January 2013

Direct and Indirect Effects of Agrochemicals on Bacterial Pathogens and Fecal Indicator Bacteria

Zachery Staley

University of South Florida, zstaley307@gmail.com

Follow this and additional works at: https://digitalcommons.usf.edu/etd

Part of the Microbiology Commons

Scholar Commons Citation
Staley, Zachery, "Direct and Indirect Effects of Agrochemicals on Bacterial Pathogens and Fecal Indicator Bacteria" (2013). USF Tampa Graduate Theses and Dissertations. https://digitalcommons.usf.edu/etd/4584

This Dissertation is brought to you for free and open access by the USF Graduate Theses and Dissertations at Digital Commons @ University of South Florida. It has been accepted for inclusion in USF Tampa Graduate Theses and Dissertations by an authorized administrator of Digital Commons @ University of South Florida. For more information, please contact digitalcommons@usf.edu.
Direct and Indirect Effects of Agrochemicals on Bacterial Pathogens and Fecal Indicator Bacteria

by

Zachery R. Staley

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
Department of Integrative Biology
College of Arts and Sciences
University of South Florida

Co-Major Professor: Valerie J. Harwood, Ph. D.
Co-Major Professor: Jason R. Rohr, Ph. D.
Kathleen M. Scott, Ph.D.
John T. Lisle, Ph. D.

Date of Approval: March 26, 2013

Keywords: Water Quality, Protozoa, Pesticides, Sediment, Viruses

Copyright © 2013, Zachery R. Staley
DEDICATION

I would like to dedicate this dissertation to my parents, Ruth Lawrence and Floyd Staley. Without your love and support this would not have been possible.

Additionally, I would like to dedicate this work to my friends, Ryan Sherman, Jake Sears, Jonathan Jeerapaet, Alex Brewer, Cory Larkin, Kyle Spidel, Jeremiah Callahan, and Emily Grennor. You all have been like family to me and I would have never gotten this far without you in my life.

This dedication is a small token of how much I value all of you and appreciate the significant contributions you have made and continue to make to my life.
ACKNOWLEDGMENTS

I would like to express my sincere thanks to Dr. Valerie J. Harwood and Dr. Jason R. Rohr, my mentors and advisors, for all the guidance, advice and direction they have given me over the years. I would also like to thank my committee members, Dr. Kathleen M. Scott and Dr. John T. Lisle for their time and suggestions throughout the course of my graduate studies. Further, I would like to acknowledge Dr. James T. Riordan for accepting to serve as the chair person for my dissertation defense.

Special thanks are also due to the former and current members of the Harwood lab (Miriam Brownell, Asja Korajkic, Brian Badgley, Shannon McQuaig, Chris Staley, Katrina Gordon, Bina Nayak, Stephaneys Leskinen, Dawn Hunter, Eva Chase, Pauline Wanjugu, and Suzanne Young) for their friendship and support. I would also like to thank my undergrads (Kennon Hydock, Melissa Tamayo, Diana Contreras, Laura Duran, Hannah Wapshott, and especially Jacob Senkbeil) for their contribution to and help on my research. Lastly, I must thank the cat, Ms. Persephone “Lumpy” Farris, for providing relief and distraction as needed.
**TABLE OF CONTENTS**

LIST OF TABLES .............................................................................................................................. iv  

LIST OF FIGURES ............................................................................................................................. v  

ABSTRACT ........................................................................................................................................ vii  

CHAPTER ONE: EFFECTS OF AGROCHEMICALS ON MICROORGANISMS ............... 1  
- Introduction ................................................................................................................................. 1  
- Direct and Indirect Effects ........................................................................................................ 5  
- Agrochemical Effects ................................................................................................................. 6  
  - Insecticides ............................................................................................................................... 7  
  - Herbicides ............................................................................................................................... 13  
  - Fungicides ............................................................................................................................... 18  
- Ecological Relevance and Areas for Further Research ......................................................... 21  
- References .................................................................................................................................. 23  

CHAPTER TWO: THE EFFECT OF AGROCHEMICALS ON INDICATOR DENSITIES IN OUTDOOR MESOCOSMS ............................................................... 35  
- Summary .................................................................................................................................. 35  
- Introduction ............................................................................................................................... 36  
- Results ....................................................................................................................................... 38  
- Discussion ................................................................................................................................. 41  
- Experimental Procedures ......................................................................................................... 45  
  - Experimental Design ............................................................................................................. 45  
  - Bacterial Inoculation .............................................................................................................. 47  
  - Sample Collection and Filtration ........................................................................................... 48  
  - Statistical Analyses ............................................................................................................... 49  
- Acknowledgments ...................................................................................................................... 51  
- References .................................................................................................................................. 51  

CHAPTER THREE: A TEST OF DIRECT AND INDIRECT EFFECTS OF AGROCHEMICALS ON THE SURVIVAL OF FECAL INDICATOR BACTERIA ................................................................................. 61  
- Summary .................................................................................................................................. 61  
- Introduction ............................................................................................................................... 62  
- Experiment Design ................................................................................................................... 66  
  - Direct Effects Experiment .................................................................................................... 66  
  - Indirect Effect Experiment .................................................................................................... 69  
- Sample Collection and Filtration ............................................................................................. 70
**CHAPTER FIVE: AGROCHEMICAL EFFECTS ON A ZOONOTIC PATHOGEN MEDIATED BYPROTOZOAN PREDATION** ..................................................118

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>118</td>
</tr>
<tr>
<td>Introduction</td>
<td>119</td>
</tr>
<tr>
<td>Experimental Design</td>
<td>121</td>
</tr>
<tr>
<td>Direct Effects Experiment</td>
<td>121</td>
</tr>
<tr>
<td>Indirect Effects Experiment</td>
<td>122</td>
</tr>
<tr>
<td>Statistical Analysis</td>
<td>124</td>
</tr>
<tr>
<td>Results</td>
<td>125</td>
</tr>
<tr>
<td>Direct Effects Experiment</td>
<td>125</td>
</tr>
<tr>
<td>Indirect Effects: <em>T. pyriformis</em> Experiment</td>
<td>126</td>
</tr>
<tr>
<td>Indirect Effects: <em>O. danica</em> Experiment</td>
<td>126</td>
</tr>
<tr>
<td>Discussion</td>
<td>127</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>130</td>
</tr>
<tr>
<td>References</td>
<td></td>
</tr>
</tbody>
</table>

**CHAPTER FOUR: LACK OF DIRECT EFFECTS OF AGROCHEMICALS ON ZOONOTIC PATHOGENS AND FECAL INDIATOR BACTERIA** .............................................96

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>96</td>
</tr>
<tr>
<td>Introduction</td>
<td>96</td>
</tr>
<tr>
<td>Experimental Design</td>
<td>98</td>
</tr>
<tr>
<td>Microcosm Establishment</td>
<td>98</td>
</tr>
<tr>
<td>Bacterial Enumeration</td>
<td>100</td>
</tr>
<tr>
<td>Growth Curves</td>
<td>100</td>
</tr>
<tr>
<td>Statistical Analysis</td>
<td>101</td>
</tr>
<tr>
<td>Results</td>
<td>102</td>
</tr>
<tr>
<td>Discussion</td>
<td>103</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>104</td>
</tr>
<tr>
<td>References</td>
<td>104</td>
</tr>
</tbody>
</table>

**E. coli** Genetic Typing .....................................................................71

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Statistical Analysis</td>
<td>73</td>
</tr>
<tr>
<td>Direct Effect Experiment</td>
<td>73</td>
</tr>
<tr>
<td>Treatment Effects on FIB Abundance</td>
<td>73</td>
</tr>
<tr>
<td>Treatment Effects on FIB Composition</td>
<td>74</td>
</tr>
<tr>
<td>Relationship Between FIB Composition in the Sediment and Water Column</td>
<td>74</td>
</tr>
<tr>
<td>Indirect Effect Experiment</td>
<td>75</td>
</tr>
<tr>
<td>Results</td>
<td>75</td>
</tr>
<tr>
<td>Direct Effects of Agrochemicals (Dark Conditions)</td>
<td>75</td>
</tr>
<tr>
<td>Indirect Effects of Agrochemicals (Light Conditions)</td>
<td>77</td>
</tr>
<tr>
<td>Discussion</td>
<td>79</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>84</td>
</tr>
<tr>
<td>References</td>
<td>84</td>
</tr>
</tbody>
</table>
CHAPTER SIX: THE EFFECTS OF AGROCHEMICALS ON BACTERIVOROUS PREDATION AND COMPETITION

Abstract .................................................................................................................. 138

Introduction ......................................................................................................... 139

Experimental Design ............................................................................................ 143
  Competition and Predation from Natural Microbial Populations ........... 143
  Sample Collection, Filtration, and Bacterial and Protozoan Enumeration .................................................................................. 145
  Statistical Analysis ............................................................................................ 145

Results.................................................................................................................... 146

Discussion ............................................................................................................. 147

References ............................................................................................................ 151
LIST OF TABLES

Table 1. List of pesticides and mechanism of action ..................................................34

Table 2. Results of multivariate analysis of variance examining the effects of spatial block, fertilizer, atrazine, chlorothalonil, malathion, and sampling date on the density of *E. coli* and enterococci in sediment. .............................................60

Table 3. Primers and probes used for qPCR quantification ........................................108

Table 4. Correlation coefficients (r) between bacterial concentrations obtained via culture-dependent methods and qPCR at T24 and T168. ...............................................109

Table 5. T90 values and decay rates with standard deviations in parentheses for all targets averaged among all agrochemical treatments ..............................................110

Table 6. Results of multivariate analysis of variance for the *Dark Microcosms* examining the effects of spatial block, agrochemical treatment, and sampling date on the density, quantified by qPCR, of *E. coli*, *Ent. faecalis*, *E. coli* O157:H7, *S. enterica*, HPyV, and adenovirus in the water column. .........................................................111

Table 7. Results of multivariate analysis of variance for the *Light Microcosms* examining the effects of spatial block, agrochemical treatment, and sampling date on the density of *E. coli*, *Ent. faecalis*, *E. coli* O157:H7, and *S. enterica* in the water column .................................................................112

Table 8. Growth rates of bacteria exposed to agrochemicals. Error bars represent standard deviations .............................................113
LIST OF FIGURES

Figure 1. Densities of IOs in sediments of mesocosms over 28 days: A) E. coli and B) enterococci .................................................................58

Figure 2. Least squares means (±1 SE) for the effects of fertilizer (left) and atrazine (right) on the density of (A) E. coli and (B) enterococci after 28 days. ............59

Figure 3. Schematic of the procedure for used to culture and process E. coli and enterococci strains for inoculation into microcosms ................................90

Figure 4. Average decay rates per day (mean Δ log CFU per100ml or 100 g ± standard error (SE), n=4) in water and sediment averaged over all four temporal blocks for A) E. coli and B) enterococci ...............................................................91

Figure 5. The relationship between strain composition in the sediment and water column for three E. coli strains (strain 14: squares, strain 19: triangles, strain 9637: circles). ...........................................................................................................92

Figure 6. E. coli densities in the A) water and B) sediment for each treatment (mean ± SE, n = 5) at days 2, 7, and 28. .................................................................93

Figure 7. Phytoplankton abundance (measured as F0) with and without atrazine (least squares mean ± SE, n = 5) on days 7, 14, and 28 when controlling for initial phytoplankton levels. ...........................................................................................................94

Figure 8. Relationship between phytoplankton abundance (controlling for initial abundance estimates) and E. coli densities (measured as CFU/100 ml or CFU/100 g) in the A) water column and B) sediment ................................................................95

Figure 9. Average concentrations for A) E. coli, B) Ent. faecalis, C) E. coli O157:H7, and D) S. enterica for all agrochemical treatments in the water column of the Dark Microcosms (least squares means ± SE, n = 4) .................................................................114

Figure 10. Average bacterial concentrations for all agrochemical treatments in the water column of the Light Microcosms (least squares means ± SE, n = 4). .........115

Figure 11. Concentrations of target microbes in the Dark Microcosms at each time point, averaged for all agrochemical treatments (± SD). ........................................116
Figure 12. Concentrations of target bacteria in the *Light Microcosms* in the A) water column and B) sediments averaged for all agrochemical treatments (± SD). 117

Figure 13. Average densities (n=3±SE) of A) T. pyriformis and B) O. danica in each agrochemical treatment. ................................................................. 134

Figure 14. Average E. coli O157:H7 and T. pyriformis densities (n=3 ± SD) in A) control and B) chlorothalonil treatments at each sampling point................................. 135

Figure 15. Average E. coli O157:H7 densities (n=3±SE) with and without predation by A) T. pyriformis and B) O. danica in each agrochemical treatment. .............. 136

Figure 16. Average E. coli O157:H7 and O. danica densities (n=3 ± SD) in A) control and B) atrazine and C) chlorothalonil treatments at each sampling point. .... 137

Figure 17. Mean densities (n=3±SD) of naturally-occurring protozoa in each agrochemical treatment with A) unamended conditions, B) cycloheximide addition and C) streptomycin addition................................................................. 156

Figure 18. Mean *E. coli* (A, D, G), *Ent. faecalis* (B, E, H), and *E. coli* O157:H7 (C, F, I) densities (n=3±SD) as a function of time in microcosms with A-C) unamended conditions, D-F) cycloheximide addition, G-I) streptomycin addition. ................................................................................................. 157

Figure 19. The effect of inhibitors and agrochemical treatments on mean FIB and pathogen densities in microcosms (n=3±SE) over the entire six-day period assessed by repeated measures ANOVA. ......................................................... 158
ABSTRACT

The presence of agrochemical residues in both urban and agricultural water bodies has become ubiquitous, often producing deleterious effects in the impacted watershed including reductions in biodiversity, alterations in species interactions, and toxicity to non-target organisms. While these effects have been studied on metazoan consumers, the consequences of agrochemical contamination on microorganisms, such as bacteria, protozoa, and viruses, are poorly understood. Agrochemicals could act directly on microorganisms, including pathogens, by either facilitating their survival or decreasing their abundance. Further, a multitude of indirect effects of agrochemicals on microorganisms are possible, whereby agrochemicals alter predation, competition, or parasitism on or available nutrient to microbes.

The primary method by which agrochemicals enter water bodies is through stormwater and agricultural runoff, which can also introduce agriculturally-associated zoonotic pathogens. Presently, regulatory standards utilize fecal indicator bacteria (FIB) to predict the presence of pathogens in contaminated watersheds. However, if agrochemicals have different effects on FIB and bacterial pathogens, then these regulatory standards might be confounded by the presence of pesticide residues in impacted water bodies. Additionally, if agrochemicals promote the survival of zoonotic pathogens, then the presence of pesticide residues could potentially increase risks to human health.
The studies in this dissertation investigated both the direct and indirect effects of agrochemicals on the growth and survival of FIBs (Escherichia coli and Enterococcus faecalis), zoonotic bacterial pathogens (E. coli O157:H7, and Salmonella enterica), and two virus groups (human polyomaviruses and adenoviruses). The agrochemicals utilized in these experiments are among the most prominently used in their respective pesticide classes and included the herbicide atrazine, the insecticide malathion, the fungicide chlorothalonil and inorganic fertilizer containing phosphate and fixed nitrogen. Initially, complex mesocosms containing zooplankton, phytoplankton, leaf litter, and vertebrate and invertebrate species were used to examine net (direct and indirect) effects of agrochemicals on FIB in sediments. Subsequent studies utilized experiments in simplified microcosms to detect direct or indirect effects (i.e., predation, competition or effects on nutrient resources) on FIBs and pathogens.

In complex mesocosms, atrazine and fertilizer significantly increased FIB densities in the sediment; however, because of the complexity of the mesocosms, it was not possible to determine whether these results were the product of direct or indirect agrochemical effects. Simplified microcosms, limited to predominantly direct effects, as well as in vitro growth curves, revealed no direct effects of any agrochemical treatment on either growth or survival of FIB or bacterial pathogens. When algal communities were allowed to establish, however, atrazine significantly reduced both phytoplankton and E. coli densities in the water column, but increased E. coli densities within the sediments. These effects on E. coli were indirect because they required the presence of algal species.
To investigate indirect effects of predation on FIBs and *E. coli* O157:H7, we manipulated the presence and absence of an obligate heterotroph, *Tetrahymena pyriformis*, a facultative heterotroph, *Ochromonas danica*, and natural protozoan populations. In both laboratory and greenhouse microcosm experiments, the fungicide chlorothalonil significantly reduced all protozoan populations, which resulted in increased densities of FIBs and *E. coli* O157:H7 because of reduced predation. Atrazine was not found to have any significant direct effect on the densities of *T. pyriformis* or natural protozoans; however, atrazine did significantly reduce *O. danica* densities in greenhouse experiments. In laboratory experiments with *O. danica*, atrazine treatments resulted in decreased densities of *E. coli* O157:H7. Presumably, atrazine prevented or reduced photosynthesis forcing *O. danica* to increase its predation on *E. coli* thus shifting its trophic level.

These studies reveal that agrochemicals can have a significant effect on microbial communities, but that these effects are often indirect and mediated through alterations of nutrient resources and predation. Atrazine application reduced FIB and pathogen densities in the water column via reduction of phytoplankton and increased predation by *O. danica*. These data suggest that the net effects of atrazine is deleterious to FIB survival in the water column and that application of this herbicide could result in an ecosystem service, reducing the abundance of zoonotic pathogens and lessening the risk to human health. However, elevation of FIB densities was observed in the sediments when atrazine was applied. The potential resuspension of increased sediment bacteria may negate or out-weigh the deleterious effects of atrazine on bacteria in the water column. Chlorothalonil application decreased protozoan densities, lessening the stress of
predation on the bacterial targets and increasing FIB and *E. coli* O157:H7 densities. The use of chlorothalonil may therefore have negative implications for human health risks, as the reduction in predation seems to facilitate the survival of zoonotic waterborne pathogens. Understanding the net effects of agrochemicals is important for public health, as pesticide applications can act to either maintain or diminish potential bacterial and protozoan pathogens of humans. These studies show that indirect effects of agrochemicals on non-target microbes tend to be more prominent than direct effects and can significantly impact the fate of bacterial pathogens in aquatic environments.
CHAPTER ONE: EFFECTS OF AGROCHEMICALS ON MICROORGANISMS

Introduction

The presence of agrochemicals in freshwater and marine ecosystems has become pervasive. In 2001, over five billion kg of chemicals were used in the United States (Kiely, Donaldson et al. 2004) and pesticides were detected in over 90% of developed watersheds (defined as any water shed dominated by agricultural, urban or mixed land use) (Gilliom 2007). Further, pesticides were detected in over 50% of wells examined and in the sediments of most streams tested (Gilliom 2007). Mixtures of pesticides were also detected in over 90% of developed watersheds and in 37 to 47% of agricultural and urban wells, respectively (Gilliom 2007).

The United States Environmental Protection Agency (USEPA) uses GENEEC v.2 software to make initial predictions of the expected environmental concentration (EEC) of many agrochemicals (for example, 102 µg/L for the herbicide atrazine, 101 µg/L for the insecticide malathion, 170 µg/L for the fungicide chlorothalonil). The software supposedly produces a high-end estimate based on laboratory data and several assumptions and variables, including: the amount of pesticide applied, the watershed is isolated from neighboring watersheds, and six inches of rainfall have resulted in runoff in the past 24 hours (U. S. Environmental Protection Agency 2004). The actual
concentration of any agrochemical in an impacted water body varies depending on which pesticide is used, the amount applied, and environmental conditions such as antecedent rainfall, and may differ from a USEPA estimate made using the GENEEC software.

Between 2000 and 2007, global pesticide usage increased from 2.27 billion to 2.36 billion kg, approximately ~1% (U. S. Environmental Protection Agency 2012). Increases in agrochemical use have resulted from agricultural intensification (Robinson and Sutherland 2002); agricultural usage (pasture and cropland) accounted for 45.4% of total land use in the United States 2002 and 42% of total land use in the European Union in 2006 (United States Department of Agriculture 2005; European Environment Agency 2010). However, agrochemical usage is not restricted to agriculture; pesticide usage has also increased in urban areas (Sprague and Nowell 2008).

Entry of agrochemicals into water bodies is facilitated primarily through agricultural and storm water runoff. Rain events have been found to remove significant amounts of agrochemicals from areas where they are applied and transport the pesticides into neighboring water bodies (Palma, Sanchez et al. 2004). Previous studies have found that upwards of 59% of applied pesticides are transported during rain events and that, relative to dry conditions, pesticide concentrations in water bodies are higher following a storm water runoff event (Gilliom 2007; Sprague and Nowell 2008; Vryzas, Vassiliou et al. 2009; Taghavi, Merlina et al. 2011).

The presence of agrochemicals in both freshwater and marine ecosystems can produce harmful effects on humans and the ecosystem as a whole. Based upon USEPA benchmarks, pesticides exceeded standards for human health in ~10% of agricultural
streams, ~7% of urban streams, and ~1% of groundwater tested (Gilliom 2007; U. S. Environmental Protection Agency 2012). Based upon the USEPA benchmark for the health of aquatic life, 57% of agricultural and 83% of urban streams exceeded the regulatory standard (U. S. Environmental Protection Agency 2004; Gilliom 2007). According to European Union (EU) directives, ~43% of median pesticide levels in surface waters of northeastern Greece (Vryzas, Vassiliou et al. 2009) and 12% of ground water tested in northern Spain exceeded regulatory standards (Hildebrandt, Guillamon et al. 2008). Based upon the ubiquity and elevated level of agrochemicals found in developed watersheds and ground water, it is important to understand the effects that these pesticides have on the biota of impacted watersheds.

While all agrochemicals are designed to target a specific pest, the use of pesticides may have additional effects on non-target species (Rohr, Kerby et al. 2006). For instance, agrochemical exposure has resulted in decreased biodiversity, toxicity to algae and diatoms resulting in harmful algal blooms, and alterations in ecosystem food webs, such as increases in heterotrophic activity, with projections suggesting that continued extension of current agricultural practices could produce further harmful effects (Tilman 1999; Robinson and Sutherland 2002; Benton, Vickery et al. 2003; Debenest, Silvestre et al. 2010). Multiple studies have investigated the harmful effects of pesticides on amphibian, arthropod and fish species (Rohr, Elskus et al. 2003; Stark and Banks 2003; Rohr and Palmer 2005; Rohr, Sager et al. 2006; Desneux, Decourtye et al. 2007; Rohr, Schotthoefer et al. 2008; Relyea 2009; Rohr and McCoy 2010; McMahon, Halstead et al. 2011); however, relatively little research has addressed the effects of agrochemicals on microorganisms, particularly heterotrophic bacterial and protozoan
populations. Of the studies on interactions between agrochemicals and microorganisms, most have examined the role of microorganisms in the biodegradation of agrochemicals (Levanon 1993; De Souza, Newcombe et al. 1998; Anderson, Wheeler et al. 2002; Wackett, Sadowsky et al. 2002; Seffernick, Aleem et al. 2007; Zeinat, Nashwa et al. 2008; Cycoń, Wójcik et al. 2009) rather than investigating the potential ecological effects of the agrochemicals on the fate of aquatic microbes, including zoonotic pathogens.

Microorganisms, including plankton, bacterial, and protozoan species, have a significant effect on aquatic ecosystems, providing crucial services such as primary production, decomposition and nutrient cycling. Therefore, any alteration of microbial communities, as a consequence of agrochemical inputs, could result in dramatic changes and damage to an impacted water body. Further, agrochemical effects could have implications for human health. Many bacterial and protozoan pathogens are waterborne, and may potentially be affected by the presence of pesticides. Agrochemicals, either directly or indirectly (see below), may facilitate pathogen survival or reduce pathogen densities, effectively “killing two birds with one stone.” Presently, the effects of agrochemicals on the fate of microorganisms, particularly bacterial and protozoan populations, are poorly understood and often, studies have found conflicting results. Additionally, while several studies have looked at the direct effects of agrochemicals on microbes, far fewer studies have looked at how top-down and bottom-up indirect effects of agrochemicals influence microbial survival. This paper will review the different effects that have been observed on microbial species as a result of pesticide exposure, as well as investigating the ecological mechanisms through which these effects were mediated.
Direct and Indirect Effects

The effects of agrochemicals on microbes may be direct or indirect and either beneficial or adverse (Clements and Rohr 2009; Verro, Finzio et al. 2009; Verro, Finzio et al. 2009). Agrochemical exposure may prove directly toxic to microbes (an adverse effect) or agrochemicals may be utilized by microbes, particularly bacterial species, as a nutrient source, facilitating growth and/or survival (a beneficial effect). Further, direct effects of agrochemicals may not be immediately apparent. Sub-lethal effects, such as certain mutations, may carry over to successive generations, resulting in decreased fitness (an adverse effect) (Rohr, Sager et al. 2006). Additionally, direct effects may be masked by indirect agrochemical effects. For example, an initial decrease in a population as a result of a directly toxic agrochemical effect may be ameliorated by an accompanying decrease in predation or competition, masking the initial adverse effect over time (Rohr, Sager et al. 2006).

