meet Assumptions
✔	!	!	✖

same time as control for most experiments. Thus, the difference in total growth observed was used instead.

In most of these single-exposure experiments, a maximum of one outlier data point was removed from any single treatment group due to meeting statistical criteria for outliers. This comes to approximately 11% for all groups except for mid-range experiments and the Clinical-range antimicrobials. The mid-range Zn group had three outlier data points (30%) removed from the 13,890 ppb Zn, two data points (16%) were removed from mid-range CIP at 168.8 ppb, four from 125 ppb OTC, and up to five (33%) from Clinical TCS. The Clinical TCS group experienced very high variability within replicates. An additional three outlier data points – one replicate – were removed from the Clinical OTC negative control due to contamination, and two full replicates were removed from all treatments of Clinical CIP also due to contamination.

For all figures below, a dagger (†) denotes published PNEC value for antimicrobials or CCC value for metals, asterisk (*) indicates statistical significance (Dunnett's test $p \leq 0.05$), positive (+) inhibition values were indicative of reduced growth, and negative (-) suggest biostimulatory growth.

In the PNEC range of antimicrobials, there was a clear dose response pattern for all three toxicants (e.g., increased growth inhibition with increasing dose) (**Figure 4.2**). For PNEC exposures this response range included 1.10% inhibition for triclosan, a 28.53% inhibition for oxytetracycline, and 3.68% for ciprofloxacin. All of the oxytetracycline exposure doses were statistically significant (Dunnett's test $p \leq 0.05$) from the negative control, as was the 2 ppb dose of triclosan and 0.256 ppb dose of ciprofloxacin (**Table 4.7**).



a. PNEC Range [Triclosan] (ppb) b. PNEC Range [Oxytetracycline] (ppb) c. PNEC Range [Ciprofloxacin] (ppb)

Figure 4.2: Inhibition of *E. faecium* following exposure to the PNEC range of antimicrobials.

Table 4.7: ANOVA statistical analysis of results from the PNEC range of antimicrobials.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control $p \leq 0.05$	Shapiro-Wilk: Normality $p \geq 0.05$	Levene's Test: Homogeneity of Variance $p \geq 0.05$	Computed Power $p \geq 0.8$
PNEC [Ciprofloxacin] (ppb)	Negative Control	9		✓ 0.0622	✓ 0.0505	✓ 0.991
	0.032 ppb CIP	9	✗ 0.2045			
	0.064 ppb CIP	9	✗ 0.1807			
	0.128 ppb CIP	9	✗ 0.0999			
	0.256 ppb CIP	9	✓ 0.0057			
PNEC [Oxytetracycline] (ppb)	Negative Control	9		✓ 0.1752	✓ 0.0862	✓ 0.999
	0.25 ppb OTC	9	✓ 0.0005			
	0.50 ppb OTC	9	✓ 0.0001			
	1.00 ppb OTC	9	✓ 0.0001			
	2.00 ppb OTC	9	✓ 0.0001			
PNEC [Triclosan] (ppb)	Negative Control	9		✓ 0.3798	✓ 0.2344	✓ 0.999
	0.25 ppb TCS	9	✗ 0.9997			
	0.50 ppb TCS	9	✗ 0.9227			
	1.00 ppb TCS	9	✗ 0.3995			
	2.00 ppb TCS	9	✓ 0.0073			

Exposure to oxytetracycline resulted in very high levels of inhibition, up to approximately 93% following exposure to just 2.0 ppb of oxytetracycline.

Exposure of *E. faecium* to a clinically relevant range of antimicrobials resulted in very high growth inhibition, ranging from 72%I - 98.4%I for triclosan, 29.96%I - 98.03%I for oxytetracycline, and 40.18%I - 89.74%I for ciprofloxacin. All treatment groups in this range experienced statistically significant (Dunnett's test $p \leq 0.05$) growth inhibition at all concentrations tested (**Figure 4.3, Table 4.8**).

In a mid-range triclosan exposure group used to better define the effects at the MEC, most treatments had negligible inhibition or very slight (0.82%) growth stimulation, but only he highest level tested at 230 ppb was statistically significant (Dunnett's test $p \leq 0.05$) from the negative control with 16.78% growth inhibition. Conversely, the mid-range ciprofloxacin exposure group for MEC determination had a well-defined dose-response curve, ranging from 4.62%I at the lowest (22.28 ppb) concentration up to 48.07%I at the highest (168.8 ppb) concentration tested. All of these ciprofloxacin results were statistically (Dunnett's test $p \leq 0.05$) significant from the negative control (**Figure 4.4, Table 4.9**).

The range of metals based on the EPA Criterion Continuous Concentration saw no statistical significance (Dunnett's test $p \leq 0.05$) compared to negative control and a varied response with maximum levels of inhibition ranging from 3.44% (Cu) to 4.95% (Zn) (**Figure 4.5, Table 4.10**).

Following exposure to a range of metals treatments based on the *Vibrio vulnificus* EC20 for each metal, results were again varied and not statistically significant (Dunnett's test $p \leq 0.05$) when compared to the negative control (**Figure 4.6, Table 4.11**). Maximum

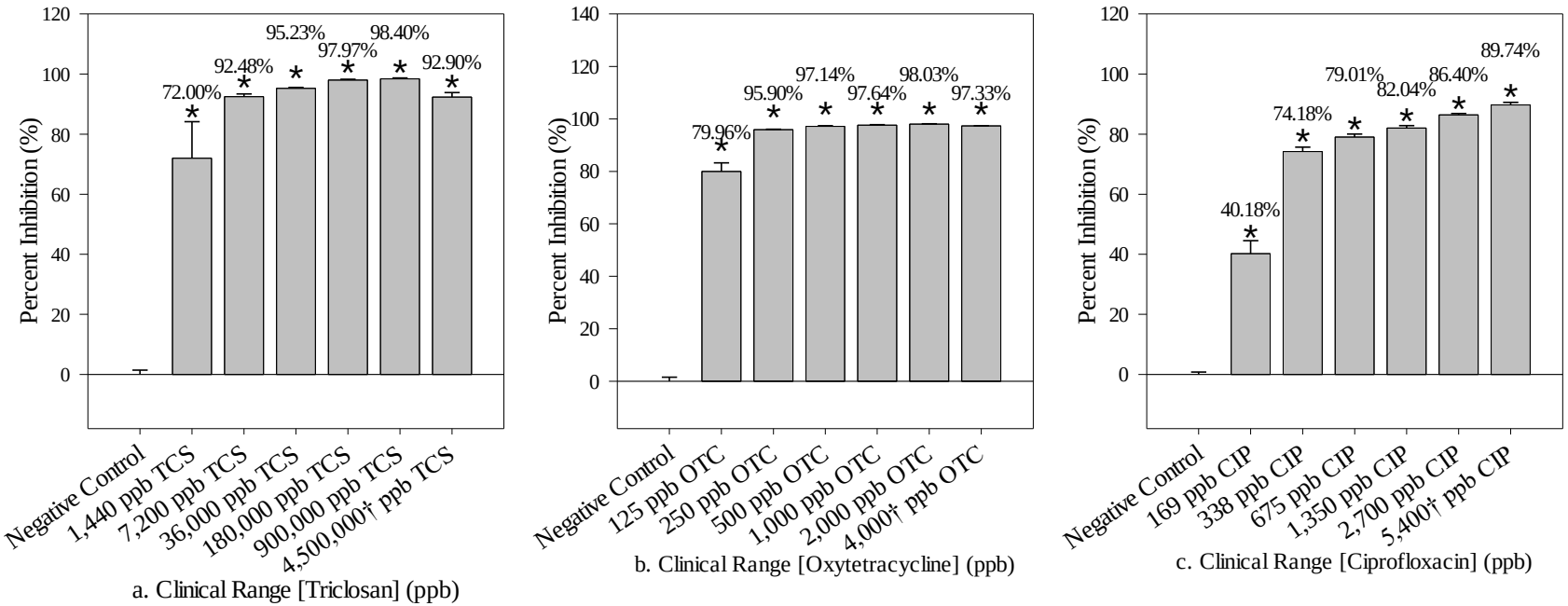


Figure 4.3: Inhibition of *E. faecium* following exposure to the clinical range of antimicrobials.

Table 4.8: ANOVA statistical analysis of results from the clinical range of antimicrobials.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control $p \leq 0.05$	Shapiro-Wilk: Normality $p \geq 0.05$	Levene's Test: Homogeneity of Variance $p \geq 0.05$	Computed Power $p \geq 0.8$
Clinical [Ciprofloxacin] (ppb)	Negative Control	9		✓ 0.2254	! 0.0196	✓ 0.999
	169 ppb CIP	9	✓ 0.0001			
	338 ppb CIP	9	✓ 1.00E-04			
	675 ppb CIP	9	✓ 1.00E-04			
	1,350 ppb CIP	9	✓ 1.00E-04			
	2,700 ppb CIP	9	✓ 1.00E-04			
	5,400† ppb CIP	9	✓ 1.00E-04			
Clinical [Oxytetracycline] (ppb)	Negative Control	12		✓ 0.5121	! 0.0076	✓ 0.999
	125 ppb OTC	11	✓ 0.0001			
	250 ppb OTC	15	✓ 0.0001			
	500 ppb OTC	15	✓ 0.0001			
	1,000 ppb OTC	15	✓ 0.0001			
	2,000 ppb OTC	15	✓ 0.0001			
	4,000† ppb OTC	15	✓ 0.0001			
Clinical [Triclosan] (ppb)	Negative Control	13		✗ 0.0001	✗ 0.0004	✓ 0.999
	1,440 ppb TCS	11	✓ 0.0001			
	7,200 ppb TCS	10	✓ 0.0001			
	36,000 ppb TCS	13	✓ 0.0001			
	180,000 ppb TCS	15	✓ 0.0001			
	900,000 ppb TCS	14	✓ 0.0001			
	4,500,000† ppb TCS	10	✓ 1.00E-04			

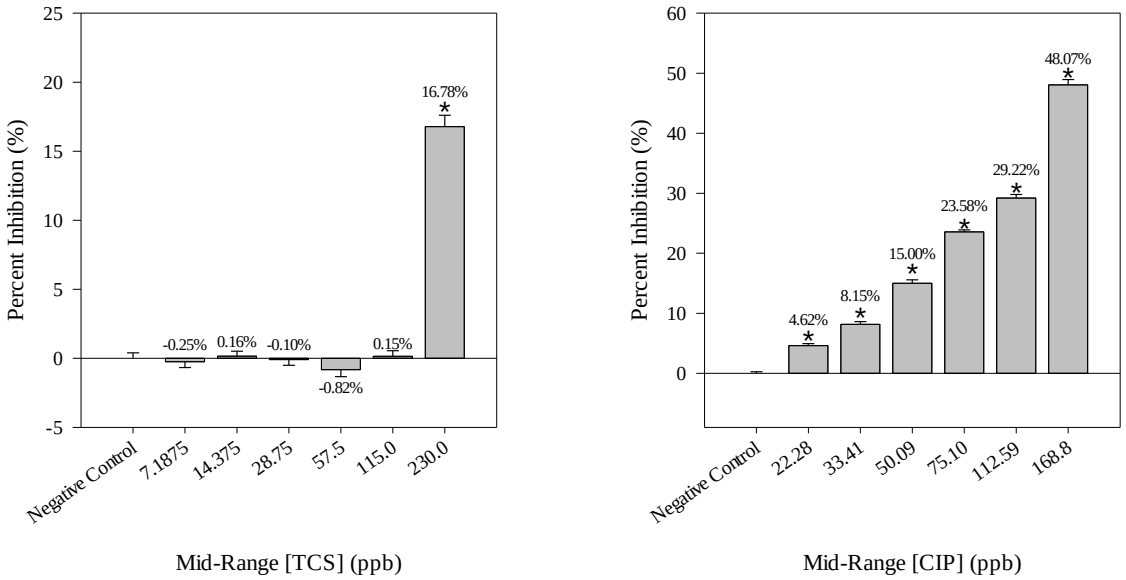


Figure 4.4: Inhibition of *E. faecium* following exposure to a range of triclosan or ciprofloxacin concentrations falling between the CCC and EC20 concentration ranges.

Table 4.9: ANOVA statistical analysis of results from a mid-range of (a) triclosan or (b) ciprofloxacin.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control $p \leq 0.05$	Shapiro-Wilk: Normality $p \geq 0.05$	Levene's Test: Homogeneity of Variance $p \geq 0.05$	Computed Power $p \geq 0.8$
Midrange [Triclosan] (ppb)	Negative Control	12		✓ 0.5235	! 0.0151	✓ 0.999
	7.1875 ppb TCS	12	✗ 0.9998			
	14.375 ppb TCS	12	✗ 1.00E+00			
	28.75 ppb TCS	12	✗ 1.00E+00			
	57.5 ppb TCS	11	✗ 9.56E-01			
	115.0 ppb TCS	12	✗ 1.00E+00			
	230.0 ppb TCS	11	✓ 1.00E-04			

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control $p \leq 0.05$	Shapiro-Wilk: Normality $p \geq 0.05$	Levene's Test: Homogeneity of Variance $p \geq 0.05$	Computed Power $p \geq 0.8$
Midrange [Ciprofloxacin] (ppb)	Negative Control	12		✓ 0.1474	! 0.0054	✓ 0.999
	22.28 ppb CIP	12	✓ 0.0006			
	33.41 ppb CIP	12	✓ 1.00E-04			
	50.09 ppb CIP	12	✓ 1.00E-04			
	75.10 ppb CIP	12	✓ 1.00E-04			
	112.59 ppb CIP	12	✓ 1.00E-04			
	168.8 ppb CIP	10	✓ 1.00E-04			

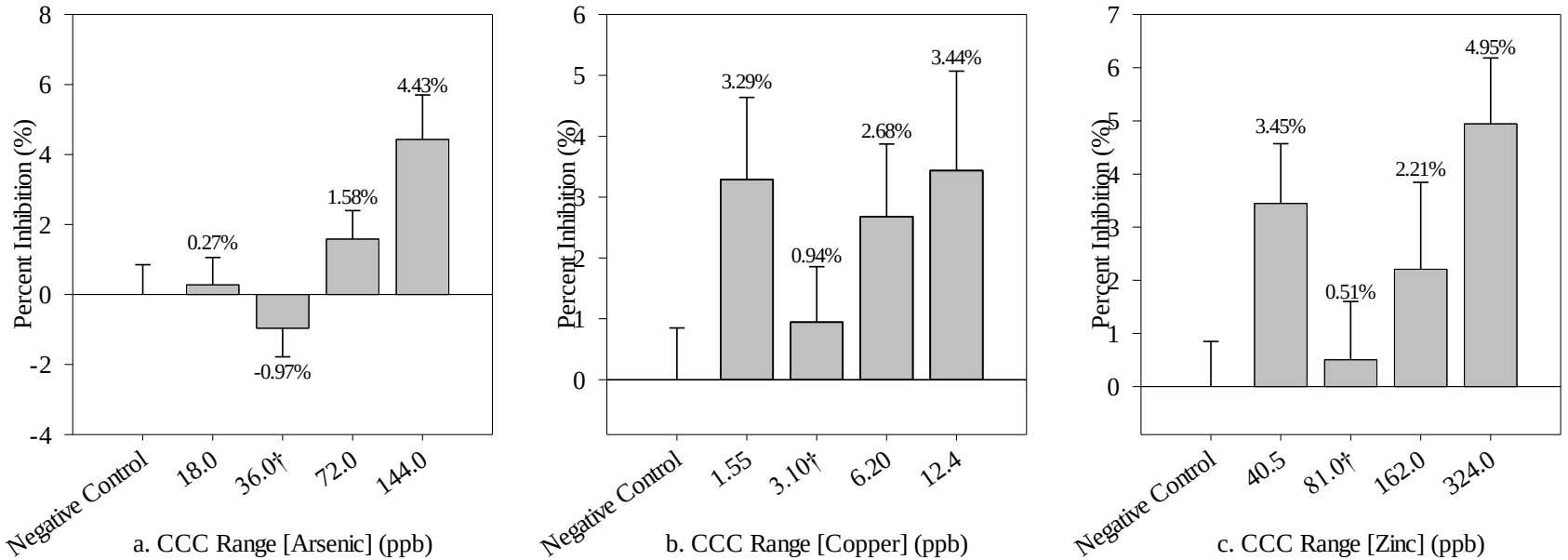


Figure 4.5: Inhibition of *E. faecium* following exposure to the CCC range of metals.

Table 4.10: ANOVA statistical analysis of results from the CCC range of metals.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control $p \leq 0.05$	Shapiro-Wilk: Normality $p \geq 0.05$	Levene's Test: Homogeneity of Variance $p \geq 0.05$	Computed Power $p \geq 0.8$
CCC [Arsenic] (ppb)	Negative Control	8		✓ 0.9042	✓ 0.0895	✓ 0.999
	18.0 ppb As	9	✗ 1			
	36.0† ppb As	9	✗ 0.9537			
	72.0 ppb As	8	✗ 0.9134			
	144 ppb As	9	✗ 0.2733			
CCC [Copper] (ppb)	Negative Control	8		✓ 0.7374	✓ 0.111	✓ 0.999
	1.55 ppb Cu	9	✗ 0.7397			
	3.10† ppb Cu	9	✗ 0.998			
	6.20 ppb Cu	9	✗ 0.852			
	12.4 ppb Cu	8	✗ 0.59			
CCC [Zinc] (ppb)	Negative Control	8		✓ 0.9016	✓ 0.0511	✓ 0.999
	40.5 ppb Zn	9	✗ 0.6889			
	81.0 ppb Zn	8	✗ 0.9999			
	162 ppb Zn	8	✗ 0.9431			
	324 ppb Zn	9	✗ 0.3976			

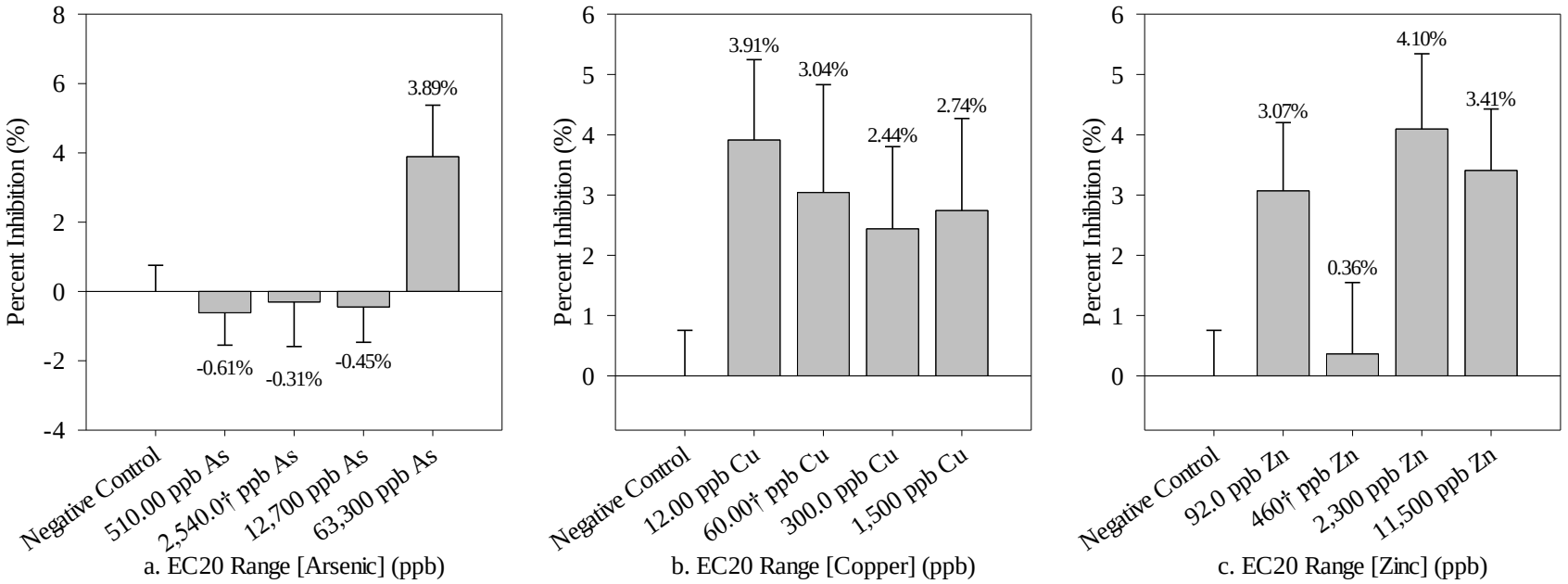


Figure 4.6: Inhibition of *E. faecium* following exposure to the EC20 range of metals.

