

UNDERSTANDING *ESCHERICHIA COLI* O157:H7 PRESENCE,
PERVASIVENESS, AND PERSISTENCE IN CONSTRUCTED
TREATMENT WETLAND SYSTEMS

by

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DEDICATION

“[H]e said to me, “My grace is sufficient for you, for my power is made perfect in weakness.” Therefore, I will boast all the more gladly about my weaknesses, so that Christ’s power may rest on me ... For when I am weak, then I am strong.”

II Corinthians 12:8-10

This is for my Dad,
for telling me to seize the
day and throttle it,

and for my Moontie,
for picking me up when the
day had throttled me;

to all the teachers
that taught me the poetry
of what research is,

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and endured with me.

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ABSTRACT

Treatment wetlands (TW) are a wastewater remediation technology that relies on the natural ability of wetland plant species and the associated microbial consortia to remove pollutants and improve water quality. Although there is substantial research on chemical pollutant remediation by TW, the removal of bacterial pathogens is much more varied and limited in scope. *Escherichia coli* O157:H7 is a bacterial pathogen that has caused numerous outbreaks and infections in the United States alone and is closely associated with improper water treatment. Understanding how *E. coli* O157:H7 could potentially persist and survive through a TW process is important in order to appropriately determine the efficacy of TW for treating water and protecting human health. This work used epifluorescent microscopy and qPCR relative DNA abundance to track *E. coli* O157:H7 tagged with a fluorescent DsRed protein in various environments pertaining to a TW. Two high performing wetland plant species, *Carex utriculata* and *Schoenoplectus acutus*, were used in hydroponic and simulated TW columns to better understand how the bacteria localize and persist. Teflon nylon strings (diameter 0.71-1.02 mm), cleaned and with established biofilm, were run hydroponically as control inert surfaces. Unplanted gravel columns were used as a nonplanted control for column experiments. *E. coli* O157:H7-DsRed were observed by microscopy on root surfaces both in hydroponic reactors and lab scale TW columns. The organisms persisted, forming microcolonies shortly after initial inoculation on both root and nylon surfaces. In the lab scale columns, cells persisted for three weeks, although strong biofilm formation was not observed. qPCR also provided evidence that *E. coli* O157:H7 was able to persist on the tested surfaces of plant roots, nylon inert surfaces, and gravel, showing higher abundance *S. acutus* roots than on the inert surface and gravel, however higher in unplanted gravel overall. For the plant types, *C. utriculata* was statistically lower for *E. coli* O157:H7 abundance than *S. acutus* over time. This work provides evidence that *E. coli* O157:H7 is able to colonize and persist in a TW environment, and plant surfaces may offer a higher inactivation than an inert matrix.

CHAPTER ONE

INTRODUCTION

Treatment Wetlands

Water is an increasingly important resource as population and urbanization grows. A healthy living environment requires both clean potable water and effective water treatment for subsequent wastewater (Kurzbaum et al. 2012). Issues with improper treatment or usage of wastewater have lead to numerous health and environmental problems, including increasing incidences of waterborne pathogen outbreaks from infected water and food (Licence et al. 2001). The need for wastewater treatment is a vital but an economically costly issue. Research into alternative methods of treatment is extensive and one promising technique is the use of constructed treatment wetlands (TW) in place of traditional secondary domestic wastewater treatment (Brown et al. 2000). TW have been implemented in small-scale plants and rural cities with successful pollutant reduction reaching many standards for clean water regulated by the United States Environmental Protection Agency (EPA) (Brix and Arias 2005, Merlin et al. 2002). They also provide an important means of controlling nonpoint source pollution to natural watersheds, such as run off from agricultural farms and cattle ranches (Hancock et al. 1998, Scott et al. 2009).

Design Characteristics

Due to the rigorous amount of research performed over the decades on TW for optimizing total pollutant removal, many variations in design and wastewater flow have

been developed (Austin 2014, Brown et al. 2000). The basic design includes an impermeable base layer to retain the wastewater within the TW for remediation, which is filled with some form of planted media and wetland species. The media type can be fine soil particulates, small grade sized gravel, or larger pebbles (Baskar et al. 2014). The media used for planting has been shown to have an effect on removal efficiencies and therefore must be taken into account when designing a TW for specific pollutants (Brisson and Chazarenc 2009, Stein and Hook 2005).

Plant type has also shown a significant effect on the subsequent reduction of common chemical and biological pollutants in wastewater (Taylor et al. 2011). Multiple studies have been performed analyzing removal rates from mixed and monoculture facultative or obligate wetland species (Allen et al. 2002, Coleman et al. 2001, Stein and Hook 2005). For all wetland species researched, there is consensus that planted TW outperformed just the media for increased rate of removal as well as final concentration of common water pollutants. This is due to the plant's ability to transport oxygen and nutrients into the subsurface environment that is normally anoxic and nutrient starved (Berendsen et al. 2012). The plant roots allow smaller aerobic niches that foster the growth of beneficial microbes essential for wastewater remediation. It is important when choosing the plant species to understand its effectiveness for wastewater remediation, but careful account must also be taken when using a natural resource such as wetlands not to introduce invasive species.

The arrangement of wastewater influent and recycling effluent is also a variable in design. A TW is generally identified by the location of the influent water entering the

planted media bed and how the wastewater travels through the media bed to the effluent channel (Austin 2014, Kurzbaum et al. 2012). There are two general methods of influent addition: vertical flow and horizontal flow. Vertical flow TW add wastewater to the top of the media bed with the effluent channel at the base, causing the wastewater to travel vertically down through the water column until eventual release (Kengne et al. 2014, Tietz et al. 2007). In horizontal flow designs, the influent channel is positioned at one side of the media bed and thus water is forced to flow horizontally from the influent channel to the effluent channel on the opposite side at equal height (Clifford et al. 2010, Decamp and Warren 2001).

TW are also divided according to free water surface flow or subsurface flow. The horizontal flow systems can operate as a free water surface flow, in which the media bed is partitioned to allow areas of open standing water for better aeration, however these two unique identifiers are not exclusive (Ibekwe et al. 2007). Alternatively, horizontal flow wetlands can be designed as subsurface flow in which water is kept at a level below the planted media to prevent added surface inoculation and contamination and also retain the water within the location of highest activity (Decamp and Warren 2001). All treatment designs have inherent advantages and disadvantages depending on the pollutants of highest concern for wastewater treatment (Austin 2014).

Residence time is also a large consideration for TW design. Depending on the age of the TW and the season, an optimal time must be identified between complete removal of chemical pollutants and the loading volume that can be treated with a TW per day (Josimov-Dunderski et al. 2013, Stein and Hook 2005). Depending on the size of the

TW and the concentration of the pollutants in the influent, residence time varies anywhere from 1-2 days to a week (Baskar et al. 2014). Many TW implement a recycling channel allowing the effluent to be pumped back to the influent channel and sent through the TW for multiple cycles before the water is released to a following treatment process (Baskar et al. 2014). This can increase performance of removal for some pollutants without increasing the wetland size.

Optimization for Various Pollutant Removal Efficiencies

Wastewater is a highly variable source of pollution that can range from high phosphorus and nitrates due to agricultural run off to fecal indicator bacteria and pharmaceuticals from domestic water sources (Karimi et al. 2014, McGarvey et al. 2005). TW have been researched extensively for effective removal of pollutants regulated by the EPA (Brown et al. 2000). One of the most common indicators of TW performance is measuring chemical oxygen demand (COD), which is an estimation of the carbon reduction occurring in the wastewater by indirectly measuring the amount of oxygen that is utilized per liter of wastewater to fully oxidize the organic compounds to carbon dioxide (Allen et al. 2002, Taylor et al. 2011).

Other molecules under scrutiny for removal in TW include nitrates, sulfates and phosphates (Bridgham et al. 1998, Howard-Williams 1985, Wiessner et al. 2005). Many wastewater treatment facilities optimize for complete removal of these molecular pollutants due to the negative impact on the environment (Grady et al. 2011, Isaacs and Henze 1995). High levels of nitrogen and phosphorus species can cause severe

eutrophication and subsequent dead zones in the receiving body of a wastewater treatment plant, affecting the ecosystem as a whole (Gleisberg et al. 1976). TW decrease the levels, however phosphorus has proven especially difficult and although the decrease is significant, most TW tested have not reached regulation levels (Brix and Arias 2005). Nitrogen has also proven to be a source of difficulty and requires further research due to incomplete denitrification to nitrous oxide gas rather than inert dinitrogen gas. Nitrous oxide is a greenhouse gas that is over 300 times more potent than carbon dioxide, thus only moving the issue from water pollution to air pollution (Ma et al. 2008, Wu et al. 2009).

One pollutant of major concern is the removal efficiency of bacteria from the wastewater. This aspect is complicated due to the bacteria's ability to survive for long periods of time retained within biofilms in the wetland without complete inactivation (Sleytr et al. 2007). This becomes problematic with fecal indicator bacteria, which are assumed to be representative of fecal contamination and thus the potential presence of wastewater pathogens (Benami et al. 2015, Wheeler Alm et al. 2003). Potential pathogens may then be subsequently released into the treated wastewater effluent. Human contact with rivers or lakes has caused health outbreaks due to waterborne pathogen contamination of these natural resources (Licence et al. 2001, Olsen et al. 2002). Contaminated food as a result of contaminated water being used for irrigation purposes has also caused major health risks and economic damage from recalls (Griffin and Tauxe 1991). In-depth knowledge of TW efficiency in complete pathogen bacteria

removal and inactivation is limited, although the results available do show multiple log reduction (Characklis et al. 2005, Hogan et al. 2012, Rogers et al. 2011).