While many direct effects may be easily predicted (i.e., an herbicide being directly toxic to an algal species), indirect effects may be more difficult to predict and are often more common and complex than direct effects (Rohr and Crumrine 2005; Rohr, Kerby et al. 2006; Relyea 2009). Indirect effects may be either density- or trait-mediated (Rohr, Kerby et al. 2006). Density-mediated indirect effects would be those that result in either the increase or decrease in abundance of a predator, competitor, parasite, or food resource, which subsequently affects the focal organism (Raffel, Martin et al. 2008; Rohr, Raffel et al. 2008; Rohr, Schotthoefer et al. 2008; Rohr, Swan et al. 2009). For instance, agrochemical application may reduce the abundance of a bacterivorous protozoan species, facilitating the survival of a target microbe (a beneficial effect). Also,
agrochemicals may act to reduce competing microorganisms, lessening survival pressure on the survivors (a beneficial effect), similar to effects seen with antibiotics (Wanjugi and Harwood 2012). In addition to density-mediated indirect effects, agrochemicals have the capacity to alter traits of organisms, such as behavior, immunity, physiology or morphology, resulting in trait-mediated indirect effects (Rohr, Elskus et al. 2003; Rohr and Crumrine 2005; Rohr and Palmer 2005; Rohr, Kerby et al. 2006; Rohr, Schotthoefer et al. 2008; Rohr, Swan et al. 2009). For example, following herbicide exposure, a facultative heterotroph may switch from an autotrophic to a heterotrophic mode, causing increased predation of the microbial prey population (an adverse effect) (Debenest, Silvestre et al. 2010). Agrochemicals may also stimulate an increase or decrease in anti-parasite or anti-predation behavior, affecting overall survival (Rohr, Swan et al. 2009). Based upon the variety and complexity of potential agrochemical effects, it is often necessary to assess beneficial and adverse effects at multiple trophic levels to understand the net effect on the impacted ecosystem (Clements and Rohr 2009).

**Agrochemical Effects**

Several of the studies reported in this review have utilized agrochemical concentrations that are well above both estimated and actual environmental concentrations. For example, while the USEPA estimates are 102 µg/L for the herbicide atrazine, 101 µg/L for the insecticide malathion, 170 µg/L for the fungicide chlorothalonil, several studies are presented using concentrations of similar pesticides in each class at concentrations of 10 mg/L and higher, with one study utilizing pesticide concentrations over 1000 mg/L (Ukeles 1962; Clegg and Koevenig 1974; Faust, Altenburger et al. 1994; Kent and Currie 1995; Azizullah, Richter et al. 2011). However,
studies are also presented where concentrations at or below EEC were also utilized (Lal, Saxena et al. 1987; Peterson, Boutin et al. 1994; Lopez, Pozo et al. 2006; Downing, DeVanna et al. 2008; Staley, Rohr et al. 2010).

**Insecticides.** The general mechanism of action of most commercial insecticides is nervous system disruption (Table 1). Organophosphate insecticides (i.e., parathion, diazinon, malathion, chlorpyrifos, etc.) and carbamate insecticides (i.e., carbaryl, carbofuran) act to hydrolyze acetylcholine and inhibit acetylcholinesterase, resulting in a build-up of the neurotransmitter acetylcholine and eventual death of the organism (Fukuto 1990). Pyrethroid insecticides and organochlorine insecticides (i.e., DDT, aldrin, dieldrin, endrin) act by binding to GABA-aminobutyric acid (GABA) receptors, preventing chloride anions from entering nerve cells (Coats 1990).

Organophosphates, as a class, have generally been found to have adverse effects on algal growth, with several exceptions. Parathion inhibited reproduction in *Chlorella fusca* at concentrations of 7.86 g/L (Faust, Altenburger et al. 1994). Malathion inhibited cyanobacteria permanently at concentrations of 200 mg/L (Torres and O'Flaherty 1976; DeLorenzo, Scott et al. 2001). Fenitrothion inhibited growth of both phytoplankton and cyanobacteria at concentrations between 0.8 and 24.4 mg/L (Kent and Currie 1995). The green alga *Scenedesmus obliquus* and the cyanobacterium *Anacystis nidulans* concentrated parathion by approximately 100-fold, with no effect to the organism, when the insecticide was applied at 1 mg/L over seven days (Gregory, Reed et al. 2007).

However, the application of chlorpyrifos has been associated with cyanobacterial blooms (Butcher, Boyer et al. 1977). The organophosphate diazinon, facilitated growth
of cyanobacteria (Singh 1973). Chlorpyrifos increased phytoplankton, cyanobacteria and periphyton growth when predation by herbivorous crustaceans was removed as a result of agrochemical application (Hurlbert, Mulla et al. 1972). Overall, there seems to be little consensus as to the direction of the effects of organophosphates on cyanobacteria and algal species, with observed effects relying heavily on the complexity of the system investigated (simplified in vitro microcosms limited to direct effects as opposed to complex systems with the potential for top-down or bottom-up indirect effects), the concentration of the agrochemical, and exposure times.

With regard to protozoa, organophosphate insecticides were generally toxic to the ciliate species, Colpidium campylum (Dive, Leclerc et al. 1980). Studies have demonstrated that parathion, at concentrations of 1 mg/L, can be concentrated approximately 100-fold by the protozoan species Euglena gracilis, Paramecium bursaria, and P. multimicronucleatum, although no effect was reported on any of the species examined (Gregory, Reed et al. 2007). Tetrahymena pyriformis was found to undergo alterations in cell morphology and concentrate parathion approximately 1000-fold (Shalesh and Anil 2007). Chlorpyrifos and fenitrothion also accumulated in T. pyriformis, however inhibition of growth (0.5-2.5 µg/ml and 1-10 µg/ml, respectively) and, at higher concentrations of fenitrothion (5-10 µg/ml), cell death also resulted (Lal, Saxena et al. 1987). Chlorpyrifos also produced toxic effects on the marine diatom Minutocellus polyorphus (Walsh, McLaughlin et al. 1988). No effect of malathion was observed on E. gracilis at concentrations ranging from 6-50 mg/L (Azizullah, Richter et al. 2011).
Organophosphate pesticides have generally had little effect on bacterial species (Williams, Robbins et al. 1963; Kerszman 1993; Pratt, Bowers et al. 1993; Staley, Rohr et al. 2010; Staley, Rohr et al. 2011; Staley, Senkbeil et al. 2012); however, any observed effects were generally directly beneficial. One study found that organophosphate insecticides at 50 µg/ml tended to increase abundance of heterotrophic bacteria in water samples collected from a natural lake (Lopez, Pozo et al. 2006). In mesocosms designed to simulate a tidal creek, chlorpyrifos applied at 10 µg/L increased cyanobacterial abundance (DeLorenzo, Lauth et al. 1999); however, another study revealed no significant effects of the agrochemical on natural protozoan, bacterial, or fungal species in microcosms, even when concentrations exceeded water quality criteria three-fold (~0.12 µg/L) (Pratt, Bowers et al. 1993). At concentrations of 3.3 and 9.9 g/kg, chlorpyrifos increased abundance of *Listeria monocytogenes*, *Salmonella* spp., *Shigella* spp., and *E. coli* O157:H7 100-1000-fold over a 96-hour period (Guan, Blank et al. 2001). Further, diazinon, which accumulated in rumen protozoa, did not diminish the abundance of rumen bacteria (Williams, Robbins et al. 1963). Malathion was also not directly toxic to *Escherichia coli* or *Bacillus subtilis* in *in vitro* tests (Kerszman 1993). At malathion concentrations of 0.44 µl/g of soil, no significant effects on soil bacteria were observed (Stanlake and Clark 1975). Similarly, malathion at 101 and 202 µg/L did not have any significant effect on *E. coli* or *Enterococcus* spp. in the sediment of complex mesocosms (Staley, Rohr et al. 2010). Further, in simplified *in vitro* microcosms, malathion at 101 and 202 µg/L produced no effect on the growth rates or survival of *E. coli*, *Ent. faecalis*, *E. coli* O157:H7, or *Salmonella typhimurium* (Staley,
Senkbeil et al. 2012). However, malathion inhibited bacterial growth in sludge at concentrations of 0.5 mg/L (Pai, Wang et al. 2009).

Carbamate insecticides have adverse effects on a variety of microorganisms, but like the organophosphates, the types of negative effects vary. Carbaryl inhibited carbon uptake in diatoms and green algae, and exhibited direct toxicity to phytoplankton at concentrations ranging from 0.1-100 mg/L (Ukeles 1962; Peterson, Boutin et al. 1994). Carbaryl application resulted in a decrease in zooplankton abundance when applied over a range of 0.1-20 µg/L, while microbial and phytoplankton concentrations increased (Downing, DeVanna et al. 2008). Affected ecosystems in this study showed signs of recovery after 40 days, but changes in zooplankton population diversity were noted (Downing, DeVanna et al. 2008). The toxic effects of carbaryl on protozoa when applied at a concentration of 30 µg/ml have also been observed (Weber, Shea et al. 1982).

Studies have shown mixed effects of carbaryl on bacterial species. Carbaryl had a half maximal effective concentration (EC50), reducing the optical density of Vibrio fisheri by 50%, at 5 mg/L, while metabolites of carbaryl had an EC50 of 3.7 mg/L (Somasundaram, Coats et al. 1990). Growth of Ent. faecalis was inhibited by a carabryl concentration of 5 mg/L, while Staphylococcus aureus and Sal. enterica Typhimurium experienced increased growth rates at the same concentration (Guthrie, Anugwelem et al. 1981). Weber and Rosenberg found that bacterial growth was inhibited at a carbaryl concentration of 100 µg/ml in isolated systems, but that the addition of organic matter attenuated the effects of the chemical and no significant effect on bacterial growth was observed in field plots (Weber and Rosenberg 1984). The carbamate insecticide carbofuran was also found to have deleterious effects on bacteria and protozoa. An EC50
of 20.5 mg/L was observed for *V. fisherii* and carbofuran produced adverse effects on the protozoan *E. gracilis*, reducing cell growth after 72 hours of exposure, at a concentration of 50 mg/L (Somasundaram, Coats et al. 1990; Azizullah, Richter et al. 2011).

Organochlorine insecticides have also been found to have diverse, and in some cases conflicting effects on microbes. DDT, at concentrations below 10 µg/L, inhibited photosynthesis in marine algae (Lee, Fang et al. 1976). DDT was concentrated approximately 1000-fold, by the green algae *S. obliquus* and the cyanobacterium *A. nidulans*, with no effect on either organism (Gregory, Reed et al. 2007). Mirex, methoxychlor and endosulfan, also inhibited algal growth (Kricher, Urey et al. 1975; DeLorenzo, Scott et al. 2001). Endosulfan was concentrated in cyanobacteria by over 1000 mg/L, with no observed effect on the organisms (Rao and Lal 1987) and generally tended to be more toxic to cyanobacteria than green algae (Mohapatra and Mohanty 1992). Despite multiple findings of adverse effects of organochlorines on algal species, several organochlorines have also been found to produce no significant effects. Aldrin, dieldrin, and endrin each had no significant effect on algal respiration at concentrations up to 101 mg/L (Vance and Drummond 1969), while another study showed that all three agrochemicals, at concentrations of 100 mg/L, lowered ATP production, but did not result in reduced algal abundance (Clegg and Koevenig 1974). A study by Hollister, Walsh, et al. also revealed no effect of mirex on marine algae, although the agrochemical was concentrated 1000-fold by the algae (Hollister, Walsh et al. 1975). Pyrethroid insecticides were also found to have no significant or lasting effects on algal communities (DeLorenzo, Scott et al. 2001).
Effects of organochlorines on (facultatively) heterotrophic protozoa have generally been adverse, but have consistently been found to bioaccumulate. DDT was found to be bioaccumulated by *T. pyriformis, E. gracilis, P. bursaria, and P. multimicronucleatum* up to 1000-fold, as well as inhibit the growth of *T. pyriformis* at concentrations from 10-100 µg/ml (Lal, Saxena et al. 1987; Gregory, Reed et al. 2007). Dieldrin was also concentrated approximately 1000-fold by *T. pyriformis* (Bhatnagar, Kumar et al. 1988). Mirex produced detrimental effects on *T. pyriformis* cell growth at 0.9 µg/L and was concentrated by 193-fold (Cooley, Keltner et al. 2007). Endosulfan reduced diatom, chrysophyte, cryptophyte and dinoflagellate abundance at concentrations of 1 and 10 µg/L (Downing, DeLorenzo et al. 2004).

Studies on the effects of organochlorines on heterotrophic bacterial species have produced mixed results. Generally, organochlorines did not have any inhibitory effects on rumen bacteria (Williams, Robbins et al. 1963), and, at a concentration of 50 µg/ml, were found to increase heterotrophic bacteria concentrations (Lopez, Pozo et al. 2006). However, bacterial abundance was diminished by endosulfan at concentrations ranging from 0.002 to 2 mg/L, with bacteria in the water column experiencing greater detrimental effects than those in the sediment (Rajendran, Rajendran et al. 1990). Bacterial abundance was reduced with endosulfan concentrations of 1 to 10 µg/L, although heterotrophic productivity (moles thymidine incorporated/L/h) was not affected (DeLorenzo, Scott et al. 1999). DDT and dieldrin also reduced bacterial densities, although this effect was dependent on the culture medium (Lal and Saxena 1982). Mirex had no effect on bacterial growth or survival (Jones and Hodges 1974). Pyrethroid insecticides had an indirect, positive effect, facilitating bacterial survival by reducing
predation by *Daphnia magna* (Foit, Chatzinotas et al. 2010). Guan, Blank, *et al.* found that a commercially available solution of pyrethroid insecticides resulted in a 100-1000-fold increase in the abundance of *L. monocytogenes, Salmonella* spp., *Shigella* spp., and *E. coli* O157:H7 after 96 hours using concentrations of 0.5 and 1.5 g/kg (Guan, Blank et al. 2001).

**Herbicides.** Herbicides comprise a vast array of differing biochemical groups and, as such, encompass a variety of different modes of action (Table 1). Herbicides may act by blocking amino acid synthesis through inhibition of 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSP synthase), acetolactate synthase, or glutamine synthetase (Duke 1990). Carotenoid, lipid, microtubule, cellulose, or folate synthase may also be inhibited (Duke 1990). The most prominent mechanism of action of herbicides is via inhibition of photosynthesis. One mechanism by which photosynthesis may be compromised is through bleaching, wherein agrochemicals act in concert with photosystem I to produce free radicals (Duke 1990). The majority of herbicides, including triazines, anilides, ureas, phenyl carbamates, etc. (Table 1), act by blocking electron transport in photosystem II (Duke 1990).

As might be expected, most herbicides are directly toxic to algal species. Phenoxyalkane herbicides (i.e., 2,4-D and 2,4,5-T), at concentrations exceeding the EEC (1400 mg/L), had low toxic effects (<10% inhibition of carbon uptake) on microscopic phototrophs, including green algae, cyanobacteria, and diatoms, however no inhibition was observed when phototrophs were exposed to bromoxynil or pyridine herbicides (Peterson, Boutin et al. 1994). Bipyridinium herbicides (i.e., diquat, bromoxynil, paraquat) were generally more toxic to cyanobacteria and chrysophytes than green algae
(Campbell, Bartell et al. 2000), which was reflected in EC50 values of 0.019-0.48 mg/L for green algae, and lower EC50 values (0.022-0.093 mg/L) for cyanobacteria and chrysophytes. Inhibition of carbon uptake by 99-100% was also observed in diatom and cyanobacteria species when exposed to diquat at the EEC (0.73 mg/L) (Peterson, Boutin et al. 1994). Kosinski found that, at 10 µg/L, paraquat significantly reduced cyanobacterial respiration (Kosinski 1984). The aldehyde herbicide acrolein, as well as the substituted urea herbicide tebuthiuron (at their respective EECs of 1 mg/L and 5.9mg/L), were reported to inhibit carbon uptake by nine algal species (Peterson, Boutin et al. 1994).

Studies have also shown that phenylurea herbicides have toxic effects on algal species. Diuron is generally the most toxic of the group, followed by monuron, neburon, and fenuron (Ukeles 1962). Diuron produced lethal effects on marine algae at a concentration of 4 µg/L (Ukeles 1962), while 4 mg/L monuron reduced Chlorella growth rates (Cho, Tchan et al. 1972), and C. fusca reproduction was inhibited at by chlortoluron at 23.4 mg/L (Faust, Altenburger et al. 1994). By contrast, sulfonylurea herbicides at the EEC did not have a significant effect on carbon uptake by algal species (Peterson, Boutin et al. 1994). The glycine derivative glyphosate had an EC50 ranging from 8.9-89 mg/L for different freshwater periphyton communities following four hours of exposure and significantly reduced carbon uptake in diatoms and cyanobacteria (Goldsborough and Brown 1988; Peterson, Boutin et al. 1994).

The acetanilide herbicide metachlor inhibited carbon uptake in green algae, and both metachlor and the imidazolinone herbicide imazethapyr reduced carbon uptake in cyanobacteria at the EEC (Peterson, Boutin et al. 1994). Multiple triazine herbicides
were tested at their EEC and all resulted in decreased carbon uptake in green algae, diatoms and cyanobacteria (Peterson, Boutin et al. 1994). Both anilazine and simazine disrupted reproduction of *C. fusca* at concentrations of 1389 mg/L and 26.1 mg/L, respectively (Ukeles 1962).

The effects of the majority of herbicides on heterotrophic bacteria and protozoa have been adverse. Phenoxyalkane herbicides 2,4-D and 2,4,5-T had EC50 values for *V. fisherii* of 100.7 and 51.7 mg/L, respectively, while their metabolites had much lower EC50 values of 5.0 and 1.8, respectively, suggesting that the metabolites of these herbicides are more toxic than their parent compounds (Somasundaram, Coats et al. 1990). Coliform growth was also reduced when exposed to 2,4-D at concentrations of 5-10 mM, although triazine herbicides had no significant effects (Higgins and Hohn 2008). At concentrations ranging from 0.3-30 mg/L, the bipyridinium herbicide diquat was decreased the abundance of freshwater algal and bacterial species and protozoan species richness (Melendez, Kepner et al. 1993). Diquat had an EC50 value of 2.9 mg/L for *E. gracilis* (Campbell, Bartell et al. 2000). Additionally, Breazeale and Camper found a significant reduction in bacterial growth rates when 25-50 µg/ml of diquat or paraquat were present (Breazeale and Camper 1972). Glyphosate, at a concentration of 0.5 mg/L, reduced the growth rate of heterotrophic bacteria (Pai, Wang et al. 2009). Phenlyurea herbicides diuron and chlortoluron also had an inhibitory concentration (IC50; the concentration required to reduce cell growth by 50%) values for *V. fisherii* at concentrations of approximately 68.2 and ~298.5 mg/L, respectively, and *T. pyriformis* at concentrations of approximately 10.7 and 8.2 mg/L, respectively (Nelieu, Bonnemoy et al. 2010). An increase of 100-1000-fold in the abundance of *E. coli* O157:H7, *L.*
monocytogenes, Salmonella spp., and Shigella spp. was reported for linuron at concentrations of 11.3 and 33.9 g/kg over a 96-hour period (Guan, Blank et al. 2001).

Atrazine, a triazine herbicide, is one of the most widely used agrochemicals in the United States and worldwide (deNoyelles, Kettle et al. 1982; Kiely, Donaldson et al. 2004) and acts to inhibit electron transport by disrupting the Hill reaction of photosystem II (Duke 1990). Multiple studies have been conducted detailing the effects of atrazine exposure on phytoplankton, revealing that negative effects are dependent on atrazine concentration, length of exposure and species of phytoplankton (Huber 1993; Solomon, Baker et al. 1996). Atrazine toxicity has also been demonstrated toward macrophytes, benthic invertebrates, zooplankton, fish and amphibian species (Solomon, Baker et al. 1996; Rohr and McCoy 2010). In contrast, periphyton growth was stimulated with atrazine concentrations of 10 µg/L or less (Pratt, Bowers et al. 1988). Atrazine affected experimental pond communities with concentrations as low as 1-5 µg/L and as high as 500 µg/L, reducing green algae and flagellate abundance while increase cryptophytes and chrysophytes (deNoyelles, Kettle et al. 1982). Another study by Hamala and Kolliq found that, at a concentration of 100 µg/L, cryptophytes and diatoms were reduced while cyanobacteria increased (Hamala and Kolliq 1985). Low concentrations of atrazine, i.e. 120-5800 ng/L, were reported to result in increased amino acid production, lower pH, and lower chlorophyll production in marine phytoplankton (Bester, Huhnerfuss et al. 1995).

While many herbicides have been found to have generally adverse effects on most heterotrophic bacterial and protozoan species, atrazine has tended to produce neutral or beneficial effects. A reduction in chlorophyll coupled with increases in cyanobacteria and bacterial abundance was reported with atrazine concentrations ranging from 40-160...
µg/L, although bacterial abundance decreased over 45 hours (DeLorenzo, Lauth et al. 1999). In the same study, atrazine had no significant effect on small ciliate abundance, although large ciliates and small flagellates increased, while large flagellate abundance declined (DeLorenzo, Lauth et al. 1999). At concentrations of 20 and 200 µg/L, chlorophytes and chrysophytes decreased, while increasing the relative abundance of diatoms and heterotrophic protists (Downing, DeLorenzo et al. 2004). Similarly, Hamala and Kolliq found that at a concentration of 100 µg/L there was an increase of heterotrophic activity (Hamala and Kolliq 1985). Stimulation of heterotrophic bacteria was also observed for atrazine concentrations ranging from 3-100 µg/L, although the examined ecosystems collapsed at concentrations ranging from 100-300 µg/L in laboratory experiments (Pratt, Melendez et al. 1997). Further, Lopez, Pozo, et al. reported increased heterotrophic bacterial abundance with an atrazine concentration of 50 µg/ml (Lopez, Pozo et al. 2006). Atrazine concentrations ranging from 0.1-100 µg/L were also reported to increase E. coli and Ent. faecalis populations (Koutsotoli, Dimou et al. 2005). In the sediments of complex outdoor mesocosms, the addition of atrazine at 102 or 204 µg/L increased abundance of E. coli and Enterococcus spp. (Staley, Rohr et al. 2010). Further, in simplified microcosms where algal populations were allowed to establish, atrazine application at 102 µg/L resulted in decreases in phytoplankton and E. coli abundance in the water column, but increases in E. coli abundance in the sediments (Staley, Rohr et al. 2011). Despite multiple studies wherein atrazine was reported to have beneficial effects on bacterial species, Higgins found no effect of atrazine, at 50 ng/L, on coliform growth (Higgins and Hohn 2008). Similarly, Breazeale and Camper (1972) also found no significant effect of atrazine on bacterial growth at 25 and 50 µg/ml. At
concentrations of 102 and 204 µg/L, atrazine was found to have no effect on *E. coli*, *Ent. faecalis*, *S. typhimurium*, or *E. coli* O157:H7 growth or survival in simplified microcosms (Staley, Senkbeil et al. 2012). The lack of effects of both atrazine and its metabolite, deethylatrazine on both bacterial and protozoan abundance have also been reported (DeLorenzo, Scott et al. 1999).

**Fungicides.** Fungicides, like herbicides, have a variety of different potential mechanisms of action (Table 1). Aromatic hydrocarbon fungicides, triazoles, and hydrochlorines generally tend to be deleterious to membrane synthesis, acting to block either lipid or sterol biosynthesis or inhibiting the biosynthesis of intracellular membrane components (Yang, Hamel et al. 2011). Additionally, fungicides (i.e., pyrimidinamines, pyridine caboxamindes, benzamides, etc.; Table 1) may act by diminishing cellular respiration, either by disrupting oxidative phosphorylation or specifically targeting NADH oxido-reductase or succinate-dehydrogenase (Yang, Hamel et al. 2011). Further, phenylpyrrole and dicarboximide fungicides act by blocking signal transduction and many antibiotic fungicides are inhibitory to amino acid biosynthesis (Yang, Hamel et al. 2011).