Table 4.11: ANOVA statistical analysis of results from the EC20 range of metals.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control p ≤ 0.05	Shapiro-Wilk: Normality p ≥ 0.05	Levene's Test: Homogeneity of Variance p ≥ 0.05	Computed Power p ≥ 0.8
EC20 [Arsenic] (ppb)	Negative Control	9		✓ 0.89	✓ 0.3477	✓ 0.999
	510.00 ppb As	9	✗ 0.9979			
	2,540.0† ppb As	8	✗ 1.00E+00			
	12,700 ppb As	8	✗ 1.00E+00			
	63,300 ppb As	7	✗ 3.76E-01			
EC20 [Copper] (ppb)	Negative Control	9		✓ 0.2498	! 0.0181	✓ 0.944
	12.00 ppb Cu	9	✗ 0.6682			
	60.00† ppb Cu	9	✗ 0.8161			
	300.0 ppb Cu	9	✗ 0.901			
	1,500 ppb Cu	9	✗ 0.861			
EC20 [Zinc] (ppb)	Negative Control	9		✓ 0.7634	✓ 0.1622	✓ 0.999
	92.0 ppb Zn	9	✗ 0.5964			
	460† ppb Zn	8	✗ 1			
	2,300 ppb Zn	8	✗ 0.4355			
	11,500 ppb Zn	9	✗ 0.5144			

growth inhibition ranged from 3.89 (As) to 4.10% (Zn) and only As had very slight growth stimulation at the three lowest doses ($\leq$ 0.61%)

Results of toxicity tests at the MIC for each metal indicated only significant growth inhibition (Dunnett's test $p \leq 0.05$) of 3.35-9.79% at the two highest doses of As and at all concentrations tested for Cu and Zn, when compared to controls (**Figure 4.7, Table 4.12**). Inhibition following treatment with copper ranged from 24.16 to 98.64% and following treatment with zinc ranged from 54.11 to 91.84%.

As a secondary goal of this experiment was to determine a minimum inhibitory concentration, copper was also tested at a mid-range dose exposure, which resulted in a classic dose-response curve ranging from 4.75%I to 98.76%I (**Figure 4.8**). All of these results were significant (Dunnett's test $p \leq 0.05$) when compared to the negative control (**Table 4.13**).

(b) Binary Exposure Experiments

Following the conclusion of the single-compound exposure experiments, concentrations of the six toxicants which caused a sublethal toxicological effect at environmentally relevant levels were identified. These concentrations will be referred to as the minimum effective concentration (MEC). The MEC values were used as constants and secondary controls in a series of experiments examining binary exposures of *E. faecium* to the sublethal toxicant in combination with the PNEC range of antimicrobials or CCC range of metals.

The majority of treatment groups had no data points removed as outliers, and those that did generally only had one (~4%) to three (~13%) removed. Four treatment groups had seven (~30%) to nine (~37%) data points removed as outliers.

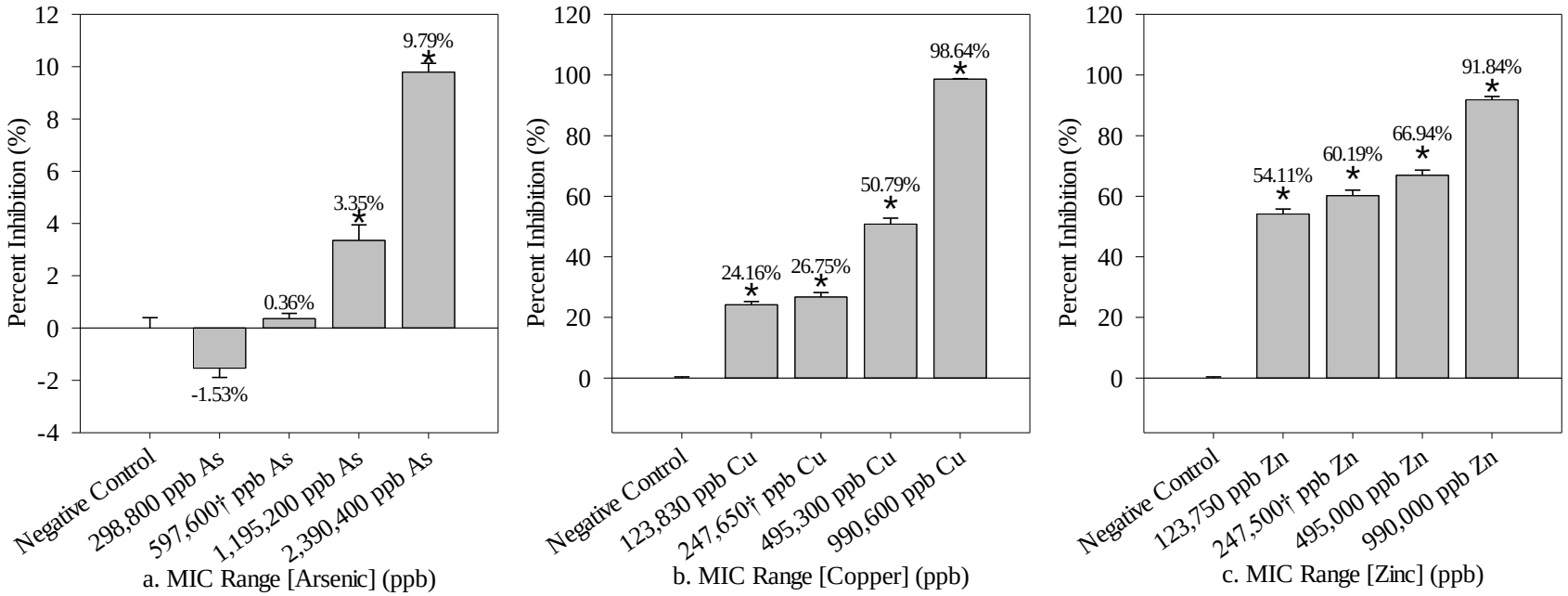


Figure 4.7: Inhibition of *E. faecium* following exposure to the MIC range of metals.

Table 4.12: ANOVA statistical analysis of results from the MIC range of metals.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control $p \leq 0.05$	Shapiro-Wilk: Normality $p \geq 0.05$	Levene's Test: Homogeneity of Variance $p \geq 0.05$	Computed Power $p \geq 0.8$
MIC [Arsenic] (ppb)	Negative Control	9		✓ 0.8652	✓ 0.0755	✓ 0.999
	298,800 ppb As	9	✗ 6.13E-02			
	597,600† ppb As	9	✗ 9.13E-01			
	1,195,200 ppb As	8	✓ 5.00E-04			
	2,390,400 ppb As	9	✓ 1.00E-04			
MIC [Copper] (ppb)	Negative Control	9		! 0.0466	! 0.0302	✓ 0.999
	123,830 ppb Cu	9	✓ 1.00E-04			
	247,650† ppb Cu	9	✓ 1.00E-04			
	495,300 ppb Cu	9	✓ 1.00E-04			
	990,600 ppb Cu	9	✓ 1.00E-04			
MIC [Zinc] (ppb)	Negative Control	9		! 0.0289	! 0.0342	✓ 0.999
	123,750 ppb Zn	9	✓ 1.00E-04			
	247,500† ppb Zn	9	✓ 1.00E-04			
	495,000 ppb Zn	9	✓ 1.00E-04			
	990,000 ppb Zn	9	✓ 1.00E-04			

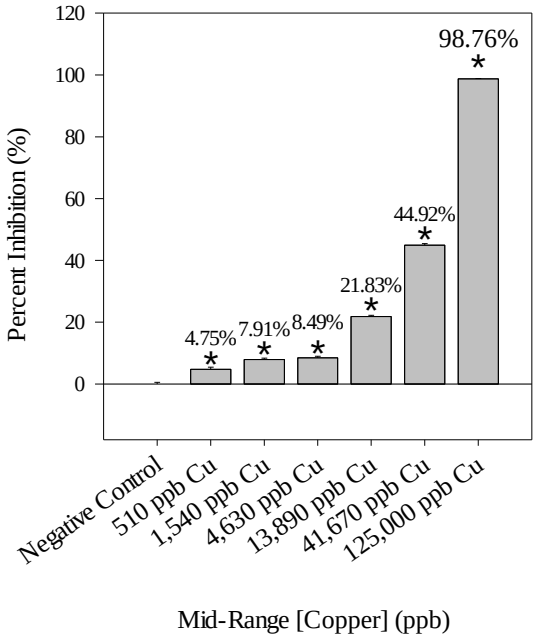


Figure 4.8: Inhibition of *E. faecium* following exposure to a range of copper concentrations falling between the CCC and EC20 concentration ranges.

Table 4.13: ANOVA statistical analysis of results from the mid-range of copper concentrations.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control $p \leq 0.05$	Shapiro-Wilk: Normality $p \geq 0.05$	Levene's Test: Homogeneity of Variance $p \geq 0.05$	Computed Power $p \geq 0.8$
Midrange [Copper] (ppb)	Negative Control	9		✓ 0.6459	! 0.0392	✓ 0.999
	510 ppb Cu	8	✓ 0.0021			
	1,540 ppb Cu	8	✓ 1.00E-04			
	4,630 ppb Cu	9	✓ 1.00E-04			
	13,890 ppb Cu	6	✓ 1.00E-04			
	41,670 ppb Cu	9	✓ 1.00E-04			
	125,000 ppb Cu	9	✓ 1.00E-04			

For all figures below, a dagger (†) denotes published PNEC value for antimicrobials or CCC value for metals, asterisk (*) indicates statistical significance (Dunnett's test $p \leq 0.05$), positive (+) inhibition values were indicative of reduced growth, and negative (-) suggest growth stimulation.

Treatment with the MEC of arsenic combined with a range of triclosan based on the PNEC was inhibited by 3.22% to 6.29%. Only the highest tested dose, MEC As + 2.0 ppb TCS, was statistically significant (Dunnett's test $p \leq 0.05$) compared to a negative control. The second group consisting of MEC Arsenic and a PNEC range of oxytetracycline responded with a clear dose-response curve, from 3.22% to 96.55%. All combinations of MEC Arsenic and PNEC ranges of oxytetracycline had significant growth inhibition compared to controls. The third treatment group, MEC arsenic with a PNEC range of ciprofloxacin, varied in its response ranging from 3.22% to 7.75%. Only the highest two doses of ciprofloxacin, 0.128 ppb and 0.256 ppb, in combination with As MEC were statistically significant compared to the negative control (**Figure 4.9, Table 4.14**).

All of the results from the binary exposures of copper at the MEC with a PNEC range of antimicrobials mixtures exhibited growth inhibition which was statistically significant (Dunnett's test $p \leq 0.05$) when compared to the negative control, although all three treatment groups failed Levene's Test for homogeneity of variance (**Table 4.15**). Exposure to a combination of MEC copper and PNEC-range triclosan mixture inhibited growth by 15.13% - 19.59%, a combination of MEC copper and PNEC-range oxytetracycline mixture inhibited growth by 18.38% - 95.49%, and a combination of MEC copper and PNEC-range ciprofloxacin mixture inhibited growth by 16.54% - 19.76% (**Figure 4.10**). These results clearly indicate that the MEC copper in combination with

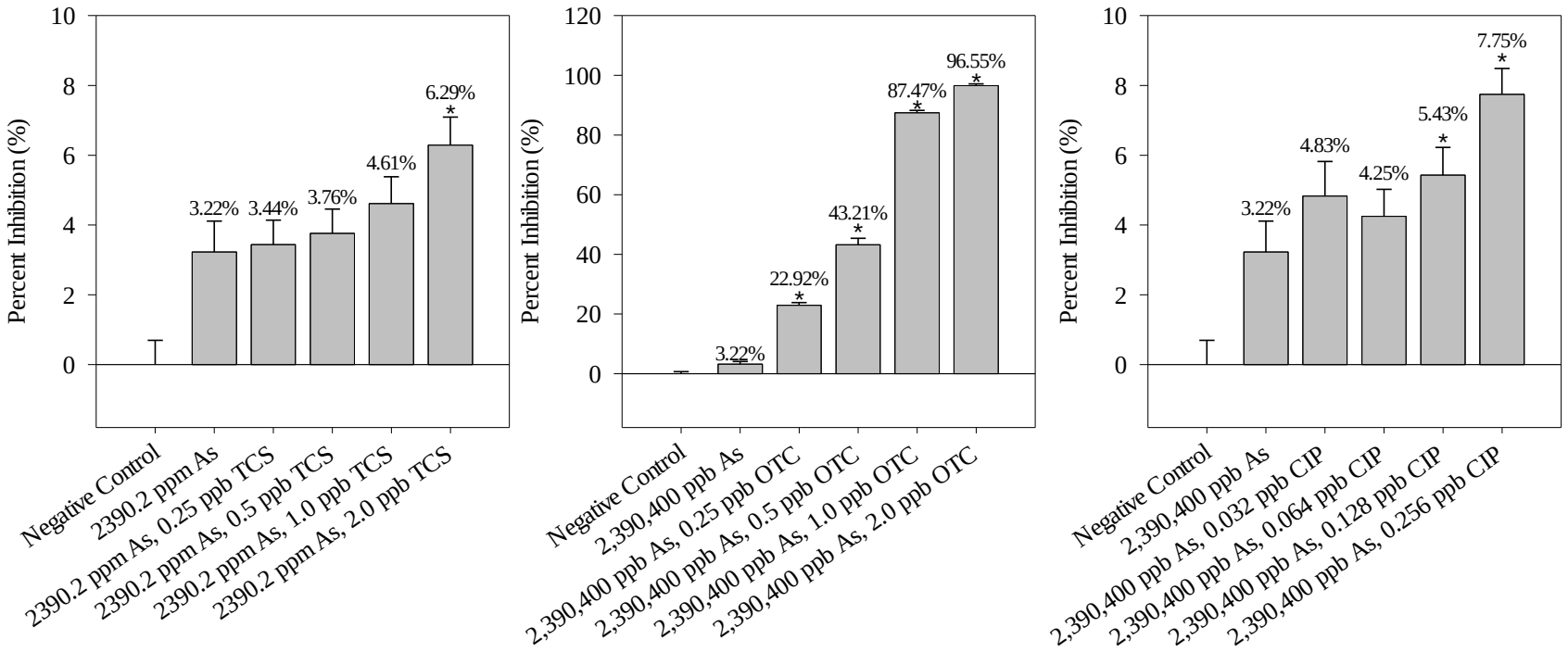
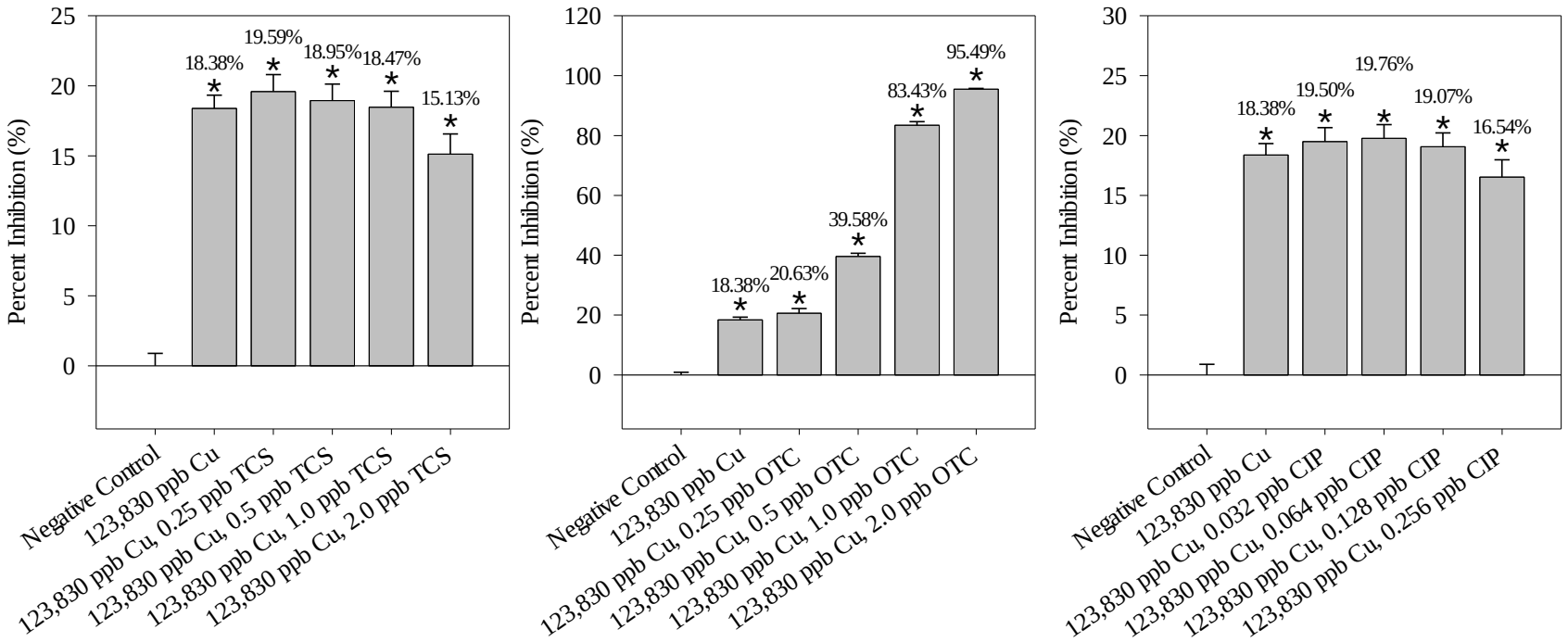


Figure 4.9: Inhibition of *E. faecium* following exposure to the Binary Group 1 Mixtures of the As MEC with a PNEC range of (a) TCS, (b) OTC, or (c) CIP.

Table 4.14: ANOVA statistical analysis of results from the Binary Group 1 Mixtures of MEC As with a PNEC range of TCS, OTC, or CIP.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control $p \leq 0.05$		Shapiro-Wilk: Normality $p \geq 0.05$		Levene's Test: Homogeneity of Variance $p \geq 0.05$		Computed Power $p \geq 0.8$	
Binary 1.1: 2,390,400 ppb As, PNEC [TCS] (ppb)	Negative Control	24			✓	0.6021	!	0.0124	✓	0.999
	2,390,400 ppb As	24	✗	0.3036						
	2,390,400 ppb As, 0.25 ppb TCS	24	✗	0.2497						
	2,390,400 ppb As, 0.5 ppb TCS	24	✗	0.1817						
	2,390,400 ppb As, 1.0 ppb TCS	24	✗	0.0699						
	2,390,400 ppb As, 2.0 ppb TCS	24	✓	0.0073						
Binary 1.2: 2,390,400 ppb As, PNEC [OTC] (ppb)	Negative Control	24			✓	0.101	!	0.0073	✓	0.999
	2,390,400 ppb As	24	✗	0.4618						
	2,390,400 ppb As, 0.25 ppb OTC	22	✓	1.00E-04						
	2,390,400 ppb As, 0.5 ppb OTC	15	✓	0.0001						
	2,390,400 ppb As, 1.0 ppb OTC	22	✓	0.0001						
	2,390,400 ppb As, 2.0 ppb OTC	24	✓	0.0001						
Binary 1.3: 2,390,400 ppb As, PNEC [CIP] (ppb)	Negative Control	24			✓	0.0741	!	0.0112	✓	0.999
	2,390,400 ppb As	24	✗	0.3494						
	2,390,400 ppb As, 0.032 ppb CIP	24	✗	0.0719						
	2,390,400 ppb As, 0.064 ppb CIP	24	✗	0.1355						
	2,390,400 ppb As, 0.128 ppb CIP	24	✓	0.0352						
	2,390,400 ppb As, 0.256 ppb CIP	24	✓	0.0014						



a. Binary Group 2.1

b. Binary Group 2.2

c. Binary Group 2.3

Figure 4.10: Inhibition of *E. faecium* following exposure to the Binary Group 2 Mixtures of the Cu MEC with a PNEC range of (a) TCS, (b) OTC, or (c) CIP.

Table 4.15: ANOVA statistical analysis of results from the Binary Group 2 Mixtures of MEC Cu with PNEC ranges of TCS, OTC, or CIP.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control $p \leq 0.05$	Shapiro-Wilk: Normality $p \geq 0.05$	Levene's Test: Homogeneity of Variance $p \geq 0.05$	Computed Power $p \geq 0.8$
Binary 2.1: 123,830 ppb Cu, PNEC [TCS] (ppb)	Negative Control	24		✓ 0.6719	✗ 0.0013	✓ 0.999
	123,830 ppb Cu	24	✓ 1.00E-04			
	123,830 ppb Cu, 0.25 ppb TCS	22	✓ 1.00E-04			
	123,830 ppb Cu, 0.5 ppb TCS	24	✓ 1.00E-04			
	123,830 ppb Cu, 1.0 ppb TCS	24	✓ 1.00E-04			
	123,830 ppb Cu, 2.0 ppb TCS	20	✓ 1.00E-04			
Binary 2.2: 123,830 ppb Cu, PNEC [OTC] (ppb)	Negative Control	24		✓ 0.2872	✗ 0.0047	✓ 0.999
	123,830 ppb Cu	24	✓ 1.00E-04			
	123,830 ppb Cu, 0.25 ppb OTC	20	✓ 1.00E-04			
	123,830 ppb Cu, 0.5 ppb OTC	21	✓ 1.00E-04			
	123,830 ppb Cu, 1.0 ppb OTC	22	✓ 1.00E-04			
	123,830 ppb Cu, 2.0 ppb OTC	24	✓ 1.00E-04			
Binary 2.3: 123,830 ppb Cu, PNEC [CIP] (ppb)	Negative Control	24		✓ 0.0904	✗ 0.0045	✓ 0.999
	123,830 ppb Cu	24	✓ 1.00E-04			
	123,830 ppb Cu, 0.032 ppb CIP	23	✓ 1.00E-04			
	123,830 ppb Cu, 0.064 ppb CIP	24	✓ 1.00E-04			
	123,830 ppb Cu, 0.128 ppb CIP	23	✓ 1.00E-04			
	123,830 ppb Cu, 0.256 ppb CIP	19	✓ 1.00E-04			

PNEC oxytetracycline mixture had the greatest growth inhibition effect on Enterococcus compared to the other antimicrobials tested.