Wetland Rhizosphere

TW were first implemented and recognized as a viable alternative for wastewater treatment centuries ago due to the natural observation that wetlands filter water along riverbanks and deltas (Jasper et al. 2013). The mechanisms for remediation were not clearly understood but merely taken advantage of for water quality purposes. Even when TW were first engineered for direct wastewater treatment, the media and plant root matrix were treated as a black box and there were differing views as to whether pollutant removal was physical (sedimentation and filtration) or biologically driven uptake and reaction (Bridgham et al. 1998). As research continued on TW, the importance of the biological factors of the plant roots and associated microbial community became increasingly evident (Faulwetter et al. 2009).

Plant-Microbe Interaction

The rhizosphere is defined as the small volume of soil or media that is influenced by the plant roots or in direct association to the roots and plant-produced material (Bringinghurst et al. 2001). Soils are characteristically nutrient starved and the microbial community is highly competitive and will scavenge what can be found (Berendsen et al. 2012). The rhizosphere in contrast is relatively nutrient rich due to the plant releasing exudates such as amino acids and sugar compounds into the environment through the roots (Bhattacharyya and Jha 2012). Up to 25% of photosynthesis energy is allocated to

root exudation in order to increase soil richness for the plant (Santos et al. 2014), and many species of bacteria take advantage and colonize the plants. In addition there is oxygen release by the roots in the subsurface, and water nutrient uptake causing further nutrient flow to the root surface (Stein and Hook 2005). Soil microbiota constitute the largest environmental biodiversity and this increases drastically, from 4000 species per gram of soil to over 30,000 unique species and upwards of 10^{11} cells per gram along the roots (Berendsen et al. 2012, Saharan and Nehra 2011).

Rather than deter microbial growth on the roots, plants have been shown to foster the microbial community in a symbiotic relationship. Bacteria in biofilm communities use a form of communication known as quorum sensing in which individual cells emit signal molecules and respond once they reach a certain threshold (Miller and Bassler 2001); plants have been shown to mimic these signal molecules (Badri et al. 2009, Loh et al. 2002, Morris and Monier 2003). By interacting with the bacterial population, the plant enhances its own innate defense against plant pathogens by allowing colonization of only those species beneficial to the plant, much like the human microbiome in the gut (Berendsen et al. 2012). Plants also gain nutrients from the bacterial population through siderophores that scavenge iron and other trace nutrients (Bhattacharyya and Jha 2012).

Plant Growth Promoting Rhizobacteria (PGPR)

The term plant growth promoting rhizobacteria (PGPR) was first introduced in 1978 to identify those bacterial species that were indispensable to the plant rhizosphere and stimulate the growth of the host (Bhattacharyya and Jha 2012, Pechy-Tarr et al. 2013). In order to be included in the PGPR classification, the microbial species has to

satisfy two of the three qualifying conditions: aggressive colonization of the root surface, enhance plant growth, and enact some form of biocontrol of other microbial species (Berendsen et al. 2012, Bhattacharyya and Jha 2012, Tyler and Triplett 2008). Stimulating these PGPR has been shown to cause significant growth compared to control groups as well as increased protection from bacterial, fungal, and even insect invasion (Berendsen et al. 2012, Pechy-Tarr et al. 2013, Santos et al. 2014). PGPR have a variety of methods for biocontrol, including but not limited to the release of antibiotics, siderophore scavenging of trace nutrients to deter competition, and stimulating plant hormones to increase defense (Boutillier et al. 2009, Tyler and Triplett 2008).

Waterborne Pathogen *Escherichia coli* O157:H7

Background and Identification

The importance of bacterial removal in TW is a human health issue. Improperly treated wastewater causes multiple infection outbreaks yearly due to waterborne pathogens, one of the most common being *Escherichia coli* O157:H7 (Lothigius et al. 2010). It was first identified as a human pathogen in 1982. *E. coli* O157:H7 is a Gram negative bacterium in the Proteobacteria phylum (Griffin and Tauxe 1991). It is a facultative anaerobe that has the ability to ferment lactose, a metabolism that is utilized in some techniques to identify fecal indicators in water samples (Huffman et al. 2003, Licence et al. 2001). The *E. coli* O157:H7 serotype is classified according to the somatic O-antigen type, which encodes for part of the lipopolysaccharide layer on the Gram-

negative outer membrane, and the H-antigen encoding for flagella (Griffin and Tauxe 1991, Perna et al. 2001).

Pathogenicity

There are many serotypes of *E. coli* that are pathogenic and are differentiated according to the symptoms they cause. *E. coli* O157:H7 is classified as part of the enterohemorrhagic *E. coli* (EHEC) strains which cause gastrointestinal illness, diarrhea, and bloody diarrhea which can develop into hemolytic uremic syndrome and kidney failure. For healthy individuals, the infection can result in hospitalization and is usually cleared within an average of a week. However, in the elderly, young, and immunocompromised, EHEC infections become life threatening and have resulted in death. *E. coli* O157:H7 has a low infectious dose of approximately 10-50 cells (Saldana et al. 2011), making it incredibly potent and therefore of high priority to obtain complete removal.

E. coli O157:H7 is also part of the Shiga-toxin producing *E. coli* strains (STEC), which are characterized by the ability to produce and release Shiga toxins, causing the immunogenic response and illness (Liu et al. 2010). There are two types of Shiga toxins, coded by the *StxA* and *StxB* genes. Most strains of STEC contain both or just the *StxA* gene. There are very few strains that contain only *StxB*. *E. coli* O157:H7 contains both (Mora et al. 2004, Schaffzin et al. 2012). The Shiga toxins are located on the pathogenicity island within STEC strains and are expressed in conjunction with attachment to the intestine lining (Mora et al. 2004, Rashid et al. 2006).

Reservoirs and Infection Routes

The main routes of infection for *E. coli* O157:H7 are contamination of water or food improperly washed or irrigated with contaminated water (Licence et al. 2001). One of the main food sources colonized with *E. coli* O157:H7 is from bovine dairy or meat products. This is due to cattle being a natural reservoir for *E. coli* O157:H7. A general survey of cattle farms and ranches showed heifers tended to be infected with the strain in just over 1% of the population, however with the low infectious dose that is still problematic (Griffin and Tauxe 1991). *E. coli* O157:H7 is found in the manure and run-off from dairy farms (Ibekwe et al. 2002, McGarvey et al. 2005). The manure used in organic farming can inoculate the produce, and research has shown that *E. coli* O157:H7 binds strongly to the plant surface and has the ability to migrate inside the stomata and lacerations in the plant tissue, thereby making washing ineffective (Deering et al. 2012e, Saldana et al. 2011). *E. coli* O157:H7 has shown prolonged survival on spinach leaves, carrots, leafy greens, and on vegetable roots (Jablasone et al. 2005, Jeter and Matthysse 2005, Patel et al. 2010, Warriner et al. 2003).

E. coli O157:H7 within TW

Current Limitations in Tracking and Verification of Removal from TW

Given the knowledge of *E. coli* O157:H7 persistence on vegetable plants and produce, it is likely that *E. coli* O157:H7 could also persist on the roots and within the plants in TW. The research is lacking with most studies performed tracking *E. coli* O157:H7 or fecal indicator organisms through log reductions from influent to effluent

and using plate counting as the sole method of verification (Quiñónez-Díaz et al. 2001, Reinoso et al. 2008, Sleytr et al. 2007). Plate counting is an inaccurate method for tracking persistent *E. coli* O157:H7 due to the cell's ability to enter a viable but non-culturable (VBNC) state where cells are still viable and infectious, however they cannot be resuscitated on agar plates directly from the environment (Liu et al. 2009, Liu et al. 2010, Oliver 2010). This underestimation using plate counts has been verified in studies that also incorporate molecular and microscopic techniques to track *E. coli* O157:H7 (Fu et al. 2005, Liu et al. 2009).

Reduction from influent to effluent in a TW also underestimates *E. coli* O157:H7 survival due to the inaccurate assumption that one residence time provides complete wash out and/or inactivation. Studies have shown *E. coli* O157:H7 survival and eventual release and reappearance in effluent water months after initial inoculation (García et al. 2008). *E. coli* O157:H7 has developed mechanisms for survival in the environment, including attaching to sediments or to plant tissue until inoculation into a new host can be completed (Boutilier et al. 2009, Tyler and Triplett 2008), giving evidence that tracking removal from the water alone is not enough to verify pathogen inactivation.