Fungicides have generally been found to have adverse effects on algal species. While cyanobacteria are generally reported to be fairly resistant to insecticides, fungicides tend to be considerably more toxic (DeLorenzo, Scott et al. 2001), and are also generally harmful to ciliate species (Dive, Leclerc et al. 1980). A triazole derivative of propiconazole at the EEC was reported to inhibit carbon uptake by as much as 31% in the green algae *S. quadricauda* and cyanobacterium *Microsystis* (Peterson, Boutin et al. 1994). Harris, White, *et al.* found that organomercury fungicides at concentrations of
less than 1 µg/L reduced growth and photosynthesis in phytoplankton and diatom species (Harriss, White et al. 1970). Pyrimethanil, fludioxonil, and procymidone were toxic to *Scenedesmus acutus* with IC50 values of 22.8, 4.8, and 4.6 mg/L, respectively (Verdisson, Couderchet et al. 2001)

Adverse effects on heterotrophic bacterial and protozoan species exposed to fungicides have also been observed in previous studies, with several exceptions. At concentrations ranging from 0.05-10 mg/L, thiocarbamate fungicides were found to inhibit growth of *E. gracilis* (Moore 1970). Chlorothalonil reduced bacterial populations in soil at a mean concentration of 1.5 mg/kg (Yu, Shan et al. 2006). Similarly, triadimefon and propiconazole both reduced microbial growth in soils at concentrations of 10-100 mg/kg (Yen, Chang et al. 2009). Fenpropimorph diminished the abundance of bacterivorous protozoa (amoeba and flagellates) at high concentrations (0.74-750 mg/L), with lower concentrations only reducing heterotrophic flagellate taxa (Ekelund 1999). Both fenpropimorph and propiconazole, with EC50 values ranging from 3-70 mg/L, were also reported to reduce soil bacteria (Milenkovski, Baath et al. 2010). Banerjee and Banjerjee observed a reduction in soil bacteria with concentrations ranging from 75-82 mg/L and 370-477 mg/L of tridemorph and vinclozolin, respectively (Banerjee and Banerjee 1991). Further, bacterial diversity in soil was found to be diminished by concentrations of carbendazim ranging from 0.94-4.70 kg/ha (Wang, Song et al. 2009). Thymol, at concentrations of 375 and 750 g/ha was found to reduce gram-negative bacterial species, with no apparent effects on gram-positive species (Minambres, Conles et al. 2010). Acriflavine was observed to have dose-dependent bactericidal effects and result in thickening of the cell walls of *S. aureus* (Kawai and Yamagishi 2009).
Additionally, dichloran, at a concentration of 0.14 µg/L, was found to have potentially mutagenic properties to *Sal. typhimurium* (DeOuveira, Sakagami et al. 2009).

In contrast to studies wherein adverse effects were observed on heterotrophic bacterial populations, several studies have demonstrated null or beneficial effects of fungicide application. In a study by Downing, DeLorenzo *et al.*, 20 µg/L of chlorothalonil was found to stimulate heterotrophic bacterial activity (Downing, DeLorenzo *et al.* 2004). Abundance of *E. coli* O157:H7, *Salmonella* spp., *Shigella* spp., and *L. monocytogenes* were reported to increase by 100-1000-fold when exposed to a commercially available chlorothalonil mixture at concentrations of 10 and 30 g/kg over 96 hours (Guan, Blank *et al.* 2001). Similarly, captan, at 50 µg/ml was found to increase heterotrophic bacterial abundance in a lake ecosystem (Lopez, Pozo *et al.* 2006). At concentrations ranging from 20-60 g/L, various fungicide application increased abundance of soil bacteria (Niewiadomska, Sawinska *et al.* 2011). While not finding a beneficial effect, Bending, Rodriguez-Cruz *et al.* reported that chlorothalonil had no effect on either biomass or community composition of soil microbes at concentrations up to 10 mg/kg (Bending, Rodriguez-Cruz *et al.* 2007). Similarly, in the sediments of complex outdoor mesocosms, chlorothalonil produced no significant effect on *E. coli* or *Enterococcus* spp. at concentrations of 170 or 340 µg/L (Staley, Rohr *et al.* 2010). Further, chlorothalonil at the same concentrations had no effect on abundance or growth rate of *E. coli, Ent. faecalis, E.coli* O157:H7, or *S. typhimurium* in simplified microcosms (Staley, Senkbeil *et al.* 2012).
Ecological Relevance and Areas for Further Research

Based upon the studies presented in this review and the ubiquity of agrochemicals in water bodies in the United States and throughout the world, it is very likely that most aquatic ecosystems are in some way affected by the presence of agrochemicals. Understanding the effects of these pesticides is important to safeguard both aquatic and human health. Multiple studies have been presented revealing the capacity for microbes to concentrate pesticides, serving as vectors for the pesticide to affect higher trophic levels (Lal, Saxena et al. 1987; Cooley, Keltner et al. 2007; Gregory, Reed et al. 2007; Jo, Kim et al. 2011). Furthermore, studies have revealed that direct effects of agrochemicals on one trophic level may indirectly affect the abundance of higher or lower trophic populations (Relyea 2009; Foit, Chatzinotas et al. 2010; Staley, Rohr et al. 2011).

Understanding the ecological effects of agrochemicals and potentially different responses among bacterial and protozoan species may also have implications for microbial regulatory criteria and human health. Presently, microbial water quality is assessed by quantifying the abundance of fecal indicator bacteria (FIB; i.e., E. coli and enterococci) in contaminated water bodies (U. S. Environmental Protection Agency 2002; U. S. Environmental Protection Agency 2002; U. S. Environmental Protection Agency 2002). Epidemiological studies have shown that elevated concentrations of FIB have been correlated with increased risk of contracting gastrointestinal disease (Wade, Pai et al. 2003; Colford, Wade et al. 2007; Wade, Calderon et al. 2008). However, not all microbial, protozoan or viral pathogens exhibit the same persistence in secondary environments as do FIB (Jenkins, Fisher et al. 2011). As agrochemicals may result in different effects on different bacterial and protozoan species, it is increasingly important
to understand whether FIB are affected identically to the pathogens they are used to predict or whether the ubiquity of agrochemicals in contaminated water bodies is confounding regulatory measures.

Much research still needs to be conducted on the impact that pesticides have on microbes. Many of the studies on the effects of agrochemicals on microorganisms have focused on the effects of agrochemicals on algal species, whereas the majority of research examining the effects of pesticides on heterotrophic bacterial and protozoan species has primarily investigated bioaccumulation or biodegradation of the pesticides. Further, the existing body of research has yet to establish a consistent effect for most pesticides. For any given agrochemical, studies have tended to show contrasting or null effects depending on the species examined, the concentration of the agrochemical, the duration of exposure, the presence of sediments and humic materials, the presence of higher or lower trophic levels and indirect effects of pesticides on such trophic levels, and the levels of abiotic factors (pH, DO, etc.) in the examined environment or mesocosm.

Based upon the lack of an established effect for many agrochemicals, more systematic research is needed to address the direct effects of prominent agrochemicals on heterotrophic bacteria and protozoan species, as well as to determine the ecological mechanism (i.e., direct or indirect effects), by which agrochemicals act on the target species.
References


Taghavi, L., G. Merlina, et al. (2011). "The role of storm flows in concentration of pesticides associated with particulate and dissolved fractions as a threat to quatic ecosystems - Case study: the agricultural watershed of Save river (Southwest of France)." *Knowledge and Management of Aquatic Ecosystems* 400(06).


U. S. Environmental Protection Agency (2002). "Implementation guidance for ambient water quality criteria for bacteria. EPA-823-B-02-003."


U. S. Environmental Protection Agency (2012). "Human Health Benchmarks for Pesticides. EPA-822-F-12-001."


Table 1. List of pesticides and mechanism of action.

<table>
<thead>
<tr>
<th>Pesticide Category</th>
<th>Groups Included</th>
<th>General Toxic Effect</th>
<th>Specific Site of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herbicide</td>
<td><strong>Glyphosate</strong></td>
<td>Amino Acid Synthesis</td>
<td>EPSP Synthase</td>
</tr>
<tr>
<td></td>
<td><strong>Sulfonyl Ureas, Imidazolinones</strong></td>
<td>Amino Acid Synthesis</td>
<td>Acetolactate Synthase</td>
</tr>
<tr>
<td></td>
<td><strong>Glufosinate</strong></td>
<td>Amino Acid Synthesis</td>
<td>Glutamine Synthetase</td>
</tr>
<tr>
<td></td>
<td>**Triazines, Anilides, Phenyl Carbamates, Ureas, Biscarbamates, Benzimidazoles,</td>
<td>Photosynthesis</td>
<td>Hill Reaction of Photosystem II</td>
</tr>
<tr>
<td></td>
<td>Hydroxynitriles**</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Bipyridiniums, Heteropentalenes</strong></td>
<td>Photosynthesis (Bleaching)</td>
<td>Photosystem I</td>
</tr>
<tr>
<td></td>
<td><strong>Diphenyl ethers, Oxadiazoles, N-phenyl imides</strong></td>
<td>Photosynthesis (Bleaching)</td>
<td>Protoporphyrinogen Oxidase</td>
</tr>
<tr>
<td></td>
<td><strong>Aryoxyphenoxy propionates, Cyclohexanenediones, Chloroacetamide</strong></td>
<td>Lipid Biosynthesis</td>
<td>Acetyl-CoA Carboxylase</td>
</tr>
<tr>
<td></td>
<td><strong>Pyridazinones, Fluridone, m-Phenoxybenzamides, 4-Hydroxypyridines</strong></td>
<td>Carotenoid Biosynthesis</td>
<td>Phytoene Desaturase</td>
</tr>
<tr>
<td></td>
<td><strong>Amino triazole</strong></td>
<td>Carotenoid Biosynthesis</td>
<td>Lycopene Cyclase</td>
</tr>
<tr>
<td></td>
<td><strong>Dichloromate</strong></td>
<td>Carotenoid Biosynthesis</td>
<td>ζ-Carotene Desaturase</td>
</tr>
<tr>
<td></td>
<td><strong>Ioxazolidinones</strong></td>
<td>Carotenoid Biosynthesis</td>
<td>IPP Isomerase and/or Prenyl Transferase</td>
</tr>
<tr>
<td></td>
<td>**Dinitroanilines, Phosphoric amides, Chlorthaldimethyl, Propyzamide, Chokhicine,</td>
<td>Microtubule Biosynthesis</td>
<td>β-Tubulin</td>
</tr>
<tr>
<td></td>
<td>Terbutol**</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Dichlobenil</strong></td>
<td>Cellulose Biosynthesis</td>
<td>Cellulose Synthase</td>
</tr>
<tr>
<td></td>
<td><strong>Asulam</strong></td>
<td>Folate Biosynthesis</td>
<td>Dihydroprteroate Synthase</td>
</tr>
<tr>
<td>Insecticides</td>
<td><strong>Carbamates</strong></td>
<td>Nervous System Inhibition</td>
<td>Acetylcholinesterase</td>
</tr>
<tr>
<td></td>
<td><strong>Organophosphates</strong></td>
<td>Nervous System Inhibition</td>
<td>GABA Receptor</td>
</tr>
<tr>
<td></td>
<td><strong>Organochlorines</strong></td>
<td>Nervous System Inhibition</td>
<td>GABA Receptor</td>
</tr>
<tr>
<td></td>
<td><strong>Cyclohexanes</strong></td>
<td>GABA Receptor</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Pyrethroids</strong></td>
<td>Nervous System Inhibition</td>
<td>GABA Receptor</td>
</tr>
<tr>
<td>Fungicides</td>
<td><strong>Aromatic hydrocarbons, Triazoles</strong></td>
<td>Membrane Biosynthesis</td>
<td>Lipid Biosynthesis</td>
</tr>
<tr>
<td></td>
<td><strong>Cinnamic acid amide, Morpholine, Triazoles</strong></td>
<td>Membrane Biosynthesis</td>
<td>Sterol Biosynthesis</td>
</tr>
<tr>
<td></td>
<td><strong>Hydrochloride</strong></td>
<td>Membrane Biosynthesis</td>
<td>Intracellular Membrane Components</td>
</tr>
<tr>
<td></td>
<td><strong>Glucopyranosyl antibiotics, Tetracycline antibiotics</strong></td>
<td>Amino Acid Synthesis</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Phenylpyroles, Dicarboximides</strong></td>
<td>Signal Transduction</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Pyrimidinamines</strong></td>
<td>Respiration</td>
<td>NADH oxido-reductase Inhibitors</td>
</tr>
<tr>
<td></td>
<td><strong>Pyridine carboxamides, Benzamides, Gxathiin carboxamides</strong></td>
<td>Respiration</td>
<td>Succinate-dehydrogenase Inhibitors</td>
</tr>
<tr>
<td></td>
<td><strong>2,6-dinitroanilines, Dinitrophenyl crotonate</strong></td>
<td>Respiration</td>
<td>Oxidative Phosphorylation Uncouplers</td>
</tr>
</tbody>
</table>
CHAPTER TWO: THE EFFECT OF AGROCHEMICALS ON INDICATOR BACTERIA DENSITIES IN OUTDOORS MESOCOSMS

Summary

Water bodies, which are monitored for microbial water quality by quantification of fecal indicator organisms (IOs), can contain various zoonotic pathogens contributed by livestock waste and other sources. Sediments can serve as reservoirs of IOs and other enteric microorganisms, including pathogens. Agrochemicals may influence the survival of these microorganisms in water bodies impacted by livestock waste by enhancing or reducing their survival. Complex, 1100L, freshwater mesocosms containing leaf litter, zooplankton, periphyton, phytoplankton, and invertebrate and vertebrate animals were used to investigate the response of \textit{E. coli} and enterococci to agrochemicals. Replicate tanks were treated with atrazine, malathion, chlorothalonil, and inorganic fertilizer, either alone at 1x or 2x their expected environmental concentrations (EEC) or in pair-wise combinations at their EECs. IOs inoculated in sediment ($\sim 10^4$ CFU/100ml) were enumerated over 28 days. IOs generally declined over time, but MANOVA revealed that addition of fertilizer and atrazine resulted in significantly greater IO densities relative to controls. Malathion, chlorothalonil, and agrochemical concentration did not

\footnote{(Published in Journal of Environmental Microbiology 2010, 12(12), 3150-3158)}
significantly affect IO densities and no significant interactions between agrochemicals were noted. The augmentation of IO densities in sediments by fertilizer and atrazine may impact their reliability as accurate predictors of water quality and human health risk, and indicates the need for a better understanding of the fate of IOs and enteric pathogens in sediments exposed to agrochemicals.

Introduction

Management of endemic and emerging human pathogens associated with agriculture, such as Salmonella, pathogenic Escherichia coli, and zoonotic influenza viruses, has become a matter of great concern to many governmental agencies and water quality managers (U. S. Environmental Protection Agency, 2005, 2007, 2009a, b). In fact, a recent review notes that the majority of known human pathogens are zoonotic, and that agricultural practices play a major role in the dynamics of disease transmission (Lloyd-Smith et al., 2009). Many agriculturally-derived pathogens are transmitted via the waterborne route and can enter water bodies via runoff that is contaminated by the waste of livestock, e.g. cattle, swine, and poultry. Further, the sediments of water bodies can serve as reservoirs for enteric microorganisms, prolonging their survival (Davies et al., 1995; Ishii et al., 2007; Badgley et al., 2010).

Testing directly for waterborne pathogens is prohibitively costly and extremely difficult because of the great diversity of potential pathogens and culturing difficulties; therefore, regulatory agencies have relied on enumeration of indicator organisms (IOs) to indicate fecal contamination and increased probability of the presence of human pathogens. IOs such as E. coli and Enterococcus spp. naturally inhabit the gastrointestinal tract of warm-blooded, and some cold-blooded, animals (Harwood et al.,
are generally commensal, and are shed in the feces with enteric pathogens. Regulatory agencies have determined that both \textit{E. coli} and \textit{Enterococcus} spp. serve as useful indicators of enteric pathogens in fresh water, while enterococci are the better indicator in marine/estuarine waters (Cabelli et al., 1979; U. S. Environmental Protection Agency, 1983; Wade et al., 2006). While regulatory standards only take into account IO densities in the water column, \textit{E. coli} and enterococci are also present in the underlying sediments of water bodies. It has been established that IOs are capable of extended persistence, and possibly growth in the environmental reservoirs represented by the sediments of estuarine and marine water bodies (Davies et al., 1995; Anderson et al., 2005; Ishii et al., 2007). Studies have shown that the resuspension of IOs in the sediment can occur as a result of natural turbulence or human influence, both of which may re-inoculate overlying water (Craig, 2004; Jin et al., 2004; Graczyk et al., 2007a; Graczyk et al., 2007b; Philip et al., 2009). In fact, resuspension of bacteria caused by wading activities has been shown to elevate \textit{E. coli} concentrations as much as 4-fold (Philip et al., 2009).

Runoff from agricultural sites also often contains agrochemicals, such as fertilizers and pesticides, which can have various effects on freshwater ecosystems, including the death of algal and some diatom species, inhibition of photosynthesis resulting in increased heterotrophic activity by protozoa, and alteration of the species diversity within the affected ecosystem (Verro, 2009a, b; Debenest et al., 2010). The effects seen on bacterial populations may be direct or indirect; beneficial or adverse (Clements and Rohr, 2009; Verro, 2009a, b). Agrochemicals can alter the traits of organisms, such as behavior, immunity, physiology and morphology, which can in turn
alter interactions with con- and heterospecifics (Rohr et al., 2003, 2004; Rohr and Crumrine, 2005; Rohr and Palmer, 2005; Rohr et al., 2006a; Rohr et al., 2008b).

Agrochemicals can also have adverse or beneficial indirect effects via direct toxicity to a focal species’ food resources, parasites, competitors, or predators (Rohr et al., 2006a; Raffel et al., 2008; Rohr et al., 2008a; Rohr et al., 2008b; Rohr et al., 2009). For instance, a chemical might reduce the number of conspecifics, reducing competition for the survivors (Rohr et al., 2006b). If agrochemicals have positive or negative direct or indirect effects on IOs, then the utility of IOs as predictors of contamination and the possible presence of human pathogens may be confounded.

To determine the effects of agrochemicals on IO populations, multiple freshwater mesocosms inoculated with IOs were dosed with inorganic fertilizer, the herbicide atrazine, the insecticide malathion, or the fungicide chlorothalonil, either alone at 1x or 2x their expected environmental concentrations (EEC) or in pair-wise combinations at their EECs. Each of these pesticides was selected because they are among the top two in usage in the United States within their pesticide type (herbicide, insecticide, synthetic fungicide; Kiely, 2004). We then quantified IO densities in these tanks through time using standard culture (membrane filtration) methods (U. S. Environmental Protection Agency, 2002a, b).

Results

The targeted 1x nominal EECs for atrazine, chlorothalonil, malathion, and fertilizer (102, 169, 101, 110 P:2338 N µg/L, respectively) closely matched the actual concentrations in the mesocosms (1x: 101, 172, 92, 140 P:2450 N; 2x:228, 351, 164, 440...
Concentrations of calcium, and total phosphorus and nitrogen in control tanks were 39000, 60, and 370 µg/L, respectively.

A representative subset of survival curves for IOs subjected the various agrochemical treatments is shown in Figure 1. *E. coli* densities in all treatments were initially \(10^4\) CFU/100 g sediment (wet weight). Populations in all mesocosms dropped about 2.5 logs over 7 days, generally decreased more slowly over the next week, and stabilized over the next two weeks (Figure 1). Enterococci densities decreased from \(10^3\) CFU/100 g at the initial time point to \(10^2\) CFU/100 g after one week. Enterococci densities decreased to their lowest point at 14 days (\(10^1 - 10^2\) CFU/100 g), and tended to increase by one log or less over the next 14 days. When IO densities were averaged over all treatments, enterococci densities on day 28 were significantly greater than *E. coli* densities.

None of the 16 treatments differed in *E. coli* or enterococci density before agrochemical applications (Treatment: Wilk’s \(F_{30,88} = 0.77, P=0.786\)). There was no treatment-by-time interaction; that is, IO densities in all treatments followed a similar trend over time. Daily decay rates were calculated for the first seven and 28 days. The initial daily decay rate ranged from -0.296 to -0.173 for enterococci and -0.386 to -0.221 for *E. coli*. The 28-day daily decay rate ranged from -0.064 to -0.030 for enterococci and -0.122 to -0.068 for *E. coli*. Decay rates for *E. coli* for all treatments combined were significantly greater (more rapid decline in concentrations; \(P < .0001\)) than they were for enterococci (Figure 1).

There was no evidence that any of the agrochemicals synergistically or antagonistically affected the densities of IOs (Agrochemical*agrochemical: Wilk’s \(F_{2,33} <\)
Given that we had no evidence for interactions between the agrochemicals or for differences between the 1x EEC and 2x EEC concentrations, our final model was a regression-based MANOVA containing just four main effects representing the presence or absence of each of the four agrochemicals (as well as block, the repeated measures factor, and interactions between the agrochemicals and the repeated measures factor). That is, the MANOVA model pooled all treatments without the chemical in question, and all treatments with the given agrochemical, including the 1x and 2x EEC treatments as well as the pair-wise combinations that contained the amendments. This model provided the greatest power to detect effects of the agrochemicals because it uses all the data and pools the 1x EEC and 2x EEC treatments, as well as incorporating both response variables (E. coli and enterococci density). This MANOVA revealed that IO density was elevated, compared to controls, by both fertilizer and atrazine and these responses were consistent through time (Figure 2, Table 2). ANOVA (considering the response variables separately) revealed that enterococci concentrations were significantly greater in the presence of both fertilizer and atrazine \((F_{1,56}=7.44, P=0.004; F_{1,56}=5.90, P=0.009; \text{respectively, one-tailed, Figure 2A})\). Similarly, E. coli concentrations were significantly greater in the presence of fertilizer at the alpha level of 0.05 \((F_{1,56}=5.84, P=0.009)\) but
were not significantly greater in the presence of atrazine at that alpha level (F_{1,56}=2.55, P=0.058; Fig. 2B); however, it should be noted that the difference is significant when alpha is set at 0.10. Neither malathion (E. coli: parameter=0.108, 95% confidence interval=±0.244; enterococci: parameter=0.106, 95% confidence interval=±0.223) nor chlorothalonil (E. coli: parameter=0.196, 95% confidence interval=±0.244; enterococci: parameter=0.203, 95% confidence interval=±0.223) significantly affected IO densities across sampling dates or at any given sampling date (Table 2).

**Discussion**

The mesocosms employed here to investigate the effect of agrochemicals on the maintenance of fecal indicator bacteria populations in an aquatic system are unusually complex for a microbiological study in that they included a community of vertebrates, invertebrates, algae and plants, as well as natural sediments. In addition to ecological complexity, sampling from 64 large mesocosms provided true replication, which also is uncommon in studies of microbial survival in the environment. We were interested in the fate of IOs in sediments impacted by agrochemicals because sediments are the environmental compartment in which IOs can be exposed long-term to the chemicals in flowing waters. While bacteria in the water column will be transported downstream and both they and agrochemicals will be successively diluted, sediments that receive agricultural runoff can be expected to retain elevated concentrations of these chemicals due to their propensity to adsorb to particles and sediments (Chung et al., 1996; Scarlators, 1997; Xu et al., 2009). Furthermore, greater IO survival in sediments compared to the water column in environmental waters is well-documented (Anderson et al., 2005; Ishii et al., 2007); and resuspension of sediments in polluted waters can result
in exposure of humans and animals to increased levels of enteric microorganisms. The overall decline observed in both *E. coli* and enterococci densities in sediments over time is consistent with observations from other studies, as is the slower decline of enterococci compared to *E. coli* (Craig, 2004; Anderson et al., 2005; Sampson, 2006; Hartz, 2008).

Significantly greater IO densities compared to controls were observed when atrazine or fertilizer was present in the mesocosms. These results are novel, in that it is the first time these effects have been seen on *E. coli* and *Enterococcus* spp. in mesocosms that attempt to reflect the complexity of natural systems. The positive effect of atrazine is corroborated by a previous study conducted *in vitro*, which found increased densities of *E. coli* and *Enterococcus* spp. in atrazine-treated cultures that was attributed to utilization of the chemical as a nutrient source (Koutsotoli, 2005). The positive effects of atrazine noted in our study could result from a direct effect, as noted above and in other studies (Yanzekontchou and Gschwind, 1994; Rhine et al., 2003). It has been shown that bacteria persisting in anaerobic wetland sediment were capable of metabolizing atrazine and other triazine herbicides to NH$_3$ and CO$_2$ (Chung et al., 1996). However, the mechanism of atrazine stimulation may be wholly or in part due to indirect effects in the complex mesocosms used here. Atrazine is toxic to phytoplankton (Rohr et al., 2008b), which, after dying following exposure to atrazine, settle upon the sediment, increasing the amount of available carbon to the heterotrophic IOs. Furthermore, a decrease in phytoplankton would allow more light to penetrate the water column, stimulating photosynthesis by periphyton in sediment biofilms and increasing available carbon (Herman et al., 1986; Pratt et al., 1997; Rohr et al., 2008b).
Inorganic fertilizer also led to greater densities of *E. coli* and enterococci after 28 days. The data are consistent with results of previous studies of soil, which showed greater persistence of *E. coli* O157:H7 and *Salmonella enterica* serovar Typhimurium as well as non-pathogenic *E. coli* and enterococci in soils amended with fertilizer (Kudva et al., 1998; Lau and Ingham, 2001; Semenov et al., 2009). Like the proposed mechanism(s) for enhancement of IO survival with atrazine, the maintenance of greater *E. coli* and enterococci densities associated with fertilizer treatment has two possible explanations: the IOs may use the fertilizer directly as an inorganic nutrient source, and/or algal populations may be altered so as to affect available organic carbon levels. It should be noted that a low density of indicator bacteria species and strains were probably introduced into the mesocosms from the algal, vertebrate and invertebrate organisms which were initially added to the tanks. However, BOX-PCR genotyping of *E. coli* and enterococci isolated from sediments showed a predominance of the originally inoculated strains after 28 days (data not shown). The greater densities of *E. coli* and enterococci maintained in mesocosms containing atrazine and fertilizer should not be taken as a generalized statement that all *E. coli* strains and all species of enterococci will benefit from exposure to these agrochemicals, as it is highly probable that certain strains survive in a culturable state longer than others under the conditions used here.