Treatment groups of MEC zinc with PNEC-ranges of triclosan and ciprofloxacin had no statistically significant reduction or stimulation of growth when compared to controls (Dunnett's test $p \leq 0.05$), and both failed Levene's test for homogeneity of variance (**Table 4.16**). Only exposure to MEC Zn with PNEC-range OTC significantly inhibited growth, ranging from 28.90% - 88.07% inhibition (**Figure 4.11**). There was a moderate departure from the assumptions of the Levine's for the MEC Zn and PNEC OTC results (**Table 4.16**).

Binary treatment with MEC TCS and a PNEC range of As, Cu, or Zn had slight variations between exposure groups. TCS alone caused a significant (Dunnett's $\leq$ p 0.05) reduction in growth compared to controls ranging from 6.93-6.97%. In the mixture of TCS and As, there was also a significant (Dunnett's test $p \leq 0.05$) reduction in growth compared to the controls ranging from 7.35-7.74%, but this additional growth reduction was not significantly different from the 6.93% growth reduction caused by TCS alone. In the TCS and Cu mixture, significant growth reductions were observed in both TCS alone (6.97%) and all copper concentrations (5.68-8.05%) tested which were significantly (Dunnett's test $p \leq 0.05$) different than the negative control but were not significantly different than the TCS exposure alone. The TCS and Zn mixture resulted in significant (Dunnett's test $p \leq 0.05$) growth compared to the negative control, with inhibition of 6.97% in TCS alone and a range from 6.56% to 9.39% in the Zn-TCS mixture. In addition, the combination of TCS and Zn caused additional growth inhibition compared to the TCS exposure alone at the three highest concentrations tested (**Figure 4.12, Table 4.17**).

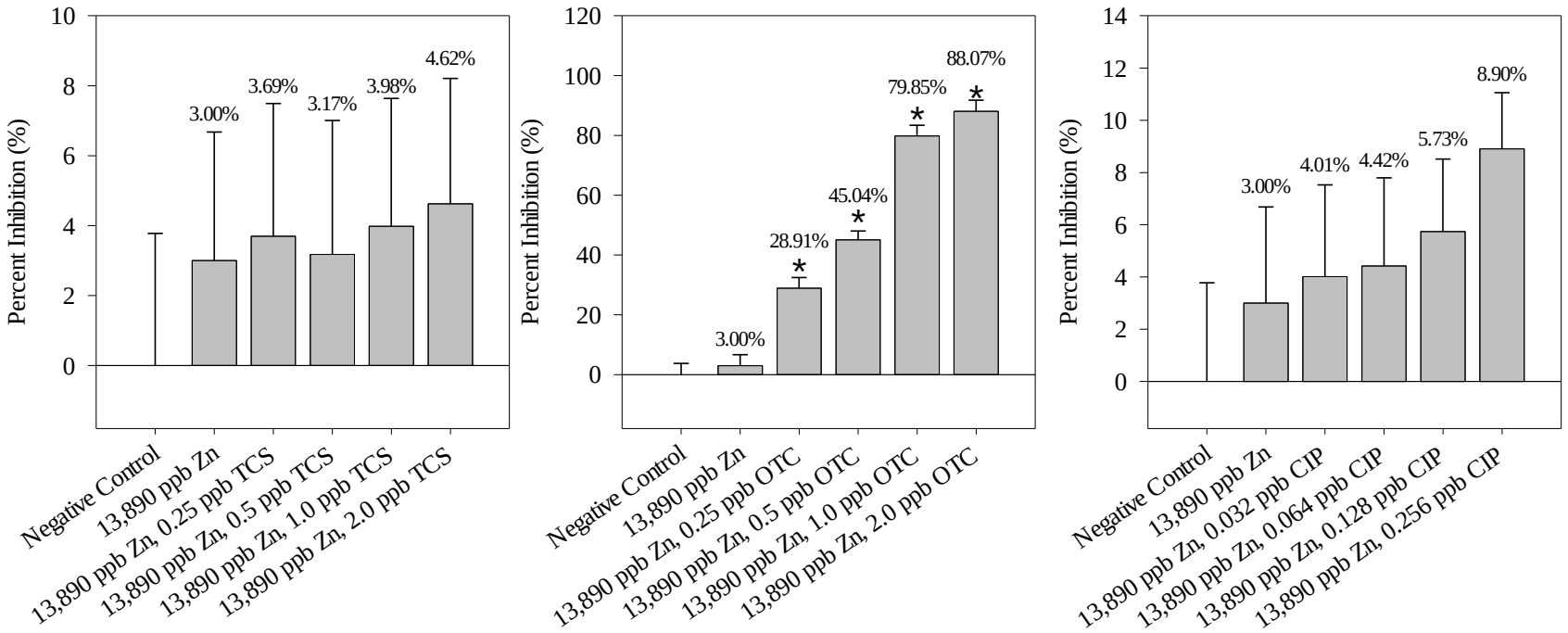
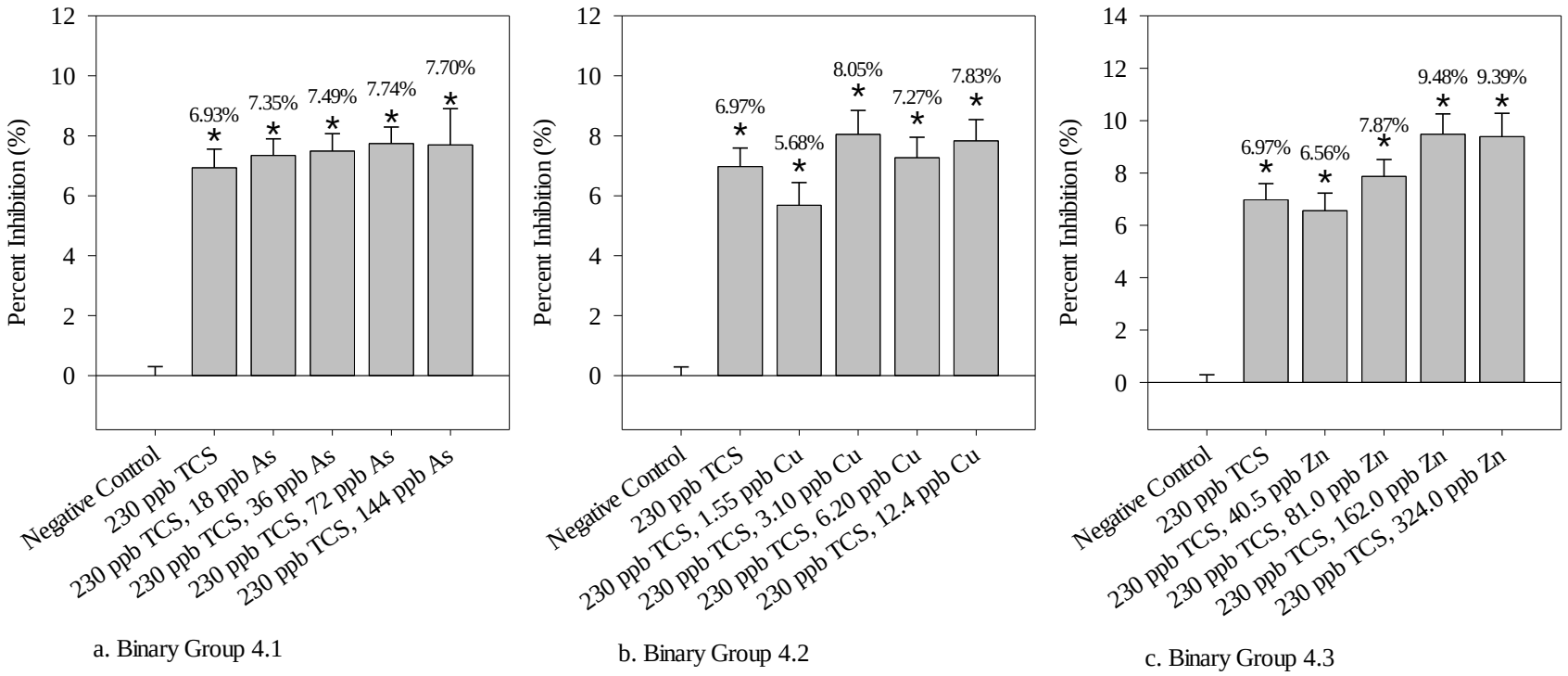


Figure 4.11: Inhibition of *E. faecium* following exposure to the Binary Group 3 Mixtures of the Zn MEC with a PNEC range of (a) TCS, (b) OTC, or (c) CIP.

Table 4.16: ANOVA statistical analysis of results from the Binary Group 3 Mixtures of MEC zinc with a PNEC range of TCS, OTC, or CIP.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control $p \leq 0.05$	Shapiro-Wilk: Normality $p \geq 0.05$	Levene's Test: Homogeneity of Variance $p \geq 0.05$	Computed Power $p \geq 0.8$
Binary 3.1: 13,890 ppb Zn, PNEC [TCS] (ppb)	Negative Control	21		✓ 0.7178	✗ 0.0026	✓ 0.999
	13,890 ppb Zn	21	✗ 0.9978			
	13,890 ppb Zn, 0.25 ppb TCS	21	✗ 0.9943			
	13,890 ppb Zn, 0.5 ppb TCS	20	✗ 0.9961			
	13,890 ppb Zn, 1.0 ppb TCS	21	✗ 0.992			
	13,890 ppb Zn, 2.0 ppb TCS	21	✗ 0.9845			
Binary 3.2: 13,890 ppb Zn, PNEC [OTC] (ppb)	Negative Control	21		✓ 0.2263	✗ 0.0034	✓ 0.999
	13,890 ppb Zn	21	✗ 0.997			
	13,890 ppb Zn, 0.25 ppb OTC	20	✓ 0.0216			
	13,890 ppb Zn, 0.5 ppb OTC	17	✓ 0.0007			
	13,890 ppb Zn, 1.0 ppb OTC	20	✓ 0.0001			
	13,890 ppb Zn, 2.0 ppb OTC	21	✓ 0.0001			
Binary 3.3: 13,890 ppb Zn, PNEC [CIP] (ppb)	Negative Control	21		✓ 0.4214	! 0.0121	✓ 0.999
	13,890 ppb Zn	21	✗ 0.9961			
	13,890 ppb Zn, 0.032 ppb CIP	21	✗ 0.9854			
	13,890 ppb Zn, 0.064 ppb CIP	21	✗ 0.9779			
	13,890 ppb Zn, 0.128 ppb CIP	21	✗ 0.9371			
	13,890 ppb Zn, 0.256 ppb CIP	20	✗ 0.8433			



a. Binary Group 4.1

b. Binary Group 4.2

c. Binary Group 4.3

Figure 4.12: Inhibition of *E. faecium* following exposure to the Binary Group 4 Mixtures of the TCS MEC with a CCC range of (a) As, (b) Cu, or (c) Zn.

Table 4.17: ANOVA statistical analysis of results from the Binary Group 4 Mixtures of MEC TCS with a CCC range of As, Cu, or Zn.

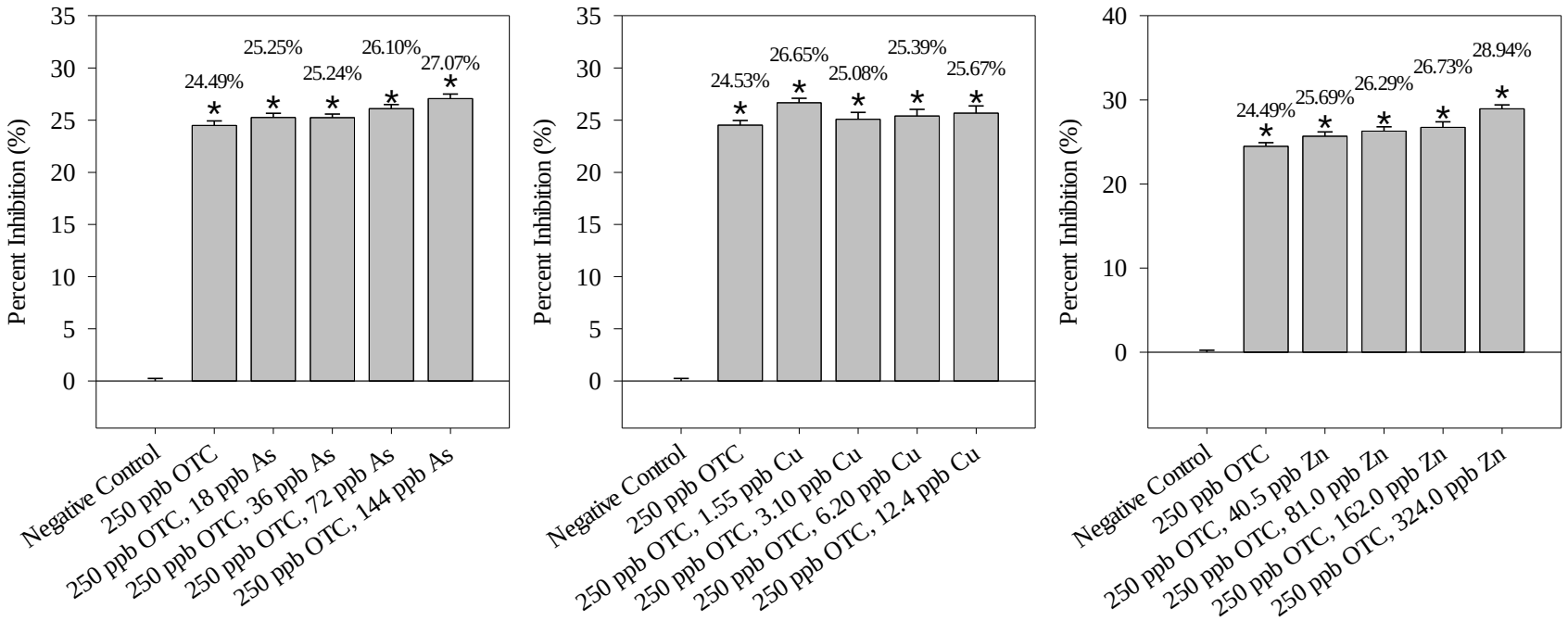
Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control $p \leq 0.05$	Shapiro-Wilk: Normality $p \geq 0.05$	Levene's Test: Homogeneity of Variance $p \geq 0.05$	Computed Power $p \geq 0.8$
Binary 4.1: 230 ppb TCS, CCC [As] (ppb)	Negative Control	23		✓ 0.7784	! 0.0134	✓ 0.999
	230 ppb TCS	24	✓ 0.0003			
	230 ppb TCS, 18 ppb As	24	✓ 0.0001			
	230 ppb TCS, 36 ppb As	24	✓ 0.0001			
	230 ppb TCS, 72 ppb As	24	✓ 0.0001			
	230 ppb TCS, 144 ppb As	17	✓ 3.00E-04			
Binary 4.2: 230 ppb TCS, CCC [Cu] (ppb)	Negative Control	24		✓ 0.0928	! 0.0167	✓ 0.999
	230 ppb TCS	24	✓ 3.00E-04			
	230 ppb TCS, 1.55 ppb Cu	24	✓ 3.10E-03			
	230 ppb TCS, 3.10 ppb Cu	24	✓ 1.00E-04			
	230 ppb TCS, 6.20 ppb Cu	23	✓ 2.00E-04			
	230 ppb TCS, 12.4 ppb Cu	22	✓ 1.00E-04			
Binary 4.3: 230 ppb TCS, CCC [Zn] (ppb)	Negative Control	24		✓ 0.6186	! 0.005	✓ 0.999
	230 ppb TCS	24	✓ 4.00E-04			
	230 ppb TCS, 40.5 ppb Zn	23	✓ 8.00E-04			
	230 ppb TCS, 81.0 ppb Zn	23	✓ 1.00E-04			
	230 ppb TCS, 162.0 ppb Zn	24	✓ 1.00E-04			
	230 ppb TCS, 324.0 ppb Zn	23	✓ 1.00E-04			

MEC OTC exposure alone caused a range of growth inhibition of 24.49-24.53% compared to controls while the different OTC-metal mixtures had only slightly increased growth inhibitions of 25.08 - 29.84%. Overall, there was less than a 5% difference in growth inhibition between the mixtures and OTC exposure alone. MEC OTC and PNEC-range As exhibited 24.49% - 27.0%; MEC OTC and PNEC-range Cu was inhibited by 24.53% - 26.65%, and MEC OTC + PNEC Zn saw inhibition from 24.49% - 28.9%. All of these treatments were statistically significant (Dunnett's test $p \leq 0.05$) from the negative control, but both OTC + Cu and OTC + Zn failed the Shapiro-Wilk test for normality and Levene's test for homogeneity of variance. MEC OTC with 144 ppb As and with 324 ppb Zn were both significant (Dunnett's test $p \leq 0.05$) when compared to MEC OTC alone, as well (**Figure 4.13, Table 4.18**).

The MEC CIP and a PNEC-range of As, Cu, or Zn mixtures had significantly (Dunnett's test $p \leq 0.05$) reduced growth at all concentrations tested compared to controls but did not exhibit much variability between or within treatment groups. CIP exposure alone reduced growth by 22.55% compared to growth inhibitions of 21.29-23.57% in the MEC CIP and trace metal mixtures. In total, there was approximately a 2% change in growth inhibition differences between the MEC CIP exposure alone and the MEC CIP - PNEC trace metal mixtures. The three treatment mixture groups all failed Levene's test for homogeneity of variance (**Figure 4.14, Table 4.19**).

(c) Kirby-Bauer Assays

Kirby-Bauer antimicrobial resistance assays were performed for each combination of MEC metals with PNEC antimicrobials, and MEC antimicrobials with CCC metals. However, the Binary Group 2 mixtures, which contained the MEC of copper for the



a. Binary Group 5.1

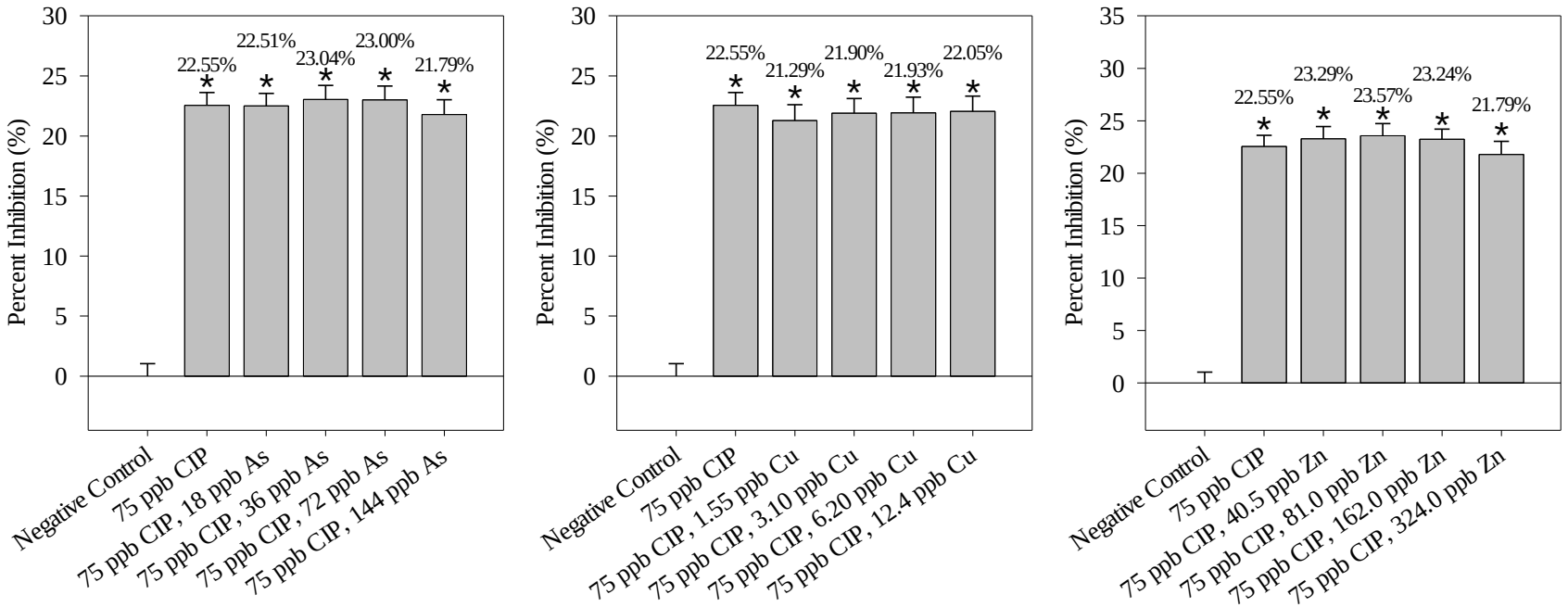
b. Binary Group 5.2

c. Binary Group 5.3

Figure 4.13: Inhibition of *E. faecium* following exposure to the Binary Group 5 Mixtures of the OTC MEC with a CCC range of (a) As, (b) Cu, or (c) Zn.