E. coli O157:H7 Attachment Mechanisms in Plants

Most studies on bacterial attachment in plants have been in the area of food microbiology and therefore a relatively recent concept in wetland science (Cooley et al. 2003, Deering et al. 2012e, Macarisin et al. 2012). *E. coli* species in general have been shown to attach securely onto plant surfaces using curli, pili, flagella, and type 3

secretion systems (T3SS) (Jeter and Matthysse 2005, Macarisin et al. 2012, Saldana et al. 2011). Interestingly, these attachment mechanisms are associated with pathogenic strains and are located on the same pathogenicity island as the verotoxins, including the Shiga toxin genes (Deering et al. 2012a, Jeter and Matthysse 2005). Knock-out mutants and nonpathogenic strains lacking these genes do not attach strongly to surfaces, however when transformed with a plasmid containing curli or T3SS genes, the binding significantly increases to the level of pathogenic strains (Saldana et al. 2011).

It has been suggested that the ability of *E. coli* O157:H7 to strongly attach and incorporate into plant tissue is indicative of plants being another viable reservoir for *E. coli* O157:H7 in the environment, similar to cattle (Tyler and Triplett 2008). *E. coli* O157:H7 has been shown to release plant hormones *in vitro*, suggesting an adapted ability to become part of PGPR (Tyler and Triplett 2008). Once colonized on roots, *E. coli* O157:H7 has been tracked microscopically to migrate into the tissue of plants through any damage or lateral root crack and enter the vascular system where it can persist until the plant is subsequently consumed and the pathogen is transferred to a host (Cooley et al. 2003, Deering et al. 2012a, Jeter and Matthysse 2005).

Research Objectives

This work provides a measurement of *E. coli* O157:H7 persistence within TW rhizosphere and gravel matrix using a molecular technique that uses relative abundance qPCR as well as epifluorescent microscopy of a DsRed protein producing strain. It is a comprehensive study to determine the fate of *E. coli* O157:H7 within a TW and if it is

inactivated or if it is surviving on the wetland plant species. Specifically, the objectives of this research were as follows:

- Determine the importance of the rhizosphere on *E. coli* O157:H7 binding and persistence as opposed to an inert surface
- Visualize *E. coli* O157:H7 attachment and possibility of growth on the root surface
- Examine survival and localization of *E. coli* O157:H7 within a lab-scale vertical batch TW gravel matrix and rhizosphere
- Using DNA metagenomic sequencing of the entire TW microbial community, determine *E. coli* O157:H7 impact on the existing rhizobacteria and possible incorporation into the root biofilm

Dissertation Structure

Chapter 2 is an accepted peer-reviewed publication through the International Water Association (IWA) that was written as a conference paper for the International Conference on Wetland Systems. Chapter 3 has been and Chapter 4 will be submitted as individual manuscripts for peer-reviewed journals. Chapter 5 states the conclusions and review of the research. There are four appendixes included in this thesis. Appendix A provides background experimental data of *E. coli* O157:H7 survival and plasmid retention in nanopure water and sterilized simulated wastewater media. Appendix B is the statistical analysis for the experimental design and set up. Appendix C gives a detailed explanation of the process for qPCR validation and cleanup for the signal-to-

noise background interference. Appendix D has confocal microscopy imaging of the *E. coli* O157:H7 attachment on plant roots.

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CHAPTER TWO

PRESENCE AND PERSISTENCE OF WASTEWATER PATHOGEN
ESCHERICHIA COLI O157:H7 IN HYDROPONIC REACTORS
OF TREATMENT WETLAND SPECIES

Contribution of Authors and Co-Authors

Manuscript in Chapter 2

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Manuscript Information Page

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Abstract

Treatment wetlands (TW) efficiently remove many pollutants including a several log order reduction of pathogens from influent to effluent; however, there is evidence to suggest that pathogen cells are sequestered in a sub-surface wetland and may remain viable months after inoculation. *Escherichia coli* is a common pathogen in domestic and agricultural wastewater and the O157:H7 strain causes most outbreaks in the United States. To assess attachment of *E. coli* to the TW rhizosphere, direct measurements of *E. coli* levels were taken. Experiments were performed in chemostats containing either Teflon nylon as an abiotic control or roots of *Carex utriculata* or *Schoenoplectus acutus*. Flow of simulated wastewater through the chemostat was set to maintain a 2-hour residence time. The influent was inoculated with *E. coli* O157:H7 containing DsRed fluorescent protein. Root samples were excised and analyzed via epifluorescent microscopy. *E. coli* O157:H7 was detected on the root surface at 2 hours after inoculation, and were visible as single cells. Microcolonies began forming at 24 hours post-inoculation and were detected for up to 1 week post-inoculation. Image analysis determined that the number of microcolonies with >100 cells increased 1 week post-inoculation, confirming that *E. coli* O157:H7 is capable of growth within biofilms surrounding wetland plant roots.

Introduction

Treatment wetlands (TW) have been used as an alternative method for treatment of domestic wastewater for many years. The general structure of TW has an impermeable membrane layer encompassing the wetland to protect the surrounding subsurface and aquifers from potential wastewater leaching. This bed is filled with some medium such as sand or gravel in which plants are established. Many substrates have been researched previously for their effect on removal rates of both chemical and bacterial contaminants (Baskar et al. 2014). Plant species have also been a point of interest and have been found to greatly influence TW remediation ability (Brisson and Chazarenc 2009, Taylor et al. 2011). Although their effects are varied, there is consensus that increased removal of pollutants occurs in planted wetlands than unplanted media or filter beds (Vymazal, 2001). This effect is likely due in part to the interaction between the plant and the microbial community within the rhizosphere. Plants have been shown to harbor increased microbial density and diversity in close connection to the root systems (Berendson et al., 2012; Vymazal, 2011). They release oxygen and exudates, such as sugars and amino acids, into the rhizosphere that provide a rich nutrient source to the microbial community as compared to the nutrient-starved soil matrix (Bhattacharyya and Jin, 2012). The associated microbial community provides a large amount of the wastewater remediation reactions including nitrification, denitrification and phosphorus uptake (Jasper et al., 2013).

Several studies have found that bacterial pathogens, either specific organisms or indicator organisms, decrease significantly from TW influent to effluent (Reinoso et al.

2008). Little is understood, however, about the mechanisms of removal, and long-term studies have indicated that bacterial contaminants can be retained within the wetlands and be released months after inoculation by wastewater. These methods have also been limited to detection by plate count alone which is deceiving given many pathogen's ability to enter a viable but non-culturable state (VBNC) (Barcena et al. 2009, Liu et al. 2009).

E. coli are Gram-negative bacterium in the phylum Proteobacteria. They are facultative anaerobes that are found most commonly in the intestinal tract of mammalian species (Griffin and Tauxe, 1991). Although many strains are commensal organisms, there are a number of serotypes that are pathogenic (Kaper et al., 2004). *E. coli* O157:H7 is an enterohemorrhagic strain that is highly infectious, with the level of infection detected to be as few as ten cells. It is a common pathogen found in non-treated water sources. A main reservoir is in cattle, which releases *E. coli* O157:H7 into the environment to contaminate water sources and subsequent crop production either through use of non-treated water irrigation or manure fertilizer (Hancock et al. 1998, Licence et al. 2001). It has been shown that *E. coli* O157:H7 is capable of long-term survival in river water as well as on the surfaces of plants, namely agricultural crops (Dinu et al. 2011, Liu et al., 2001). These studies have been performed using direct counting or molecular methods due to the VBNC ability of *E. coli* O157:H7.

This study attempts to gain a better mechanistic view of the survival and persistence of *E. coli* O157:H7 within a TW to determine the risk of subsequent release of pathogens to the environment. Given the inaccuracy of plate counts, microscopy was

employed as a direct cell counting method for the presence of *E. coli* O157:H7 within the root rhizosphere. The complexity of a full or pilot scale TW was scaled down and the medium removed as a potential variable in this study, instead focusing on *E. coli* O157:H7 interaction with the roots of plants. Roots have been shown to provide an ideal environment for biofilm growth and sustainability with the release of oxygen and exudates to the subsurface (Allen et al. 2002). It was hypothesized the added benefit of a nutrient rich environment would aid in attachment and survival of *E. coli* O157:H7 to a root as opposed to an inert surface within the TW.

Materials and Methods

E. coli O157:H7 Strains and Growth

E. coli O157:H7 was originally isolated from an environmental outbreak by Dr. Barry Pyle of Montana State University (Pyle et al. 1995). The DsRed fluorescent protein plasmid was previously inserted into the wild-type strain and constitutively expressed for all subsequent microscopy. A loopful of *E. coli* O157:H7 frozen stock was inoculated into 80 mL of custom Tryptic Soy Broth (TSB) + 250 mg/L carbenicillin and grown overnight. The TSB media consisted of: 24 g/L non-casein enzymatic digest of protein, 5 g/L NaCl, and 2.5 g/L K₂HPO₄. Three replicates of 20 mL overnight culture were spun down at 4816 x g for 20 minutes, washed with nanopure H₂O, and concentrated in 1 mL nanopure H₂O for inoculation into replicate reactors.