Although previous studies revealed the ability of certain bacteria to use the insecticide malathion as a carbon and/or phosphate source (Karpouzas, 2006; Abo-Amer., 2007; Cycoń, 2009), no significant increase in *E. coli* or enterococci densities compared to controls was observed in mesocosms containing malathion. Malathion’s non-significant effect on IO density may have been due to an inability of the IOs to utilize this
chemical as a nutrient or because of inadequate statistical power. The fungicide chlorothalonil also had no significant effect on IO density in this study at either 1x or 2x the EEC, although previous studies demonstrated adverse effects of chlorothalonil on microbial community structure and microbial densities after repeated exposures or high doses (Singh, 2002; Yu, 2006; Chu, 2008; Podio, 2008).

The introduction of agrochemical run-off into rural water bodies may have unintended consequences for the survival of enteric microorganisms in aquatic habitats. Like IOs, human pathogens such as toxigenic *E. coli* and *Salmonella* are chemoorganoheterotrophs and belong to the same species or are closely related to one of the IOs used here (*E. coli*), therefore it is reasonable to hypothesize that their response to agrochemicals would be similar. Alternatively, agrochemicals may affect the persistence of IOs and certain pathogens disproportionally, causing a disconnect in the IO-pathogen relationship. Epidemiological studies have demonstrated correlations between elevated IO levels and the risk of gastroenteritis (Barrell, 2000; Craun, 2006; Colford, 2007; Kite-Powell et al., 2008; Wade, 2008). While risk assessment and epidemiological research has focused on analysis of the water column, a better understanding of the relationship between environmental influences, pathogen densities and IO densities in sediments is necessary to better predict the impact that resuspension may have on water quality and human health risk. To that end, several recent studies have focused on IO and pathogen densities in sand and sediment, and their relationships to those measurements in the water (Abdelazhar et al., 2010; Badgley et al. 2010; Ishii et al 2007; Yamahara et al 2007, 2009); finding extended persistence and elevated densities in these substrates compared to the water column.
Presently, the effects of fertilizer and atrazine on enteric pathogens are largely unknown, and further studies are needed to establish the relationship between these chemicals and their effects on the growth and persistence of pathogens in secondary habitats, such as environmental waters and sediments. This mesocosm study was designed to capture both direct and indirect effects of agrochemicals on IOs, either or both of which may have generated the observed results. The next logical step would be to conduct a direct toxicity study, where simplified mesocosms contain the agrochemicals and inoculated organisms only. Understanding the effects of agrochemicals on enteric microorganisms is necessary to determine the most effective system of indicators of fecal contamination of water, and the best management practices for application of agrochemicals in order to protect human and animal health.

**Experimental Procedures**

**Experimental Design.** Sixty-four outdoor mesocosms were established at the University of Florida’s Institute of Food and Agricultural Sciences Gulf Coast Research and Education Center (Ruskin, FL). The mesocosms consisted of round cattle water tanks that were each 6 feet in diameter and two feet deep. Each tank contained 1100L of municipal water, 300g of leaf litter (predominantly live oak, *Quercus virginiana*), and local zooplankton, phytoplankton, periphyton, tadpole, insect, gastropod, and crayfish species collected from several ponds in the region. Zooplankton, periphyton, and phytoplankton collected from four ponds was homogenized and used as the source of initial inoculations into each mesocosm. Three weeks was allowed for zooplankton, periphyton, and phytoplankton populations to establish before agrochemical applications. Just before agrochemical applications, tadpoles, insects, gastropods, and crayfish were
counted and distributed among the tanks to ensure that each tank had the same starting communities. Zooplankton and chlorophyll a in the periphyton and phytoplankton were quantified throughout the experiment in two week intervals, and tadpole, insect, gastropod, and crayfish survival were quantified at the end of the experiment. Densities of zooplankton, periphyton, and phytoplankton were similar among tanks prior to agrochemical applications and community-level effects of the treatments on species other than bacteria will be presented elsewhere. All mesocosms were covered with 60% shade cloth.

An uncovered plastic box (11cm x 27cm x 37cm) containing sediment (~5cm high) from the lower Hillsborough River (Tampa, FL) was placed in each tank. The sediment had been dried for one week before placement into the tanks to allow any endogenous indicator bacteria to die. Culturing on selective-differential media (as described below) verified that the sediment contained undetectable levels of *E. coli* and enterococci. Each of these sediment boxes was inoculated with known *E. coli* and *Enterococcus* spp. strains (see below). Hence, these mesocosms contained most of the abiotic and biotic characteristics of a community in Florida ponds.

Tanks were arranged in a randomized block design with four replicates of each of 16 treatments. There were two control treatments, with addition of only 50 ml of water or acetone (used as a solvent for all agrochemicals). Tanks in the remaining 14 treatments received agrochemicals. Our representative agrochemicals were the herbicide atrazine, the insecticide malathion, the fungicide chlorothalonil, and inorganic fertilizer (sodium nitrate and sodium phosphate). All pesticides were technical grade (purity >98%, Chemservice, West Chester, PA). Six of the agrochemical treatments were
pair-wise combinations of the agrochemicals at their expected environmental concentrations (EEC) calculated using the Environmental Protection Agency’s GENECC v2 software. Fertilizer was added at an expected environmental concentration of 220 µg/L P: 4675 µg/L N, a concentration found commonly in a pond survey (Chase, 2003). The remaining eight treatments were each agrochemical alone, either at its EEC (1x EEC) or at double its EEC (2x EEC). The 2x EEC treatments were incorporated in an effort to control for the fact that mixtures had twice the concentration of agrochemicals as the single-chemical treatments. Lone agrochemicals and agrochemical mixtures were dissolved in 50 ml of acetone so that each agrochemical tank received the same amount of acetone. To quantify actual concentrations, pooled samples from each lone pesticide treatment were analyzed by the Mississippi State Chemical Laboratory (Starkville, MS) and samples from each lone fertilizer treatment were analyzed by the Hillsborough County Water Resource Services Environmental Laboratory (Tampa, FL).

Bacterial Inoculation. Five *E. coli* strains and five *Enterococcus* spp. strains were selected for use in this experiment. Three isolates each of *E. coli* and enterococci were selected based on prolonged survival in the culturable state in a previous mesocosm study along with two isolates of each that rapidly became unculturable (Anderson et al., 2005). Four of the *E. coli* strains used for inoculation were originally obtained from wastewater and the fifth was *E. coli* 9637 (American Type Culture Collection). Inoculated *Enterococcus* strains included environmental water isolates (two *Ent. casseliflavus*, one *Ent. faecium*, and one *Ent. faecalis*) as well as *Ent. faecalis* 19433 (American Type Culture Collection).
The five *E.coli* and *Enterococcus* spp. strains were each streaked for isolation on trypticase soy agar (TSA) and incubated for 24 h at 37°C. Isolated colonies were then inoculated into 10 ml cultures of brain heart infusion broth (BHI) and were incubated for 24 hr at 37°C. The 10 ml cultures of the five *E. coli* and *Enterococcus* strains were then added to two separate flasks, one for *E.coli* and one for enterococci, of one liter each of sterile buffered water (0.0425 g · L\(^{-1}\) KH\(_2\)PO\(_4\) and 0.4055 g L\(^{-1}\) MgCl\(_2\); American Public Health Association, 1995). Prior to inoculation and submersion in the mesocosm, 1L of water from each cattle tank was placed in the respective sediment-containing box, which would be submerged in the tank after inoculation. From their respective one-liter flasks, 500µL of *E. coli* and 1mL of enterococci were then inoculated into each sediment box (~10\(^4\) CFU/100m for *E. coli* and ~10\(^3\) for enterococci, taking into account only the volume of water in each sediment box). The boxes were covered and bacteria were allowed to settle for 1 h before they were submerged in the cattle tanks. The boxes were placed into each tank and the lids were carefully removed to avoid disturbing the sediment. Hence, each sediment box in each tank was inoculated with approximately 10\(^4\) CFU/100ml of each of the five strains of *E. coli* and approximately 10\(^3\) CFU/100ml each of the five strains of *Enterococcus* spp.

**Sample Collection and Filtration.** Sediment boxes in each tank were inoculated on July 14, 2008 and sediment samples were taken an hour after inoculation. Pesticides were applied after these sediment samples were taken and thus we had an estimate of *E. coli* and *Enterococcus* spp. density before treatment applications. Additional sediment samples were collected once a week for the next four weeks. The amount of sediment collected varied based upon the bacterial counts obtained from the previous sampling,
ranging from 10 to 40 g wet weight. To sample the sediment, a sterile 50ml centrifuge tube was used to scoop the top 1-2 cm of the sediment, across the length of the sediment box, until filled. Samples were placed on ice for transport to the laboratory. Prior to filtration, sediment samples were weighed out and diluted 1:10 in sterile buffered water (American Public Health Association, 1995) and sonicated for 30s on setting 4 (Sonic Dismembrator Model 100, Fisher Scientific) to release bacterial particles attached to the sediment (Anderson et al., 2005; Korajkic, 2009). Sediment suspensions were allowed to settle for several minutes before the supernatant was pipetted off and filtered through a nitrocellulose filter (0.45 μm pore-size, 47 mm diameter). The volume filtered ranged from 0.1 ml on the first day to 100 ml after two weeks.

Indicator organisms were enumerated by standard membrane filtration methods. Enterococci were enumerated on mEI agar after 24 h incubation at 41° C (U. S. Environmental Protection Agency, 2002a); E. coli were enumerated on mTEC media at 35° C for 2 h, followed by 22 h incubation at 44.5° C (U. S. Environmental Protection Agency, 2002b). Typical colonies on plates were counted and densities were reported as CFU · 100 g⁻¹ (wet weight).

Statistical Analyses. All response variables were log-transformed and spatial block was included in all analyses because it was always significant. The residuals were always carefully scrutinized to ensure that we met the assumptions of the analysis. We conducted repeated measures analyses for all data taken after agrochemical applications, where the repeated measures factor was IO density on the three sampling dates. In these analyses, we always included interactions between among- and within-tank (repeated measures) factors. This allowed us to test for treatment-by-time interactions. We also
conducted regression-based analysis of variance (ANOVA) after regression-based multivariate analysis of variance (MANOVA) to ensure that we did not miss any significant univariate effects. In all MANOVAs, the response variables were the density of *E. coli* and enterococci on each sampling date.

Given the complexity of the experiment, we conducted a series of analyses in a step-wise hierarchical manner. We first conducted a MANOVA to test whether the density of *E. coli* and enterococci differed among any of the 16 treatments before the agrochemicals were applied in order to insure that initial densities were similar. We then tested whether any of the agrochemicals had synergistic or antagonistic effects on *E. coli* and enterococci density by conducting a repeated measures MANOVA that included the presence or absence of each the agrochemicals as four main effects and that included all two way interactions between agrochemicals. In our third analysis, we tested for a difference between the 1x EEC and 2x EEC treatments and their dependence on agrochemical treatment by conducting a 4 x 2 repeated measures MANOVA. That is, there were the four main effects of agrochemicals crossed by whether the agrochemical was applied at one times or two times the EEC. If there was any evidence of interactions between agrochemicals or differences among the 1x and 2x treatments, then these effects would have to be included in the final model. If there was no evidence of interactions or differences among the 1x and 2x treatments, then we could justify using a final model that contained just the four main effects representing the presence or absence of each of the four agrochemicals (as well as block, the repeated measures factor, and interactions between the agrochemicals and the repeated measures factor).
Initial (7-day) and overall (28-day) decay rates (decrease in cell concentration) were calculated for each tank for both *E. coli* and enterococci. The initial decay rate was obtained by subtracting the bacterial density (log_{10} / 100g) immediately after inoculation from the bacterial density seven days following inoculation. This value was then divided by seven to obtain the initial daily decay rate. The overall daily decay rate was calculated by subtracting the bacterial density immediately after inoculation from the bacterial density 28-days after inoculation. This value was then divided by 28 to obtain the overall daily decay rate.

**Acknowledgments**

We would like to thank the Rohr lab, Steven A. Johnson and the University of Florida's Gulf Coast Research and Education Center for help with setting up and maintaining mesocosms. Funding was provided by USDA-NIFA Water and Watershed program grant 2009-35102-05043.

**References**


U. S. Environmental Protection Agency (2009a) Review of Published Studies to Characterize Relative Risks from Different Sources of Fecal Contamination in Recreational Water. EPA 822-R-09-001.

U. S. Environmental Protection Agency (2009b) Review of Zoonotic Pathogens in Ambient Waters. EPA 822-R-09-002.


Figure 1. Densities of IOs in sediments of mesocosms over 28 days: A) *E. coli* and B) enterococci. Results from a subset of agrochemical treatments are shown for each IO.
Figure 2. Least squares means (±1 SE) for the effects of fertilizer (left) and atrazine (right) on the density of (A) *E. coli* and (B) enterococci after 28 days. The effect of fertilizer and atrazine was averaged across 1x and 2x EEC concentrations and mixtures containing the focal agrochemical because there was no evidence for differences between the 1x EEC and 2x EEC concentrations or for interactions between the agrochemicals. Furthermore, treatments without the focal agrochemical include both the water and solvent controls. White bars indicate IO density (log$_{10}$-transformed CFU 100 g$^{-1}$) in mesocosms without agrochemicals; black bars indicate IO density in mesocosms with agrochemicals.
Table 2. Results of multivariate analysis of variance examining the effects of spatial block, fertilizer, atrazine, chlorothalonil, malathion, and sampling date on the density of *E. coli* and enterococci in sediment.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Wilk’s F</th>
<th>df effect</th>
<th>df error</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>66.76</td>
<td>2</td>
<td>55.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Block</td>
<td>6.95</td>
<td>6</td>
<td>110.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fertilizer</td>
<td>5.45</td>
<td>2</td>
<td>55.0</td>
<td>0.007</td>
</tr>
<tr>
<td>Atrazine</td>
<td>3.53</td>
<td>2</td>
<td>55.0</td>
<td>0.036</td>
</tr>
<tr>
<td>Chlorothalonil</td>
<td>1.22</td>
<td>2</td>
<td>55.0</td>
<td>0.304</td>
</tr>
<tr>
<td>Malathion</td>
<td>0.35</td>
<td>2</td>
<td>55.0</td>
<td>0.708</td>
</tr>
<tr>
<td>Sampling date</td>
<td>1.47</td>
<td>4</td>
<td>53.0</td>
<td>0.226</td>
</tr>
<tr>
<td>Date*block</td>
<td>1.32</td>
<td>12</td>
<td>140.5</td>
<td>0.215</td>
</tr>
<tr>
<td>Date*fertilizer</td>
<td>0.80</td>
<td>4</td>
<td>53.0</td>
<td>0.532</td>
</tr>
<tr>
<td>Date*atrazine</td>
<td>1.40</td>
<td>4</td>
<td>53.0</td>
<td>0.248</td>
</tr>
<tr>
<td>Date*chlorothalonil</td>
<td>1.25</td>
<td>4</td>
<td>53.0</td>
<td>0.303</td>
</tr>
<tr>
<td>Date*malathion</td>
<td>1.79</td>
<td>4</td>
<td>53.0</td>
<td>0.144</td>
</tr>
</tbody>
</table>
CHAPTER THREE: A TEST OF DIRECT AND INDIRECT EFFECTS OF AGROCHEMICALS ON THE SURVIVAL OF FECAL INDICATOR BACTERIA

Summary

Water bodies often simultaneously receive agrochemicals and animal waste carrying faecal indicator bacteria (FIB) and zoonotic pathogens, but we know little about the effects of agrochemicals on these microbes of human relevance. In this study, we assessed the direct effects of the pesticides atrazine, malathion, and chlorothalonil, and inorganic fertilizer (all at expected environmental concentration) on *Escherichia coli* and enterococci survival in water and sediments of simplified microcosms held in the dark. There was a positive correlation between *E. coli* strain composition in the sediment and the water column, but none of the four agrochemicals had detectable direct effects on *E. coli* strain composition or on densities of culturable FIBs. In a companion study, microcosms with non-disinfected pond water and sediments were exposed to or shielded from sunlight to examine potential indirect effects of atrazine and inorganic fertilizer on *E. coli*. The herbicide atrazine had no effect on *E. coli* in dark-exposed microcosms

\(^1\) (Published in Journal of Applied and Environmental Microbiology 2011, 77(24), 8765-8774)
containing natural microbial and algal communities. However, in light-exposed microcosms, atrazine significantly lowered \textit{E. coli} densities in the water column and significantly increased densities in the sediment compared to controls. This effect appears to be mediated by the effects of atrazine on algae given that atrazine significantly reduced phytoplankton, which was a positive and negative predictor of \textit{E. coli} densities in the water column and sediment, respectively. Our results suggest that the tested agrochemical do not directly affect the survival of FIBs but that atrazine indirectly alters the relative distribution and abundance of \textit{E. coli} by altering the altering phytoplankton and periphyton communities. These results improve our understanding of how agricultural practices can influence faecal indicator bacterial densities in water bodies impacted by agricultural runoff.

\textbf{Introduction}

Within the last century, agriculture has become a significant, and in some areas the dominant, form of land use (Robinson and Sutherland 2002). This agricultural intensification has had detrimental effects on neighboring freshwater and marine ecosystems leading to the loss of biodiversity, blooms of harmful algal species, and shifts in the composition of food webs (Tilman 1999). In recent years, agricultural practices have been subjected to increased scrutiny for their potential contribution to human health risks (Kudva, Blanch et al. 1998; Fratamico 2004; Semenov, van Overbeek et al. 2009; Ziemer, Bonner et al. 2010). For instance, many endemic and emerging human pathogens are derived from or associated with livestock, including pathogenic \textit{E. coli} strains, \textit{Salmonella enterica}, \textit{Campylobacter jejuni}, \textit{Cryptosporidium} spp. and zoonotic influenza viruses (U. S. Environmental Protection Agency 2005; U. S. Environmental Protection
Many of these agriculturally-derived pathogens are waterborne and can enter water bodies via introduction of runoff containing waste from livestock, such as cattle, swine, and poultry (Fratamico 2004; Berry 2007; Brooks, Adeli et al. 2009).

Due to the multitude of potential fecal-derived pathogens in water, which include bacteria, protozoa, and viruses, testing directly for each pathogen is prohibitively costly and time-consuming. Consequently, regulatory agencies have relied on the density of indicator organisms (FIB) for over a century to detect fecal contamination and thus an increased likelihood of the presence of human pathogens (Wolf 1972). Many epidemiological studies in recreational waters have supported the association between elevated FIB densities and the risk of contracting gastroenteritis (Craun and Calderon 2006; Kite-Powell, Fleming et al. 2008; Wade, Calderon et al. 2008).

Storm water and agricultural runoff regularly introduce both microbial contaminants and agrochemicals, such as fertilizers and pesticides, to water bodies. Through direct or indirect mechanisms, agrochemicals may affect bacterial populations in beneficial or adverse ways (Clements and Rohr 2009). Possible direct effects include agrochemicals acting as nutrients (beneficial) or toxic compounds (adverse). Agrochemicals can also have indirect effects that are mediated by another species, or by interactions with other factors. For instance, agrochemicals can alter the traits of an organism, such as behavior, immunity, physiology, or morphology, thereby affecting its interactions with other species. This effect has been seen in multicellular organisms (Rohr, Elskus et al. 2003; Rohr and Crumrine 2005; Rohr and Palmer 2005; Rohr, Kerby et al. 2006; Rohr, Schotthoefer et al. 2008) and microorganisms (DeLorenzo, Scott et al.
agrochemicals may cause density-mediated, rather than trait-mediated, mediated indirect effects via direct toxicity to a target species’ food resources, parasites, competitors, or predators (Dive, Leclerc et al. 1980; Ekelund 1999; Sumpono, Belean et al. 2003; Rohr, Kerby et al. 2006; Raffel, Martin et al. 2008; Rohr, Raffel et al. 2008; Rohr, Schotthoefer et al. 2008; Rohr, Swan et al. 2009; Foit, Chatzinotas et al. 2010). FIBs, pathogens, and other organisms ranging from bacterivorous protozoa, to algae, to aquatic animals are exposed to agrochemicals in environmental waters that receive agricultural runoff, but we know very little about the effect of the chemicals on the fate of microorganisms in such systems.

Here, we assess the direct effects of the pesticides atrazine, malathion, and chlorothalonil, and inorganic fertilizer (all at expected environmental concentration) on *Escherichia coli* and enterococci survival in simplified microcosms held in the dark. Based on our previous work (Staley et al. 2010), atrazine and fertilizer only were predicted to have indirect effects on *E. coli*. Hence, in a separate study, we assessed whether these agrochemicals where having indirect effects on *E. coli* densities by incubating half the microcosms in the dark to prevent the growth of phototrophs, while the other half were exposed to ultraviolet (UV) light. Each pesticide was selected because it was among the top two in usage within the United States for its pesticide type [e.g. herbicide, insecticide, synthetic fungicide (Kiely, Donaldson et al. 2004)].

Fertilizer and atrazine both had positive effects on FIBs in our previous outdoor mesocosms experiment where direct and indirect effects were combined and thus we hypothesized that fertilizer and atrazine might impact FIB densities through either direct
or indirect mechanisms (Staley, Rohr et al. 2010). Previous research showed that soils amended with fertilizer resulted in greater persistence of *E. coli* O157:H7, *Salmonella enterica* serovar Typhimurium, non-pathogenic *E. coli* and enterococci (Kudva, Blanch et al. 1998; Lau and Ingham 2001; Semenov, van Overbeek et al. 2009), suggesting a potential direct mechanism. Similarly, positive direct effects were anticipated for atrazine, as past *in vitro* studies have shown that *E. coli* is chemotactic toward atrazine (Liu and Parales 2009) and they can directly utilize atrazine as a nutrient (Koutsotoli, 2005; Yanzekontchou, 1994; Rhine, 2003). Atrazine and fertilizer might also cause indirect effects by influencing the algal populations within water bodies. Fertilizer often increases algal growth, potentially increasing the reservoir for bacteria and nutrient availability to heterotrophs (Byappanahalli, Shively et al. 2003; Whitman, Shively et al. 2003; Badgley, Nayak et al. 2010; Badgley, Thomas et al. 2010). Atrazine is typically associated with decreases in phytoplankton that result in an increase in UV light penetration (Rohr, Schotthoefer et al. 2008), which could stress bacteria in the water column (Sinton, Hall et al. 2002; Noble, Lee et al. 2004). Additionally, atrazine is often associated with increases in periphyton that could provide additional nutrients to bacteria in the sediment. Therefore, we hypothesize that atrazine will alter the algal community under light-exposed conditions, which in turn will result in a reduction in bacterial densities in the water column but an increase in densities in the sediment. Neither malathion nor chlorothalonil had significant effects on FIBs in our previous outdoor mesocosms experiment where direct and indirect effects were combined and thus we hypothesized that they would not have direct effects on FIBs in this experiment.
Experimental Design

Direct Effect Experiment. We established microcosms at the University of South Florida Botanical Garden (Tampa, FL) in an outdoor greenhouse to examine exclusively direct effects of four agrochemicals (atrazine, chlorothalonil, fertilizer and malathion), and all their pair-wise combinations, on the survival of FIBs. Replication was achieved by repeating each 12-microcosm block (controls, single agrochemical treatments and pair-wise combinations) four times (four temporal blocks). The first temporal block (Block A) was run from June 3, 2009 through June 10th, Block B was run from June 17th through June 24th, Block C was run from July 1st through the 8th, and Block D was run from July 29th through August 5th. Overall, 48 separate microcosms were established to examine direct effects. Air temperatures during the temporal blocks ranged from 21-34°C for Block A, 23-40°C for Block B, 22-37°C for Block C and 18-39°C for Block D. The microcosms consisted of 11.3 L Rubbermaid plastic trash cans with opaque sides (29.97 x 22.86 x 33.65cm) and each contained disinfected water (2 L) and sediment (1 L). Sediment was collected from the lower Hillsborough River (Tampa, FL). Each microcosm was disinfected prior to bacterial inoculation via addition of 2 L of a 30% (V/V) bleach solution made with de-ionized water. The bleach solution was then neutralized through the addition of 45 ml of a 10% sodium thiosulfate (Na₂S₂O₃) solution (0.225% final concentration), and served as the water column for each microcosm (U. S. Environmental Protection Agency 2001). Culturing on selective-differential media (as described below) verified that the sediment and water column contained undetectable levels of E. coli and enterococci. Microcosms were covered individually with aluminum foil and a dark tarp covered all microcosms to prevent exposure to light. The water
column of each microcosm was inoculated with three *E. coli* strains and five *Enterococcus* spp. strains. Two of the *E. coli* strains were originally isolated from wastewater in Tampa, FL and the third was *E. coli* 9637 (American Type Culture Collection). Inoculated *Enterococcus* strains included environmental water isolates (two *Ent. casseliflavus*, and one *Ent. faecalis* isolated from Siesta Key, FL and one *Ent. faecium* isolated from a University of South Florida pond, Tampa, FL) as well as *Ent. faecalis* 19433 (American Type Culture Collection). *E. coli* and enterococci species/strains were selected based on differential survival observed in a previous study (Anderson, Whitlock et al. 2005). This combination of “survivor” (strains exhibiting prolonged survival) and “non-survivor” (strains which became unculturable in a relatively short time frame) strains was selected to determine whether or not strains previously demonstrated to exhibit differing degrees of robustness in secondary environments would all react similarly to the application of agrochemical treatments. One of the wastewater strains and ATCC 9637 were previously been shown to be “survivors,” while the other wastewater strain was a “non-survivor.” The two *Ent. casseliflavus* strains and the *Ent faecium* strain were observed to be “survivors,” while both *Ent faecalis* strains were “non-survivors.”