Table 4.18: ANOVA statistical analysis of results from the Binary Group 5 Mixtures of MEC OTC with a CCC range of As, Cu, or Zn.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control $p \leq 0.05$	Shapiro-Wilk: Normality $p \geq 0.05$	Levene's Test: Homogeneity of Variance $p \geq 0.05$	Computed Power $p \geq 0.8$
Binary 5.1: 250 ppb OTC, CCC [As] (ppb)	Negative Control	24		✓ 0.3297	! 0.0055	✓ 0.999
	250 ppb OTC	24	✓ 0.0001			
	250 ppb OTC, 18 ppb As	24	✓ 0.0001			
	250 ppb OTC, 36 ppb As	24	✓ 0.0001			
	250 ppb OTC, 72 ppb As	24	✓ 0.0001			
	250 ppb OTC, 144 ppb As	23	✓ 0.0001			
Binary 5.2: 250 ppb OTC, CCC [Cu] (ppb)	Negative Control	24		✗ 0.0001	✗ 0.0001	✓ 0.999
	250 ppb OTC	23	✓ 0.0001			
	250 ppb OTC, 1.55 ppb Cu	24	✓ 0.0001			
	250 ppb OTC, 3.10 ppb Cu	24	✓ 0.0001			
	250 ppb OTC, 6.20 ppb Cu	24	✓ 0.0001			
	250 ppb OTC, 12.4 ppb Cu	24	✓ 0.0001			
Binary 5.3: 250 ppb OTC, CCC [Zn] (ppb)	Negative Control	24		✗ 0.0004	✗ 0.0003	✓ 0.999
	250 ppb OTC	24	✓ 0.0001			
	250 ppb OTC, 40.5 ppb Zn	23	✓ 0.0001			
	250 ppb OTC, 81.0 ppb Zn	23	✓ 0.0001			
	250 ppb OTC, 162.0 ppb Zn	24	✓ 0.0001			
	250 ppb OTC, 324.0 ppb Zn	24	✓ 0.0001			



a. Binary Group 6.1

b. Binary Group 6.2

c. Binary Group 6.3

Figure 4.14: Inhibition of *E. faecium* following exposure to the Binary Group 6 Mixtures of the CIP MEC with a CCC range of (a) As, (b) Cu, or (c) Zn.

Table 4.19: ANOVA statistical analysis of results from the Binary Group 6 Mixtures of MEC CIP with a CCC range of As, Cu, or Zn.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control $p \leq 0.05$	Shapiro-Wilk: Normality $p \geq 0.05$	Levene's Test: Homogeneity of Variance $p \geq 0.05$	Computed Power $p \geq 0.8$
Binary 6.1: 75 ppb CIP, CCC [As] (ppb)	Negative Control	24		✓ 0.8947	✗ 0.002	✓ 0.999
	75 ppb CIP	24	✓ 0.0001			
	75 ppb CIP, 18 ppb As	24	✓ 0.0001			
	75 ppb CIP, 36 ppb As	24	✓ 0.0001			
	75 ppb CIP, 72 ppb As	24	✓ 0.0001			
	75 ppb CIP, 144 ppb As	21	✓ 0.0001			
Binary 6.2: 75 ppb CIP, CCC [Cu] (ppb)	Negative Control	24		✓ 0.5889	✗ 0.0012	✓ 0.999
	75 ppb CIP	24	✓ 0.0001			
	75 ppb CIP, 1.55 ppb Cu	24	✓ 1.00E-04			
	75 ppb CIP, 3.10 ppb Cu	24	✓ 0.0001			
	75 ppb CIP, 6.20 ppb Cu	24	✓ 0.0001			
	75 ppb CIP, 12.4 ppb Cu	24	✓ 0.0001			
Binary 6.3: 75 ppb CIP, CCC [Zn] (ppb)	Negative Control	24		✓ 0.9347	✗ 0.0022	✓ 0.999
	75 ppb CIP	24	✓ 0.0001			
	75 ppb CIP, 40.5 ppb Zn	24	✓ 0.0001			
	75 ppb CIP, 81.0 ppb Zn	24	✓ 0.0001			
	75 ppb CIP, 162.0 ppb Zn	23	✓ 0.0001			
	75 ppb CIP, 324.0 ppb Zn	24	✓ 0.0001			

constant exposure, did not undergo visible growth in the overnight culture, and when streaked on Muller-Hinton agar did not grow on the plate. The starting stock of copper was re-made for these experiments and may have been miscalculated, or the wrong amount of copper stock may have been added to the overnight cultures.

For all figures below, an asterisk (*) indicates statistical significance (Dunnett's test $p \leq 0.05$). Positive (+) susceptibility values indicate that the antimicrobial challenge was more toxic for the exposure group than the negative control, and negative (-) susceptibility values indicate that the antimicrobial challenge was less toxic for the exposure group than the negative control. No data points were removed as outliers from this data set, although some plates had antimicrobial discs fall from the agar, preventing the antimicrobial to diffuse into the agar. One replicate from Binary Group 4.3, containing three discs of each antimicrobial, was removed from analysis due to contamination. Additionally, one replicate from Binary Group 5.1, containing two filter discs of CIP and OTC and three of VAN, froze during overnight storage in a 4°C refrigerator after removal from the incubator. Ice crystals forming in the agar made the ZOI impossible to measure for this plate and it was therefore removed from analysis.

As evidenced in Table **4.20(b),** all treatment groups were susceptible to all three challenges, both before and after the 24-hour acclimation period.

In a series of experiments which allowed *E. faecium* to acclimate for 24 hours to the probable PNEC of all three antimicrobials of interest (TCS, OTC, or CIP) or the CCC of the three metals (As, Cu, Zn), the change in susceptibility to CIP, VAN, and OTC varied widely when compared to the negative control, though none were statistically significant (Dunnett's test $p \leq 0.05$). Only one treatment – 23 ppb As – induced slight (-0.64%)

Table 4.20(a): AMR breakpoints for *E. faecium* exposure to ciprofloxacin (CIP), vancomycin (VAN), and oxytetracycline (OTC) (CLSI 2017).

Antimicrobial Challenge	Breakpoints Zone of Inhibition (ZOI) [mm]		
Legend	▲ Susceptible	– Intermediate	▼ Resistant
Ciprofloxacin (5 µg)	> 21	16-20	<15
Vancomycin (30 µg)	>17	15-16	<14
(Oxy)tetracycline (30 µg)	>19	15-18	<14

Table 4.20(b): Post-exposure Zones of Inhibition with susceptibility designations.

Average Zone of Inhibition (mm)				
Treatment Group	**Treatment**	**CIP Challenge**	**VAN Challenge**	**OTC Challenge**
Negative Control	Negative Control	26.4	25.2	28.1
Low Doses	36 ppb As	26.2	25.4	28.4
	3.10 ppb Cu	27.6	25.9	28.5
	81.0 ppb Zn	27.3	25.8	29.1
	0.5 ppb TCS	27.1	25.9	29.3
	0.5 ppb OTC	28.4	26.4	28.3
	0.064 ppb CIP	27.1	25.9	29.0
High Doses	2,390,400 ppb As	27.4	25.8	28.3
	13,890 ppb Zn	27.4	25.2	28.1
	230 ppb TCS	26.7	25.8	27.8
	250 ppb OTC	31.6	28.6	32.7
	75 ppb CIP	27.5	26.0	28.9
Binary Group 1	2,390,400 ppb As, 0.5 ppb TCS	26.1	25.7	28.4
	2,390,400 ppb As, 0.5 ppb OTC	28.4	26.0	29.7
	2,390,400 ppb As, 0.064 ppb CIP	26.8	25.0	27.8
Binary Group 3	13,890 ppb Zn, 0.5 ppb TCS	28.0	26.0	29.0
	13,890 ppb Zn, 0.5 ppb OTC	29.1	26.7	29.0
	13,890 ppb Zn, 0.064 ppb CIP	28.0	26.1	29.5
Binary Group 4	230 ppb TCS, 36 ppb As	27.2	25.4	29.1
	230 ppb TCS, 3.10 ppb Cu	28.1	25.9	29.2
	230 ppb TCS, 81.0 ppb Zn	27.9	26.2	28.9
Binary Group 5	250 ppb OTC, 36 ppb As	32.9	28.7	35.1
	250 ppb OTC, 3.10 ppb Cu	33.8	29.8	35.4
	250 ppb OTC, 81.0 ppb Zn	33.1	30.0	34.5
Binary Group 6	75 ppb CIP, 36 ppb As	28.7	26.0	29.8
	75 ppb CIP, 3.10 ppb Cu	28.6	26.7	28.7
	75 ppb CIP, 81.0 ppb Zn	27.7	26.2	29.5

reduction in susceptibility to a ciprofloxacin challenge, and no exposures reduced susceptibility to either vancomycin or oxytetracycline. The highest change in susceptibility to any of these antimicrobials was 4.95% increase in susceptibility to vancomycin following 24-hour acclimation to the PNEC (0.5 ppb) of OTC, which was not statistically significant. There was also a 4.59% increase in susceptibility to oxytetracycline following acclimation to 0.5 ppb TCS, while overnight acclimation to 0.5 ppb OTC increased susceptibility to oxytetracycline by just 0.78%. All three challenge groups failed the Shapiro-Wilk test for normality, and the vancomycin challenge also failed Levene's test for homogeneity of variance (**Figure 4.15, Table 4.21**).

Following acclimation to the MEC of As, Zn, TCS, OTC, or CIP, and a challenge by CIP, VAN, and OTC, the only significant (Dunnett's test $p \leq 0.05$) results were from acclimation to 250 ppb OTC, in all three challenge experiments. These different challenge experiments resulted in a 16.67% increased susceptibility to oxytetracycline, a 19.69% increase in susceptibility to ciprofloxacin and a 13.67% increase in susceptibility to vancomycin, which were significantly (Dunnett's test $p \leq 0.05$) different than the negative control. There was a very slight (0.87%) decrease in susceptibility to oxytetracycline after acclimation to 230 ppb TCS. The rest of the challenges following acclimation to the MEC of each toxicant of interest ranged from 1.25% - 4.01% increase in susceptibility to ciprofloxacin, 0.18% - 3.09% increase in susceptibility to vancomycin, and 0.41% - 3.01% increase in susceptibility to oxytetracycline (**Figure 4.16, Table 4.22**).

Following a binary mixture of MEC As with PNEC TCS CIP and OTC exposure prior to challenge experiments with CIP, VAN and OTC, there were no significant (Dunnett's test $p \leq 0.05$) changes in susceptibility observed in any treatment when

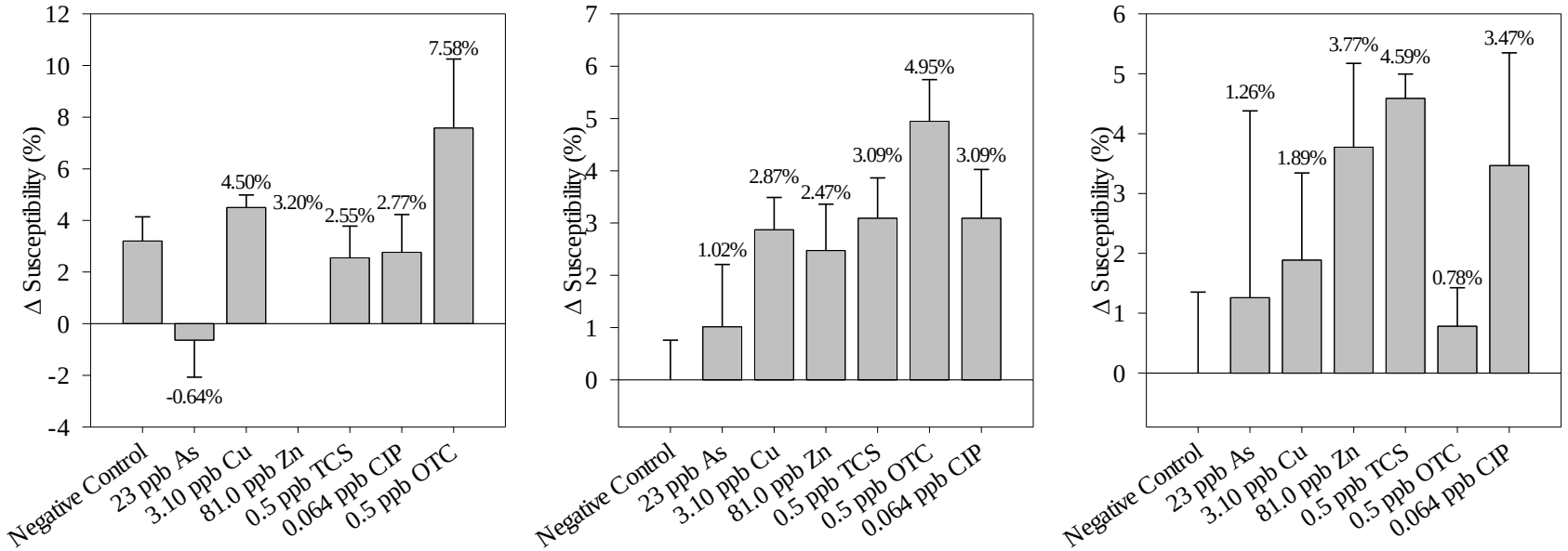
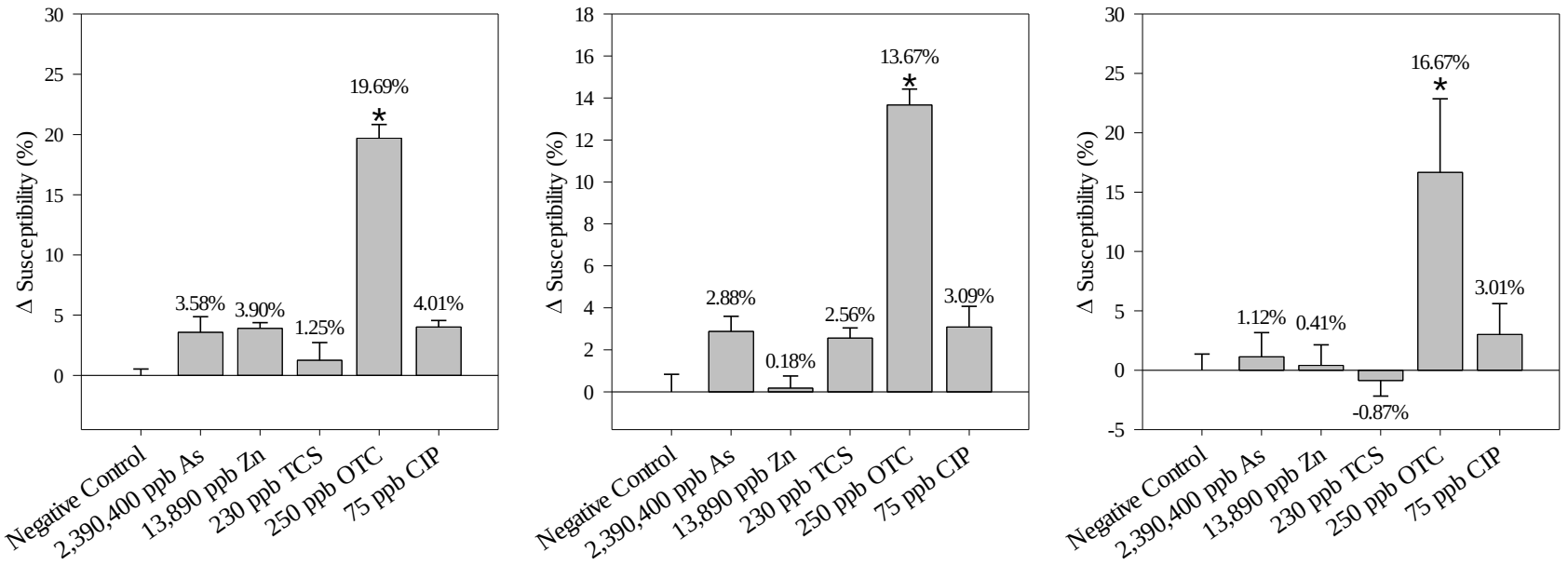


Figure 4.15: *E. faecium* change in susceptibility to (a) ciprofloxacin (-0.64% - 7.58%), (b) vancomycin (1.02% - 4.95%), and (c) oxytetracycline (0.78% - 4.59%) following 24-hour acclimation to the EPA Criterion Continuous Concentration of As, Cu, or Zn, or the Probable No Effects Concentration of TCS, OTC, or CIP.

Table 4.21: ANOVA statistical analysis of the results for Kirby-Bauer Low Dose Exposures to CCC doses of As, Cu, and Zn and PNEC doses of TCS, OTC, or CIP.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control p ≤ 0.05	Shapiro-Wilk: Normality p ≥ 0.05	Levene's Test: Homogeneity of Variance p ≥ 0.05	Computed Power p ≥ 0.8
Low Doses: CIP Challenge	Control	8		✗ 0.0001	✓ 0.0694	✓ 0.999
	36 ppb As	7	✗ 1			
	3.10 ppb Cu	7	✗ 0.566			
	81.0 ppb Zn	7	✗ 0.749			
	0.5 ppb TCS	7	✗ 0.923			
	0.5 ppb OTC	7	✗ 0.164			
	0.064 ppb CIP	7	✗ 0.949			
Low Doses: VAN Challenge	Control	9		✗ 0.0001	✗ 0.0003	✓ 0.999
	36 ppb As	9	✗ 0.9935			
	3.10 ppb Cu	9	✗ 0.6298			
	81.0 ppb Zn	9	✗ 0.7475			
	0.5 ppb TCS	9	✗ 0.5643			
	0.5 ppb OTC	9	✗ 0.1681			
	0.064 ppb CIP	9	✗ 0.5643			
Low Doses: OTC Challenge	Control	7		✗ 0.0004	✓ 0.18	✓ 0.977
	36 ppb As	6	✗ 1			
	3.10 ppb Cu	7	✗ 0.9152			
	81.0 ppb Zn	7	✗ 0.7913			
	0.5 ppb TCS	7	✗ 0.5943			
	0.5 ppb OTC	6	✗ 0.9998			
	0.064 ppb CIP	7	✗ 0.6278			



a. Ciprofloxacin Challenge b. Vancomycin Challenge c. Oxytetracycline Challenge

Figure 4.16: *E. faecium* change in susceptibility to (a) ciprofloxacin (1.25% - 19.69%), (b) vancomycin (0.18% - 14.57%), and (c) oxytetracycline (-0.87 – 16.67%) following 24-hour acclimation to the MEC of As, Zn, TCS, OTC, or CIP.

Table 4.22: ANOVA statistical analysis of results for the Kirby-Bauer High Dose Exposures to MEC doses of As, Cu, Zn, TCS, OTC, or CIP.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control $p \leq 0.05$	Shapiro-Wilk: Normality $p \geq 0.05$	Levene's Test: Homogeneity of Variance $p \geq 0.05$	Computed Power $p \geq 0.8$
High Doses: CIP Challenge	Negative Control	8		✗ 0.0008	✓ 0.0666	✓ 0.999
	2,390,400 ppb As	7	✗ 0.4307			
	13,890 ppb Zn	7	✗ 0.2909			
	230 ppb TCS	7	✗ 0.9092			
	250 ppb OTC	7	✓ 0.0001			
	75 ppb CIP	7	✗ 0.2336			
High Doses: VAN Challenge	Negative Control	8		! 0.0311	✓ 0.1147	✓ 0.999
	2,390,400 ppb As	8	✗ 0.587			
	13,890 ppb Zn	9	✗ 1			
	230 ppb TCS	9	✗ 0.648			
	250 ppb OTC	9	✓ 0.0001			
	75 ppb CIP	9	✗ 0.47			
High Doses: OTC Challenge	Negative Control	7		✗ 0.0001	✓ 0.0686	✓ 0.999
	2,390,400 ppb As	7	✗ 1			
	13,890 ppb Zn	7	✗ 1			
	230 ppb TCS	7	✗ 0.9998			
	250 ppb OTC	6	✓ 0.0373			
	75 ppb CIP	7	✗ 0.9859			

compared to controls. Following a binary mixture of MEC As with PNEC TCS, there was only a 4.65% decrease in susceptibility to ciprofloxacin, no change in susceptibility to vancomycin, and negligible change in susceptibility to ciprofloxacin. The mixture of MEC As with PNEC OTC also had mixed results, with a 3.65% increase in susceptibility to ciprofloxacin, negligible change in susceptibility to vancomycin, and 4.79% increase in susceptibility to oxytetracycline. The binary mixture of MEC As with PNEC CIP, on the other hand, resulted in only slight decreased susceptibility to all three antimicrobial challenges. For this treatment, there was a decrease in susceptibility to ciprofloxacin of 2.14%, 3.73% for vancomycin, and 1.92% for oxytetracycline. None of these results were statistically significant (Dunnett's test $p \leq 0.05$) when compared to the negative control, and all three failed the Shapiro-Wilk test for normality (**Figure 4.17, Table 4.23**).