Plant Strains and Growth

Plant material was sourced from mesocosms containing monocultures of *Carex utriculata* and *Schoenoplectus acutus* that had been established for over ten years in the greenhouse laboratory facilities at Montana State University. The plants were kept in a controlled environment and fed with simulated wastewater media (Table 2.1) diluted with treated Bozeman tap water to maintain nonpathogenic bacterial populations in the mesocosms. Shoots and roots were harvested with substantial root base and washed with tap water to remove excess dirt and sloughed biofilm cells. The extracted plants were transferred to 300 mL chemostat reactors open to the air in a laboratory environment under 24 hour plant growth lighting and allowed to grow hydroponically for 3-7 days in a

Table 2.1. Properties of the growth media

Nutrient	Chemical Compound	Concentration (g/L)
Organic carbon	$C_{12}H_{22}O_{11}$	0.0997
Iron	$FeCl_3$	0.0004
Magnesium	$MgSO_3 \cdot 7 H_2O$	0.062
Ammonia	NH_4Cl	0.0191
Copper	$CuSO_4$	0.0008
Manganese	$MnSO_4$	0.0078
Zinc	$ZnSO_4$	0.0078
Calcium	$CaCl_2$	0.0019
Phosphorus	K_2HPO_4	0.044
Nitrate	$NaNO_3$	0.1214
Boron	H_3BO_3	0.01
Potassium	KI	0.0019
Molybdenum	Na_2MoO_4	0.004
Primatone	complex	0.111

simulated wastewater media (Table 2.1). Plants were established under constant flow conditions (3mL/min), which continued during the reactor series experiments. The amount of plant material placed in each reactor was determined by the mass of displaced water ($49.3\text{mL} \pm 26.4\text{mL}$).

An abiotic treatment was also used to determine the effect of a system of living roots as opposed to an inert surface of similar dimensions. Teflon nylon strings, used to simulate roots, were added to chemostats with simulated wastewater flow already established immediately prior to *E. coli* O157:H7 inoculation. Total weight of the nylon strings, cut into 14.5cm lengths, was 2.94 g, which ranged in diameter from 0.79-1.01 mm.

Reactor Series

After plant establishment, 1 mL of concentrated overnight inoculum of *E. coli* O157:H7 was added to the open reactor system in three replicates of the three treatments (*Carex*, *Schoenoplectus* and nylon) and mixed via pipetting and rotating the plant or nylon “roots”. One additional reactor per treatment was not inoculated as a control for a total of four reactors per treatment. The control was kept on simulated wastewater media and Bozeman city tap water free of any pathogenic bacterial strains. The experiment was run in 300 mL chemostat reactors with a flow of 3 mL/min for the course of one week under 24 hour growth lighting and an ambient air temperature approximately 22 °C. The experiment was repeated three times. An initial water sample, considered time point zero, was taken from each reactor immediately after inoculation and analyzed for *E. coli* O157:H7 concentration. Effluent samples were collected from the three inoculated

reactors on 45-minute intervals over the first six hours unless the replicate was destructively sampled within six hours. The uninoculated control was sampled at only 0 and 6 hours to verify no *E. coli* O157:H7 was contaminated in the system. All water samples were immediately frozen until further analysis. After two hours, corresponding to approximately one residence time of the reactor, one of the triplicate inoculated reactors was destructively sampled. Root and nylon samples were excised in half and frozen for subsequent DNA extraction. A representative root length was pulled through a 2 mm square glass capillary (Wale Apparatus, PA) and observed using epifluorescent microscopy to view initial attachment. Another water sample was taken at 24 hours and a second replicate was extracted and destructively sampled as previously described. The third replicate and uninoculated control were destructively sampled at one week.

Epifluorescent Microscopy

The root excision within the glass capillary was rinsed with water and viewed under 600X magnification with water optical focus. The root was viewed under 558nm wavelength absorption for the DsRed plasmid excitation. Images were taken along the entire length of the root, focusing on the root tip, mid-root, and top root portions. The photosystem II chlorophyll enzymes within the root naturally fluoresce under a range of wavelengths allowing the surface to be viewed under a contrasting condition to the *E. coli* O157:H7 DsRed plasmid. The images were semi quantitatively analyzed using MetaMorph ® Image Analysis Software to determine amount and colony size using average *E. coli* O157:H7 single cell fluorescence.

Results and Discussion

Microscopy

E. coli O157:H7 was inoculated successfully into the triplicate reactors. It was verified that *E. coli* O157:H7 is able to persist but not proliferate within the simulated wastewater media designed for this experiment (Figure 2.1) by tracking the survival in sterile wastewater media batch cultures. Given this observation, it was assumed that *E. coli* O157:H7 cell counts and cell concentration would not be affected by cell division of the original inoculum. In addition, due to the low replication rate, the 100 minute residence time limits the retention of planktonic cells in the chemostat and encourages attachment to the root biofilm. One replicate was sampled in its entirety at two hours to determine initial attachment, and the other two at 24 hours and 1 week respectively to assess the ability of *E. coli* O157:H7 to persist over a duration typical of a TW residence time.

At two hours, one residence time for washout, one replicate of the plants and nylon string were destructively sampled. A control root or nylon sample was also taken and verified there was no contamination with *E. coli* O:157:H7-DsRed. Images were taken of the root and string under epifluorescent microscopy (Figure 2.2A). *C. utriculata*, *S. acutus* and the nylon string all had visible single cells of *E. coli* O157:H7 attached on the surface. There was no significant difference between each plant species and the abiotic nylon, suggesting that initial attachment is based primarily on attachment surface availability and less dependent on any effect of a living root. The nylon string “roots” were added just prior to *E. coli* O157:H7 inoculation, therefore no initial biofilm

was present, also providing support that an established bacterial community on the surface does not have a strong effect on initial pathogen attachment.

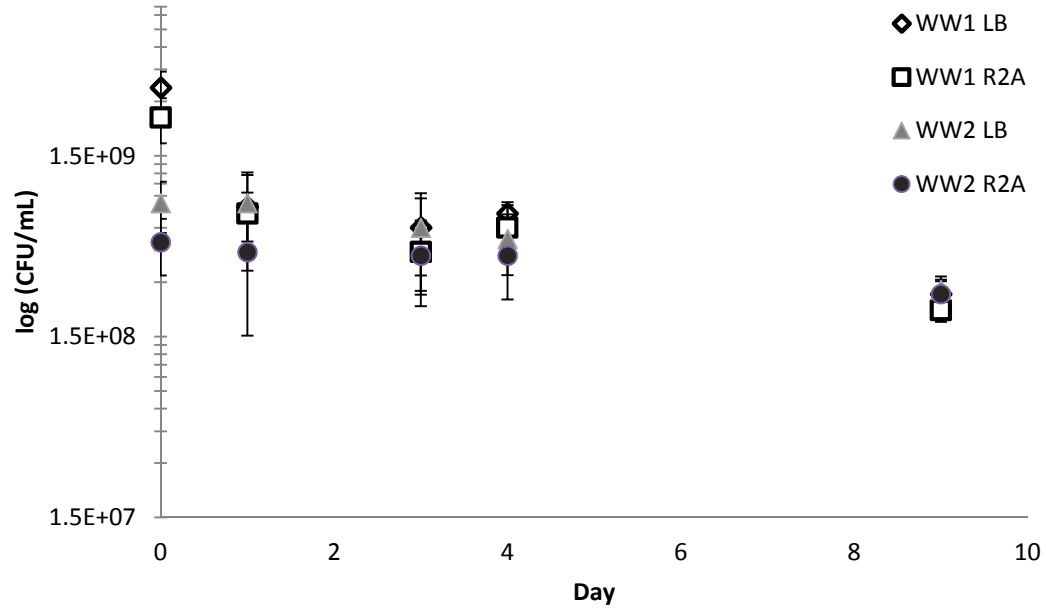


Figure 2.1. *E.coli* O157:H7 survival in sterile simulated wastewater media. Plate counts were tracked on two nutrient agar types. Triplicate technical replicates in two experimental replicates are represented. WW1 = first replicate in sterile simulated wastewater; WW2 = second replicate; LB = Luria broth agar plates used in plate count detection; R2A = Reasoner's 2A agar plates.