The *E. coli* and *Enterococcus* spp. strains were streaked for isolation on trypticase soy agar (TSA) and incubated for 24h at 37°C (see Figure 3 for flow chart). Isolated colonies were inoculated into 10ml of brain heart infusion broth (BHI) and incubated for 24h at 37°C. Each of these cultures was then centrifuged at 5000 RPM for 5 min (IEC Multi, Thermo Scientific), and the supernatant was discarded. Each culture was then resuspended and washed in 10 ml of sterile buffered water (0.0425 g · L⁻¹ KH₂PO₄ and
0.4055 g L\(^{-1}\) MgCl\(_2\) twice (American Public Health Association 1995). Each of these 10 ml suspensions was then added to a respective bottle containing 90 ml of sterile buffered water. Bottles containing *E. coli* strains were then combined, as were bottles containing *Enterococcus* spp. strains. From both the bottles containing *E. coli* and the bottle containing enterococci, 3 ml of mixed culture was aseptically added to each microcosm (~10\(^7\) CFU/100ml for both *E. coli* and enterococci).

One hour after FIB inoculations, the 12 agrochemical treatments were assigned randomly to the microcosms. There were two control treatments, one consisting of DI water and a second in which DI water was amended with 0.002% acetone (used as a solvent for all agrochemicals). The remaining 10 microcosms received technical grade agrochemicals: the herbicide atrazine, the insecticide malathion, the fungicide chlorothalonil, or inorganic fertilizer (sodium nitrate and sodium phosphate; Chemservice, West Chester, PA, Purity>97%), either alone at their expected environmental concentration (EEC, 102 µg/L for atrazine, 101 µg/L for malathion, 170 µg/L for chlorothalonil, and 4400 µg/L N and 440 µg/L P for fertilizer, calculated using the U.S. Environmental Protection Agency’s GENECC v2 software) or in pair-wise combinations at their EEC. Each microcosm was then covered with a layer of aluminum foil and all 12 were then covered with a dark tarp (1.676m x 2.286 m) to prevent light penetration. The experiment was carried out in four sequential temporal blocks (see above for the specific dates for each temporal block). All microcosms were thoroughly cleaned and disinfected with 10% bleach between following sampling at one week, prior to the establishment of the another replication of the twelve treatments.
Water and sediment samples were collected from each microcosm one hour after FIB inoculations but immediately before agrochemical applications to obtain baseline (pre-agrochemical) measurements of FIB densities (Time 0). Water and sediment samples were also collected from each microcosm 24 h and 1 week after inoculation. In total, microcosms were sampled three times (T0, T24, and T168) in each of the four temporal blocks.

**Indirect Effect Experiment.** We established 30 microcosms at the University of South Florida in an outdoor greenhouse, as described above, to test for indirect effects of atrazine and fertilizer on E. coli. These microcosms consisted of 2L glass beakers containing autoclaved DI water (1.5 L), sediment collected from the lower Hillsborough River (0.5 L disinfected by baking at 350°F for 24h) and pond water collected from a eutrophic pond at the University of South Florida (0.5 L). Half of the microcosms were completely covered with aluminum foil to prevent light penetration, while the other half were covered only at the top with plastic wrap. Algal populations from the seeded pond water were given two weeks to establish before treatment and E. coli applications.

In addition to bacteria already present in the pond water, the water column of each microcosm was inoculated with four strains of E. coli: E. coli 9637 (American Type Culture Collection) and three strains isolated from wastewater in Tampa, FL. These strains were prepared as described in the Direct Effect Experiment (~10^7 CFU/100ml). Water and sediment samples were collected from each microcosm prior to agrochemical additions.
One hour after FIB inoculations, the microcosms were randomly assigned one of three agrochemical treatments: a water control, inorganic fertilizer or atrazine at 1x EEC (as described above). Microcosms were arranged so that there were five replicates (in spatial blocks conducted concurrently) of each agrochemical treatment for both light-exposed and dark conditions.

Water and sediment samples were collected from each microcosm one hour after FIB inoculations but immediately before agrochemical applications to obtain baseline (pre-agrochemical) measurements of FIB densities (Time 0). Water and sediment samples were also collected from each microcosm after 2, 7, 9, 14, and 28 days after inoculation. A longer sampling period was used for this experiment than the Direct Effect Experiment because any indirect effects on the algal populations would require a longer period of time to become evident, as observed in our previous study (Staley, Rohr et al. 2010). We used an AquaPen AP100 meter (Photon Systems Instruments, Brno, Czech Republic) to quantify the effects of the treatments on suspended algae (phytoplankton). We measured chlorophyll a (F0) and photosynthetic efficiency (QY) initially from each microcosm and then at day 7, 14, and 28.

Sample Collection and Filtration. For both the Direct and Indirect Effect Experiments, water samples were aseptically collected in centrifuge tubes. Sediment samples were collected using a sterile 50-ml centrifuge tube to scoop the top 1-2 cm of the sediment across the length of the microcosm. Sterile gloves were worn when collecting samples and the microcosms were shallow enough that no connection between human skin and the microcosms occurred. Samples were placed on ice for transport to
the laboratory. Transit time to the laboratory was approximately 10 min and samples were processed immediately upon arrival.

Water samples were filtered through a nitrocellulose membrane filter (0.45 µm pore-size, 47 mm diameter, Fisher Scientific). Before filtration, sediment samples were weighed and diluted 1:10 (w/v) in sterile buffered water (American Public Health Association 1995) and sonicated for 30s at 14 watts (Sonic Dismembrator Model 100, Fisher Scientific) to dislodge bacterial particles attached to the sediment for the direct effects microcosms (Anderson, Whitlock et al. 2005; Korajkic, Badgley et al. 2009). For the *Indirect Effect Experiment*, sediment samples were shaken for 2 min instead of being subjected to sonication (Boehm, Griffith et al. 2009). Sediment suspensions were allowed to settle for several minutes before the supernatant was pipetted off and filtered as detailed above.

Culturable bacteria were enumerated by standard membrane filtration methods. For the direct effects microcosms, *E. coli* colonies were enumerated on mTEC agar at 35°C for 2h, followed by 22h incubation at 44.5°C (U. S. Environmental Protection Agency 2002); enterococci colonies were enumerated on mEI agar after 24h incubation at 41°C (U. S. Environmental Protection Agency 2002). For the *Indirect Effect Experiment*, fecal coliforms were enumerated on mFC agar at 44.5°C for 24h (American Public Health Association 1999).

*E. coli* Genetic Typing. No genetic typing was done on colonies isolated from the *Indirect Effect Experiment*, however, *E. coli* isolates cultured from the *Direct Effect Experiment* after one week of incubation were selected for genetic typing from each time
block. Twenty isolates were randomly selected for each agrochemical treatment in both water and sediment samples, where possible. Colonies were picked with sterile toothpicks and transferred into wells of microtiter plates containing EC broth amended with 4-methylumbelliferyl-β-d-glucuronide (MUG; 50 μg/ml). Fluorescence under UV light was used to assess MUG cleavage, which is characteristic of *E. coli* and differentiates it from the remainder of the coliform group (Bitton, Koopman et al. 1995; Anderson, Whitlock et al. 2006). Cultures in microtiter plates were stored at -80°C after the addition of 3 drops of glycerol, used as a cryopreservant, to each well of the microtiter plate.

Strains were revived from freezer storage by streaking for isolation on TSA, followed by incubation for 24 h at 37°C. A single, well-isolated colony from each plate was then inoculated into 750 µl BHI and incubated for 24 h at 37°C. To extract DNA, suspensions were centrifuged at 14000 RPM for 1 min and the supernatant was discarded. Cells were washed with autoclaved buffered water twice and then resuspended in 500 µl autoclaved DI water and boiled for 5 min. This suspension was used as the template for PCR reactions. Negative extraction controls consisted of sterile DI water were run with each group of cultures subjected to DNA extraction.

Horizontal fluorophore-enhanced repetitive extragenic palindromic PCR (HFERP) was used to provide unique DNA banding patterns for each isolate (Johnson, Brown et al. 2004). A positive control (E. coli 9637) and negative controls, including the extraction control described above and a no-template control, were run with each set of reactions. PCR products were loaded onto 1.5% agarose gels and run at 90V for 4 h at room temperature. Gels were visualized using a Typhoon 9410 variable mode imager.
(Molecular Dynamics/Amersham Biosciences, Sunnyvale, CA). Banding patterns were compared using BioNumerics (Applied Maths) and verified by eye. Typing of the water column isolates was done for the first three temporal blocks and typing of the sediment samples was done for only the third temporal block because of financial and time constraints.

**Statistical Analysis.** All response variables were log-transformed and block was included in all analyses. The residuals were always carefully scrutinized to ensure that the assumptions of the analysis were met.

**Direct Effect Experiment.**

**Treatment Effects on FIB Abundance.** We conducted repeated measures, regression-based, multivariate analysis of variance (MANOVA), where the repeated measures factor was FIB density on the three sampling intervals [times 0, 24 h and 168 h (Bray and Maxwell 1985)]. In these analyses, we always included interactions between among- and within-tank (repeated measures) factors. This allowed us to test for treatment-by-time interactions. In all MANOVAs, the response variables were the density of *E. coli* and enterococci on each sampling date. We first conducted a MANOVA to ensure that there were no differences in FIB densities among microcosms before agrochemical applications. We then tested for a difference between the water and solvent controls to determine whether we could pool these treatments. Finally, our full model included the main effects of sample location (sediment or water column) and the four agrochemicals, two-way and three-way interactions (excluding three-way interactions among agrochemicals given that we only had pair-wise combinations), and
repeated-measures main effects and interactions. ANOVAs were also conducted on each FIB to ensure that we did not miss any significant univariate effects (Armstrong and Hilton 2004).

**Treatment Effects on FIB Composition.** To test for treatment effects on FIB composition, we arcsine-square-root transformed the proportion of *E. coli* colonies that were of the three inoculated strains and conducted a MANOVA testing for the main effects of agrochemicals and their two-way interactions (Steel and Torrie 1960). Isolates from only three out of four of the blocks were typed (isolates from the fourth temporal block, Block D, were not genetically typed).

**Relationship Between FIB Composition in the Sediment and Water Column.** To test for a relationship between FIB composition in the sediment and water column, for each microcosm in the third temporal block, we calculated the proportion of total colonies from the sediment and water column that were each of the three *E. coli* strains. Treating these three strains as independent of one another would have inappropriately tripled our sample size. Hence, we bootstrapped the relationship between FIB composition in the sediment and water column by randomly selecting paired sediment and water column samples, with replacement, until we reached the sample size for the block. We then calculated the Pearson’s correlation coefficient for these randomly selected, paired sediment and water column samples. We did this 1000 times and attained our probability value for the null model by calculating the proportion of these 1000 correlation coefficients that were ≤ 0.
**Indirect Effect Experiment.** We conducted repeated measures, regression-based, analysis of variance (ANOVA), where the repeated measures factor was *E. coli* density on the five sampling intervals (times 2, 7, 9, 14, and 28 days). In these analyses, we always included interactions between among- and within-tank (repeated measures) factors, as well as utilizing the day 0 densities as a continuous covariate to control for stochastic differences at the outset of the experiment. This allowed us to test for treatment-by-time interactions. In all ANOVAs, the response variables were the density of *E. coli* on each sampling date. Our full model included the main effects of light (present or absent) and the three agrochemical treatments (water, fertilizer or atrazine), two-way interactions and repeated-measures main effects and interactions. Separate ANOVAs were conducted for densities in the water column and the sediment. We used a repeated measures multivariate analysis of variance (MANOVA) to evaluate the factorial effects of the treatments and light exposure on phytoplankton F0 and QY measurements on days 7, 14, and 28, controlling for initial phytoplankton abundance. We used a regression analysis to test whether phytoplankton abundance on day 28 (end of the experiment) was predictive of *E. coli* densities in the water column and sediment on day 28. We always conducted separate ANOVAs and MANOVAs excluding the fertilizer treatment to verify that significant differences were driven by a difference between the water control and atrazine and not differences between fertilizer and atrazine treatments.

**Results**

**Direct Effects of Agrochemicals (Dark Conditions).** Initial densities of *E. coli* in dark conditions before the addition of agrochemicals were \(~10^7\) CFU/100ml in the water column and \(~10^5\) CFU/100g (wet weight) in the sediment. Enterococci densities were
initially \(\sim 10^7\) CFU/100ml in the water column and \(\sim 10^6\) CFU/100g (wet weight) in the sediment. Average decay rates per day were calculated for \(E.\ coli\) and enterococci in both water and sediment by subtracting the density at T168 from the initial density and dividing by seven. Decay rates for \(E.\ coli\) (Figure 4A) and enterococci (Figure 4B) were not significantly different among treatments in either the water column or sediment.

Mean values from all temporal blocks showed \(E.\ coli\) densities in the water column and sediment ranging from \(\sim 10^6\) to \(\sim 10^9\) CFU per 100 ml or 100 g across all treatments. Enterococci densities after one week in the water column ranged from \(\sim 10^4\) to \(\sim 10^6\) CFU/100ml and sediment densities ranged from \(\sim 10^4\) to \(\sim 10^8\) CFU/100g. When averaged over all treatments and all blocks after a week, \(E.\ coli\) densities were significantly higher than enterococci densities in both water and sediment matrices.

We found no significant differences in FIB density among microcosms before agrochemical applications and no significant difference between the water and solvent controls after agrochemical applications (\(P>0.05\)). Hence, we pooled the water and solvent controls for subsequent analyses. There were significant multivariate effects of block \((F_{6,152}=28.96, P<0.001)\), sampling intervals \((F_{2,76}=38.43, P<0.001)\), and sampling location \((F_{2,76}=7.69, P<0.001)\), i.e., the sediment had higher densities than the water column for enterococci \((F_{1,76}=28.23, P<0.001)\) but not for \(E.\ coli\) \((F_{1,76}=0.82, P=0.367)\). There were no significant main effects or interaction associated with any of the agrochemicals (all \(P>0.115\)).

MANOVA revealed that agrochemical treatments did not influence the genotype composition of \(E.\ coli\) strains (number of isolates of any given genotype recovered), nor the proportion of each genotype recovered (all main effects and interactions had
However, bootstrapping analysis revealed that there was a significant positive multivariate correlation between the genotypes recovered from the sediment and water column (mean $r = 0.532$, $P=0.018$, Figure 5).

**Indirect Effects of Agrochemicals (Light Conditions).** Initially, and prior to agrochemical additions, *E. coli* densities in the water column were $\sim 10^7$ CFU/100ml. After 2 days, *E. coli* densities in the water column had dropped $\sim 3$ logs in the light-exposed tanks and $\sim 2$ logs in the darkened tanks (Figure 6A), demonstrating the adverse effects of light exposure on water column *E. coli*. The decline in the *E. coli* densities slowed over the remainder of the experiment with declines of $\sim 0.5$-1 log/wk for all tanks in either light or dark conditions.

Within the water column (Figure 6A), ANOVA revealed significant main effects of light ($F_{1,19}=5.36$, $P=0.031$), block ($F_{4,19}=4.29$, $P=0.012$), and a significant interaction between light exposure and treatment ($F_{2,19}=4.05$, $P=0.034$). An additional ANOVA was run excluding the fertilizer treatment and a significant effect of light*treatment was still observed ($F_{1,11}=8.24$, $P=0.015$), meaning that the effect was the result of a difference between the water control and atrazine treatments, not the result of differences between the atrazine and fertilizer treatments. Under lit conditions, atrazine treatments resulted in significantly lower *E. coli* densities in the water column compared to control treatments, but atrazine had no significant effect on *E. coli* densities in the dark (for dark treatments at day 7: $P = 0.28$; at day 24 $P = 0.10$; Figure 6A). No significant effect was observed for fertilizer treatments relative to controls. No significant effects were seen for the repeated measures main effect or interactions.
E. coli densities within the sediments of all treatments were initially ~10^7 CFU/100g prior to agrochemical additions. No decline was seen between day 0 and day 2, though between days 2 and 7 a decline of ~half a log was observed in the sediments of all treatments, and E. coli densities continued to decline more slowly than in the water column (Figure 6B). E. coli densities in the sediments of the dark fertilizer treatment experienced a slightly slower, albeit not statistically significant, decay rate relative to other treatments. ANOVA revealed no significant main effect of light or block for the sediment densities, however there was a significant main effect of treatment (F_{2,19}=3.57, P=0.048) and a significant light-by-treatment interaction (F_{2,19}=7.77, P=0.003). An additional ANOVA excluding fertilizer revealed a significant main effect of treatment (F_{1,11}=5.71, P=0.036) and light-by-treatment interaction (F_{1,11}=6.10, P=0.031). In other words, the significant effects of treatment and light*treatment were driven by differences between the water control and atrazine treatments, not by differences between the atrazine and fertilizer treatments. Under lit conditions, atrazine treatments resulted in significantly higher E. coli densities in the sediment after 28 days compared to control treatments, but atrazine had no significant effect on E. coli densities in the dark. No significant effect was observed for fertilizer treatments relative to controls. A significant main effect was observed for the repeated measures factor (F_{2,38}=4.94, P=0.012) as well as a significant interaction between time*light (F_{2,38}=14.72, P<0.001), time*treatment (F_{4,38}=4.95, P=0.003), and time*light*treatment (F_{4,38}=7.64, P<0.001).

We quantified phytoplankton levels (chlorophyll a) in each microcosm to evaluate whether treatment effects on E. coli densities could be explained by indirect effects mediated by algae. There was a significant time-by-treatment-by-light interaction for the
algal responses \((F_{8,40}=2.20, \ P=0.048)\) and this interaction remained significant when the fertilizer treatment was excluded \((F_{4,12}=4.17, \ P=0.024)\), indicating that phytoplankton were responding differently in atrazine-treated than control microcosms. This interaction was driven by atrazine having no effect on phytoplankton abundance in dark or lit conditions until day 28, when chlorophyll a measures were lower in the atrazine-treated microcosms than the control microcosms under lit conditions only (Figure 7). Furthermore, algal abundance at the end of the experiment was a significant positive predictor of *E. coli* densities in the water column regardless of whether an outlier was included or excluded (Figure 8A). In contrast, algal abundance at the end of the experiment was negatively associated with *E. coli* densities in the sediment (Figure 8B).

**Discussion**

The overall net effect (direct + indirect mechanisms) of agrochemicals on the fate of allochthonous bacteria in water bodies and the underlying sediment has been greatly understudied. Our previous research suggested that the presence of fertilizer or atrazine has positive effects on FIB densities in the sediment (Staley, Rohr et al. 2010). However, our previous study did not investigate potential impacts of agrochemicals in the water column and, due to the complexity of the mesocosms used in that study, it was not possible to say definitively whether the effects observed were due to direct mechanisms, indirect mechanisms, or a combination of the two. The present study utilized simple microcosms, inoculated with several known strains of *E. coli* and *Enterococcus* spp., to further examine the impact that agrochemicals have on the fate of bacteria in both water and sediment, as well as to distinguish between direct and indirect mechanisms. The initial bacterial densities (~10^7 CFU/100 ml), though certainly elevated, have been
observed in environmental water samples in a previous study (Weidass, Tamzen et al. 2011). While the addition of bacteria to the microcosms would add some nutrients to the microcosms, cells were washed prior to inoculation into the microcosms to limit the transfer of excess nutrients, and were added to all of the microcosms at similar densities.

To examine direct effects of agrochemicals, our microcosms were designed specifically to exclude potential indirect confounders, specifically algal growth and protozoan predation. Under these conditions, none of the agrochemicals, alone or in combinations, had significant effects on the densities of *E. coli* or enterococci in either the water column or the underlying sediment. These results suggest that the agrochemicals used in this experiment do not have any direct impact, positive or negative, on FIB densities in either the water or sediment matrix. However, when seeded with pond water (containing algae and protozoan predators), significant effects were observed on *E. coli* densities in both the water column and the sediment when atrazine was present and microcosms were exposed to light, indicating that the effects of atrazine on *E. coli* were indirect because they depended on the presence of other species.

Given that the effect of atrazine on *E. coli* depended on light availability, the indirect effect is almost certainly at least partially mediated by phototrophs. Atrazine is directly toxic to phytoplankton (Rohr, Schotthoefer et al. 2008). The present experiment supports this finding as phytoplankton density decreased in lit microcosms containing atrazine relative to controls (Figure 7). Further, light attenuates exponentially as a function of chlorophyll a in phytoplankton, potentially resulting in an exponential decrease in light penetration in response to a small increase in chlorophyll a (Gallegos 1990). Therefore, as the phytoplankton dies off, the water column receives increased
light, which would result in greater UV stress to bacteria in the water column, explaining the significantly lower density of *E. coli* in the water column of the light-exposed atrazine tanks (Sinton, Hall et al. 2002; Noble, Lee et al. 2004). The algal data support this conclusion, as greater amounts of phytoplankton were predictive of higher *E. coli* densities in the water column (Figure 8A).

Increased light penetration has also been shown to stimulate the growth of periphyton in sediment biofilms, thus increasing available carbon (Herman, Kaushik et al. 1986; Pratt, Melendez et al. 1997; Rohr, Raffel et al. 2008). Therefore, as phytoplankton decreased in the lit atrazine microcosms, periphyton likely increased (although this was not measured). In support of this assertion, we observed significantly higher *E. coli* densities in the sediments of lit atrazine microcosms and a strong negative trend for the correlation between phytoplankton in the water column and *E. coli* densities in the sediments (Figure 8B). All of these findings support the conclusion that algal dynamics mediated the indirect effects of atrazine on *E. coli*. However, it should be noted that not all algal species respond identically to atrazine. As algal species can differ in sensitivity to atrazine, the initial algal community present in a water body may well modify the direct and indirect algal-mediated effects on the entire community (Rohr and Crumrine 2005). Further, it should be noted that atrazine, beyond phytotoxic effects, can exert effects on higher trophic levels in impacted communities. While the ability of atrazine to directly cause mortality is controversial, a meta-analysis has shown that atrazine consistently influences metamorphosis, anti-predator behavior, and immunity of freshwater fish and amphibians (Rohr and McCoy 2010). These wide-ranging effects of
atrazine exposure could have further implications for microbial fate in water bodies exposed to the herbicide.

In contrast to findings from our previous study conducted with nutrient poor sediments and water (Staley, Rohr et al. 2010), no significant increase in FIB densities was observed for the fertilizer treatment. The sediments used in this experiment were taken from the Hillsborough River, and would, therefore, be expected to contain large amounts of phosphorous as well as multiple nitrogen species (Wang, Martin et al. 1999). Additionally, the pond water seeded into the microcosms was taken from a eutrophic pond at the University of South Florida. Therefore, we hypothesize that the seeded pond water and sediments resulted in microcosms that were neither phosphorous nor nitrogen limited, preventing us from detecting an increase in FIBs with fertilizer addition.

Although we detected no direct effect of the agrochemicals on overall *E. coli* and enterococci densities, we also hypothesized that changes in strain composition may be induced by agrochemical treatments. Particular strains of *E. coli* and *Enterococcus* spp. have been shown in previous studies to exhibit extended survival in microcosms (Anderson, Whitlock et al. 2005), so it was thought that individual strains may respond differently to agrochemical treatments. However, these experiments did not support this hypothesis, as the proportion of each *E. coli* strain recovered from water and sediment in light-free microcosms was independent of treatments, and no one strain persisted better overall than any other. We do not suggest that these results should be generalized to all *E. coli* strains, as only three were included in this study.
Despite the lack of direct effects of the agrochemicals on the abundance or population structure of FIBs, we did detect a positive multivariate correlation between *E. coli* genotype abundance (observation of a particular genotype) in the sediment and water column. These results suggest that the distribution of *E. coli* genotypes in the sediment influence the population structure within the water column, and/or vice versa. Regulatory standards only consider FIB densities in the water column (U. S. Environmental Protection Agency 1983; U. S. Environmental Protection Agency 2002), but *E. coli* and enterococci are clearly also present in the sediments, which can act as important reservoirs of FIBs, and possibly of pathogens (Anderson, Whitlock et al. 2005; Droppo, Liss et al. 2009; Badgley, Nayak et al. 2010; Badgley, Thomas et al. 2010).