Similar results were obtained in the MEC Zn with PNEC TCS, OTC, or CIP exposures followed by CIP, VAN, and OTC challenge. None of these MEC Zn and PNEC TCS, OTC, and CIP mixtures resulted in significant (Dunnett's test $p \leq 0.05$) changes in susceptibility compared to controls in any of the antimicrobial challenge experiments. MEC Zn with PNEC TCS resulted in only 2.08%, 2.99%, and 3.15% increases in susceptibility to ciprofloxacin, vancomycin, and oxytetracycline, respectively. MEC Zn with PNEC OTC caused 6.09% increase in susceptibility to ciprofloxacin, 5.63% increase in vancomycin, and 3.00% increase in oxytetracycline. The final treatment in this group, MEC Zn with PNEC CIP, resulted in 3.08%, 3.26%, and 5.03% increase in susceptibility to ciprofloxacin, vancomycin, and oxytetracycline, respectively. None of these results were statistically significant (Dunnett's test $p \leq 0.05$), all three challenges failed the Shapiro-

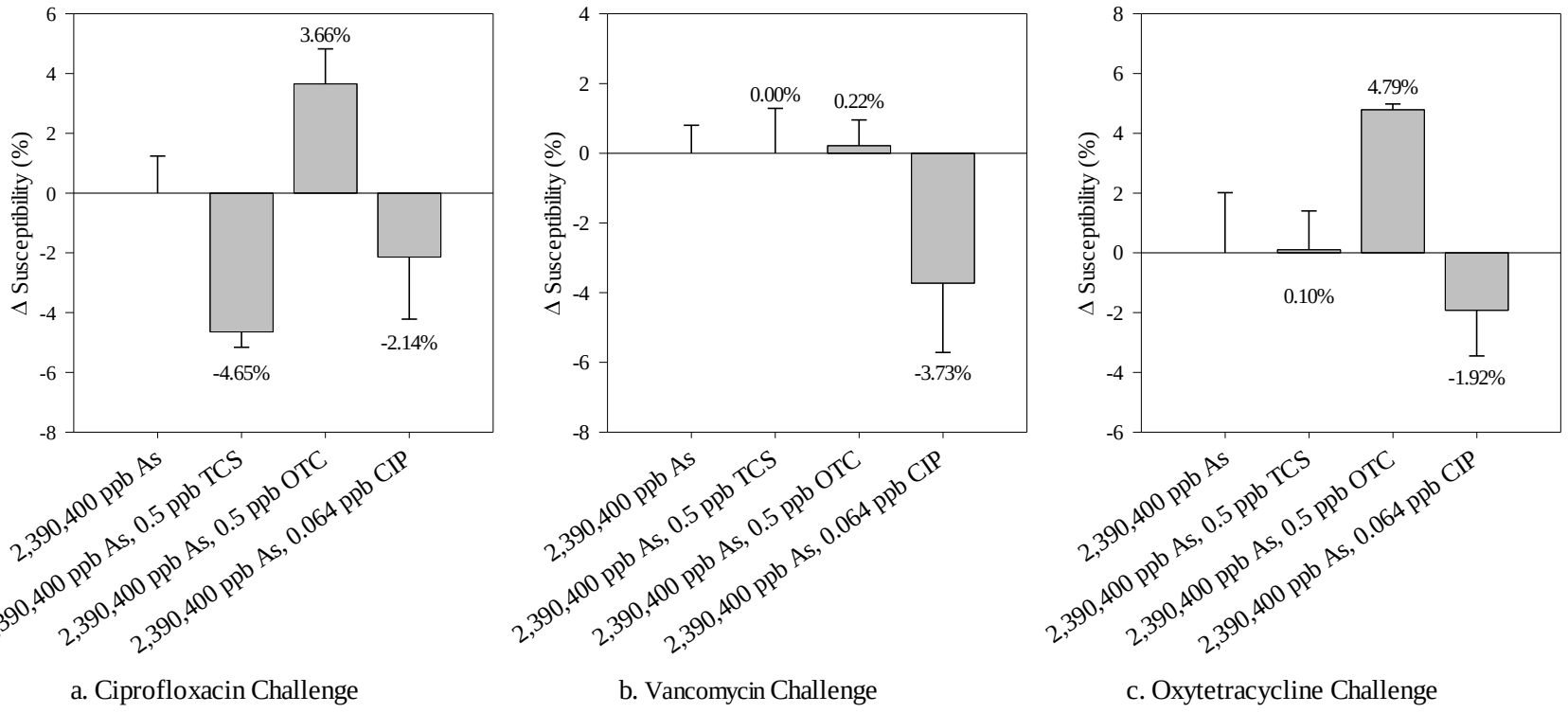


Figure 4.17: *E. faecium* change in susceptibility to (a) ciprofloxacin (-4.65 – 3.66%), (b) vancomycin (-3.73% - 0.22%), and (c) oxytetracycline (-1.92% - 4.79%) following 24-hour acclimation to the Binary Group 1 Mixtures of the As MEC with the PNEC of TCS, OTC, or CIP.

Table 4.23: ANOVA statistical analysis of results for Kirby-Bauer Binary Group 1 Mixtures of MEC As with PNEC ranges of TCS, OTC, or CIP.

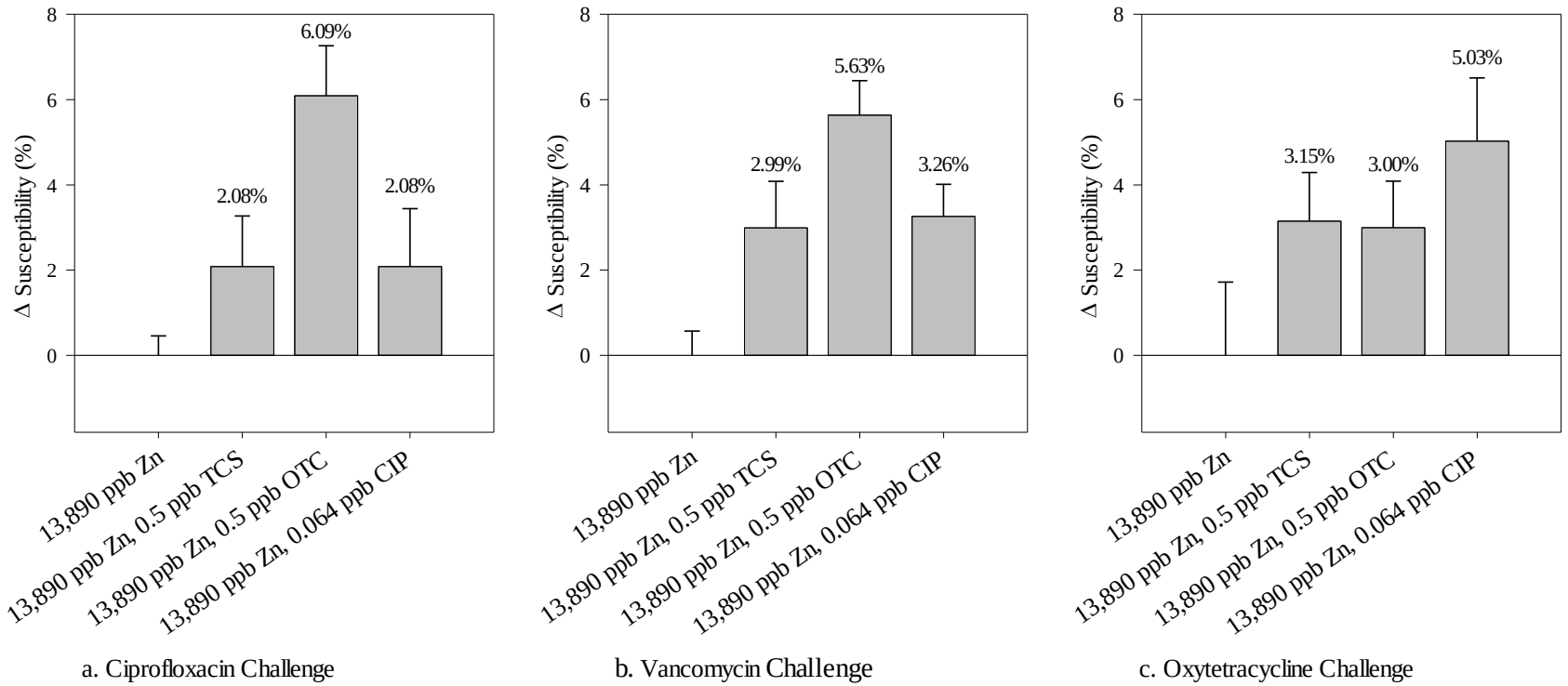
Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control $p \leq 0.05$	Shapiro-Wilk: Normality $p \geq 0.05$	Levene's Test: Homogeneity of Variance $p \geq 0.05$	Computed Power $p \geq 0.8$
Binary Group 1: CIP Challenge	2,390,400 ppb As	7		✗ 0.001	✓ 0.2078	✓ 0.999
	2,390,400 ppb As, 0.5 ppb TCS	7	✗ 0.4546			
	2,390,400 ppb As, 0.5 ppb OTC	7	✗ 0.4277			
	2,390,400 ppb As, 0.064 ppb CIP	7	✗ 0.9483			
Binary Group 1 VAN Challenge	2,390,400 ppb As	7		✗ 0.0001	✓ 0.0517	✓ 0.999
	2,390,400 ppb As, 0.5 ppb TCS	7	✗ 0.9863			
	2,390,400 ppb As, 0.5 ppb OTC	9	✗ 0.9964			
	2,390,400 ppb As, 0.064 ppb CIP	8	✗ 0.8243			
Binary Group 1 OTC Challenge	2,390,400 ppb As	7		✗ 0.0001	✓ 0.0524	✓ 0.999
	2,390,400 ppb As, 0.5 ppb TCS	7	✗ 0.9913			
	2,390,400 ppb As, 0.5 ppb OTC	7	✗ 0.2872			
	2,390,400 ppb As, 0.064 ppb CIP	6	✗ 1			

Wilk test for normality, and the vancomycin challenge also failed Levene's test for homogeneity of variance (**Figure 4.18, Table 4.24**).

The mixture of MEC TCS with CCC As, Cu, or Zn resulted in slight increases in susceptibility in almost every challenge. The exception is in the vancomycin challenge following exposure to MEC TCS and CCC As, which saw a slight 1.68% decrease in susceptibility. The rest ranged from 1.60% - 4.33% increases in susceptibility to ciprofloxacin, 0.17% - 1.53% increases in vancomycin, and 4.06% - 5.14% increases in oxytetracycline. None of these data were statistically significant (Dunnett's test $p \leq 0.05$) when compared to the negative control, and both the ciprofloxacin and oxytetracycline challenges failed the Shapiro-Wilk test for normality (**Figure 4.19, Table 4.25**).

No combinations of MEC OTC with CCC metals mixtures resulted in significant (Dunnett's test $p \leq 0.05$) alterations in susceptibility compared to controls for any of the three antimicrobial challenges. Slight increases in susceptibility to ciprofloxacin ranged from 4.19% - 6.87%, to vancomycin ranged from 0.87% to 5.22%, and to oxytetracycline ranged from 0.22% - 2.88%. None of these results were statistically significant (Dunnett's test $p \leq 0.05$) from control, and both the vancomycin and oxytetracycline challenges failed the Shapiro-Wilk test for normality (**Figure 4.20, Table 4.26**).

Co-exposure to MEC CIP with CCC As, Cu, or Zn did not result in greater than a 5% increase in susceptibility to any of the three antimicrobial challenges. The MEC CIP + CCC Cu treatment, however, saw a very slight (0.64%) decrease in susceptibility to oxytetracycline. None of the results from these exposure groups were statistically significant (Dunnett's test $p \leq 0.05$) when compared to the negative control, all three

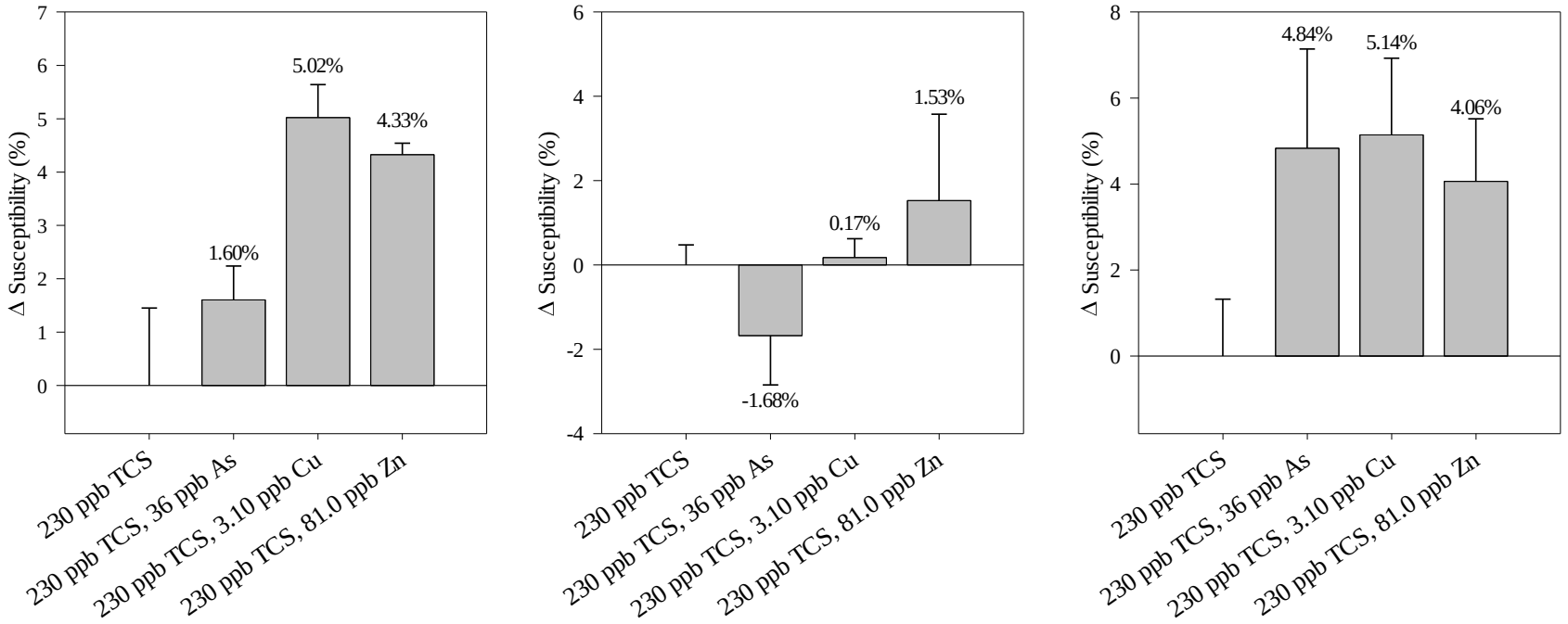


a. Ciprofloxacin Challenge b. Vancomycin Challenge c. Oxytetracycline Challenge

Figure 4.18: *E. faecium* change in susceptibility to (a) ciprofloxacin (2.08% - 6.09%), (b) vancomycin (2.99% - 5.63%), and (c) oxytetracycline (3.00% - 5.03%) following 24-hour acclimation to the Binary Group 3 mixture of the Zn MEC with the PNEC of TCS, OTC, or CIP.

Table 4.24: ANOVA statistical analysis of results for Kirby-Bauer Binary Group 3 Mixtures of MEC Zn with PNEC ranges of TCS, OTC, or CIP.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control p ≤ 0.05	Shapiro-Wilk: Normality p ≥ 0.05	Levene's Test: Homogeneity of Variance p ≥ 0.05	Computed Power p ≥ 0.8
Binary Group 3: CIP Challenge	13,890 ppb Zn	7		✗ 0.001	✓ 0.1043	✓ 0.998
	13,890 ppb Zn, 0.5 ppb TCS	7	✗ 0.7208			
	13,890 ppb Zn, 0.5 ppb OTC	7	✗ 0.1171			
	13,890 ppb Zn, 0.064 ppb CIP	7	✗ 0.7568			
Binary Group 3: VAN Challenge	13,890 ppb Zn	9		✗ 0.0001	✗ 0.0028	✓ 0.999
	13,890 ppb Zn, 0.5 ppb TCS	9	✗ 0.464			
	13,890 ppb Zn, 0.5 ppb OTC	9	✗ 0.0947			
	13,890 ppb Zn, 0.064 ppb CIP	9	✗ 0.4035			
Binary Group 3: OTC Challenge	13,890 ppb Zn	7		✗ 0.0001	✓ 0.0519	✓ 0.999
	13,890 ppb Zn, 0.5 ppb TCS	7	✗ 0.5997			
	13,890 ppb Zn, 0.5 ppb OTC	7	✗ 0.6517			
	13,890 ppb Zn, 0.064 ppb CIP	7	✗ 0.3234			



a. Ciprofloxacin Challenge b. Vancomycin Challenge c. Oxytetracycline Challenge

Figure 4.19: *E. faecium* change in susceptibility to (a) ciprofloxacin (1.60% - 5.02%), (b) vancomycin (-1.68% - 1.53%), and (c) oxytetracycline (4.06% - 5.14%) following 24-hour acclimation to the Binary Group 4 mixture of the TCS MEC with the CCC of As, Cu, or Zn.

Table 4.25: ANOVA statistical analysis of results for the Kirby-Bauer Binary Group 4 Mixtures of MEC TCS with CCC range of As, Cu, or Zn.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control p ≤ 0.05	Shapiro-Wilk: Normality p ≥ 0.05	Levene's Test: Homogeneity of Variance p ≥ 0.05	Computed Power p ≥ 0.8
Binary Group 4: CIP Challenge	230 ppb TCS	7		✗ 0.0001	✓ 0.0815	✓ 0.999
	230 ppb TCS, 36 ppb As	7	✗ 0.8084			
	230 ppb TCS, 3.10 ppb Cu	7	✗ 0.1673			
	230 ppb TCS, 81.0 ppb Zn	4	✗ 0.3097			
Binary Group 4: VAN Challenge	230 ppb TCS	9		✓ 0.4383	✓ 0.1043	✓ 0.922
	230 ppb TCS, 36 ppb As	9	✗ 0.838			
	230 ppb TCS, 3.10 ppb Cu	9	✗ 0.9997			
	230 ppb TCS, 81.0 ppb Zn	5	✗ 0.9903			
Binary Group 4: OTC Challenge	230 ppb TCS	7		✗ 0.0001	✓ 0.0787	✓ 0.999
	230 ppb TCS, 36 ppb As	7	✗ 0.6585			
	230 ppb TCS, 3.10 ppb Cu	7	✗ 0.5917			
	230 ppb TCS, 81.0 ppb Zn	4	✗ 0.7134			

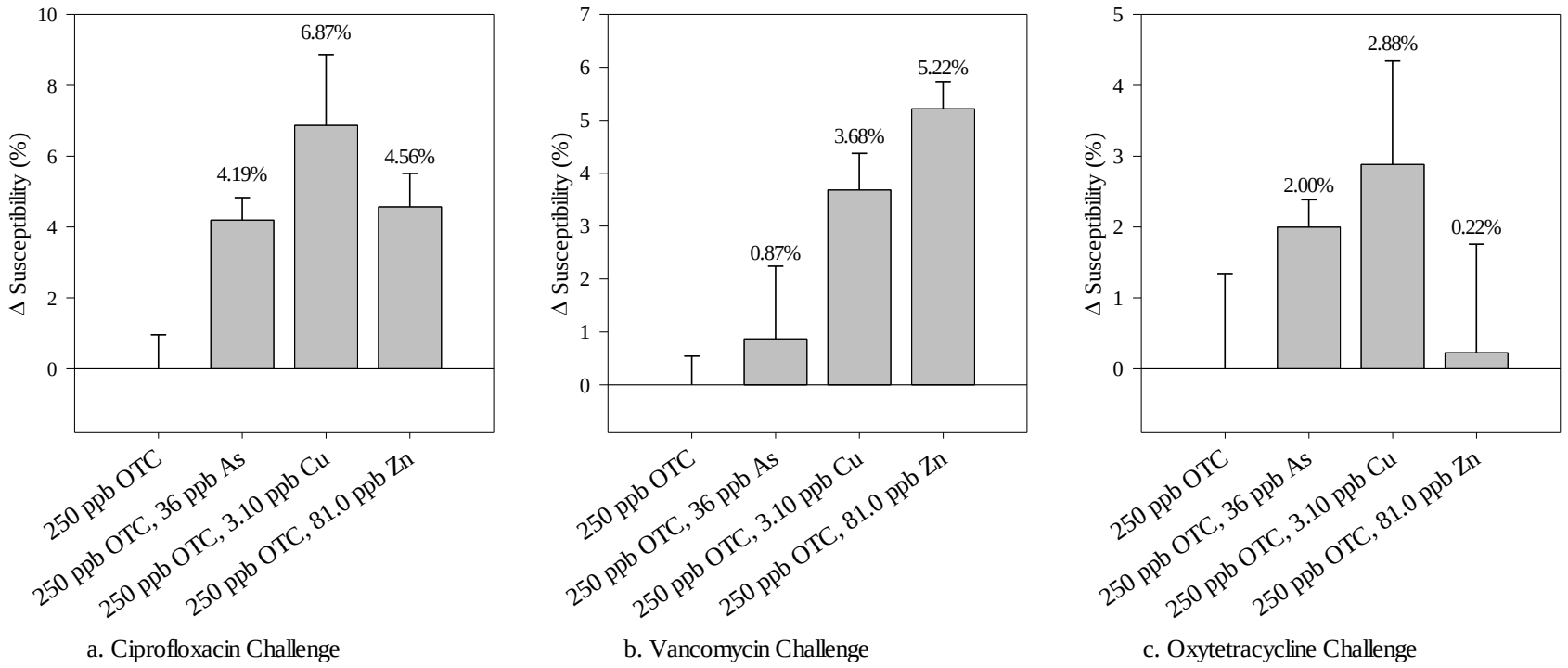


Figure 4.20: *E. faecium* change in susceptibility to (a) ciprofloxacin (4.19% - 6.87%), (b) vancomycin (0.87% - 5.22%), and (c) oxytetracycline (0.22% - 2.88%) following 24-hour acclimation to the Binary Group 5 mixture of the OTC MEC with the CCC of As, Cu, or Zn.