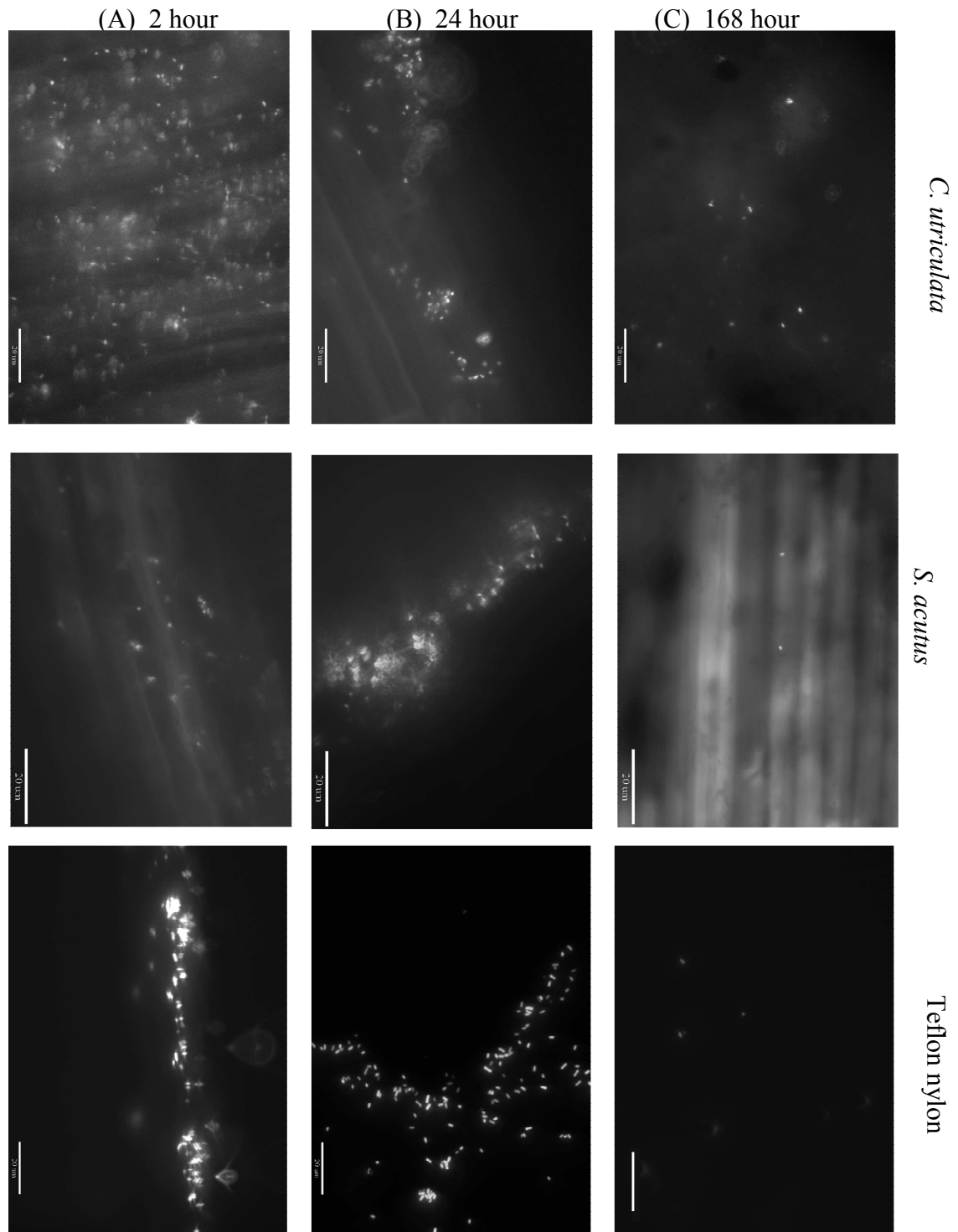


Figure 2.2. Microscopy images of *C. utriculata*, *S. acutus*, and Teflon nylon at two hours (A), 24 hours (B), and one week (C) post-inoculation of *E. coli* O157:H7. Images were taken at 600x optical zoom and 558nm wavelength. Scale bar represents 20 μ m.

Another sampling point was taken at 24 hours post-inoculation (Figure 2.2B). On all three experimental surfaces, *E. coli* O157:H7 cell abundance was seen to increase. Microcolonies are seen forming in addition to a high abundance of single cells retained on the surface. A pattern of increased attachment and active growth continued at the one week sampling point (Figure 2.2C). Much larger microcolonies were formed and three-dimensionality could be detected in portions. There was no difference in the level of persisting cells between the biotic and abiotic surfaces.

Cell Clump Analysis

Using MetaMorph ® Image Analysis Software, colony size was determined as a percentage of total cells counted over each sampling point (Figure 2.3). In all surfaces,

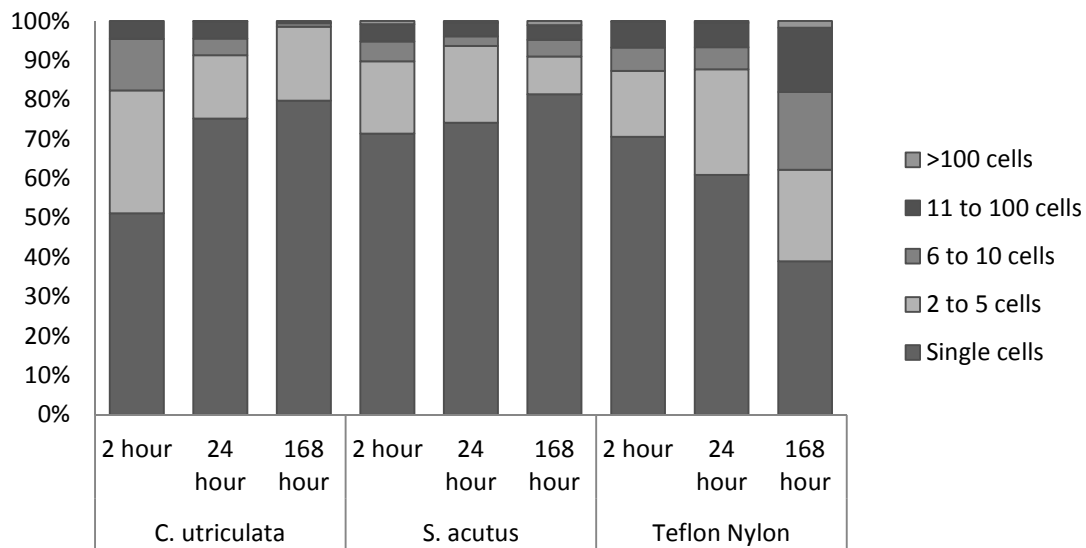


Figure 2.3. Analysis of cell population per microcolony over the course of three sampling points for *C. utriculata*, *S. acutus*, and nylon control, respectively. Populations are normalized as percentage of total cells counted.

the majority of cells remained as single cells. In *C. utriculata*, the total counts increased and colony sizes >100 cell counts were found at 24 hour and one week post-inoculation. *S. acutus* showed a smaller increase in cell counts and colony size and viable cells and colonies were detectable on nylon roots at sampling times. The data suggest that for all surfaces, *E. coli* O157:H7 was able to remain persistent over the course of one week under constant flow conditions and possibly allow for the proliferation of attached cells into larger colony structures.

Conclusion

Initial experimental results show no significant difference in attachment of *E. coli* O157:H7 between the biological surfaces, *C. utriculata* and *S. acutus* root systems, and the abiotic control surface, Teflon nylon string. *E. coli* O157:H7 was able to attach to all surfaces and was detected up to a week after inoculation in a continuous flow reactor system. In most cases viable colony growth was observed. These data suggest the mechanism for survival and growth may not be influenced significantly by the added benefit of oxygen and/or exudates provided by plant roots or by the existence of an established biofilm on the attachment site.

Future studies are planned to determine *E. coli* O157:H7 presence in pilot-scale full TW systems, including the gravel matrix. Molecular studies will also be employed to determine quantitatively the percentage of *E. coli* O157:H7 that is able to persist and if there is significant proliferation of cells attached in biofilms.

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CHAPTER THREE

ESCHERICHIA COLI O157:H7 ATTACHMENT AND PERSISTENCE WITHIN
ROOT BIOFILM OF COMMON TREATMENT WETLANDS PLANTS

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

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Contributions: Experimental design, laboratory experiments, data analysis, and manuscript writing

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Contributions: PI advisor of the project, experimental design, and manuscript editing.

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Abstract

Alternative methods for wastewater treatment have been developed and researched to find appropriate new techniques that are both economically and environmentally feasible. One such technique is treatment wetlands (TW), which is the use of natural wetland plants to remediate water pollutants, including human pathogens. *Escherichia coli* O157:H7 with a DsRed plasmid insertion was used as a model organism to track potential pathogen attachment onto roots of two obligate wetland species, *Carex utriculata* and *Schoenoplectus acutus*. Teflon nylon string was used as an inert surface control to determine any attachment advantage on living surfaces. Plants and nylon were placed in open-air chemostat reactors under constant flow with simulated wastewater media. Epifluorescent microscopy and qPCR were used to determine attachment and relative abundance of *E. coli* O157:H7 through 1 week. Attachment and microcolony growth was seen for all surface types after 24 hours, which decreased in abundance at 1 week. qPCR showed relative abundance of *E. coli* O157:H7 was preferential to roots than nylon control. There was also significant difference between root type, which may indicate preferential binding to the zones of cell elongation and differentiation than to tap root structures. *S. acutus* also showed significantly higher binding than *C. utriculata*, showing a possible plant species effect. *E. coli* O157:H7 binding and prolonged attachment on root surfaces may be a means of survival in TW and a need for further research into pathogen retention and subsequent release as this may be a concern for human health.

Introduction

Wastewater treatment is an important concern for environmental and human health. In recent years many alternative methods of treatment have been developed and researched (Antoniadis et al. 2010, García et al. 2008, Tonderski et al. 2009). One method of particular promise is the use of treatment wetlands (TW) for wastewater remediation. TW are the adoption and adaptation of a natural resource to take advantage of the plant and bacterial removal of common contaminants in water. In general, TW consist of a nonpermeable layer to separate wastewater from leaching into the ground. The wetland is then constructed with single or mixed species plants in a medium of gravel or soil (Baskar et al. 2014). In terms of wastewater loading and retention, there are several design variations. These include vertical and horizontal water flow where the wastewater enters either from the top of the wetland to exit the bottom or from one side to travel to the other. These can both additionally be surface or subsurface flow, which depends on whether the wastewater has contact with the air (Austin 2014). Each design has its own advantages and disadvantages in terms of the contaminants needing removal, and the best-case pilot wetlands combine design flows in multi-step series in order to best optimize for the variety of pollutants being treated (Austin 2014).