The effects of agrochemicals on the survival of FIBs in environmental waters are greatly understudied. Based on the results of this study, agrochemicals had no direct effect on FIB growth, although the presence of atrazine did mediate significant indirect effects. The significant effects observed in the presence relative to the absence of light and the associations between phytoplankton abundance and *E. coli* densities suggest that the presence of atrazine alters algal dynamics that affect light and nutrient levels leading to reductions in *E. coli* in the water column but increases in the sediment. While these results are novel and suggest a mechanism of action for the effect of atrazine on FIB densities, it is unknown whether agrochemical treatments will have any direct or indirect effects on other bacterial pathogens or viruses. Further examination of both the direct and indirect effects of agrochemicals on pathogen survival in the environment is needed to better understand and manage potential risks to human health.
Acknowledgments

We would like to thank Laurie Walker, Dr. Gordon Fox and the USF Botanical Gardens for greenhouse space and logistical support. Funding was provided by USDA-NIFA Water and Watershed program grant 2009-35102-05043.

References


U. S. Environmental Protection Agency (2002). "Implementation guidance for ambient water quality criteria for bacteria. EPA-823-B-02-003."


U. S. Environmental Protection Agency (2009). "Review of Published Studies to Characterize Relative Risks from Different Sources of Fecal Contamination in Recreational Water. EPA 822-R-09-001."

U. S. Environmental Protection Agency (2009). "Review of Zoonotic Pathogens in Ambient Waters. EPA 822-R-09-002."


Figure 3. Schematic of the procedure for used to culture and process *E. coli* and enterococci strains for inoculation into microcosms.
**Figure 4.** Average decay rates per day (mean Δ log CFU per 100 ml or 100 g ± standard error (SE), n=4) in water and sediment averaged over all four temporal blocks for A) *E. coli* and B) enterococci.
**Figure 5.** The relationship between strain composition in the sediment and water column for three *E. coli* strains (strain 14: squares, strain 19: triangles, strain 9637: circles). The three strains are not independent of one another because they were sampled from the same mesocosms. To calculate the multivariate correlation between composition in the water column and sediment, we bootstrapped the relationship, ensuring that our sample size for each of the 1000 iterations matched the number of replicates. These results revealed an average Pearson’s *r* of 0.532 and a probability value for the null model of 0.018.
Figure 6. E. coli densities in the A) water and B) sediment for each treatment (mean ± SE, n = 5) at days 2, 7, and 28.
Figure 7. Phytoplankton abundance (measured as F0) with and without atrazine (least squares mean ± SE, n = 5) on days 7, 14, and 28 when controlling for initial phytoplankton levels.
Figure 8. Relationship between phytoplankton abundance (controlling for initial abundance estimates) and *E. coli* densities (measured as CFU/100 ml or CFU/100 g) in the **A** water column and **B** sediment.
CHAPTER FOUR: LACK OF DIRECT EFFECTS OF AGROCHEMICALS ON ZOONOTIC PATHOGENS AND FECAL INDICATOR BACTERIA

Abstract

Agrochemicals, fecal indicator bacteria (FIB) and pathogens frequently contaminate water simultaneously. No significant direct effects of fertilizer, atrazine, malathion and chlorothalonil on the survival of Escherichia coli, Enterococcus faecalis, Salmonella enterica, Enterococcus faecalis, human polyomaviruses, and adenovirus were detected, supporting the assertion that previously observed effects of agrochemicals on FIB were indirect.

Introduction

Many endemic and emerging zoonotic pathogens, such as Escherichia coli strains, Salmonella enterica, Cryptosporidium spp., Giardia spp., and zoonotic influenza viruses (U. S. Environmental Protection Agency, 2005, U. S. Environmental Protection Agency, 2009, U. S. Environmental Protection Agency, 2009, Lebbad, et al., 2010), can be of agricultural origin and shed in the feces of livestock (Fratamico, 2004, Berry, 2007, Brooks, et al., 2009). Many of these pathogens are waterborne and are, therefore, capable of entering water bodies via storm water and agricultural runoff, as well as via

1(Published as a shortform paper in Journal of Applied and Environmental Microbiology 2012, 78(22), 8146-8150)
subsurface transport. Testing directly for each pathogen in fecal-impacted water bodies would be prohibitively costly and time consuming. Consequently, regulatory standards have relied on the quantification of FIB for water quality assessment (U. S. Environmental Protection Agency, 2004). The ability of FIB to predict human health risks has been supported by epidemiological studies (Cabelli, et al., 1979, Dufour, 1984, Wade, et al., 2006, Wade, et al., 2008, Heaney, et al., 2009).

The association between FIB levels and the risk of pathogens in recreational waters may be impeded by agricultural practices. In addition to fecal contamination, agricultural runoff is likely to contribute agrochemicals (i.e., fertilizers and pesticides), which have wide-ranging effects on ecosystems (DeLorenzo, et al., 2001, Downing, et al., 2004, Rohr & Crumrine, 2005, Rohr, et al., 2008, Clements & Rohr, 2009, Rohr & McCoy, 2010, McMahon, et al., 2011). However, the effects of agrochemicals on the fate of pathogen and FIB populations has received little attention (Downing, et al., 2004). Agrochemicals may have adverse or beneficial effects on bacteria through direct mechanisms, such as through direct toxicity or by directly providing nutrients, or through indirect mechanisms, such as by altering predator-prey dynamics or biotic food sources. Further, FIB and waterborne pathogens may have similar or diverging response to agrochemicals, potentially disconnecting the FIB-pathogen relationship.

Here, the direct effects of inorganic fertilizer, atrazine (herbicide), malathion (insecticide), and chlorothalonil (fungicide) on the concentrations of FIB (E. coli ATCC 9637 and Enterococcus faecalis ATCC 19433), zoonotic bacterial pathogens (E. coli O157:H7 EDL 933 and S. enterica serovar Typhimurium CBD777) and viruses (human polyomavirus [HPyV]) BK and adenovirus type 2 [ATCC VR-846]) were investigated.
Factors that could lead to indirect effects on survival, such as phytoplankton shading, competition from native bacteria, and predation by protozoa, were intentionally excluded from this study because the focus was strictly on direct effects. To isolate direct effects, two experiments were conducted, one in the dark and one exposed to light, using simplified microcosms that contained only autoclaved deionized water, disinfected sediments, and an agrochemical treatment. The use of sterilized water and disinfected sediment excluded algal and biofilm communities which may have resulted in indirect agrochemical effects seen previously in microcosms exposed to sunlight (Staley, et al., 2010, Staley, et al., 2011).

**Experimental Design**

**Microcosm Establishment.** For both experiments, microcosms were established in a greenhouse at the University of South Florida Botanical Gardens (Tampa, FL). In the first, dark experiment, microcosms consisted of 11.3 L Rubbermaid plastic trash cans with opaque sides (29.97 x 22.86 x 33.65cm) containing 1 L of sediment disinfected by baking at 176.67°C and 2 L of autoclaved deionized water. These microcosms were covered with aluminum foil to prevent light penetration. In the second, light experiment, microcosms consisted of 2L glass beakers containing 0.5 L of sediment disinfected as above and 1.5 L of autoclaved de-ionized water. In both experiments, target bacteria were inoculated at densities of approximately $10^7$ CFU/100 ml and target viruses at densities of approximately $10^3$ copy numbers/ml.

In the first experiment, microcosms were treated with atrazine, malathion, chlorothalonil, or inorganic fertilizer singularly at the estimated environmental
concentration (EEC; 102 µg/L for atrazine, 101 µg/L for malathion, 170 µg/L for chlorothalonil, and 4400 µg/L N and 440 µg/L P for fertilizer) or with one of all possible pair-wise combinations. Solvent and water controls were also included, which resulted in 12 treatments. These agrochemicals were chosen because, in the U.S., they are among the top two in usage for their agrochemical class (Kiely, et al., 2004). Every microcosm received all six of the focal microbial taxa (4 bacteria and 2 viruses). Microcosms were covered to prevent light penetration and the microbes were enumerated in both the water and sediment immediately before agrochemical application (T0), and 24 hours (T24) and one week (T168) after agrochemical applications. The bacteria were quantified using both culture-dependent methods and qPCR, whereas the viruses were only quantified using qPCR (Table 3).

In the environment, agrochemicals are also subject to photolytic degradation, resulting in intermediate compounds which may impact organisms differently than parent compounds (De Souza, et al., 1998, DeLorenzo, et al., 2001, Wackett, et al., 2002). To examine the potential effect of photolytic-derived intermediates as well as parent compounds, the second experiment used the same microcosm set-up as in the first experiment except that the microcosms were exposed to natural light. There were other minor differences from Experiment 1. For instance, treatments for this experiment consisted only of a singular agrochemical treatment at the EEC. Also, due to logistical constraints and the lack of observed significant effects observed in the dark microcosms (see below), viruses were excluded from this experiment and the four inoculated bacteria were only enumerated via culturable membrane filtration methods (Novicki, et al., 2000, U. S. Environmental Protection Agency, 2002, U. S. Environmental Protection Agency,
Further, previous research has indicated that viruses are significantly less susceptible to UV radiation than bacteria, therefore it was not expected that the virus concentrations would be significantly different in the light microcosms (Hijnen, et al., 2006).

**Bacterial Enumeration.** Culture-based methods of bacterial enumeration utilized selective differential media for each bacterial target, *E. coli* was enumerated on mTEC agar following incubation for 2 hours at 35°C and then at 44.5°C for 22 h (U. S. Environmental Protection Agency, 2002), *Enterococcus faecalis* was enumerated on mEI agar after 24 h incubation at 41°C (U. S. Environmental Protection Agency, 2002), *E. coli* O157:H7 was enumerated on Sorbitol MacConkey agar following 24 h incubation at 37°C (Novicki, et al., 2000), and *S. enterica* was enumerated on XLT-4 agar following incubation for 24 h at 37°C (Kornacki, et al., 2003). Quantification via qPCR (preformed only for the dark microcosms) utilized the primers and probes found in Table 3.

**Growth Curves.** Growth curves were conducted by inoculating an isolated colony of *E. coli* ATCC 9637, *Ent. faecalis* ATCC 19433, *S. enterica* serovar Typhimurium, or *E. coli* O157:H7 EDL 933 into a centrifuge tube containing 20ml of M9 Minimal Media (supplemented with 12g/L of yeast extract for *Ent. faecalis* growth) and incubated for 24h at 37°C. The optical density at 600 nm (OD$_{600}$) was measured for each overnight culture using a NanoDrop 2000 Spectrophotometer (Thermo Scientific) and then diluted 1:20 into individual tubes containing M9 Minimal Media. The OD$_{600}$ was measured for each of the tubes to get a baseline prior to agrochemical addition. Three replicates of each singular agrochemical treatment (water and solvent controls, inorganic fertilizer, atrazine, malathion, or chlorothalonil) were established at 1x EEC. The OD$_{600}$ was measured after
1 h and then after every subsequent 30 min for ~5 h. This procedure was repeated until growth curves had been conducted for all target bacteria at 1x and 2x EEC. Growth curves were also conducted as above for each of the four target bacteria as well as *E. coli* WW6 (isolated from a wastewater treatment plant in Tampa, FL) using agrochemical treatments including a water control, atrazine, atrazine-2-hydroxy, cyanuric acid, and atrazine exposed to 365 nm UV lamps for ~18h.

**Statistical Analysis.** Concentrations of target microbes were log-transformed for all analyses. Repeated measures statistical analysis was used to assess changes in bacterial concentrations over the one-week experiment while taking into account the non-independence of sampling the same microcosms over multiple time intervals. Specifically, multivariate analysis of variance (MANOVA) was employed, where the repeated measures factor was the microbial concentration on each of the three sampling intervals (T0, T24 and T168). In these analyses, interactions between among- and within-microcosm (repeated measures) factors were always included, allowing for analysis of treatment-by-time interactions. In all MANOVAs, the response variables were the concentration of the six (Experiment 1) or four (Experiment 2) microorganisms. Water and sediment were sampled and analyzed separately. Culturable and qPCR concentrations were also analyzed separately (Table 4), but these responses were significantly correlated in general and were consistent with previously observed correlations (Noble, *et al.*, 2010, Harwood, *et al.*, 2011). Statistical analyses were also conducted where the response variables were the differences between initial concentration and T24 and T168 concentrations divided by the number of days which had passed (decay rates; Table 5). Further, T90 (the amount of time required for a bacterial
concentration to decrease by 90%) was calculated for the enterococci (Easton, *et al.*, 1999).

**Results**

No significant difference in T90 was observed among treatments in either experiment, therefore the values were averaged among all agrochemical treatments. The average T90 for enterococci in the water columns of the dark microcosms was ~21 h and ~9 h in the light microcosm experiments. No other target microbe experienced a log decline throughout either experiment, likely as a result of the absence of predation and competition (Table 5).

None of the agrochemicals significantly affected microbial concentrations at any of the time periods, regardless of whether the response variables used were the concentrations or the decay rates (Figures 9 and 10, Table 5). Furthermore, these results were consistent across quantification methods (culture or qPCR), sample location (water or sediment), and dark or light exposure (Tables 6 and 7). Notably, in both dark and light microcosms, *E. coli* and *E. coli* O157:H7 exhibited greater survival than *Ent. faecalis* or *S. enterica* (Figures 11 and 12). Based upon decay rates, the survival of adenovirus and HPyV genetic material was so similar that the data overlay one another precisely on the graph (Figure 11). Viral survival more closely resembled that of the *E. coli* strains than *Ent. faecalis* and *S. enterica* (Figure 11). *T*-tests revealed significantly higher bacterial concentrations (*P* = 0.007) in dark microcosms compared to light microcosms. As the deleterious effects of sunlight on bacterial survival have been well documented, this result is not surprising (Sinton, *et al.*, 2002, Noble, *et al.*, 2004).
Discussion

Previous experiments found no significant direct effects of agrochemicals on FIB survival (Staley, et al., 2011), however the present experiments expand on this conclusion and demonstrate that there are no significant direct effects of agrochemicals on some bacterial pathogens and viruses as well. Furthermore, actively replicating bacterial cultures exposed to agrochemicals (\textit{E. coli}, \textit{E. coli} O157:H7, \textit{Ent. faecalis} or \textit{S. entericia}) showed no significant difference in growth rates, and thus there was no evidence that these agrochemicals either facilitated or hindered the growth of these organisms (Table 8). In previous research, atrazine was found to significantly affect \textit{E. coli} concentrations in microcosms exposed to light. However, in this previous work, pond water was used and algal and biofilm communities were allowed to establish, while in the present work, we excluded algal and biofilm communities. These results suggest that exposure to light alone does not result in agrochemical effects on microbial survival, and that the previously observed effects on \textit{E. coli} levels were more likely the result of an indirect effect of atrazine on the phytoplankton and biofilm communities (Staley, et al., 2011).

While our present study indicates that these agrochemicals have no direct impact on the tested bacterial pathogens and viruses, a limited suite of pathogens is included here. Furthermore, the indirect effect of agrochemicals on pathogens has not been tested thoroughly. Further studies are essential to understanding the impact of agricultural practices on potential human health risks, and the relationship between FIB and the various waterborne pathogens that can impact human health.
Acknowledgments

We would like to thank Laurie Walker, Dr. Gordon Fox and the USF Botanical Gardens for greenhouse space and logistical support. Also, we would like to thank Kennon Hydock and Diana Contreras for their assistance on this project. Finally, we would like to thank Dr. James T. Riordan for providing *E. coli* O157:H7 EDL933 and the USF Center for Biological Defense for providing *S. enterica* serovar Typhimurium CDB777. Funding was provided by USDA-NIFA Water and Watershed program grant 2009-35102-05043.

References


Dufour AP (1984) Health effects criteria for fresh recreational waters. EPA-600/1-84-004 Washington, D.C.


U. S. Environmental Protection Agency (2009) Review of Published Studies to Characterize Relative Risks from Different Sources of Fecal Contamination in Recreational Water. EPA 822-R-09-001.


## Table 3. Primers and probes used for qPCR quantification.

<table>
<thead>
<tr>
<th>Target</th>
<th>Orientation</th>
<th>Primer or Probe</th>
<th>Sequence (5'-3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPyV</td>
<td>Forward</td>
<td>SM2</td>
<td>AGT CTT TAG GGT CTT CTA CCT TT</td>
<td>(McQuaig, et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>P6</td>
<td>GGT GCC AAC CTA TGG AAC AG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>KGJ3</td>
<td>(FAM)-TCA TCA CTG GCA AAC AT-(MGBNFQ)</td>
<td></td>
</tr>
<tr>
<td>Adenovirus</td>
<td>Forward</td>
<td>JTVXF</td>
<td>GGA CGC CTC GGA GTA CCT GAG</td>
<td>(Jothikumar, et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>JTVXR</td>
<td>ACI GTG GGG TTT CTG AAC TTG TT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>JTVXP</td>
<td>(FAM)-CTG GTG CAG TTC GCC CTG GCC A-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(MGBNFQ)</td>
<td></td>
</tr>
<tr>
<td>E. coli O157:H7</td>
<td>Forward</td>
<td>EcoOH-F</td>
<td>TCG AGC GGA CCA TGA TCA</td>
<td>(Lee, et al., 2005, Shannon, et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>EcoOH-R</td>
<td>GGC GGC TGC TGA GAT AAC A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>EcoOH-PR</td>
<td>(FAM)-AGA ACT TCA AAT CCA TCA TT-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(MGBNFQ)</td>
<td></td>
</tr>
<tr>
<td>Salmonella enterica</td>
<td>Forward</td>
<td>Sal-F</td>
<td>CGT TTC CTG CGG TAC TGT TAA TT</td>
<td>(Lee, et al., 2005, Shannon, et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>Sal-R</td>
<td>AGA CGG CTG GTA CTG ATC GAT AA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>Sal-probe</td>
<td>(FAM)-CCA CGC TCT TTC GTC T-(MGBNFQ)</td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>Forward</td>
<td>Eco-F</td>
<td>GTC CAA AGC GGC GAT TTG</td>
<td>(Lee, et al., 2005, Shannon, et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>Eco-R</td>
<td>CAG GCC AGT AGT TCT TTT TCC A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>Eco-PR</td>
<td>(FAM)-ACG GCA GAG AAG GTA-(MGBNFQ)</td>
<td></td>
</tr>
<tr>
<td>Enterococcus</td>
<td>Forward</td>
<td>EnteroF1A</td>
<td>GAG AAA TTC CAA ACG AAC TTG</td>
<td>(Ludwig &amp; Schleifer, 2000)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>EnteroR1</td>
<td>CAG TGC TCT ACC TCC ATC ATT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>GPL813TQ</td>
<td>(FAM)-TGG TTC TCT CCG AAA TAG CTT TAG GGC TA-(TAMRA)</td>
<td></td>
</tr>
</tbody>
</table>
Table 4. Correlation coefficients ($r$) between bacterial concentrations obtained via culture-dependent methods and qPCR at T24 and T168.

<table>
<thead>
<tr>
<th>Target</th>
<th>Water</th>
<th></th>
<th>Sediment</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T 24</td>
<td>T 168</td>
<td>T 24</td>
<td>T 168</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>0.86</td>
<td>0.70</td>
<td>0.83</td>
<td>0.65</td>
</tr>
<tr>
<td><em>Ent. faecalis</em></td>
<td>0.66</td>
<td>0.45</td>
<td>0.41</td>
<td>0.65</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>0.84</td>
<td>0.60</td>
<td>0.67</td>
<td>0.61</td>
</tr>
<tr>
<td><em>S. enterica</em></td>
<td>0.70</td>
<td>0.80</td>
<td>0.72</td>
<td>0.72</td>
</tr>
</tbody>
</table>
Table 5. T90 values and decay rates with standard deviations in parentheses for all targets averaged among all agrochemical treatments. Data are presented for both matrices in both dark and light microcosms.