Table 4.26: ANOVA statistical analysis of results for the Kirby-Bauer Binary Group 5 Mixtures of MEC OTC with CCC range of As, Cu, or Zn.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control p ≤ 0.05	Shapiro-Wilk: Normality p ≥ 0.05	Levene's Test: Homogeneity of Variance p ≥ 0.05	Computed Power p ≥ 0.8
Binary Group 5: CIP Challenge	250 ppb OTC	7		✓ 0.2006	✓ 0.2287	✓ 0.995
	250 ppb OTC, 36 ppb As	5	✗ 0.3284			
	250 ppb OTC, 3.10 ppb Cu	7	✗ 0.0819			
	250 ppb OTC, 81.0 ppb Zn	7	✗ 0.24			
Binary Group 5: VAN Challenge	250 ppb OTC	8		✗ 0.0001	✓ 0.0612	✓ 0.999
	250 ppb OTC, 36 ppb As	5	✗ 0.9997			
	250 ppb OTC, 3.10 ppb Cu	8	✗ 0.1728			
	250 ppb OTC, 81.0 ppb Zn	8	✗ 0.0584			
Binary Group 5: OTC Challenge	250 ppb OTC	5		✗ 0.0001	✓ 0.0787	✓ 0.999
	250 ppb OTC, 36 ppb As	3	✗ 0.932			
	250 ppb OTC, 3.10 ppb Cu	7	✗ 0.789			
	250 ppb OTC, 81.0 ppb Zn	7	✗ 0.999			

antimicrobial challenges failed the Shapiro-Wilk test for normality, and the vancomycin challenge also failed Levene's test for homogeneity of variance (**Figure 4.21, Table 4.27**).

Discussion

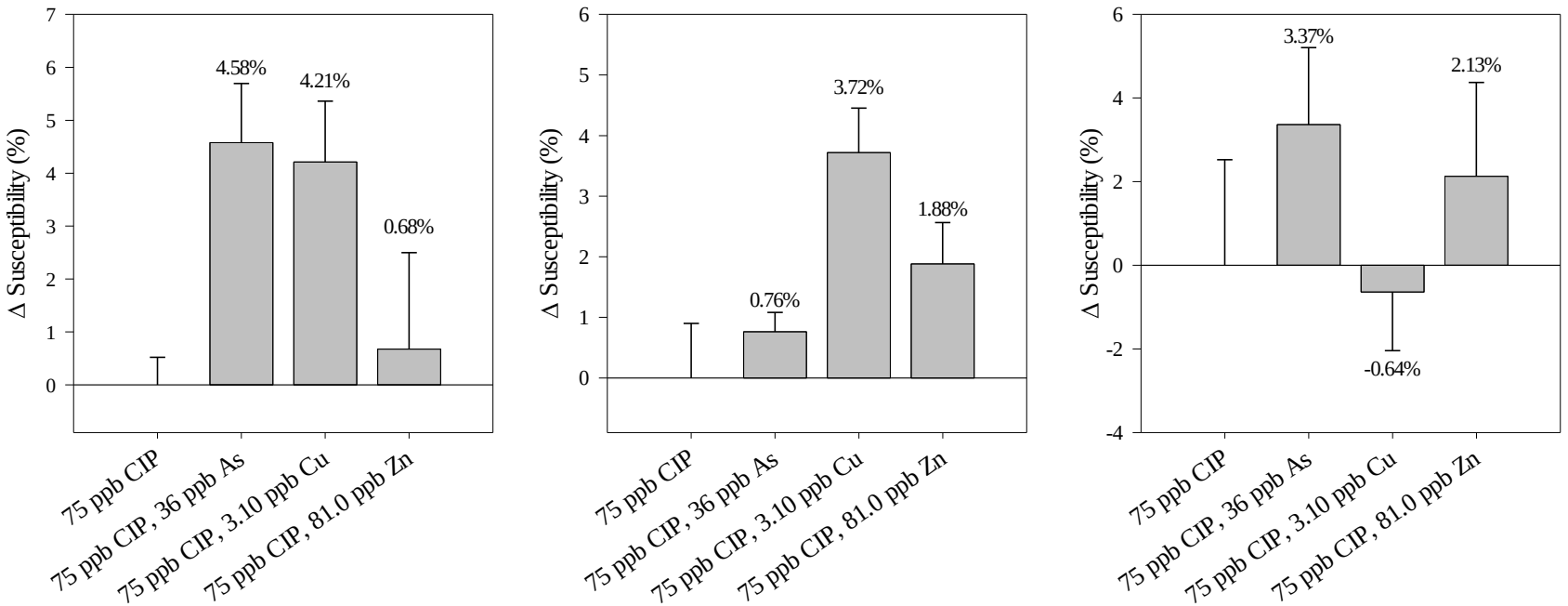
(a) Key Points

This strain of Efm appears to already be resistant to arsenic: The bacteria experienced only 9.79%I at 400% exceedance of published *Enterococcus* MIC. This is not surprising given the widespread nature of As concentrations in sediments throughout the southeastern US, with sediment quality guideline exceedances of > 28% in NOAA NERRS Sites in SC (Sanger et al. 1999). Thus, Enterococcus appears to be highly tolerant to As exposure. Additionally, there does not appear to be much difference in the binary mixture exposure groups that cannot be accounted for in single-exposure experiments. However, more experiments are needed to demonstrate repeatability of the binary mixture exposures as well as the antimicrobial resistance analysis.

(b) Confounding Factors

There are several potential confounding factors which may affect the statistical results in these data. As evidenced by some considerable variability between replicates in the single and binary exposure experiments, minute changes in treatment dilutions or inoculation density, likely stemming from material loss during pipetting, may exert an effect on overall responses to exposure. Smudges or minor scratches on polystyrene 96-well plates may change the optical density recorded by the spectrophotometer, as can settling of dead bacterial cells.

The Kirby-Bauer assay also has several points in the protocol which may introduce variability within and between replicates. Minor differences in the depth of Muller-Hinton



a. Ciprofloxacin Challenge b. Vancomycin Challenge c. Oxytetracycline Challenge

Figure 4.21: *E. faecium* change in susceptibility to (a) ciprofloxacin (0.68% - 4.58%), (b) vancomycin (0.76% - 3.72%), and (c) oxytetracycline (-0.64% - 3.37%) following 24-hour acclimation to the Binary Group 6 mixture of the CIP MEC with the CCC of As, Cu, or Zn.

Table 4.27: ANOVA statistical analysis of results for Kirby-Bauer Binary Group 6 Mixtures of MEC CIP with CCC range of As, Cu, or Zn.

Treatment Group	Treatment	*n*	Dunnett's Test: Significance from Control p ≤ 0.05	Shapiro-Wilk: Normality p ≥ 0.05	Levene's Test: Homogeneity of Variance p ≥ 0.05	Computed Power p ≥ 0.8
Binary Group 6: CIP Challenge	75 ppb CIP	7		✗ 0.0004	✓ 0.1852	✓ 0.998
	75 ppb CIP, 36 ppb As	7	✗ 0.2773			
	75 ppb CIP, 3.10 ppb Cu	7	✗ 0.4227			
	75 ppb CIP, 81.0 ppb Zn	7	✗ 0.9998			
Binary Group 6: VAN Challenge	75 ppb CIP	7		✗ 0.0001	✗ 0.0001	✓ 0.999
	75 ppb CIP, 36 ppb As	8	✗ 0.9974			
	75 ppb CIP, 3.10 ppb Cu	7	✗ 0.1784			
	75 ppb CIP, 81.0 ppb Zn	8	✗ 0.8409			
Binary Group 6: OTC Challenge	75 ppb CIP	7		✗ 0.0001	✓ 0.0522	✓ 0.985
	75 ppb CIP, 36 ppb As	7	✗ 0.8306			
	75 ppb CIP, 3.10 ppb Cu	7	✗ 0.9999			
	75 ppb CIP, 81.0 ppb Zn	7	✗ 0.9598			

agar from plate to plate will affect the diffusion of antibiotics through the media. Additionally, if the antimicrobial filter discs are not pressed onto the plate with the same pressure throughout, the antibiotic diffusion will again be affected. As with the prior set of experiments, slight changes in treatment dilutions or inoculation density may affect both the acclimation of the bacteria to the toxicants as well as the growth on the plate and interactions with the antimicrobial discs. Additionally, blurry margins on some inhibition zones made measurement difficult. If the protocol in this study is used for future work, the author recommends using a 150 mm susceptibility disc dispenser for more consistent application of discs and therefore more even diffusion of antimicrobials.

A high degree of variability and non-normal distribution indicate a need for more data points to successfully indicate repeatability of these experiments.

(c) Conclusions

The most significant takeaway from this series of experiments is that the effect of co-exposure to antimicrobials and trace metals is both distinctly dose-dependent and compound dependent. Additionally, it is evident that the Probable No-Effect Concentration of oxytetracycline, in particular, does have a drastic effect on the growth of *E. faecium*.

Studies conducted at WWTPs in SC discharging into impaired waters of the state in the 1990's had a dominant *E. coli* pattern of AMR of COT (Chlor-, Oxy-, and Tetracycline). Our results show similar tetracycline resistance to OTC in *Enterococcus*. Most importantly, oxytetracycline has drastic effects at environmentally relevant PNEC levels. *Enterococcus* interactions with oxytetracycline should be further studied to determine whether the levels of oxytetracycline in aquatic systems affect its effectiveness as a water quality indicator.

Chapter 5: Conclusions and Future Work

Summary

This study demonstrates that exposure of *Vibrio vulnificus* to antimicrobial and trace metal contaminants exerts enough stress for growth to be inhibited, even at concentrations below those which are not expected to have any considerable effect. Additionally, co-exposure to these two divergent contaminant classes results in often very different levels of inhibition, sometimes taking the bacteria from growth inhibition when exposed to one of the contaminants to growth stimulation in the presence of two together. The same binary mixture exposures may affect the susceptibility to clinically relevant antimicrobial products which are often detected in coastal waterways. Due to these factors, the authors are confident in rejecting both null hypotheses set forth in this study in relation to *V. vulnificus*.

As with the *Vibros*, *Enterococcus faecium* experienced significant growth inhibition following exposure to very low doses of either antimicrobials or trace metals. As such, the authors can reject the null hypothesis for Goal 1. Co-exposure to these two classes of toxins also sometimes had varying effects, although very few exhibited effects that were considerably different than the single exposures alone. These co-exposures also came with slight changes to susceptibility to clinically relevant antimicrobial products. However, due to nonparametric statistical analyses and low statistical significance, more experiments are required to confidently reject the second null hypothesis for this data set.

When comparing the results of the two microbes of interest, oxytetracycline appears to be the most important toxicant tested for both organisms. For instance, the *V. vulnificus* experiments exhibited very distinct interactions between oxytetracycline and copper at doses which may be frequently encountered in aquatic systems, especially near mariculture and agriculture operations. Meanwhile, very high inhibition was seen at PNEC doses in *E. faecium*.

Future Work

(a) Factors for Further Consideration

The findings in this study bring forward several points for deliberation as planning for future experiments moves forward.

First, chemical risk assessments may need to include microbes as a "most sensitive organism." Most aquatic risk assessments look at early life stage fish and shellfish as their most sensitive organism, but levels of toxicants in this study largely fall in the allowable range. With this in mind, researchers should start looking more deeply into how aquatic toxicants affect the microbial community, especially in terms of antimicrobial resistance, before declaring them safe.

Second, when antimicrobial risks are evaluated, researchers should be looking at extremely low concentrations rather than the minimum inhibitory concentration. It is well established that high doses of antimicrobials tend to be toxic to bacteria. However, as shown in this study, even Probable No-Effect Concentrations of antimicrobials can elicit effects on microbial growth. These effects may be an increase in growth compared to a negative control, or they may come in the form of changes in susceptibility levels to clinically important antibiotics.

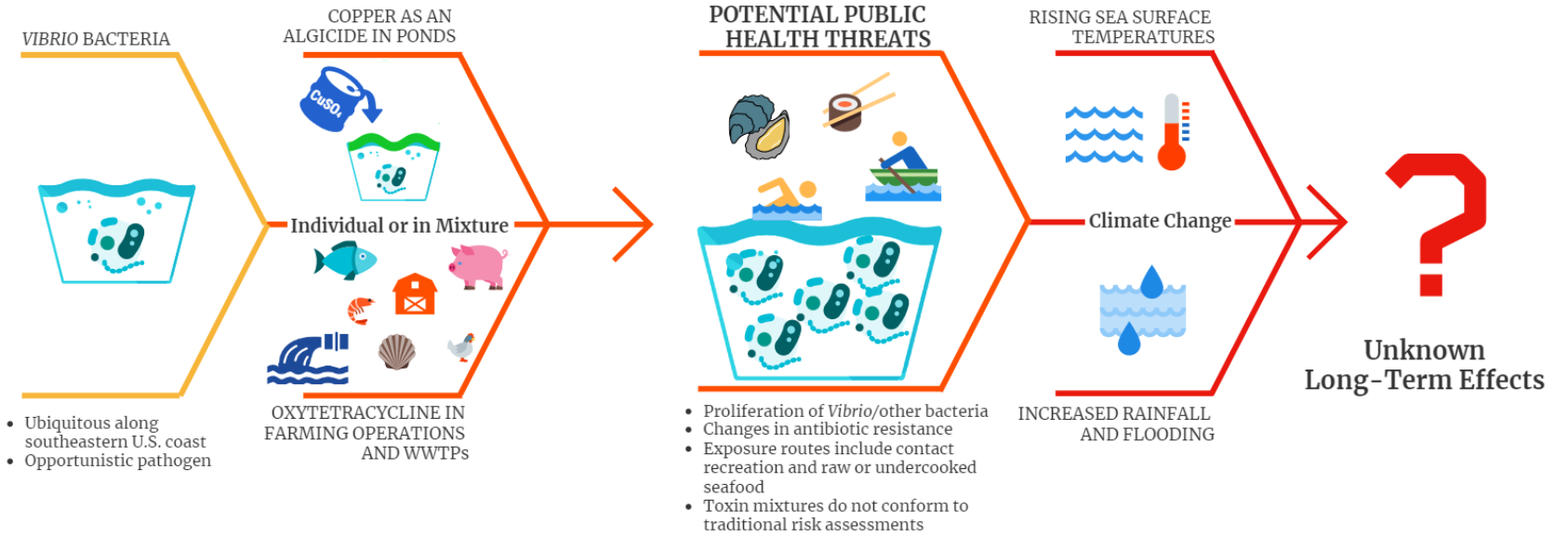


Figure 5.1: Summary of findings and potential future implications.

Third, the levels of inhibition seen in *E. faecium* exposure to oxytetracycline is concerning when taken in consideration with its status as a common water quality indicator. These bacteria are highly susceptible to a contaminant very commonly used in mariculture and agriculture operations and are frequently detected in WWTPs, at a concentration which may well be found in the same waterways being tested for water quality. It can then be inferred from our results that measured levels of *Enterococcus* bacteria in water quality analyses may be artificially suppressed, which suggests that this important water quality indicator may under-report altered water quality conditions for microbial pollution and thus not fully protect public health. Environmental managers and planners may need to re-evaluate how these bacteria are used in determining the safety of waterways for shellfish harvesting and recreation. The regulation of molluscan shellfish has continued to use fecal coliforms in lieu of *Enterococcus* species due to concerns about its relationship to sediment scour and its effectiveness as an indicator in highly turbid waters, including waters containing high levels of phytoplankton (Mote et al. 2012). This study further shows that the sensitivity of *Enterococci* to low levels of OTC in the environment – levels which may inhibit its growth – bring the reliability of this water quality indicator further into question.

Finally, oxytetracycline has been found in mariculture effluents at concentrations up to 2.3 ppb and will quickly be diluted in runoff associated with rainfall and as the land-based discharges merge with larger waterways. Meanwhile, copper in the form of copper sulfate is a common weapon in the fight against harmful algal blooms (HABs) in ponds and lakes. The conclusion may be drawn that increased aquaculture practices and HAB treatments could become important factors in predicting future microbial hazards in aquatic ecosystems in terms of important ecosystem services: swimability and fishability. The co-

occurrence of Cu and OTC in aquatic ecosystems will have a major impact on bacterial water quality in terms of enhancing antibiotic resistance in microbes. This effect is occurring as more and more people are moving to the coast, particularly senior citizens who may have altered or reduced immune systems, making them more susceptible to microbial exposure in seafood and via contact recreation. Continued vigilance in educating the public about this threat and in reducing discharges of Cu and OTC into aquatic ecosystems is paramount to reducing/managing this threat to public health.

(b) Genotypic Analysis for Antimicrobial Resistance

Future studies should build on the information gained from the phenotypic data contained herein by examining the genotypic effects exerted by exposure to these combinations of toxicants, especially those which were synergistic. During the course of this project, three 1 mL stocks of the bacterial suspensions used to inoculate the Kirby-Bauer plates were kept at -80°C with a final concentration of 25% glycerol to preserve them for such future research.

Specifically, an analysis of changes in virulence gene expression is crucial to understanding the full impact of these data on public health. Genes such as *PilF* in *V. vulnificus* or *esp* in *E. faecium* are excellent candidates for further study. These genes are frequently detected in disease-causing strains of the bacteria and are often utilized as estimates of virulence in humans (Vankerckhoven et al. 2004; Sanjuan et al. 2009; Roig et al. 2010; Baker-Austin et al. 2012; Al-Talib et al. 2015; Haghi et al. 2019).

Incorporation of genetic virulence into current *Vibrio* forecast models is needed to protect public health, as those existing models only predict bacterial abundance. In 1854, Sir John Snow fashioned a new way of thinking about a public health issue, which we know

today was *Vibrio cholerae*, by using spatial mapping of a disease outbreak to discern how to identify methods to control the associated illness. Today, we need similar innovation to create 21st century gene maps of *Vibrios* to improve predictions of where high levels of virulence and antibiotic resistance occur as well as what environmental and climate factors increase their abundance. This mapping will also forecast antimicrobial resistance and virulence and provide the ability to develop an early warning system of key growth characteristics and gene expression changes, which will enable high risk individuals to be notified and avoid exposure.

(c) Climate Change

With the worsening of climate change emerge potential further problems with stormwater ponds. Increases in the frequency and severity of extreme rainfall events (Risser and Wehner 2017; Patricola and Wehner 2018) and sea level rise is resulting in a subsequent increase in contaminant discharge and loading to receiving water catchments (Sharma et al. 2016). Flooding events following extreme rainfall can flush both new and sequestered contaminants to nearby aquatic ecosystems (Baalousha et al. 2015), where bacteria acclimated to those toxicants may survive, replicate, and pass on acquired resistance genes (Aminov 2010; McDaniel et al. 2010). In addition, evidence is emerging indicating a relationship between increased occurrence of microplastic pollution in aquatic ecosystems and the prevalence of bacterial growth and biofilms on plastic surfaces, especially *Vibrio* bacteria (Amaral-Zettler et al. 2020). In addition, increased nutrient loads will increase the abundance of *Vibrios* (Conrad and Harwood 2022) and increase the expression of biofilm production genes in *Vibrio* bacteria, generally associated with higher levels of antibiotic resistance (Correa Velez and Norman 2021). If increased exposure to

trace metals and antimicrobials continues, increased antibiotic resistance effects may be magnified, particularly in *Vibrio* bacteria.