The driving factor behind the success of TW as a wastewater remediation option is the bacterial populations that grow within the rhizosphere and medium matrix. Whereas a completely engineered system, such as a conventional wastewater treatment plant, requires a substantial investment of time and money into operations and continued optimization of aerobic and anaerobic zones, TW provide both anaerobic and

aerobic microniches within the matrix due to the roots (Boutilier et al. 2009, DeCamp and Warren 2001, Dong et al. 2010, Faulwetter et al. 2009, Reinoso et al. 2008, Sleytr et al. 2009, Stottmeister et al. 2003, Zhang et al. 2010). Plant roots are able to release oxygen as well as other exudates into the subsurface, providing an environment for aerobic bacteria to survive at depths below usual oxygenic zones in water. This allows processes such as nitrification and phosphorus removal to be possible, leaving the surrounding anaerobic matrix to continue denitrification and other anaerobic processes (Allen et al. 2002, Hench et al. 2003, Taylor et al. 2011).

The movement of exudates and oxygen leaving the roots as well as water intake to the plant constantly brings fresh sources of nutrients to the root biofilm. The flow also encourages attachment to the surface thus giving a more stable community especially in well-established, long-term TW (Josimov-Dunderski et al. 2013). The root biofilm is not only beneficial for water quality but has been shown to have beneficial effects for the plants as well (Wang et al. 2014). Previous research has shown that biofilms can provide protection against plant pathogens by acting as an extra layer of security to the surface (Péchy-Tarr et al. 2012). There is evidence that plants are able to release a form of quorum sensing molecules to encourage bacterial defense systems under certain stress conditions (Morris et al. 2003). This is advantageous in a TW setting given that optimal performance is achieved with both a healthy plant and biofilm community.

This environment is also suitable for the reduction of pathogenic bacteria present in the wastewater (Boutilier et al. 2009, Jasper et al. 2013, Licence et al. 2001). Existing biofilm competition limits pathogenic bacterial attachment and settling out within the

wetlands. Common routes of pathogenic bacteria removal include predation, competition and aggregation (Boutilier et al. 2009). Despite these factors working against human pathogen survival, there is extensive research that reveals that pathogens may survive and be released in effluent water to impact downstream use (Barcina et al. 2009, van Elsas et al. 2011, Liu et al. 2009, Paruch et al. 2011). Even a low count release of as few as 100 cells for *Escherichia coli* O157:H7 enterohemorrhagic strain is enough of an infectious dose to cause human immune response and disease (Bitton 2005, EPA 2010).

E. coli O157:H7 is a problematic organism for human health. It is a common food- and waterborne pathogen that is transmitted via the fecal-oral route, usually through contaminated water or vegetables (EPA 2010, Dinu et al. 2011, Licence et al. 2001, Olsen et al. 2002). Previous research has shown a decrease of *Escherichia coli* O157:H7 through TW, however these studies are solely based on plate counts (Liu et al. 2009, Jasper et al. 2013, Ongeng et al. 2011). When using direct microscopy or molecular techniques for enumeration of the pathogen, the results reveal much higher survival, as well as the ability for later release. This is due to a phenomenon known as the viable but non-culturable (VBNC) state in which *E. coli* O157:H7 is no longer culturable on a plate but is able to be resuscitated from the unfavorable environment when returned to favorable conditions (Barcina et al. 2009, Dinu et al. 2011, Liu et al. 2010, Paruch et al. 2011).

Another serious limitation to current methods is the focus on influent to effluent measurements only with the assumption that *E. coli* O157:H7 is not surviving within the wetland in any capacity. Long-term research with effluent sampling has shown

differently, however, with *E. coli* O157:H7 present in effluent water months after initial inoculation (Liu et al. 2009). Not only could *E. coli* O157:H7 be surviving within the biofilm matrix already established, it could be incorporated into the plants, such as is shown in vegetable production (Deering et al. 2012, Dinu et al. 2011, Fu et al. 2005, Jabsalone et al. 2005, Patel et al. 2010). *E. coli* O157:H7 can invade plant tissues of leafy greens and stalks, as confirmed by microscopy and direct molecular methods of DNA extraction and quantification (Deering et al. 2012). Although this is not the health concern in TW as it is with edible plants, the survival of *E. coli* O157:H7 within vascular openings of plants could provide means of survival and subsequent release after a change to more favorable conditions.

The goal of this paper is to quantitatively analyze the importance of existing biofilm and living plant tissue on the survival of *E. coli* O157:H7 in a wetland system. Two plant species, *Carex utriculata* and *Schoenoplectus acutus*, were chosen for testing in hydroponic reactors to determine interactions with the roots without the added factor of supporting media. These two species represent two of the top performing obligate wetland species found in previous research for removal efficiency of carbon, nitrogen and sulfate (Taylor et al. 2011). The *C. utriculata* root system is densely rhizomatous whereas *S. acutus* has a single thick rhizome that grows horizontally below the surface and root hairs that descend from it, so two unique root variations are represented (USDA.gov). Inert Teflon nylon material of the average size of plant roots was used to create an abiotic environment and compared to the living plant roots that provide protection and a nutrient source. The Teflon was used as a sterile control for

surface attachment alone as well as for an inert substratum for prior biofilm formation to evaluate competition against *E. coli* O157:H7 attachment. Through microscopy and molecular techniques, the relationship between plant root surface, biofilm of indigenous organisms, and *E. coli* O157:H7 has been explored to elucidate survival within TW.

Materials and Methods

Growth of *E. coli* O157:H7

The strain of *E. coli* O157:H7 used in these experiments was isolated from an outbreak in 1993 (Pyle *et al.*, 1995). A DsRed plasmid with a carbenicillin resistance selective gene (Clonotech) was incorporated into the strain (Klayman *et al.* 2009) to allow constitutive expression for fluorescent microscopy detection. Due to the sensitivity of the plasmid expression, growth of *E. coli* O157:H7 was in media lacking peptone from casein. Tryptic Soy Broth (TSB) media was handmade using non-casein enzymatic digest of protein (24 g/L), NaCl (5 g/L), and K₂HPO₄ (2.5 g/L). 250 mg/L carbenicillin was added prior to inoculation for plasmid retention.

E. coli O157:H7 survival and plasmid stability in simulated wastewater was tested by growing *E. coli* O157:H7-DsRed and inoculating into sterilized simulated wastewater to track over the course of two weeks using plate counts, qPCR, and epifluorescent microscopy (VanKempen-Fryling *et al.* 2015).

Plant Growth and Strain Collection

Two plant strains were used for the reactor series experiments. Mesocosms containing monocultures of *Carex utriculata* and *Schoenoplectus acutus* have been

established for over ten years in the greenhouse facilities at Montana State University. Shoots with substantial root mass were harvested and washed with tap water to remove excess dirt and sloughed biofilm cells. The cleaned plants were transferred to 300 mL chemostat reactors open to the air and allowed to grow hydroponically for 3-7 days in a simulated wastewater medium (Supplementary Table S.3.1). The reactors had a constant flow (3mL/min), which continued during the series experiments. The amount of plant material placed in each reactor was $49.3\text{mL} \pm 26.4\text{mL}$, as determined by the volume displacement in water. Calculated hydraulic resistance time was 100 minutes.

The abiotic treatments were used to compare the effect of living roots system to an inert surface of similar dimensions. Teflon nylon strings (D'Addario; GHS strings), which ranged in diameter from 0.79-1.01 mm, were used to simulate roots. Two experimental systems were designed: clean nylon and nylon with established biofilm. These treatments were performed to find the distinction between surface attachment and biofilm competition alone. For the clean nylon series, Teflon nylon strings were added to chemostats with simulated wastewater flow immediately prior to *E. coli* O157:H7 inoculation. For the biofilm series, the strings were added to effluent of the established plant mesocosms and allowed to sit in batch for 24 hours, followed by flow for 1 week prior to *E. coli* O157:H7 inoculation. Total mass of the nylon strings, cut into 17.5cm lengths, was 2.94 g.

Determination of Surface Area

In order to estimate the amount of *E. coli* O157:H7 per surface area of the roots, standard curves were generated relating wet mass to root surface area. Representative

roots were excised, imaged, and analyzed with the Rootscan ® program which analyzed the approximate surface area for each root. The root was then weighed in water and the mass graphed against surface area to generate the curve. Wet mass was used as opposed to dry mass because the root excisions from the reactor series were taken wet, washed, and used for DNA extraction. The standard curves for *S. acutus* and *C. utriculata* are shown in Supplementary Figure S.3.1. *C. utriculata* had two distinct root types that formed unique standard curves for the root to mass ratio. For this reason the DNA extraction from roots were separated by root type for *C. utriculata* samples.