<table>
<thead>
<tr>
<th></th>
<th><strong>Dark Microcosms</strong></th>
<th></th>
<th><strong>Light Microcosms</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Matrix</strong></td>
<td><strong>Decay Rate T0-T24</strong></td>
<td><strong>Decay Rate T0-T168</strong></td>
<td><strong>T90</strong></td>
<td><strong>Decay Rate T0-T24</strong></td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>-0.16 (1.1)</td>
<td>0.11 (1.7)</td>
<td>N/A</td>
<td>0.87 (0.6)</td>
</tr>
<tr>
<td>Sediment</td>
<td>-0.51 (1)</td>
<td>-0.07 (1.2)</td>
<td>N/A</td>
<td>0.02 (0.7)</td>
</tr>
<tr>
<td><strong>E. coli O157:H7</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>-0.39 (0.8)</td>
<td>0.03 (1)</td>
<td>N/A</td>
<td>0.24 (1.8)</td>
</tr>
<tr>
<td>Sediment</td>
<td>-0.61 (0.7)</td>
<td>-0.09 (1)</td>
<td>N/A</td>
<td>-0.19 (1)</td>
</tr>
<tr>
<td><strong>HPyV</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>-0.12 (0.3)</td>
<td>0.15 (0.4)</td>
<td>N/A</td>
<td>-0.03 (0.2)</td>
</tr>
<tr>
<td>Sediment</td>
<td>-0.15 (0.2)</td>
<td>0.00 (0.2)</td>
<td>N/A</td>
<td>0.08 (0.1)</td>
</tr>
<tr>
<td><strong>Adenovirus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>-0.02 (0.4)</td>
<td>-0.50 (0.7)</td>
<td>N/A</td>
<td>2.98 (1.6)</td>
</tr>
<tr>
<td>Sediment</td>
<td>-0.28 (0.9)</td>
<td>1.59 (0.8)</td>
<td>N/A</td>
<td>0.30 (0.9)</td>
</tr>
<tr>
<td><strong>S. enterica</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>-0.84 (1.9)</td>
<td>-1.24 (1.7)</td>
<td>N/A</td>
<td>-1.26 (3.4)</td>
</tr>
<tr>
<td>Sediment</td>
<td>-0.91 (1.5)</td>
<td>-1.93 (1.8)</td>
<td>N/A</td>
<td>-1.36 (3.1)</td>
</tr>
</tbody>
</table>
Table 6. Results of multivariate analysis of variance for the *Dark Microcosms* examining the effects of spatial block, agrochemical treatment, and sampling date on the density, quantified by qPCR, of *E. coli*, *Ent. faecalis*, *E. coli* O157:H7, *S. enterica*, HPyV, and adenovirus in the water column.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Wilk's $F$</th>
<th>df effect</th>
<th>df error</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>1192.23</td>
<td>6</td>
<td>29</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Block</td>
<td>7.43</td>
<td>18</td>
<td>85.51</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td>1.32</td>
<td>60</td>
<td>157</td>
<td>0.09</td>
</tr>
<tr>
<td>Sampling date</td>
<td>45.38</td>
<td>12</td>
<td>23</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Date*Block</td>
<td>16.55</td>
<td>36</td>
<td>68.68</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Date*Treatment</td>
<td>1.25</td>
<td>120</td>
<td>193.24</td>
<td>0.09</td>
</tr>
</tbody>
</table>
**Table 7.** Results of multivariate analysis of variance for the *Light Microcosms* examining
the effects of spatial block, agrochemical treatment, and sampling date on the density of
*E. coli, Ent. faecalis, E. coli O157:H7, and S. enterica* in the water column.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Wilk's $F$</th>
<th>$df$ effect</th>
<th>$df$ error</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>32427.80</td>
<td>4</td>
<td>12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Block</td>
<td>76.71</td>
<td>12</td>
<td>32.04</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td>1.21</td>
<td>20</td>
<td>40.75</td>
<td>0.30</td>
</tr>
<tr>
<td>Sampling date</td>
<td>587.48</td>
<td>8</td>
<td>8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Date*Block</td>
<td>263.18</td>
<td>24</td>
<td>23.80</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Date*Treatment</td>
<td>1.29</td>
<td>40</td>
<td>37.67</td>
<td>0.22</td>
</tr>
</tbody>
</table>
### Table 8. Growth rates of bacteria exposed to agrochemicals. Error bars represent standard deviations.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>E. coli 9637</th>
<th></th>
<th>E. coli WW6</th>
<th></th>
<th>E. coli O157:H7</th>
<th></th>
<th>Ent. faecalis 19433</th>
<th></th>
<th>S. enterica</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Time⁻¹</td>
<td>Deviation</td>
<td>Time⁻¹</td>
<td>Deviation</td>
<td>Time⁻¹</td>
<td>Deviation</td>
<td>Time⁻¹</td>
<td>Deviation</td>
<td>Time⁻¹</td>
<td>Deviation</td>
</tr>
<tr>
<td>Water</td>
<td>1x EEC</td>
<td>6.5E⁻⁰¹</td>
<td>1.03E⁻⁰¹</td>
<td>9.6E⁻⁰¹</td>
<td>8.17E⁻²⁰</td>
<td>7.5E⁻⁰¹</td>
<td>8.16E⁻²⁰</td>
<td>7.0E⁻⁰¹</td>
<td>9.76E⁻²⁰</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2x EEC</td>
<td>8.5E⁻⁰¹</td>
<td>9.62E⁻⁰²</td>
<td>6.1E⁻⁰¹</td>
<td>2.00E⁻²⁰</td>
<td>6.1E⁻⁰¹</td>
<td>7.7E⁻²⁰</td>
<td>9.3E⁻⁰¹</td>
<td>3.39E⁻²⁰</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intermediates</td>
<td>1.1E+⁰⁰</td>
<td>5.83E⁻⁰²</td>
<td>7.2E⁻⁰¹</td>
<td>7.18E⁻²⁰</td>
<td>5.9E⁻⁰¹</td>
<td>3.47E⁻²⁰</td>
<td>1.0E+⁰⁰</td>
<td>1.50E⁻²⁰</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solvent</td>
<td>1x EEC</td>
<td>6.4E⁻⁰¹</td>
<td>9.18E⁻⁰²</td>
<td>1.0E+⁰⁰</td>
<td>1.13E⁻⁰¹</td>
<td>3.9E⁻⁰¹</td>
<td>2.24E⁻²⁰</td>
<td>7.5E⁻⁰¹</td>
<td>3.58E⁻²⁰</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2x EEC</td>
<td>8.2E⁻⁰¹</td>
<td>3.81E⁻⁰²</td>
<td>6.3E⁻⁰¹</td>
<td>4.66E⁻²⁰</td>
<td>7.1E⁻⁰¹</td>
<td>3.11E⁻²⁰</td>
<td>8.4E⁻⁰¹</td>
<td>2.83E⁻²⁰</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fertilizer</td>
<td>1x EEC</td>
<td>6.2E⁻⁰¹</td>
<td>5.86E⁻⁰²</td>
<td>9.5E⁻⁰¹</td>
<td>2.10E⁻²⁰</td>
<td>6.0E⁻⁰¹</td>
<td>1.02E⁻²⁰</td>
<td>6.8E⁻⁰¹</td>
<td>6.96E⁻²⁰</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2x EEC</td>
<td>7.9E⁻⁰¹</td>
<td>3.59E⁻⁰²</td>
<td>6.1E⁻⁰¹</td>
<td>4.66E⁻²⁰</td>
<td>7.1E⁻⁰¹</td>
<td>3.58E⁻²⁰</td>
<td>8.3E⁻⁰¹</td>
<td>3.70E⁻²⁰</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malathion</td>
<td>1x EEC</td>
<td>6.3E⁻⁰¹</td>
<td>1.04E⁻⁰¹</td>
<td>1.0E+⁰⁰</td>
<td>1.13E⁻⁰¹</td>
<td>5.4E⁻⁰¹</td>
<td>1.06E⁻²⁰</td>
<td>7.1E⁻⁰¹</td>
<td>9.98E⁻²⁰</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2x EEC</td>
<td>8.2E⁻⁰¹</td>
<td>5.69E⁻⁰²</td>
<td>6.0E⁻⁰¹</td>
<td>2.92E⁻²⁰</td>
<td>7.1E⁻⁰¹</td>
<td>3.12E⁻²⁰</td>
<td>8.0E⁻⁰¹</td>
<td>2.21E⁻²⁰</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorothalonil</td>
<td>1x EEC</td>
<td>6.8E⁻⁰¹</td>
<td>9.68E⁻⁰²</td>
<td>9.0E⁻⁰¹</td>
<td>1.90E⁻²⁰</td>
<td>5.5E⁻⁰¹</td>
<td>1.54E⁻²⁰</td>
<td>7.6E⁻⁰¹</td>
<td>1.15E⁻²⁰</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2x EEC</td>
<td>7.7E⁻⁰¹</td>
<td>3.10E⁻⁰²</td>
<td>6.4E⁻⁰¹</td>
<td>7.60E⁻²⁰</td>
<td>6.6E⁻⁰¹</td>
<td>6.53E⁻²⁰</td>
<td>1.0E+⁰⁰</td>
<td>1.26E⁻⁰¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atrazine</td>
<td>1x EEC</td>
<td>6.3E⁻⁰¹</td>
<td>7.73E⁻⁰²</td>
<td>9.0E⁻⁰¹</td>
<td>7.31E⁻²⁰</td>
<td>6.5E⁻⁰¹</td>
<td>2.10E⁻²⁰</td>
<td>7.5E⁻⁰¹</td>
<td>1.36E⁻²⁰</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2x EEC</td>
<td>8.3E⁻⁰¹</td>
<td>1.08E⁻⁰¹</td>
<td>5.3E⁻⁰¹</td>
<td>3.50E⁻²⁰</td>
<td>7.2E⁻⁰¹</td>
<td>8.86E⁻²⁰</td>
<td>7.9E⁻⁰¹</td>
<td>1.70E⁻²⁰</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intermediates</td>
<td>1.1E+⁰⁰</td>
<td>4.89E⁻⁰²</td>
<td>6.9E⁻⁰¹</td>
<td>1.56E⁻²⁰</td>
<td>5.8E⁻⁰¹</td>
<td>1.78E⁻²⁰</td>
<td>1.0E+⁰⁰</td>
<td>9.04E⁻²⁰</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV-atrazine</td>
<td>Intermediates</td>
<td>9.3E⁻⁰¹</td>
<td>4.13E⁻⁰²</td>
<td>7.6E⁻⁰¹</td>
<td>3.22E⁻²⁰</td>
<td>7.1E⁻⁰¹</td>
<td>4.47E⁻³⁰</td>
<td>5.5E⁻⁰¹</td>
<td>4.14E⁻²⁰</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atrazine-2-hydroxy</td>
<td>Intermediates</td>
<td>9.1E⁻⁰¹</td>
<td>5.79E⁻⁰²</td>
<td>7.1E⁻⁰¹</td>
<td>5.85E⁻²⁰</td>
<td>6.8E⁻⁰¹</td>
<td>4.88E⁻²⁰</td>
<td>5.4E⁻⁰¹</td>
<td>5.37E⁻²⁰</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyanuric Acid</td>
<td>Intermediates</td>
<td>9.9E⁻⁰¹</td>
<td>3.01E⁻⁰²</td>
<td>6.3E⁻⁰¹</td>
<td>2.18E⁻²⁰</td>
<td>6.9E⁻⁰¹</td>
<td>3.70E⁻²⁰</td>
<td>5.9E⁻⁰¹</td>
<td>2.02E⁻²⁰</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 9. Average concentrations for A) *E. coli*, B) *Ent. faecalis*, C) *E. coli* O157:H7, and D) *S. enterica* for all agrochemical treatments in the water column of the *Dark Microcosms* (least squares means ± SE, *n* = 4). This figure shows results from a repeated measures analysis.
Figure 10. Average bacterial concentrations for all agrochemical treatments in the water column of the *Light Microcosms* (least squares means ± SE, n = 4). This figure shows data from a repeated measures analysis.
Figure 11. Concentrations of target microbes in the *Dark Microcosms* at each time point, averaged for all agrochemical treatments (± SD). A) Culturable concentrations of target bacteria in the water column. B) Culturable concentrations of target bacteria in the sediments. C) Concentrations of viruses in both matrices.
Figure 12. Concentrations of target bacteria in the *Light Microcosms* in the A) water column and B) sediments averaged for all agrochemical treatments (± SD).
CHAPTER FIVE: AGROCHEMICAL EFFECTS ON A ZOONOTIC PATHOGEN MEDIATED BY PROTOZOA PREDATION

Abstract

Agrochemical residues in water bodies have become ubiquitous and frequently have deleterious effects on non-target organisms. The effects of these pesticides on microbial communities, including direct and indirect effects on zoonotic pathogens, are poorly understood. In this study, an obligate heterotrophic protozoan, *Tetrahymena pyriformis*, and a facultative heterotroph, *Ochromonas danica*, were exposed to either the herbicide atrazine or the fungicide chlorothalonil to examine both direct effects of the agrochemicals on the protozoan species as well as the indirect effects on bacterivorous predation of the zoonotic pathogen, *E. coli* O157:H7. Chlorothalonil significantly reduced the densities of both protozoan species and thus predation on *E. coli* O157:H7. This reduced predation resulted in higher densities of *E. coli* O157:H7 (a density-mediated indirect effect). Conversely, the herbicide atrazine had no effect on the density of the protozoa but altered the traits of *O. danica*, reducing its photosynthesis and thus increasing its need for bacterial predation. Consequently, atrazine significantly decreased densities of *E. coli* O157:H7 (a trait-mediated indirect effect). These results suggest that if chlorothalonil contaminates water bodies, it could reduce bacterivorous predation and
prolong the survival of pathogens. Conversely, atrazine has the potential to increase heterotrophic activity among protozoa, increasing predation of bacterial pathogens.

**Introduction**

Agrochemical contamination of water bodies has become a ubiquitous phenomenon in the United States and worldwide, affecting both rural and urban watersheds (Gilliom 2007; Sprague and Nowell 2008; Vryzas, Vassiliou et al. 2009; Taghavi, Merlina et al. 2011). Pesticide contamination can lead to adverse effects on non-target organisms (Rohr, Kerby et al. 2006). Agrochemical effects can be either direct or indirect (Clements and Rohr 2009; Verro, Finzio et al. 2009; Verro, Finzio et al. 2009). Directly, agrochemicals could be toxic to the organism or be utilized as a nutrient source. Indirect effects can be density- or trait-mediated. Density-mediated indirect effects occur when a contaminant reduces or increases the abundance of a competitor, predator, parasite, or food resource (Raffel, Martin et al. 2008; Rohr, Raffel et al. 2008; Rohr, Schotthoefer et al. 2008; Rohr, Swan et al. 2009). Trait-mediated indirect effects occur when a contaminant causes alterations in the traits (e.g., morphology, immune response, physiology or behavior) of natural enemy or food resource that then have adverse or beneficial effects on the focal organism (Rohr, Elskus et al. 2003; Rohr and Crumrine 2005; Rohr and Palmer 2005; Rohr, Kerby et al. 2006; Rohr, Schotthoefer et al. 2008; Rohr, Swan et al. 2009).

Research on how pesticides affect microorganisms, including pathogenic bacterial and protozoan species, has been sparse, with most studies focusing on the capacity of microbes to biodegrade agrochemicals (Levanon 1993; Wackett, Sadowsky et al. 2002;
Radosevich and Tuovinen 2004; Zeinat, Nashwa et al. 2008). Understanding the effects of agrochemicals on the survival of microbes in impacted watersheds is of particular interest as many endemic and emerging pathogens, such as pathogenic *Escherichia coli* strains, *Salmonella enterica*, *Cryptosporidium* spp., *Giardia* spp., and zoonotic influenza viruses, are agriculturally-associated and are likely to be present, along with pesticide contaminants, in agricultural and storm water runoff (Palma, Sanchez et al. 2004; U. S. Environmental Protection Agency 2005; Gilliom 2007; Sprague and Nowell 2008; U. S. Environmental Protection Agency 2009; U. S. Environmental Protection Agency 2009).

Previous studies have shown that the herbicide atrazine and the fungicide chlorothalonil have no direct effects on *Pseudomonas* spp., *Bacillus* spp., fecal indicator bacteria and the pathogens *E. coli* O157:H7 and *Salmonella enterica* (Breazeale and Camper 1972; Staley, Senkbeil et al. 2012). However, direct effects of agrochemicals on bacterivorous protozoan species have not been well studied. As predation can significantly reduce bacterial populations, including pathogens, the effects of agrochemicals on bacterivorous protozoa could significantly influence bacterial densities (Wanjugi and Harwood 2012; Korajkic, McMinn et al. 2013). Previous research has shown that agrochemicals, notably the herbicide atrazine, were found to stimulate heterotrophic activity in protist species (Debenest, Pinelli et al. 2009; Debenest, Silvestre et al. 2010). Similarly, several fungicides have been found to reduce the abundance of heterotrophic protist species that depredate bacteria (Moore 1970; Ekelund 1999).

In this study, we examined the effects of a synthetic fungicide, chlorothalonil, and an *s*-triazine herbicide, atrazine on two different protozoan species: the obligate heterotroph, *Tetrahymena pyriformis* and the facultative heterotroph *Ochromonas danica*.
These two agrochemicals were selected because they are among the top in usage for their pesticide class within the United States (Kiely, Donaldson et al. 2004). Direct effects of both agrochemicals were first examined on both protozoan species in isolation, with the expectation that chlorothalonil would be directly toxic, but that atrazine would have only an adverse effect for the photosynthetic *O. danica*. A follow-up experiment was conducted where *E. coli* O157:H7 and both protozoan species were allowed to interact to test for indirect effects of the agrochemicals on the abundance of this zoonotic pathogen. Based upon the previously cited literature, chlorothalonil was expected to reduce protozoan densities, lessening predation and consequently increasing densities of *E. coli* O157:H7. In contrast, atrazine (only for *O. danica*) was expected to inhibit photosynthesis and thus increase predation by the facultative heterotroph *O. danica*. Consequently, atrazine was expected to reduce the abundance of *E. coli* O157:H7.

**Experimental Design**

**Direct Effects Experiment.** Microcosms were established *in vitro* consisting of 2 L glass beakers containing 500 ml of autoclaved de-ionized (DI) water. Beakers were inoculated with 50 ml *T. pyriformis* and covered with plastic wrap to prevent debris or contaminants from entering the microcosms. In order to prepare *T. pyriformis* (obtained from Carolina Biological Supply Company, Burlington, NC) for inoculation, cultures were grown for five days in 2:1 proteose peptone yeast extract (PPY; 5 g L\(^{-1}\) proteose peptone, 5 g L\(^{-1}\) tryptone, 0.2 g L\(^{-1}\) K2HPO4, pH 7.2) solution and Page’s Amoeba Saline (PAS) solution (Rowbotham 1983; Steinberg and Levin 2007; Wanjugi and Harwood 2012). Following the 5-d growth period, protozoa were centrifuged (IEC Multi, Thermo Scientific) at 3000 RPM for three minutes and the pelleted protozoa were resuspended in
PAS overnight. Fifty ml of the overnight protozoa cultures was used as the inoculum for the microcosms.

Thirty minutes following inoculation, baseline (T0) samples were taken, after which one of three agrochemical treatments was applied. Agrochemical treatments consisted of either a solvent control (DI water amended with 0.002% acetone, used as a solvent for all agrochemicals), the herbicide atrazine at 1x the estimated environmental concentration (EEC) or the fungicide chlorothalonil at 1x the EEC (102 µg/L for atrazine, 170 µg/L for chlorothalonil). EECs were calculated using the U.S. Environmental Protection Agency’s GENECC v2 software. Each agrochemical treatment was replicated in triplicate for a total of nine microcosms. Microcosms were arranged in randomized spatial blocks.

Ten mL samples were taken from each microcosm just prior to agrochemical application (Time 0), and after one, two, three, six, and seven days (T1-T7), for a total of six sampling events. These samples were places into sterile 15 ml centrifuge tubes. Protozoal densities were quantified from these samples using a haemocytometer (HyClone, Pittsburg, PA). Half of the limit of detection was used as a data point on sampling events where no protozoa were detected using the haemocytometer. The experiment was repeated using *O. danica* (obtained from UTEX The Culture Collection of Algae, Austin, TX), which was cultured and inoculated in the same manner as *T. pyriformis*.

**Indirect Effects Experiment.** To examine the indirect effects of agrochemicals on bacterivorous predation, 18 microcosms were prepared identically to those used in the
Direct Effects Experiment, with the exception that microcosms contained 900 ml DI water instead of 500 ml and nine of the microcosms received an additional 100 ml of autoclaved DI water instead of 100 ml of *O. danica*. This was done to allow for an equal volume in microcosms that were to serve as no-predation control treatments. Protozoa were cultured and inoculated as in the Direct Effects Experiment. Thirty minutes following inoculation of both protozoa (where applicable) and bacteria (see below), microcosms were dosed with one of the three agrochemical treatments used in the Direct Effects Experiment, for a total of three replicates for each protozoa-by-agrochemical treatment. Microcosms were arranged in randomized spatial blocks.

All microcosms were inoculated with *E. coli* O157:H7 EDL 933. Prior to inoculation, a culture was streaked for isolation on trypticase soy agar and incubated overnight at 37°C. A single colony was then inoculated in brain heart infusion broth and incubated, with shaking, at 37°C overnight. The overnight culture was then centrifuged at 5000 RPM for 5 min (IEC Multi, Thermo Scientific) and the pellet was resuspended in sterile buffered water (0.0425 g L\(^{-1}\) KH\(_2\)PO\(_4\) and 0.4055 g L\(^{-1}\) MgCl\(_2\)) twice (American Public Health Association 1999). The suspension was then diluted 1:10 in sterile buffered water and 1 ml of suspension was aseptically inoculated into every microcosm (~10\(^7\) CFU/100ml).

Water samples were collected, as described in the previous experiment, just prior to agrochemical application to obtain a baseline (pre-agrochemical measurement; Time 0) and after one, two, and five days (T1-5). Protozoa were quantified as detailed above. To quantify bacterial densities, water samples were filtered through a nitrocellulose membrane filter (0.45 µm pore-size, 47 mm diameter, Fisher Scientific) and bacteria
were enumerated via standard membrane filtration methods (American Public Health Association 1999). *E. coli* O157:H7 was enumerated on sorbitol MacConkey agar after 24 h incubation at 35°C (Novicki, Daly et al. 2000).

This experiment was repeated using *T. pyriformis* with 12 identically established microcosms. The atrazine treatment was excluded for *T. pyriformis* because no direct effects of atrazine were observed on *T. pyriformis* and there was no reason to expect that atrazine application would affect predation of an obligate heterotroph.

**Statistical Analysis.** Prior to analysis, all response variables (bacterial and protozoan densities) were log-transformed. To assess the effects of the agrochemicals on protozoan and bacterial densities, a repeated-measures, regression-based analysis of variance (ANOVA) was used. The residuals were always scrutinized to ensure that the assumptions of the analysis were met. For all analyses, initial densities were used as continuous covariates to account for stochastic variation in the initial inoculation of bacteria and protozoa. Block was never included in the final model of any analysis as it was never found to be a significant factor. Where a significant effect of agrochemical treatment was found, Dunnett’s post-hoc test was used to assess which agrochemical treatment(s) were significantly different from the control treatment. Analysis of agrochemical effects on *T. pyriformis* and *O. danica* was done separately for both direct and indirect effects experiments.

To assess direct effects of agrochemicals on protozoan species, an ANOVA was conducted where the repeated measures factor was the protozoan density on the five sampling intervals (T1, T2, T3, T6, and T7). Interactions between among- and within-
tank (repeated measures) factors were always included to allow treatment-by-time interactions. In these analyses, the response variables were the densities of either *T. pyriformis* or *O. danica* (analyzed separately) on each sampling date. Main effects included agrochemical treatment, the repeated measures factor (time), and the interaction between the agrochemical treatment and time. 

To assess indirect effects of agrochemicals on bacterivorous predation, ANOVAs were first conducted on the protozoan densities as described above, but with only three sampling intervals (T1, T2, and T5). All main and interaction effects were the same as those described for the *Direct Effects Experiment*. To assess the effects on bacterial densities, ANOVAs were conducted as described above. Response variables were the density of *E. coli* O157:H7 on each sampling date. Main effects included agrochemical treatment, predation (presence or absence), and the repeated measures factor (time). Interaction effects included time-by-treatment and time-by-predation as well as the three-way interaction of time, treatment and predation.

**Results**

**Direct Effects Experiment.** There was a significant effect of agrochemical treatment on densities of *T. pyriformis* (*F*<sub>2,5</sub>=32.97, *P*=0.001; Fig 13A). Densities of *T. pyriformis* in chlorothalonil treatments were significantly lower than densities in control or atrazine treatments (*P*=0.002, *P*=0.001; respectively), which were not different from one another (*P*=0.294; Fig 13A).

There was a significant effect of agrochemical treatment on densities of *O. danica* (*F*<sub>2,5</sub>=10.26, *P*=0.017; Fig 13B). Densities of *O. danica* in chlorothalonil treatments were
significantly lower than densities in control treatments ($P=0.023$; Fig 13B). No significant difference between atrazine and control treatments was observed ($P=0.985$; Fig 13B).

**Indirect Effects: T. pyriformis Experiment.** *E. coli* densities were significantly lower in the presence than absence of *T. pyriformis* predation ($F_{1,7}=9.40$, $P=0.018$; Fig 14). Chlorothalonil significantly reduced densities of *T. pyriformis* relative to controls ($F_{1,3}=34.09$, $P=0.010$; Fig 14B) as only two agrochemical treatments were used, no post-hoc tests were conducted). In the absence of *T. pyriformis* predation, chlorothalonil had no effect on densities of *E. coli* 0157:H7, but in the presence of *T. pyriformis* predation, chlorothalonil increased densities of *E. coli* (chlorothalonil*-*T. pyriformis: $F_{1,7}=17.54$, $P=0.004$; Fig 14). In fact, densities of *E. coli* in chlorothalonil plus *T. pyriformis* treatments were not significantly different from densities of *E. coli* in treatments with chlorothalonil but not *T. pyriformis* (Fig 15B).

**Indirect Effects: O. danica Experiment.** *E. coli* densities were significantly lower in the presence than absence of *O. danica* predation ($F_{1,11}=24.77$, $P<0.001$). There also was a significant effect of agrochemical treatment on densities of *O. danica* ($F_{2,5}=60.58$, $P<0.001$). Densities of *O. danica* in chlorothalonil treatments were significantly lower than in control treatments ($P<0.001$), but there was no significant difference in densities of *O. danica* between control and atrazine treatments ($P=0.651$). There was a significant interaction between agrochemical and *O. danica* treatments ($F_{2,11}=9.37$, $P=0.004$). There was no significant difference between *E. coli* densities in any agrochemical treatment when predation was absent (Fig 15B and 16). However, as predicted, when predation was present, chlorothalonil treatments had significantly higher densities of *E. coli* relative
to control (P=0.007; Fig 15B and 16C) and atrazine treatments had significantly lower densities relative to controls (P=0.034; Fig 15B and 16B).

Discussion

The research on how pesticide contamination influences aquatic ecosystems has, to date, tended to focus on metazoan species rather than the direct and indirect effects that these agrochemicals have on microorganisms and their interactions with other microbial species. This study utilized simplified microcosms to assess direct effects of agrochemicals on two protozoan species, as well as indirect effects on bacterial densities via their effect on bacterivorous predation. The fungicide chlorothalonil had deleterious effects to both an obligate heterotroph, *T. pyriformis* and a facultative heterotroph, *O. danica*. As far as we are aware, this is the first study to demonstrate deleterious effects of chlorothalonil on protist species, however, previous research found toxic effects of other fungicides, nabam, maneb, thiram and fenpropimorph, on protozoan species (Moore 1970; Ekelund 1999). It should be noted that much of the protozoan quantification in chlorothalonil treatments, particularly toward the end of the sampling period, relied on using half the limit of detection as no protozoa were observed via the haemocytometer. Consequently, the decline in protozoan abundance caused by chlorothalonil treatments may be more pronounced than we were able to calculate in this study, potentially killing all protists within the microcosms.

The lack of a direct effect of the herbicide atrazine on *T. pyriformis* was not surprising. Atrazine is an s-triazine herbicide that blocks electron transport in photosystem II (Duke 1990), which would not be expected to directly impact an obligate
heterotroph. However, there were no declines in *O. danica* densities associated with atrazine exposure. The capacity of *O. danica* to utilize heterotrophy may have compensated for the diminished ability to photosynthesize. Additionally, as the *Direct Effects* experiment was conducted in the laboratory, full sunlight was not available which may have reduced to the importance of photosynthesis from the beginning of the experiment.

We also examined the indirect effects of agrochemicals on bacterivorous predation. Previous research has shown that the direct effects of agrochemicals on one trophic level can produce indirect effects on lower or higher trophic levels (Debenest, Silvestre et al. 2010; Foit, Chatzinotas et al. 2010). In the absence of predation, bacterial levels in atrazine and chlorothalonil treatments were not significantly different from control treatments, supporting previous research that agrochemicals have no direct effects on *E. coli* O157:H7 (Staley, Senkbeil et al. 2012). While no direct effect was observed, previous research has suggested that indirect effects may be more common and complex (Rohr, Kerby et al. 2006; Relyea 2009).

Density-mediated indirect effects can reduce the abundance of predators, lessening predation and promoting survival of prey species. As chlorothalonil had directly toxic effects to both protozoan species, it is likely that the greater bacterial densities in chlorothalonil treatments (relative to predation-present control treatments) were the result of a diminished abundance of bacterivorous predators. In a previous study, where pond water was introduced containing autochthonous protozoa, a decline in *E. coli* abundance in the water column was observed when microcosms were dosed with atrazine (Staley, Rohr et al. 2011). While this effect was previously attributed to a
decline in phytoplankton and increased ultra-violet light penetration, the presence of heterotrophic protozoa may have also been a significant factor in the diminished bacterial densities.