Aquatic bacteria, meanwhile, are experiencing something of a renaissance stemming from increasing sea surface temperatures and saltwater intrusion into freshwater sources. For example, *Vibrio* bacteria flourish in warm, brackish environments like those found in the estuaries of the southeastern U.S. (Randa et al. 2004; Chase and Harwood 2011). As global sea surface temperatures rise, *Vibrio* bacteria are being found at higher latitudes and further inland than ever before, as well as over longer periods of each year (Baker-Austin et al. 2013; Vezzulli et al. 2013; Vezzulli et al. 2016; Baker-Austin et al. 2017; Deeb et al. 2018; King et al. 2019). Expanding range combined with an influx of nutrients from agricultural and residential fertilizer use as well as sublethal stressors in the form of chemical contaminants provide ample opportunity for aquatic microbes -- including potential pathogens like *Vibrio* and *Enterococcus* bacteria -- to improve resilience and develop resistance to a number of chemical stressors.

Climate change will also affect the biogeochemical cycling of these chemical contaminants within the environment, particularly relating to the effect of trace metal cycling (McComb et al. 2014; Hassett et al. 2018). In the future, it will be important to build upon the data presented in this study by determining different environmental effects on the organisms under a variety of climate change scenarios including increased temperature, salinity, and pH, along with increased nutrient levels associated with increased urbanization (Sandifer and Scott 2021). Only with further investigation will the full impacts of antimicrobial resistance within a changing coastal environment in the 21st century truly begin to be understood.

References

Aarestrup FM, Hasman H. 2004. Susceptibility of different bacterial species isolated from food animals to copper sulphate, zinc chloride and antimicrobial substances used for disinfection. Vet Microbiol. 100:83–89. doi:10.1016/j.vetmic.2004.01.013.

Abdalkader D, Al-Saedi F. Antibacterial Effect of Different Concentrations of Zinc Sulfate on Multidrug Resistant Pathogenic Bacteria. Syst Rev Pharm. 11:2020. doi:10.5530/srp.2020.3.32.

Agwuh KN, MacGowan A. 2006. Pharmacokinetics and pharmacodynamics of the tetracyclines including glycylcyclines. J Antimicrob Chemother. 58(2):256–265. doi:10.1093/jac/dkl224.

Al-Talib H, Zuraina N, Kamarudin B, Yean C. 2015. Genotypic Variations of Virulent Genes in *Enterococcus faecium* and *Enterococcus faecalis* Isolated from Three Hospitals in Malaysia. Adv Clin Exp Med. 24(1):121–127. doi:10.17219/acem/38162. http://10.0.67.67/acem/38162.

Albert A, Falk JE, Rubbo SD. 1944. Antibacterial Action of Arsenic. Nat 1944 1533893. 153(3893):712–713. doi:10.1038/153712a0. [accessed 2022 Jan 25]. https://www.nature.com/articles/153712a0.

Amaral-Zettler LA, Zettler ER, Mincer TJ. 2020. Ecology of the plastisphere. Nat Rev Microbiol 2020 183. 18(3):139–151. doi:10.1038/s41579-019-0308-0. [accessed 2022 Jun 24]. https://www.nature.com/articles/s41579-019-0308-0.

American Meteorological Society (AMS). 2012. Flashy stream - Glossary of Meteorology. Gloss Meteorol. [accessed 2022 Jul 5]. https://glossary.ametsoc.org/wiki/Flashy_stream.

Aminov RI. 2010. A brief history of the antibiotic era: lessons learned and challenges for the future. Front Microbiol. 1:134. doi:10.3389/fmicb.2010.00134. [accessed 2017 Apr 19]. http://www.ncbi.nlm.nih.gov/pubmed/21687759.

Apeti D, Wirth E, Leight A, Mason A, Pisarski E. 2018. An Assessment of Contaminants of Emerging Concern in Chesapeake Bay, MD and Charleston Harbor, SC. NOAA Tech Memo NOS NCCOS 240. [accessed 2022 Aug 3]. https://drive.google.com/file/d/18PC32q8uZyTAKQJxEQO7DvR2YHGoqQNu/view.

Applerot G, Lellouche J, Lipovsky A, Nitzan Y, Lubart R, Gedanken A, Banin E. 2012. Understanding the antibacterial mechanism of CuO nanoparticles: revealing the route of

induced oxidative stress. Small. 8(21):3326–3337. doi:10.1002/SMLL.201200772. [accessed 2022 Jan 26]. https://pubmed.ncbi.nlm.nih.gov/22888058/.

Baalousha M, Mcneal S, Scott GI. 2015. " An Assessment of Nonpoint Source Pollution Contaminants in Stormwater Pond Systems in South Carolina " Prepared For : The South Carolina Sea Grant Consortium 287 Meeting Street Charleston , SC 29401 Prepared By : Center for Environmental Nanomaterials R.

Baer SK. 2019. Scientists Say Climate Change Is Driving An Increase In Deadly Flesh-Eating Bacteria. BuzzFeed News. [accessed 2020 Jan 8]. https://www.buzzfeednews.com/article/skbaer/flesh-eating-bacteria-climate-change-vibrio-warming.

Baker-Austin C, Lemm E, Hartnell R, Lowther J, Onley R, Amaro C, Oliver JD, Lees D. 2012. pilF polymorphism-based real-time PCR to distinguish *Vibrio vulnificus* strains of human health relevance. Food Microbiol. 30(1):17–23. doi:10.1016/j.fm.2011.09.002. http://10.0.3.248/j.fm.2011.09.002.

Baker-Austin C, Mcarthur J V, Lindell AH, Wright MS, Tuckfield RC, Gooch J, Warner L, Oliver J, Stepanauskas R. 2009. Multi-site Analysis Reveals Widespread Antibiotic Resistance in the Marine Pathogen *Vibrio vulnificus*. :151–159. doi:10.1007/s00248-008-9413-8.

Baker-Austin C, Trinanes J, Gonzalez-Escalona N, Martinez-Urtaza J. 2017. Non-Cholera Vibrios: The Microbial Barometer of Climate Change. Trends Microbiol. 25(1):76–84. doi:10.1016/j.tim.2016.09.008. http://dx.doi.org/10.1016/j.tim.2016.09.008.

Baker-Austin C, Trinanes JA, Taylor NGH, Hartnell R, Siitonen A, Martinez-Urtaza J. 2013. Emerging Vibrio risk at high latitudes in response to ocean warming. Nat Clim Chang. 3(1):73–77. doi:10.1038/nclimate1628.

Baker-Austin C, Wright MS, Stepanauskas R, Mcarthur J V. 2006. Co-selection of antibiotic and metal resistance. Trends Microbiol. 14(4):176–182. doi:10.1016/j.tim.2006.02.006.

Bayer Pharmaceuticals. 2004. CIPRO ® I.V. West Haven, CT.

Bengtsson-Palme J, Larsson DGJ. 2016. Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation. Environ Int. 86:140–149. doi:10.1016/j.envint.2015.10.015.

Benhalima L, Amri S, Bensouilah M, Ouzrout R. 2019. Antibacterial effect of copper sulfate against multi-drug resistant nosocomial pathogens isolated from clinical samples. Pakistan J Med Sci. 35(5):1322. doi:10.12669/PJMS.35.5.336. [accessed 2022 Jan 26]. /pmc/articles/PMC6717487/.

Bier N, Bechlars S, Diescher S, Klein F, Hauk G, Duty O, Strauch E, Dieckmann R. 2013. Genotypic Diversity and Virulence Characteristics of Clinical and Environmental *Vibrio*

vulnificus Isolates from the Baltic Sea Region. Appl Environ Microbiol. 79(12):3570–3581. doi:10.1128/aem.00477-13. http://10.0.4.104/aem.00477-13.

Boehm AB, Sassoubre LM. 2014. Enterococci as Indicators of Environmental Fecal Contamination. Enterococci From Commensals to Lead Causes Drug Resist Infect.:1–21. [accessed 2017 Jun 20]. http://www.ncbi.nlm.nih.gov/pubmed/24649503.

Booth DB, Hartley D, Jackson R. 2002. Forest cover, impervious-surface area, and the mitigation of stormwater impacts. J Am Water Resour Assoc. 38(3):835–845. doi:10.1111/j.1752-1688.2002.tb01000.x.

Borne KE, Fassman-Beck EA, Tanner CC. 2014. Floating Treatment Wetland influences on the fate of metals in road runoff retention ponds. Water Res. 48(1):430–442. doi:10.1016/j.watres.2013.09.056. http://dx.doi.org/10.1016/j.watres.2013.09.056.

Bradford SA, Segal E, Zheng W, Wang Q, Hutchins SR. 2008. Reuse of concentrated animal feeding operation wastewater on agricultural lands. J Environ Qual. 37(SUPPL. 5). doi:10.2134/jeq2007.0393.

Bureau USC. 2020. U.S. Census Bureau QuickFacts: Charleston County, South Carolina. [accessed 2022 Jan 25]. https://www.census.gov/quickfacts/fact/table/dorchestercountysouthcarolina,berkeleycountysouthcarolina,charlestoncountysouthcarolina/POP010220.

Burridge L, Weis JS, Cabello F, Pizarro J, Bostick K. 2010. Chemical use in salmon aquaculture: A review of current practices and possible environmental effects. doi:10.1016/j.aquaculture.2010.05.020.

Byappanahalli MN, Nevers MB, Korajkic A, Staley ZR, Harwood VJ. 2012. Enterococci in the Environment. Microbiol Mol Biol Rev. 76(4):685–706. doi:10.1128/mmbr.00023-12.

Carey DE, McNamara PJ. 2014. The impact of triclosan on the spread of antibiotic resistance in the environment. Front Microbiol. 5(DEC):1–11. doi:10.3389/fmicb.2014.00780.

CDC. 2019a. Antibiotic Resistance Threats in the United States, 2019. Atlanta, GA. [accessed 2020 Jan 8]. www.cdc.gov/DrugResistance/Biggest-Threats.html.

CDC. 2019b. *Vibrio vulnificus* & Wounds | Vibrio Illness (Vibriosis) | CDC. [accessed 2020 Jan 8]. https://www.cdc.gov/vibrio/wounds.html.

Chase E, Harwood VJ. 2011. Comparison of the effects of environmental parameters on growth rates of *Vibrio vulnificus* biotypes I, II, and III by culture and quantitative PCR analysis. Appl Environ Microbiol. 77(12):4200–4207. doi:10.1128/AEM.00135-11.

Chase E, Young S, Harwood VJ. 2015. Sediment and Vegetation as Reservoirs of *Vibrio vulnificus* in the Tampa Bay Estuary and Gulf of Mexico. Appl Environ Microbiol.

81(7):2489. doi:10.1128/AEM.03243-14. [accessed 2022 Jan 25]. /pmc/articles/PMC4357930/.

Chaudhry SB, Veve MP, Wagner JL. 2019. Cephalosporins: A Focus on Side Chains and β-Lactam Cross-Reactivity. Pharmacy. 7(3):103. doi:10.3390/PHARMACY7030103.

Clary J, Pitt R, Steets B. 2014. Pathogens in Urban Stormwater Systems. Denver, CO. http://higherlogicdownload.s3.amazonaws.com/EWRINSTITUTE/c3dac190-e71a-44cc-a432-3ee9a640acfd/UploadedImages/Final Pathogens Paper August 2014 _MinorRev9-22-14.pdf.

CLSI. 2016. Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria, Volume 3. Wayne, PA. [accessed 2020 Jan 29]. www.clsi.org.

CLSI. 2017. M100 Performance Standards for Antimicrobial Susceptibility Testing An informational supplement for global application developed through the Clinical and Laboratory Standards Institute consensus process. 27th Edition. Wayne, PA. [accessed 2020 Jan 29]. www.clsi.org.

Conrad JW, Harwood VJ. 2022. Sewage Promotes *Vibrio vulnificus* Growth and Alters Gene Transcription in *Vibrio vulnificus* CMCP6. Microbiol Spectr. 10(1). doi:10.1128/SPECTRUM.01913-21. [accessed 2022 Aug 4]. /pmc/articles/PMC8849060/.

Cooper ER, Siewicki TC, Phillips K. 2008. Preliminary risk assessment database and risk ranking of pharmaceuticals in the environment. Sci Total Environ. 398(1–3):26–33. doi:10.1016/j.scitotenv.2008.02.061.

Correa Velez KE, Norman RS. 2021. Transcriptomic Analysis Reveals That Municipal Wastewater Effluent Enhances *Vibrio vulnificus* Growth and Virulence Potential. Front Microbiol. 12. doi:10.3389/FMICB.2021.754683. [accessed 2022 Aug 4]. https://pubmed.ncbi.nlm.nih.gov/34759904/.

Dash P, Avunje S, Tandel RS, Sandeep KP, Panigrahi A. 2017. Biocontrol of Luminous Vibriosis in Shrimp Aquaculture: A Review of Current Approaches and Future Perspectives. Rev Fish Sci Aquac. 25(3):245–255. doi:10.1080/23308249.2016.1277973.

Deeb R, Tufford D, Scott GI, Moore JG, Dow K. 2018. Impact of Climate Change on *Vibrio vulnificus* Abundance and Exposure Risk. Estuaries and Coasts. 41(8):2289–2303. doi:10.1007/s12237-018-0424-5. https://doi.org/10.1007/s12237-018-0424-5.

DeLorenzo ME, Brooker J, Chung KW, Kelly M, Martinez J, Moore JG, Thomas M. 2016. Exposure of the grass shrimp, *Palaemonetes pugio*, to antimicrobial compounds affects associated Vibrio bacterial density and development of antibiotic resistance. Environ Toxicol. 31(4):469–477. doi:10.1002/tox.22060. https://doi.org/10.1002/tox.22060.

Diamond JM, Latimer HA, Munkittrick KR, Thornton KW, Bartell SM, Kidd KA. 2011. Prioritizing contaminants of emerging concern for ecological screening assessments.

Environ Toxicol Chem. 30(11):2385–2394. doi:10.1002/ETC.667. [accessed 2022 Aug 4]. https://pubmed.ncbi.nlm.nih.gov/22002713/.

Donadio S, Sosio M. 2009 Jan 1. Glycopeptides, Antimicrobial. Encycl Microbiol.:455–471. doi:10.1016/B978-012373944-5.00040-7.

Dunham B. 2013. Withdrawal of Approval of New Animal Drug Applications; Carbarsone; Roxarsone. [accessed 2022 Mar 14]. https://www.govinfo.gov/content/pkg/FR-2013-11-22/pdf/2013-27917.pdf.

Eghianruwa K. 2014. Ciprofloxacin. In: Essential Drug Data for Rational Therapy in Veterinary Practice - Kingsley Eghianruwa - Google Books. Bloomington, IN: AuthorHouse UK Ltd. p. 99. [accessed 2022 Mar 15]. https://books.google.com/books?id=CtfIAgAAQBAJ&pg=PA97&lpg=PA97&dq=ciprofloxacin+stability+in+water+refrigerated&source=bl&ots=bCb3uHiFUH&sig=ACfU3U0x10ND6xSjwImfz-SbKMIzHxrsdQ&hl=en&sa=X&ved=2ahUKEwiD2d252cj2AhWVJTQIHRTjATo4ChDoAXoECBIQAw#v=onepage&q&f=false.

EPA. 2004. National Recommended Water Quality Criteria. Washington, D.C.

Ermini ML, Voliani V. 2021. AntimicrobialNano-Agents: The Copper Age. ACS Nano. 15(4):6008. doi:10.1021/ACSNANO.0C10756. [accessed 2022 Jan 26]. /pmc/articles/PMC8155324/.

FDA. 2014. Summary Report on Antimicrobials Sold or Distributed for Use in Food-Producing Animals.

FDA. 2017. Safety and Effectiveness of Health Care Antiseptics; Topical Antimicrobial Drug Products for Over-the-Counter Human Use. Fed Regist. 82(243):60474–60503. [accessed 2022 Aug 4]. www.regulations.gov.

Froelich BA, Noble RT. 2016. Vibrio bacteria in raw oysters: Managing risks to human health. Philos Trans R Soc B Biol Sci. 371(1689). doi:10.1098/rstb.2015.0209.

Fulladosa E, Murat JC, Martínez M, Villaescusa I. 2005. Patterns of metals and arsenic poisoning in Vibrio fischeri bacteria. Chemosphere. 60(1):43–48. doi:10.1016/j.chemosphere.2004.12.026.

Garbarino JR, Bednar AJ, Rutherford DW, Beyer RS, Wershaw RL. 2003. Environmental Fate of Roxarsone in Poultry Litter. I. Degradation of Roxarsone during Composting. Environ Sci Technol. 37(8):1509–1514. doi:10.1021/ES026219Q. [accessed 2022 Mar 14]. https://pubs.acs.org/doi/abs/10.1021/es026219q.

Grass G, Rensing C, Solioz M. 2011. Metallic Copper as an Antimicrobial Surface. Appl Environ Microbiol. 77(5):1541. doi:10.1128/AEM.02766-10. [accessed 2022 Jan 26]. /pmc/articles/PMC3067274/.

Haghi F, Lohrasbi V, Zeighami H. 2019. High incidence of virulence determinants, aminoglycoside and vancomycin resistance in enterococci isolated from hospitalized patients in Northwest Iran. BMC Infect Dis. 19(1). doi:10.1186/s12879-019-4395-3. http://10.0.4.162/s12879-019-4395-3.

Hassett BA, Sudduth EB, Somers KA, Urban DL, Violin CR, Wang SY, Wright JP, Cory RM, Bernhardt ES. 2018. Pulling apart the urbanization axis: patterns of physiochemical degradation and biological response across stream ecosystems. https://doi.org/101086/699387. 37(3):653–672. doi:10.1086/699387. [accessed 2022 Aug 17]. https://www.journals.uchicago.edu/doi/10.1086/699387.

Hedgespeth ML, Sapozhnikova Y, Pennington P, Clum A, Fairey A, Wirth E. 2012. Pharmaceuticals and personal care products (PPCPs) in treated wastewater discharges into Charleston Harbor, South Carolina. Sci Total Environ. 437:1–9. doi:10.1016/j.scitotenv.2012.07.076. http://dx.doi.org/10.1016/j.scitotenv.2012.07.076.

Hudzicki J. 2009. Kirby-Bauer Disk Diffusion Susceptibility Test Protocol. [accessed 2020 Jan 8]. www.atcc.org.

Jones IA, Joshi LT. 2021. Biocide Use in the Antimicrobial Era: A Review. Molecules. 26(8). doi:10.3390/MOLECULES26082276. [accessed 2022 Jan 25]. /pmc/articles/PMC8071000/.

Jones MK, Oliver JD. 2009. *Vibrio vulnificus*: Disease and pathogenesis. Infect Immun. 77(5):1723–1733. doi:10.1128/IAI.01046-08.

Jones MK, Warner E, Oliver JD. 2008. Survival of and in situ gene expression by *Vibrio vulnificus* at varying salinities in estuarine environments. Appl Environ Microbiol. 74(1):182–187. doi:10.1128/AEM.02436-07.

Kelly KR, Brooks BW. 2018. Global Aquatic Hazard Assessment of Ciprofloxacin: Exceedances of Antibiotic Resistance Development and Ecotoxicological Thresholds. 1st ed. Elsevier Inc. http://dx.doi.org/10.1016/bs.pmbts.2018.07.004.

King M, Rose L, Fraimow H, Nagori M, Danish M, Doktor K. 2019. *Vibrio vulnificus* Infections From a Previously Nonendemic Area. Ann Intern Med. doi:10.7326/L19-0133. https://login.pallas2.tcl.sc.edu/login?url=http://search.ebscohost.com/login.aspx?direct=true&db=mnh&AN=31207614&site=eds-live.

Kitiyodom S, Khemtong S, Wongtavatchai J, Chuanchuen R. 2010. Characterization of antibiotic resistance in Vibrio spp. isolated from farmed marine shrimps (Penaeus monodon). FEMS Microbiol Ecol. 72(2):219–227. doi:10.1111/j.1574-6941.2010.00846.x.

Koh T, Tan J, Hong C, Wang W, Nather A. 2017. Early clinical manifestations of vibrio necrotising fasciitis: a case series. Singapore Med J. doi:10.11622/smedj.2017055. http://10.0.45.102/smedj.2017055.

Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: A national reconnaissance. Environ Sci Technol. 36(6):1202–1211. doi:10.1021/es011055j.

Landers TF, Cohen B, Wittum TE, Larson EL. 2012. A review of antibiotic use in food animals: perspective, policy, and potential. Public Health Rep. 127(1):4–22. doi:10.1177/003335491212700103. [accessed 2017 Apr 25]. http://www.ncbi.nlm.nih.gov/pubmed/22298919.

Long ER. 1995. Incidence of Adverse Biological Effects Within Ranges of Chemical Concentratoins in Marine and Esuarine Sediments. Environ Manage. 19(1):81–97.

Long ER, Morgan Lee G. 1991. The Potential for Biological Effects of Sediment-Sorbed Contaminants Tested in the National Status and Trends Program. Seattle.

Loria K. 2018. Woman dies of vibrio necrotizing fasciitis infection from raw oysters. Bus Insid. [accessed 2020 Jan 8]. https://www.businessinsider.com/woman-dies-of-vibrio-necrotizing-fasciitis-infection-eating-raw-oysters-2018-1.