Due to the manufactured nature of the Teflon nylon strings, surface area was estimated by considering the strings as cylinders. The strings were cut in 17.5cm lengths and rested halfway into the wastewater to allow undisturbed retrieval of the biofilm on the lower half. Three diameters were chosen to simulate root variation: 0.70 mm, 0.81 mm, and 1.01mm. Given the equation for the surface area of a cylinder excluding one capped end to estimate only the half submerged,

$$A = 2\pi rh + \pi r^2$$

the total surface area of the 24 sample strings, 8 of each diameter, comes to a total surface area of 53.32 cm².

Reactor Series

E. coli O157:H7 was grown overnight in custom TSB + 250 mg/L carbenicillin. 20mL of overnight culture was spun down at 4816 x g in triplicate, washed in nanopure water, and concentrated into 1 mL nanopure water. For each experimental condition (*C. utriculata*, *S. acutus* and nylon, with and without established biofilm), after

the plant or nylon string biofilm established for one week, the 1 mL of concentrated *E. coli* O157:H7 was added to the open reactor system in three replicates and mixed via pipetting and rotating the plant or nylon strings. One additional reactor per treatment was not inoculated as a control for a total of four reactors per treatment. The experiments were run in 300 mL chemostat reactors with a flow of 3 mL/min for one week for 100 minute hydraulic retention times (HRT). Each experiment was repeated three times. An initial water sample, considered time point zero, was taken from each reactor immediately after inoculation and analyzed for *E. coli* O157:H7 concentration. Effluent samples were collected from the three inoculated reactors on 45-minute intervals over the first six hours. One replicate was destructively sampled after three intervals at 135 minutes to show initial attachment after approximately one residence time in the reactor. The uninoculated control was sampled at 0 and 6 hours. All water samples were immediately frozen for further analysis. Roots were excised in half lengthwise for differentiation of root tip and basal roots, collected into 2mL microcentrifuge tubes and frozen for subsequent DNA extraction. The nylon samples were taken in entirety and collected into 50 mL falcon tubes, then frozen for DNA extraction. A representative root or nylon sample was pulled through a 2 mm square glass capillary approximately 90-110 mm in length (Wale Apparatus, PA) using a wire hook (Aircraft Spruce), the interior rinsed with water to slough off unattached cells, and observed using epifluorescent microscopy to view initial attachment. Another water sample was taken at 24 hours and a second replicate was extracted and destructively sampled as previously described. The third replicate and uninoculated control were destructively sampled at one week.

Epifluorescent Microscopy

The root excisions and nylon strings within the glass capillary were rinsed with water and viewed under 600X magnification with water optical focus. The samples were viewed under 558 nm wavelength absorption for the DsRed plasmid excitation. Images were taken along the entire length of the root, focusing on the root tip, mid-root, and top root portions. The photosystem II chlorophyll enzymes within the root naturally fluoresce allowing the surface to be viewed under a contrasting condition to the *E. coli* O157:H7 DsRed plasmid. The contrasting condition was at 400 nm wavelength. The images were semi-quantitatively analyzed using MetaMorph® Image Analysis Software to determine number of cells and colony size using average *E. coli* O157:H7 single cell fluorescence taken from 30 single cell images and averaging the pixel area.

DNA Extraction and qPCR Analysis

Water samples collected at the previously stated time points were thawed and spun down at 4816 x g for 20 minutes at 4°C. The supernatant was discarded and the pelleted sample was resuspended in the remaining liquid and transferred to a PowerBead Tube from the PowerSoil® DNA Isolation Kit (MoBio Laboratories). The basal roots and root tips for all root types were collected and also transferred to a PowerBead tube. The mass was taken to calculate specific surface area. DNA extraction followed protocol except in place of the MO BIO Vortex Adapter, a Fastprep®-24 bead beater (M.P. Biomedicals) was used at 5.5 m/sec for 45 seconds. DNA was isolated in 100 µL of DEPC water (Ambion®) and quantified using a Nanodrop® ND-1000 spectrophotometer (Nanodrop). Average quantification and purity per sample type is listed in Table 3.1.

Table 3.1. Quantity and Purity of DNA Extraction Samples

Sample Type	Concentration (ng/uL)	Absorbance ^{260/230}
<i>C. utriculata</i> water	80.08 ± 40.56	2.01 ± 0.21
<i>C. utriculata</i> root	96.38 ± 30.02	2.02 ± 0.15
<i>S. acutus</i> water	57.34 ± 51.16	1.49 ± 0.61
<i>S. acutus</i> root	72.09 ± 37.17	1.88 ± 0.25
Nylon clean water	59.81 ± 29.91	2.04 ± 0.21
Nylon clean surface	48.37 ± 36.63	1.94 ± 0.70
Nylon biofilm water	72.22 ± 45.80	1.95 ± 0.48
Nylon biofilm surface	40.03 ± 24.86	1.99 ± 0.34

Cell material on the nylon strings was removed by adding glass beads to the 5mL mark of the 40mL conical falcon tube with 10mL autoclaved nanopure H₂O, and vortexing for 60 seconds. The liquid fraction was removed and centrifuged at 4816 x g for 5 minutes. The supernatant was removed and the pellet resuspended and added to a PowerBead Tube for extraction as previously described.

qPCR was used to determine the amount of *E. coli* O157:H7 in isolated water and surface samples. Primers were used targeting the Shiga toxin gene unique to *E. coli* O157:H7 serotype (Table 3.2). Cells generally have one copy of *Stx1* and *Stx2* genes (Shaikh and Tarr, 2003). These primers target *Stx2*, and the assumption is made that one copy equates one cell, however as this specific outbreak strain has not been fully sequenced to confirm, units are left as gene copies. A 25 µL reaction mixture was prepared using SYBR Green Master Mix (Kapa biosystems), 0.5mM primer concentrations and 5 µL DNA diluted to 1 ng/uL from the extraction concentration. The Rotor-Gene Q PCR machine (Qiagen) was used with the following program: Hold 15 minutes at 95 degrees Celsius, hold 15 minutes at 50 degrees Celsius, cycle 38 times with

Table 3.2: Primers for detection of *E. coli* O157:H7

Target	Sequence (5'→ 3')	Position	Source
<i>stx2</i> ^a			Jothikumar
JMS2F	CGACC CCTCT TGAAC ATA	6261–6278	and Griffiths,
JMS2R	GATAG ACATC AAGCC CTCGT	6368–6349	2002

^aproduct size, 108 bp

30 second melt at 92 degrees Celsius, 30 second annealing at 56 degrees Celsius and 30 second extension at 72 degrees Celsius, then perform a melt curve analysis.

qPCR interference was a significant obstacle to overcome in the environmental samples and multiple approaches were taken to optimize the signal to noise ratio with the non *E. coli* O157:H7 sample controls. Non-specific binding was observed in plant and biofilm qPCR as observed through a multiple peak melting curve. Subsequent DNA clean-up kits were tested, specifically PowerClean® Pro DNA clean-up kit (MoBio) and Zymo *OneStep*TM PCR inhibitor removal kit (Zymo Research). Neither kits gave significant change in control reactivity and were not used in sample prep. Sample dilution gave the best results. The positive samples showed accurate dilution while the random interference decreased further in diluted samples (data not shown). Subsequent qPCR copy number was taken as a difference from positive to control.

Data Analysis

After data collection was completed, multifactor analysis of variance (ANOVA) was performed to determine statistical significance between experiments. The data were

analyzed to determine the difference between plants and Teflon nylon strings, location along the root, established biofilm vs. clean nylon, and time. General linear models (GLM) were used to compare interactions using Minitab 17®. Statistical significance between specific factors for each variable were identified by pair-wise Tukey's *post hoc* multiple comparison test using a 0.05 p-value cut-off.

Results

Water Sampling

E. coli O157:H7 water samples were collected to estimate the initial wash-out of *E. coli* O157:H7 from the bulk phase of the chemostat within four HRT (Figure 3.1). For all four experimental surfaces, the wash-out curves showed a slight increase in cells before declining as near total removal is achieved, which was expected with respect to what has been previously shown for chemostat behavior (Butler et al. 1985). The HRT was sufficiently short to prevent planktonic growth. The initial inoculum concentration as well as the initial concentration in each reactor indicates that there are cells remaining within the reactor after four complete washout cycles and that previously attached cells may potentially be released. A twenty-four hour time point was taken on a number of experiments to verify continued washout of *E. coli* O157:H7 from the inoculated chemostats due to release from the root or nylon surface. On average, plants roots gave 2.4×10^3 gene copies/mL and nylon samples 3.83×10^4 gene copies/mL after 24 hours in the effluent.