We also observed a trait-mediated indirect effect of atrazine. In microcosms where *O. danica* was exposed to atrazine, bacterial densities were significantly lower than control treatments with predation, likely as a result of increased heterotrophic activity by the protozoan. Previous research has similarly found that facultatively heterotrophic diatom species will increase heterotrophic activity in the presence of herbicides (Debenest, Silvestre et al. 2010). Several other studies have reported increases of heterotrophic protist species and increased heterotrophic activity in the presence of atrazine (Hamala and Kolliq 1985; Downing, DeLorenzo et al. 2004).

Based upon the indirect effects observed in this study, the presence of agrochemicals in aquatic ecosystems may have important effects on bacterial survival in aquatic habitats, particularly zoonotic bacterial pathogens such as *E. coli* O157:H7. As agrochemicals and agriculturally-associated pathogens are likely to be present in agricultural runoff, atrazine may provide an ecosystem service by stimulating bacterivorous predation and lessening the abundance of agriculturally-derived zoonotic bacterial pathogens. Conversely, as chlorothalonil is directly toxic to protozoan species and indirectly facilitates bacterial survival, chlorothalonil application may increase human health risks by lessening the stress on bacterial pathogens. The effects of agrochemicals (both direct and indirect) are still not well understood for the majority of pesticides, pathogens and protozoan species. However, direct and indirect effects of agrochemicals have been shown to have potentially significant consequences to
ecosystem functions and services and the protection of human health (McMahon, Halstead et al. 2012). Further research is still needed to understand the varying mechanisms by which agrochemicals act on bacterial and protozoan survival, particularly with regard to pathogens, as well as the overall net (direct + indirect) effect these have on impacted aquatic ecosystems.

References


Taghavi, L., G. Merlina, et al. (2011). "The role of storm flows in concentration of pesticides associated with particulate and dissolved fractions as a threat to aquatic ecosystems - Case study: the agricultural watershed of Save river (Southwest of France)." *Knowledge and Management of Aquatic Ecosystems* **400**(06).

U. S. Environmental Protection Agency (2009). "Review of Published Studies to Characterize Relative Risks from Different Sources of Fecal Contamination in Recreational Water. EPA 822-R-09-001."

U. S. Environmental Protection Agency (2009). "Review of Zoonotic Pathogens in Ambient Waters. EPA 822-R-09-002."


Figure 13. Average densities (n=3±SE) of A) *T. pyriformis* and B) *O. danica* in each agrochemical treatment. Half of the limit of detection was used when no protozoa were detected, potentially over-estimating value. Results are based upon a repeated-measures ANOVA.
Figure 14. Average *E. coli* O157:H7 and *T. pyriformis* densities (*n*=3 ± SD) in A) control and B) chlorothalonil treatments at each sampling point.
Figure 15. Average *E. coli* O157:H7 densities (n=3±SE) with and without predation by A) *T. pyriformis* and B) *O. danica* in each agrochemical treatment. Half of the limit of detection was used when no protozoa were detected, potentially overestimating value. Results are based on a repeated-measures ANOVA.
Figure 16. Average *E. coli* O157:H7 and *O. danica* densities (*n=3 ± SD*) in A) control and B) atrazine and C) chlorothalonil treatments at each sampling point.
CHAPTER SIX: THE EFFECTS OF AGROCHEMICALS ON BACTERIVOROUS PREDATION AND COMPETITION

Abstract

Storm water and agricultural runoff frequently contain agrochemicals, such as herbicides and fungicides, as well as fecal indicator bacteria (FIB) and pathogens derived from livestock, such as Escherichia coli O157:H7. Agrochemicals may indirectly affect bacterial densities by increasing or decreasing densities of protozoans that prey upon bacteria or by altering competition dynamics, but little research has been conducted on the effects of agrochemicals on these environmental stressors. This study utilized microcosms composed of water taken from a Florida river containing natural protozoan and bacterial populations to test the hypothesis that agrochemicals indirectly affect bacterial densities by altering densities of protozoan predators or by altering competition dynamics. Streptomycin-resistant strains of Escherichia coli, Enterococcus faecalis, and E. coli O157:H7 were inoculated into microcosms dosed with either the herbicide atrazine or the fungicide chlorothalonil. Cycloheximide, an inhibitor of protein synthesis in eukaryotes, or the antibiotic streptomycin were also added to isolate the effects of predation or competition, respectively. Protozoan densities were significantly reduced by cycloheximide and chlorothalonil, and accompanying bacterial densities were significantly elevated relative to controls. Based upon these results, chlorothalonil has an
indirect, density-mediated effect that promotes bacterial survival by decreasing predation. Atrazine was not found to have any significant effects on protozoan or bacterial densities. In all treatments, \textit{E. coli} O157:H7 survived better than \textit{E. coli} or \textit{Ent. faecalis}. While no significant alteration of competition dynamics was observed, these results suggest that agrochemicals have significant effects on bacterivorous predation which can facilitate the survival of agriculturally-associated zoonotic pathogens in aquatic habitats and increase health risks for humans who are exposed to the water.

\textbf{Introduction}

In recent years, agricultural practices such as wide-scale application of agrochemicals have become increasingly scrutinized for their potential contributions to human health risks (Kudva, Blanch et al. 1998; Fratamico 2004; Semenov, van Overbeek et al. 2009; Lebbad, Mattsoon et al. 2010; Ziener, Bonner et al. 2010). Agriculturally-associated pathogens, such as pathogenic \textit{Escherichia coli} strains, \textit{Salmonella enterica}, \textit{Cryptosporidium} spp., \textit{Giardia} spp., and zoonotic influenza viruses, are endemic or emerging human pathogens in many parts of the world (U. S. Environmental Protection Agency 2005; U. S. Environmental Protection Agency 2009; U. S. Environmental Protection Agency 2009; Lebbad, Mattsoon et al. 2010). Many of these agriculturally-derived pathogens are waterborne and can enter water bodies via storm water and agricultural runoff containing fecal material from livestock, such as cattle, swine, and poultry (Fratamico 2004; Berry 2007; Brooks, Adeli et al. 2009). In addition to fecal contamination, runoff frequently introduces agrochemicals into water bodies, which could potentially affect the survival of zoonotic pathogens, as well as autochthonous microbes and fecal indicator bacteria (FIB).
Agrochemicals have been shown to alter population densities, community composition, predator-prey relationships, and ecosystem dynamics of freshwater systems (DeLorenzo, Scott et al. 2001; Downing, DeVanna et al. 2008; Verro, Finzio et al. 2009; Verro, Finzio et al. 2009; Debenest, Silvestre et al. 2010). The mechanisms by which agrochemicals affect changes in impacted ecosystems can be either direct or indirect and either beneficial or harmful. Previous research has shown that there are no direct effects of the herbicide atrazine or the fungicide chlorothalonil, either positive or negative, on selected FIB or zoonotic pathogens (Breazeale and Camper 1972; Staley, Senkbeil et al. 2012). However, through indirect effects, agrochemicals may affect bacterial populations in beneficial or adverse ways (Clements and Rohr 2009; Staley, Rohr et al. 2011). Indirect effects of agrochemicals could be trait-mediated, altering behavior, immunity, physiology, or morphology. These effects have been seen on multicellular organisms, particularly amphibians (Rohr, Elskus et al. 2003; Rohr and Crumrine 2005; Rohr and Palmer 2005; Rohr, Kerby et al. 2006; Rohr, Schotthoefer et al. 2008), as well as microbes, such as protozoa (DeLorenzo, Scott et al. 1999; DeLorenzo, Scott et al. 2001; De Laender, Soetaert et al. 2010; Debenest, Silvestre et al. 2010). Alternatively, indirect effects of agrochemicals may be density-mediated, altering the abundance of a target species’ food resources, parasites, competitors, or predators. These effects have been seen in both multicellular organisms (Rohr, Kerby et al. 2006; Raffel, Martin et al. 2008; Rohr, Raffel et al. 2008; Rohr, Schotthoefer et al. 2008; Rohr, Swan et al. 2009) as well as protozoa and bacteria (Dive, Leclerc et al. 1980; Ekelund 1999; Sumpono, Belean et al. 2003; Foit, Chatzinotas et al. 2010; Staley, Rohr et al. 2011). As both agriculturally-derived zoonotic pathogens and agrochemical residues are likely to be
present in agricultural runoff, understanding the mechanism (direct or indirect) and direction (beneficial or adverse) of agrochemical effects in impacted water bodies and the underlying sediment is essential to utilizing agricultural practices which best safeguard human and ecosystem health.

Presently, regulatory standards for microbial water quality only consider densities of non-pathogenic FIB, such as *E. coli* and *Enterococcus* spp., in the water column (U. S. Environmental Protection Agency 1983; U. S. Environmental Protection Agency 2002). However, not all pathogens are as susceptible to environmental stressors as FIB. A recent study has found that *E. coli* O157:H7 persists longer in the presence of protozoan predation and UV stress compared to non-pathogenic *E. coli* and enterococci (Jenkins, Fisher et al. 2011). Also, even closely-related agriculturally-derived pathogens have physiological differences from FIB and are also subjected to different rates of predation from bacterivorous protozoan predators (Hayashi, Makino et al. 2001; Jenkins, Fisher et al. 2011). Given the differences in physiology, predation and stress-response, it is reasonable to believe that FIB and pathogens exhibit different responses to agrochemicals. Therefore, it is necessary to gain a better understanding of the factors that influence the survival of both FIB and pathogens in environmental waters and sediments.

In addition to understanding the effects of agrochemicals on target bacteria, potential effects on biotic stressors, such as protozoan predation and competition with native bacterial populations, also need to be considered. Predation by bacterivorous protozoa and competition with native bacteria can drastically decrease the survival of target bacterial populations (Wanjugi and Harwood 2012; Korajkic, McMinn et al. 2013). Previous studies have shown that protozoan densities are reduced when exposed to
cycloheximide, a chemical which inhibits protein synthesis in eukaryotic organisms, and, as a consequence, the populations of their bacterial prey increase (Marino and Gannon 1991; Davies, Long et al. 1995). Streptomycin and kanamycin, antibiotics which inhibit bacterial protein synthesis via binding to 16S rRNA, have been shown to reduce the competition and increase the densities of bacteria resistant to these antibiotics (Wanjugi and Harwood 2012). Agrochemicals which are toxic to protozoan predators may exhibit similar effects as cycloheximide, reducing protozoan populations and increasing bacterial densities. Also, agrochemicals may affect bacterial populations differently, potentially conferring a competitive advantage on unaffected or beneficially affected species.

To investigate the effects of agrochemicals on predation and competition with autochthonous bacteria, simplified microcosms were established using river water and disinfected sediment. Streptomycin-resistant (streptomycin$^\text{R}$) FIB and pathogens ($E. \text{coli}$, $\text{Ent. faecalis}$, and $E. \text{coli}$ O157:H7) were then inoculated and microcosms were dosed with atrazine or chlorothalonil to examine effects of agrochemicals on bacterivorous predation by a natural protozoan population and indirect effects of agrochemicals on competition with natural bacterial populations. Additionally, these microcosms were amended with either cycloheximide, to reduce protozoan predation, or streptomycin, to reduce autochthonous competition (hereafter referred to as inhibitors). Control microcosms were maintained under unamended (no addition of either cycloheximide or streptomycin) conditions. Atrazine and chlorothalonil were selected because, in the U.S., they are both within the top two agrochemicals in usage within their agrochemical class (Kiely, Donaldson et al. 2004).
Previous research has shown that application of atrazine can increase heterotrophic activity (Hamala and Kolliq 1985; DeLorenzo, Scott et al. 2001). Further, atrazine would likely inhibit photosynthesis in facultatively heterotrophic protozoa, resulting in increased heterotrophic behavior (Debenest, Pinelli et al. 2009; Debenest, Silvestre et al. 2010). Atrazine has also been shown to increase heterotrophic protist species (Downing, DeLorenzo et al. 2004). Consequently, we hypothesize that atrazine treatments will stimulate bacterivory, thereby reducing bacterial densities. Conversely, chlorothalonil has been shown to reduce levels of heterotrophic protist species (Downing, DeLorenzo et al. 2004) and may be toxic to a variety of protozoa (Bending, Rodriguez-Cruz et al. 2007). We would therefore expect chlorothalonil to have adverse effects on the protozoa, similar to the effects of cycloheximide, thereby decreasing bacterivory and allowing greater persistence of bacterial populations relative to control treatments.

**Experimental Design**

**Competition and Predation from Natural Microbial Populations.** Microcosms were established in the University of South Florida Botanical Gardens (Tampa, FL) in an outdoor greenhouse. Microcosms consisted of 0.95 L glass mason jars containing 600 ml of water collected from the Hillsborough River (Tampa, FL) containing autochthonous bacteria and protozoan species and 100 ml of sediment, also collected from the Hillsborough River, which was disinfected via baking for 24h at 177°C. One third of microcosms were dosed with cycloheximide (200 µg/ml) to reduce protozoan predation, one third were dosed with streptomycin (100 µg/ml) to diminish autochthonous competition, and the remaining third were unamended. All microcosms were covered
with plastic wrap to allow for light penetration, but prevent contaminants from entering the system.

Prior to inoculation, *E. coli* MG6155, *Ent. faecalis* 19433 (American Type Culture Collection), and *E. coli* O157:H7 EDL933 were passaged on Luria-Britani agar (Fisher Scientific), supplemented with streptomycin (100 µg/ml) in order to select for spontaneous streptomycin-resistant mutants. Streptomycin R strains were prepared for inoculation by streaking for isolation on trypticase soy agar and incubating overnight at 37°C. Isolated colonies were inoculated into brain heart infusion broth and incubated at 37°C overnight. Each individual culture was then centrifuged at 5000 RPM for 5 min (IEC Multi, Thermo Scientific) and the pellet was resuspended in sterile buffered water (0.0425 g L⁻¹ KH₂PO₄ and 0.4055 g L⁻¹ MgCl₂) twice (American Public Health Association 1999). Each suspension was then diluted 1:10 in sterile buffered water and 0.7 ml of each diluted bacterial suspension was aseptically inoculated into every microcosm (~10⁷ CFU/100ml for all bacterial species).

One hour following inoculation, one of three treatments was randomly applied to each microcosm: a solvent control (DI water amended with 0.002% acetone, used as a solvent for all agrochemicals), the herbicide atrazine or the fungicide chlorothalonil at 1x the expected environmental concentration (EEC; 102 µg/L for atrazine, 170 µg/L for chlorothalonil, calculated using the U.S. Environmental Protection Agency’s GENEEC v2 software). Within each different inhibitor condition (diminished predation, diminished competition, or unamended conditions) each agrochemical treatment was represented and replicated thrice in separate spatial blocks for a total of 27 separate microcosms divided among three spatial blocks.
Water samples were collected from each microcosm one hour following bacterial inoculation but immediately prior to agrochemical application (Time 0). Water samples were collected from every microcosm again after one day (T1), two days (T2), three days (T3) and six days (T6).

**Sample Collection, Filtration, and Bacterial and Protozoan Enumeration.** Water samples were collected by pipetting 10 ml volumes into sterile centrifuge tubes. Samples were placed on ice for transport to the laboratory and processed within 30 min of collection. Samples were filtered through a nitrocellulose membrane filter (0.45 µm pore-size, 47 mm diameter, Fisher Scientific) and bacteria were enumerated via standard membrane filtration methods (American Public Health Association 1999). *E. coli* colonies were enumerated on mTEC agar at 35°C for 2h, followed by incubation at 41°C for 22h (U. S. Environmental Protection Agency 2002); enterococci were enumerated on mEI agar at 41°C for 24h (U. S. Environmental Protection Agency 2002); and *E. coli* O157:H7 was enumerated on sorbitol MacConkey agar at 35°C for 24h (Novicki, Daly et al. 2000). Protozoa were quantified by haemocytometer as per manufacturer’s instructions (HyClone, Pittsburg, PA) using unfiltered water samples. If no protozoa were visible under the haemocytometer, half of the limit of detection (LOD) was recorded (the LOD was 2.5 X 10³ cells/ml, 1.25 X 10³ cells/ml was the value used for statistical analysis).

**Statistical Analysis.** All response variables (bacterial and protozoan densities) were log-transformed and block was included in all analysis. The residuals were always carefully scrutinized to ensure that the assumptions of the analysis were met. To assess agrochemical effects on bacterial densities with natural predators, a repeated measures,
regression-based, multivariate analysis of variance (MANOVA) was conducted, where the repeated measures factor was the bacterial density on the four sampling intervals (T1, T2, T3 and T6). The T0 densities for both bacterial and protozoan densities were used as continuous covariates to control for stochastic variation upon inoculation. In these analyses, interactions between among- and within-tank (repeated measures) factors were always included. This allowed us to examine treatment-by-time interactions. In these analyses, the response variables were the densities of *E. coli*, *Ent. faecalis*, and *E. coli* O157:H7 on each sampling date. The full model included the main effects of inhibitors (unamended, diminished predation, or diminished competition), the three agrochemical treatments (control, atrazine, chlorothalonil), two-way interactions, and repeated measures main effects and interactions. ANOVAs were also conducted on each individual bacterial species to ensure no univariate effects were missed.

Protozoa were analyzed separately using ANOVA. The response variable was the log-transformed protozoan densities, including a repeated measures factor consisting of the protozoan densities on each sampling interval (T1, T2, T3, and T6). The full model included the main effects of inhibitors (unamended, diminished predation, or diminished competition), the three agrochemical treatments (control, atrazine, chlorothalonil), two-way interactions, and repeated measures main effects and interactions.

**Results**

For protozoan densities, there were significant main effects of inhibitor ($F_{2,17}=16.60, P<0.001$) and agrochemical treatments ($F_{2,17}=16.47, P<0.001$), as protozoan densities were significantly reduced in cycloheximide microcosms and chlorothalonil.
treatments (Fig 17). A significant interaction between the agrochemical*inhibitor treatments ($F_{4,17}=5.93, P=0.004$), driven by the reduction of protozoan densities in unamended conditions when chlorothalonil was present, but no further reduction when chlorothalonil was added to the cycloheximide-amended treatments. Additions of streptomycin and atrazine were not found to significantly affect protozoan densities relative to control treatments.

For bacterial densities, there was a significant main effect of the inhibitor treatment ($F_{6,22}=27.96, P<0.005$), as densities were significantly higher in streptomycin and cycloheximide treatments than controls (Fig 18 and 19). There was also a significant main effect of agrochemical treatment ($F_{6,22}=9.89, P<0.005$), with significantly higher bacterial densities in chlorothalonil treatments relative to controls (Fig 19). Additionally, there was a significant interaction between agrochemical and inhibitor treatments ($F_{12,29,39}=7.08, P=0.030$), because bacterial densities in the unamended condition were significantly elevated in the presence of chlorothalonil, however increased bacterial densities were not observed when cycloheximide- or streptomycin-amended treatments were dosed with chlorothalonil (Fig 18 A-C and Fig 19).

**Discussion**

The environmental stress of predation and competition on bacterial survival has been well established, (Barcina, Lebaron et al. 1997; Byappanahalli, Nevers et al. 2012; Wanjugi and Harwood 2012; Korajkic, McMinn et al. 2013), however the effect agrochemicals have on these stressors, and the ensuing result on bacterial survival, is
poorly understood. Agrochemicals could prolong the survival of FIB and bacterial pathogens by lessening predatory or competitive stress, increasing the perceived or real risks to human health. Conversely, pesticide residues could increase predation or competition, having detrimental effects on bacterial persistence, effectively “killing two birds with one stone.”

Effects which indirectly influence bacterial survival, either through density- or trait-mediated effects on predators, competitors or nutrient resources, are more common and complex than direct effects (Rohr, Kerby et al. 2006; Relyea 2009). For example, while previous research has indicated that there are no direct effects of atrazine or chlorothalonil on the bacterial targets investigated in this experiment (Staley, Senkbeil et al. 2012), an indirect effect of agrochemicals, mediated by altering algal populations, has been shown to significantly affect FIB densities (Staley, Rohr et al. 2011). Therefore, understanding the indirect effects of agrochemicals on the survival of FIB and pathogens, mediated via effects on predation or competition, is important for a better understanding of how pesticides can influence risks to human health.

The top-down pressure of predation as well as the pressure of competition can have significant negative impacts on bacterial survival (Wanjugi and Harwood 2012; Korajkic, McMinn et al. 2013). Similar results were obtained in this study, as bacterial densities were significantly elevated, relative to unamended conditions, when either competition or predation was removed using inhibitors or, in the case of predation, the fungicide chlorothalonil. Further, predation was found to be a more prominent stressor on bacterial populations than competition, as bacterial densities were highest when protozoan predation was diminished in cycloheximide-amended treatments (Fig 19).
Previous studies support these results, as bacterivorous predation has been found to be a more significant stressor than competition, accounting for upwards of 90% of bacterial decay in lake systems (Anderson, Larsson et al. 1986; Gurijala and Alexander 1990; Byappanahalli, Nevers et al. 2012).

The application of chlorothalonil significantly influenced both bacterial and protozoan densities. In unamended conditions (neither competition or predation was inhibited), bacterial densities remained elevated in chlorothalonil treatments, relative to controls, and protozoan densities were significantly reduced after 24h. The elevated bacterial densities were similar to those observed in the cycloheximide-amended treatments where protozoan densities remained undetectable after 24h. These results suggest that chlorothalonil had a density-mediated indirect effect, similar to cycloheximide, wherein bacterial densities remain elevate as a consequence of decreased protozoan predation. However, protozoan densities in unamended chlorothalonil conditions (no cycloheximide) rebounded over the six day sampling period, possibly because resistance to chlorothalonil was developed or because the fungicide had decayed over the duration of the study. In cycloheximide treatments, where predation was presumably diminished by the inhibitor, no significant effect on bacterial persistence was seen for any agrochemical treatment. In this case, agrochemical effects would likely be limited to direct effects on FIB and pathogen populations. The lack of effect of agrochemicals therefore supports our previous observation that indirect effects of agrochemicals mediate the impact on FIB and pathogen survival (Staley, Senkbeil et al. 2012).
Whereas an increase in FIB and pathogen densities was observed in chlorothalonil treatments under unamended conditions (where competition and predation were undiminished), the same increase was not observed in streptomycin-amended microcosms (where predation should have been unaffected and competition was diminished). In streptomycin-amended microcosms, we hypothesized that a similar increase in bacterial densities and reduction of protozoan densities would be observed, however no significant difference was observed in either bacterial or protozoan densities when chlorothalonil was applied, relative to controls. These results suggest that there may be an interaction between streptomycin and chlorothalonil which inhibits the toxic effects of the fungicide on protozoan populations. Previous research has suggested this interaction, as the effects of chlorothalonil have previously been shown to be diminished in the presence of streptomycin (Habte 1985).

Whereas the indirect effects of agrochemicals on bacterivorous predation have been poorly studied, the results of this experiment reveal that agrochemicals do have a significant effect on bacterivorous predation, resulting in an indirect, density-mediated effect increasing bacterial survival. Most notably, chlorothalonil, when present in freshwater systems, reduces heterotrophic protozoan densities, thereby decreasing predation which results in elevated bacterial densities and persistence. Particularly with regard to *E. coli* O157:H7, the application of chlorothalonil with the resulting increase in bacterial survival, could result in the propagation and prolongation of potential zoonotic pathogens in impacted water bodies, increasing risks to human health. Further, densities of pathogenic *E. coli* O157:H7 remained significantly higher than densities of FIB in all microcosms, not only those treated with chlorothalonil. Extended persistence of *E. coli*
O157:H7 relative to FIB, even in the presence of competition and predation, has been reported previously (Jenkins, Fisher et al. 2011; Staley, Rohr et al. 2011; Staley, Senkbeil et al. 2012), suggesting that the present regulatory standards, which rely on FIB densities, may underestimate the risk posed by *E. coli* O157:H7.

References


U. S. Environmental Protection Agency (2002). "Implementation guidance for ambient water quality criteria for bacteria. EPA-823-B-02-003.".


U. S. Environmental Protection Agency (2009). "Review of Published Studies to Characterize Relative Risks from Different Sources of Fecal Contamination in Recreational Water. EPA 822-R-09-001."

U. S. Environmental Protection Agency (2009). "Review of Zoonotic Pathogens in Ambient Waters. EPA 822-R-09-002.".


Figure 17. Mean densities ($n=3\pm SD$) of naturally-occurring protozoa in each agrochemical treatment with A) unamended conditions, B) cycloheximide addition and C) streptomycin addition. The double horizontal line (═) represents $\frac{1}{2}$ the LOD, and values at that level represent samples in which protozoa were undetectable.
Figure 18. Mean *E. coli* (A, D, G), *Ent. faecalis* (B, E, H), and *E. coli* O157:H7 (C, F, I) densities ($n=3\pm SD$) as a function of time in microcosms with A-C) unamended conditions, D-F) cycloheximide addition, G-I) streptomycin addition.
Figure 19. The effect of inhibitors and agrochemical treatments on mean FIB and pathogen densities in microcosms ($n=3\pm SE$) over the entire six-day period assessed by repeated measures ANOVA.