Lund B, Billström H, Edlund C. 2006. Increased conjugation frequencies in clinical *Enterococcus faecium* strains harbouring the enterococcal surface protein gene esp. Clin Microbiol Infect. 12(6):588–591. doi:10.1111/j.1469-0691.2006.01436.x. http://10.0.4.87/j.1469-0691.2006.01436.x.

Macdonald DD, Carr RS, Calder FD, Long ER, Ingersoll CG. 1996. Development and evaluation of sediment quality guidelines for Florida coastal waters. Ecotoxicology. 5(4):253–278. doi:10.1007/BF00118995. [accessed 2022 Aug 4]. https://pubmed.ncbi.nlm.nih.gov/24193815/.

Maruya KA, Schlenk D, Anderson PD, Denslow ND, Drewes JE, Olivieri AW, Scott GI, Snyder SA. 2014. An adaptive, comprehensive monitoring strategy for chemicals of emerging concern (CECs) in California's Aquatic Ecosystems. Integr Environ Assess Manag. 10(1):69–77. doi:10.1002/IEAM.1483. [accessed 2022 Aug 4]. https://experts.arizona.edu/en/publications/an-adaptive-comprehensive-monitoring-strategy-for-chemicals-of-em.

McComb J, Alexander TC, Han FX, Tchounwou PB. 2014. Understanding Biogeochemical Cycling of Trace Elements and Heavy Metals in Estuarine Ecosystems. J bioremediation Biodegrad. 5(03). doi:10.4172/2155-6199.1000E148. [accessed 2022 Aug 16]. /pmc/articles/PMC4326106/.

McDaniel LD, Young E, Delaney J, Ruhnau F, Ritchie KB, Paul JH. 2010. High Frequency of Horizontal Gene Transfer in the Oceans. Science (80-). 330(6000). [accessed 2017 Apr 25]. http://science.sciencemag.org/content/330/6000/50.

Merck/Werth. 2020. Cephalosporins - Infectious Diseases - Merck Manuals Professional Edition. [accessed 2022 Jan 25]. https://www.merckmanuals.com/professional/infectious-

diseases/bacteria-and-antibacterial-drugs/cephalosporins.

Montero DA, Arellano C, Pardo M, Vera R, Gálvez R, Cifuentes M, Berasain MA, Gómez M, Ramírez C, Vidal RM. 2019. Antimicrobial properties of a novel copper-based composite coating with potential for use in healthcare facilities 06 Biological Sciences 0605 Microbiology 11 Medical and Health Sciences 1117 Public Health and Health Services. Antimicrob Resist Infect Control. 8(1):1–10. doi:10.1186/S13756-018-0456-4/TABLES/4. [accessed 2022 Jan 26]. https://aricjournal.biomedcentral.com/articles/10.1186/s13756-018-0456-4.

Morandi S, Brasca M, Alfieri P, Lodi R, Tamburini A. 2005. Influence of pH and temperature on the growth of *Enterococcus faecium* and *Enterococcus faecalis*. 85(3):181–192. doi:10.1051/lait:2005006. [accessed 2022 Jun 28]. http://dx.doi.org/10.1051/lait:2005006.

Mote BL, Turner JW, Lipp EK. 2012. Persistence and Growth of the Fecal Indicator Bacteria Enterococci in Detritus and Natural Estuarine Plankton Communities. Appl Environ Microbiol. 78(8):2569. doi:10.1128/AEM.06902-11. [accessed 2022 Aug 17]. /pmc/articles/PMC3318816/.

Muhling BA, Jacobs J, Stock CA, Gaitan CF, Saba VS. 2017. Projections of the future occurrence, distribution, and seasonality of three Vibrio species in the Chesapeake Bay under a high-emission climate change scenario. GeoHealth. 1(7):278–296. doi:10.1002/2017GH000089. [accessed 2022 Aug 4]. https://pubmed.ncbi.nlm.nih.gov/32158993/.

Murray CJ, Shunji Ikuta K, Sharara F, Swetschinski L, Robles Aguilar G, Gray A, Han C, Bisignano C, Rao P, Wool E, et al. 2022. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet. 0(0). doi:10.1016/S0140-6736(21)02724-0. [accessed 2022 Jan 22]. http://www.thelancet.com/article/S0140673621027240/fulltext.

National Research Council. 2002. Biosolids Applied to Land: Advancing Standards and Practices. Washington, D.C.: National Academic Press.

Nietch CT, Quinlan EL, Lazorchak JM, Impellitteri CA, Raikow D, Walters D. 2013. Effects of a chronic lower range of triclosan exposure on a stream mesocosm community. 32(12):2874–2887. doi:10.1002/etc.2385. http://10.0.3.234/etc.2385.

O'Kane C. 2019. Flesh-eating bacteria death: Man dies from necrotizing fasciitis infection 48 hours after beach trip in Florida, family says. CBS News. [accessed 2020 Jan 8]. https://www.cbsnews.com/news/man-dies-from-flesh-eating-bacteria-48-hours-after-florida-beach-trip-family-says-2019-07-13/.

O 'Neill J. 2016. TACKLING DRUG-RESISTANT INFECTIONS GLOBALLY: FINAL REPORT AND RECOMMENDATIONS THE REVIEW ON ANTIMICROBIAL RESISTANCE. [accessed 2017 Apr 20]. https://amr-review.org/sites/default/files/160525_Final paper_with cover.pdf.

Panagiotaras D, Nikolopoulos D. 2015. Arsenic Occurrence and Fate in the Environment; A Geochemical Perspective. J Earth Sci Clim Chang 2015 64. 6(4):1–9. doi:10.4172/2157-7617.1000269. [accessed 2022 Mar 14]. https://www.omicsonline.org/open-access/arsenic-occurrence-and-fate-in-the-environment-a-geochemical-perspective-2157-7617-1000269.php?aid=51452.

Papich MG. 2016. Oxytetracycline. Saunders Handb Vet Drugs.:595–598. doi:10.1016/B978-0-323-24485-5.00433-2. [accessed 2022 Jan 25]. https://linkinghub.elsevier.com/retrieve/pii/B9780323244855004332.

Pasquet J, Chevalier Y, Pelletier J, Couval E, Bouvier D, Bolzinger MA. 2014. The contribution of zinc ions to the antimicrobial activity of zinc oxide. Colloids Surfaces A Physicochem Eng Asp. 457(1):263–274. doi:10.1016/J.COLSURFA.2014.05.057. [accessed 2022 Jan 26]. https://www.researchgate.net/publication/263285014_The_contribution_of_zinc_ions_to_the_antimicrobial_activity_of_zinc_oxide.

Patricola CM, Wehner MF. 2018. Anthropogenic influences on major tropical cyclone events. Nature. 563(7731):339–346. doi:10.1038/s41586-018-0673-2. http://dx.doi.org/10.1038/s41586-018-0673-2.

Pennington P. 2022. Personal Correspondence.

PubChem. 2004a Sep 16. Copper sulfate | CuSO4. NCBI PubMed. [accessed 2022 Feb 7]. https://pubchem.ncbi.nlm.nih.gov/compound/24462#section=2D-Structure.

PubChem. 2004b Sep 16. Zinc sulfate | ZnSO4. NCBI PubChem. [accessed 2022 Feb 7]. https://pubchem.ncbi.nlm.nih.gov/compound/24424#section=2D-Structure.

PubChem. 2005a Mar 25. Ciprofloxacin | C17H18FN3O3. NCBI PubChem. [accessed 2022 Feb 7]. https://pubchem.ncbi.nlm.nih.gov/compound/2764.

PubChem. 2005b Mar 25. Triclosan | C12H7Cl3O2. NCBI PubChem. [accessed 2022 Feb 7]. https://pubchem.ncbi.nlm.nih.gov/compound/5564.

PubChem. 2005c Mar 25. Roxarsone | C6H6AsNO6. NCBI PubChem. [accessed 2022 Feb 7]. https://pubchem.ncbi.nlm.nih.gov/compound/5104.

PubChem. 2005 Jun 24. Vancomycin | C66H75Cl2N9O24. NCBI PubChem. [accessed 2022 Feb 7]. https://pubchem.ncbi.nlm.nih.gov/compound/14969#section=NCI-Thesaurus-Code.

PubChem. 2005 Aug 1. Cefotaxime | C16H17N5O7S2. NCBI PubChem. [accessed 2022 Feb 7]. https://pubchem.ncbi.nlm.nih.gov/compound/5742673.

PubChem. 2011 Dec 26. Oxytetracycline | C22H24N2O9. NCBI PubChem. [accessed 2022 Feb 7]. https://pubchem.ncbi.nlm.nih.gov/compound/54675779.

PubChem. 2013 May 27. Sodium arsenate As 74 | AsH2NaO4. NCBI PubChem. [accessed 2022 Feb 7]. https://pubchem.ncbi.nlm.nih.gov/compound/71449004.

Ralston EP, Kite-Powell H, Beet A. 2011. An estimate of the cost of acute health effects from food- and water-borne marine pathogens and toxins in the USA. J Water Health. 9(4):680–694. doi:10.2166/wh.2011.157. http://10.0.8.118/wh.2011.157.

Randa MA, Polz MF, Lim E. 2004. Effects of Temperature and Salinity on. Microbiology. 70(9):5469–5476. doi:10.1128/AEM.70.9.5469.

Rebelo A, Vanessa J, Melro C, Freitas AR, Gonzalez TMC, Maria L, Peixe V, Antunes P. 2012. P0454 Widespread copper , mercury and arsenic tolerance genes among multidrug-resistant Enterococcus spp . from human , animal and environmental origins.

Risser MD, Wehner MF. 2017. Attributable Human-Induced Changes in the Likelihood and Magnitude of the Observed Extreme Precipitation during Hurricane Harvey. Geophys Res Lett. 44(24):12,457-12,464. doi:10.1002/2017GL075888.

Rodricks J V, Swenberg JA, Borzelleca JF, Maronpot RR, Shipp AM. 2010. Triclosan: A critical review of the experimental data and development of margins of safety for consumer products. 40(5):422–484. doi:10.3109/10408441003667514. http://10.0.12.37/10408441003667514.

Roig FJ, Sanjuan E, Llorens A, Amaro C. 2010. pilF Polymorphism-Based PCR To Distinguish *Vibrio vulnificus* Strains Potentially Dangerous to Public Health. Appl Environ Microbiol. 76(5):1328–1333. doi:10.1128/aem.01042-09. http://10.0.4.104/aem.01042-09.

Rutherford DW, Bednar AJ, Garbarino JR, Needham R, Staver KW, Wershaw KW. 2003. Environmental Fate of Roxarsone in Poultry Litter. Part II. Mobility of Arsenic in Soils Amended with Poultry Litter. Environ Sci Technol. 37(8):1515–1520. doi:10.1021/es026222. [accessed 2022 Mar 14]. https://pubs.acs.org/sharingguidelines.

Sandifer PA, Scott GI. 2021. Coastlines, Coastal Cities, and Climate Change: A Perspective on Urgent Research Needs in the United States. Front Mar Sci. 8:207. doi:10.3389/FMARS.2021.631986/BIBTEX.

Sanger DM, Holland AF, Scott GI. 1999. Tidal creek and salt marsh sediments in South Carolina coastal estuaries: II. Distribution of organic contaminants. Arch Environ Contam Toxicol. 37(4):458–471. doi:10.1007/S002449900540.

Sanjuan E, Fouz B, Oliver JD, Amaro C. 2009. Evaluation of Genotypic and Phenotypic Methods To Distinguish Clinical from Environmental *Vibrio vulnificus* Strains. Appl Environ Microbiol. 75(6):1604–1613. doi:10.1128/aem.01594-08. http://10.0.4.104/aem.01594-08.

Santo CE, Lam EW, Elowsky CG, Quaranta D, Domaille DW, Chang CJ, Grass G. 2011. Bacterial killing by dry metallic copper surfaces. Appl Environ Microbiol. 77(3):794–802.

doi:10.1128/AEM.01599-10. [accessed 2022 Jan 26]. https://pubmed.ncbi.nlm.nih.gov/21148701/.

Satter H. 2022 Mar 10. Paul Ehrlich. Encycl Br. [accessed 2022 Aug 4]. https://www.britannica.com/biography/Paul-Ehrlich.

SC DNR. 2020. Planktonic Algae. Aquat Plant Manag.

Scott G. 2017. Pharmaceuticals in US Surface Waters : Frequency of Detection Concentrations of Perfluorinated Chemicals in Wildlife & Humans.

Scott G, Holland A, Sandifer P. 2006. Managing coastal urbanization and development in the 21st century: the need for a new paradigm. p. 285–299.

Scott GI, Porter DE, Norman RS, Scott CH, Uyaguari-Diaz MI, Maruya KA, Weisberg SB, Fulton MH, Wirth EF, Moore J, et al. 2016. Antibiotics as CECs: An overview of the hazards posed by antibiotics and antibiotic resistance. Front Mar Sci. 3(APR):24. doi:10.3389/FMARS.2016.00024/BIBTEX.

SCSGC. 2018. Executive summary of: Stormwater Ponds in Coastal South Carolina - 2018 State of Knowledge Report. Charleston, SC.

Seiler C, Berendonk TU. 2012. Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. Front Microbiol. 3(DEC):1–10. doi:10.3389/fmicb.2012.00399.

Shamard C. 2019. Is flesh-eating bacteria on the rise? Everything you need to know about necrotizing fasciitis. NBC Today. [accessed 2020 Jan 8]. https://www.today.com/health/flesh-eating-bacteria-rise-everything-you-need-know-about-necrotizing-t159093.

Silva F, Lourenço O, Queiroz JA, Domingues FC. 2011. Bacteriostatic versus bactericidal activity of ciprofloxacin in *Escherichia coli* assessed by flow cytometry using a novel far-red dye. J Antibiot 2011 644. 64(4):321–325. doi:10.1038/ja.2011.5. [accessed 2022 Jan 25]. https://www.nature.com/articles/ja20115.

Sirelkhatim A, Mahmud S, Seeni A, Kaus NHM, Ann LC, Bakhori SKM, Hasan H, Mohamad D. 2015. Review on Zinc Oxide Nanoparticles: Antibacterial Activity and Toxicity Mechanism. Nano-Micro Lett. 7(3):219. doi:10.1007/S40820-015-0040-X. [accessed 2022 Aug 4]. /pmc/articles/PMC6223899/.

Stacey NE, Lewis RW, Davenport JR, Sullivan TS. 2019. Composted biosolids for golf course turfgrass management: Impacts on the soil microbiome and nutrient cycling. Appl Soil Ecol. 144(October 2018):31–41. doi:10.1016/j.apsoil.2019.06.006. https://doi.org/10.1016/j.apsoil.2019.06.006.

Stepanauskas R, Glenn TC, Jagoe CH, Tuckfield RC, Lindell AH, King CJ, McArthur J V. 2006. Coselection for microbial resistance to metals and antibiotics in freshwater

microcosms. Environ Microbiol. 8(9):1510–1514. doi:10.1111/j.1462-2920.2006.01091.x.

Thurman EM. 2003. Occurrence of antibiotics in water from fish hatcheries. US Department of the Interior, US Geological Survey.

U.S. FDA. 2021 Apr 30. Questions and Answers on Arsenic-based Animal Drugs | FDA. [accessed 2022 Mar 14]. https://www.fda.gov/animal-veterinary/product-safety-information/questions-and-answers-arsenic-based-animal-drugs.

UMN-Extension. 2016. Zinc for crop production. [accessed 2022 Jan 26]. https://extension.umn.edu/micro-and-secondary-macronutrients/zinc-crop-production.

US EPA. Indicators: Enterococci | National Aquatic Resource Surveys | US EPA. [accessed 2020 Jan 8]. https://www.epa.gov/national-aquatic-resource-surveys/indicators-enterococci.

Uyaguari M, Key P, Moore J, Jackson K, Scott G. 2009. Acute effects of the antibiotic oxytetracycline on the bacterial community of the grass shrimp, *Palaemonetes pugio*. Environ Toxicol Chem. 28(12):2715. doi:10.1897/08-514.1. [accessed 2017 Apr 18]. http://doi.wiley.com/10.1897/08-514.1.

Uyaguari MI, Fichot EB, Scott GI, Norman RS. 2011. Characterization and quantitation of a novel β-lactamase gene found in a wastewater treatment facility and the surrounding coastal ecosystem. Appl Environ Microbiol. 77(23):8226–33. doi:10.1128/AEM.02732-10. [accessed 2017 Apr 18]. http://www.ncbi.nlm.nih.gov/pubmed/21965412.

Uyaguari MI, Scott GI, Norman RS. 2013. Abundance of class 1-3 integrons in South Carolina estuarine ecosystems under high and low levels of anthropogenic influence. Mar Pollut Bull. 76(1–2):77–84. doi:10.1016/j.marpolbul.2013.09.027. http://dx.doi.org/10.1016/j.marpolbul.2013.09.027.

Vankerckhoven V, Van Autgaerden T, Vael C, Lammens C, Chapelle S, Rossi R, Jabes D, Goossens H. 2004. Development of a Multiplex PCR for the Detection of asa1, gelE, cylA, esp, and hyl Genes in Enterococci and Survey for Virulence Determinants among European Hospital Isolates of *Enterococcus faecium*. J Clin Microbiol. 42(10):4473–4479. doi:10.1128/jcm.42.10.4473-4479.2004. http://10.0.4.104/jcm.42.10.4473-4479.2004.

Ventola CL. 2015. The antibiotic resistance crisis: part 1: causes and threats. P T. 40(4):277–83. [accessed 2017 Apr 25]. http://www.ncbi.nlm.nih.gov/pubmed/25859123.

Vezzaro L, Eriksson E, Ledin A, Mikkelsen PS. 2011. Modelling the fate of organic micropollutants in stormwater ponds. Sci Total Environ. 409(13):2597–2606. doi:10.1016/j.scitotenv.2011.02.046. http://dx.doi.org/10.1016/j.scitotenv.2011.02.046.

Vezzulli L, Colwell RR, Pruzzo C. 2013. Ocean Warming and Spread of Pathogenic Vibrios in the Aquatic Environment. Microb Ecol. 65(4):817–825. doi:10.1007/s00248-012-0163-2.

Vezzulli L, Grande C, Reid PC, Hélaouët P, Edwards M, Höfle MG, Brettar I, Colwell RR, Pruzzo C. 2016. Climate influence on *Vibrio* and associated human diseases during the past half-century in the coastal North Atlantic. Proc Natl Acad Sci. 113(34):E5062–E5071. doi:10.1073/pnas.1609157113. http://www.pnas.org/lookup/doi/10.1073/pnas.1609157113.

Van Wamel WJB, Hendrickx APA, Bonten MJM, Top J, Posthuma G, Willems RJL. 2007. Growth Condition-Dependent Esp Expression by *Enterococcus faecium* Affects Initial Adherence and Biofilm Formation. Infect Immun. 75(2):924–931. doi:10.1128/iai.00941-06. http://10.0.4.104/iai.00941-06.

WHO. 2014. Antimicrobial resistance: global report on surveillance. [accessed 2016 Dec 10]. http://apps.who.int/iris/handle/10665/112642.

WHO. 2019. The Selection and Use of Essential Medicines Report of the WHO Expert Committee on Selection and Use of Essential Medicines, 2019 (including the 21st WHO Model List of Essential Medicines and the 7th WHO Model List of Essential Medicines for Children) The.

WHO. 2021. Electronic Essential Medicines List. [accessed 2022 Aug 4]. https://list.essentialmeds.org/.

Willems RJL, Top J, Van Santen M, Robinson DA, Coque TM, Baquero F, Grundmann H, Bonten MJM. 2005. Global spread of vancomycin-resistant *Enterococcus faecium* from distinct nosocomial genetic complex. Emerg Infect Dis. 11(6):821–828. doi:10.3201/1106.041204.

Woosten Jr. CR. 2016. 'Like something out of a horror movie': Flesh-eating bacteria infection kills man in four days. Washington Post. [accessed 2020 Jan 8]. https://www.washingtonpost.com/news/to-your-health/wp/2016/10/23/like-something-out-of-a-horror-movie-flesh-eating-bacteria-infection-kills-man-in-four-days/?noredirect=on.

Xu Y, Xu J, Mao D, Luo Y. 2017. Effect of the selective pressure of sub-lethal level of heavy metals on the fate and distribution of ARGs in the catchment scale. Environ Pollut. 220:900–908. doi:10.1016/j.envpol.2016.10.074. http://dx.doi.org/10.1016/j.envpol.2016.10.074.

Yamazaki K, Kashimoto T, Morita M, Kado T, Matsuda K, Yamasaki M, Ueno S. 2019. Identification of in vivo Essential Genes of *Vibrio vulnificus* for Establishment of Wound Infection by Signature-Tagged Mutagenesis. Front Microbiol. 10. doi:10.3389/fmicb.2019.00123. http://10.0.13.61/fmicb.2019.00123.

Zar J. 1999. Biostatistical Analysis. 4th ed. Upper Saddle River, New Jersey: Prentice Hall.