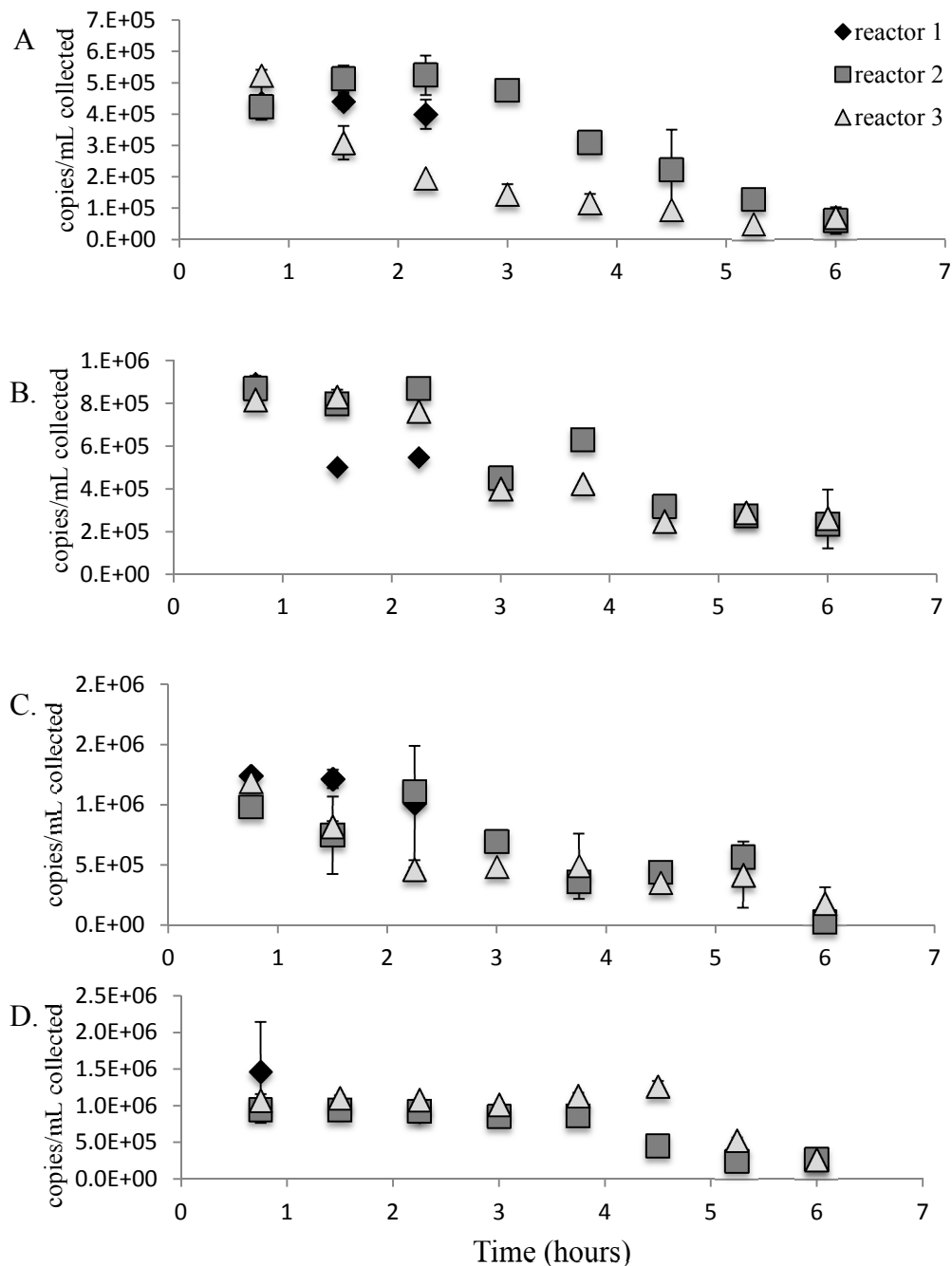


Figure 3.1. Washout curves for (A) *C. utriculata*, (B) *S. acutus*, (C) clean nylon, and (D) biofilm nylon. Effluent was collected over the duration from the previous time point or initial inoculation. Cells were collected through centrifugation, DNA extracted, and analyzed via qPCR, then normalized to mL water collected. Error bars represent technical error. Reactors 1, 2, and 3 are the 2 hours, 24 hours, and 1 week destructive sampling replicates.

Microscopy Cell Attachment and Clumping Formation

Since the focal point of the root or nylon surface through the capillary tube varied, only partial surface images were taken rather than a comprehensive sweep. Twenty images were taken in locations where *E. coli* O157:H7 was visible to determine the density of cells. Representative images are shown in Figure 3.2. Observations were done at 2, 24 and 168 hours to determine persistence. Due to the destructive sampling of each surface for each time point, the images are representative of an average attachment and are not the same surface imaged repeatedly over the course of the experiment. *E. coli* O157:H7 was present on the surface at every time point taken. Initial attachment was seen on all surfaces (Figure 3.2) as single cells and there was no observable difference in density according to location or surface type. At 24 hours, prolonged attachment was still observed on every surface type, and microcolony formation was seen. There was a distinct change in pattern from initial single cell attachment to large clusters with three-dimensional structure, which was determined visually through focused z-layers (data not shown). The final time point at 168 hours showed sustained *E. coli* O157:H7 cell attachment, however the appearance of microcolony formation was more variable than seen at 24 hours.

These observations were substantiated when total cell clumping was analyzed with the Metamorph® Image Analysis software. Total counts for each area of fluorescence were taken and average cell number extracted by division according to average single *E. coli* O157:H7 cell size as described previously (Behnke et al. 2011, Wilson et al. 2004). The means with variance are plotted in Figure 3.3 as a percent of the

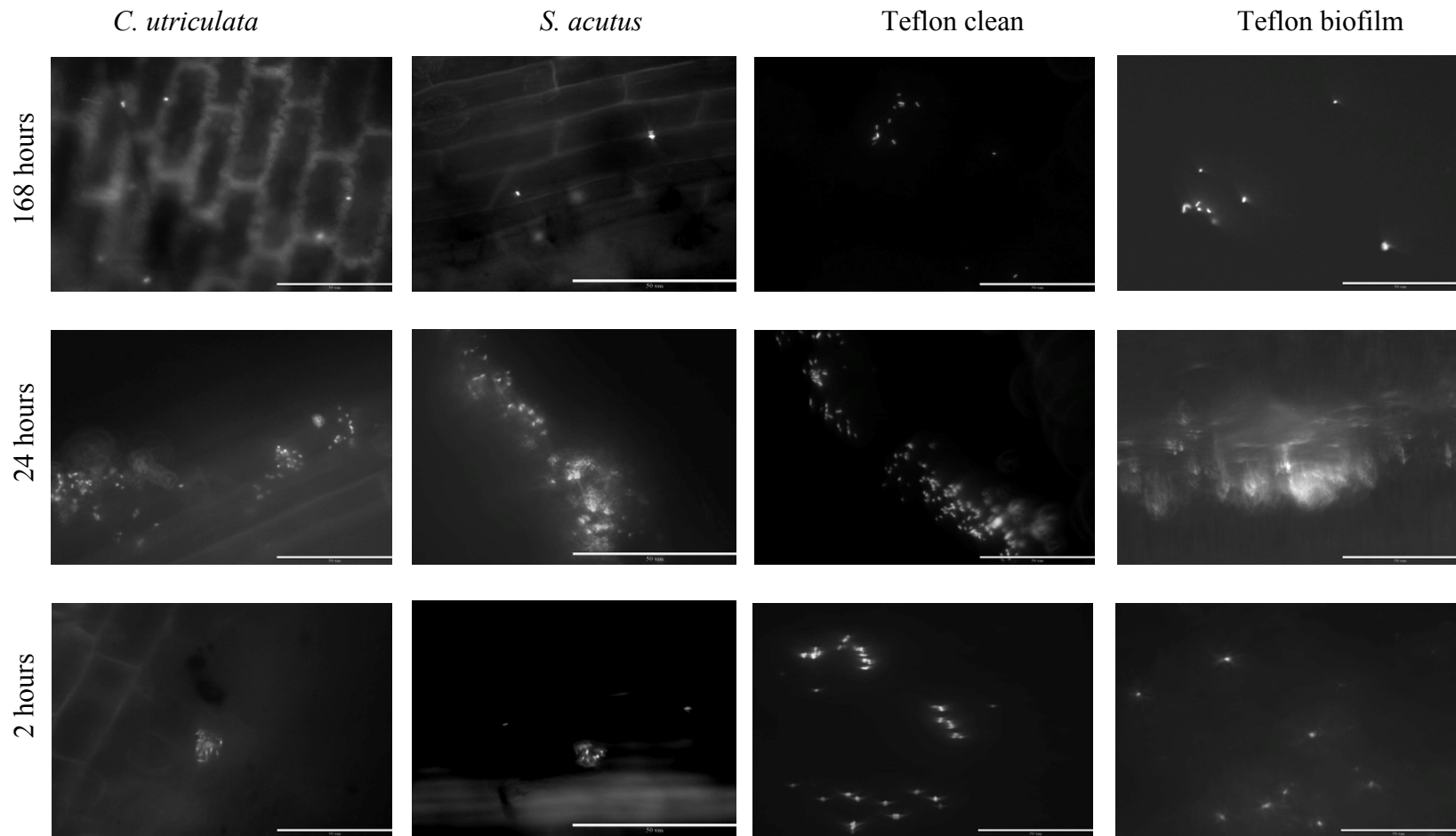


Figure 3.2: Representative images of the four experimental surface types taken at 2, 24 and 168 hours. Fluorescence was observed under 558nm wavelength. Due to natural fluorescence of photosystem II chlorophyll in the plant tissue, plant surface is shown in *C. utriculata* and *S. acutus* images. No stains or dyes were used in Teflon nylon imaging under the assumption stationary cells were attached to a surface. Scale bar represents 50 μ m

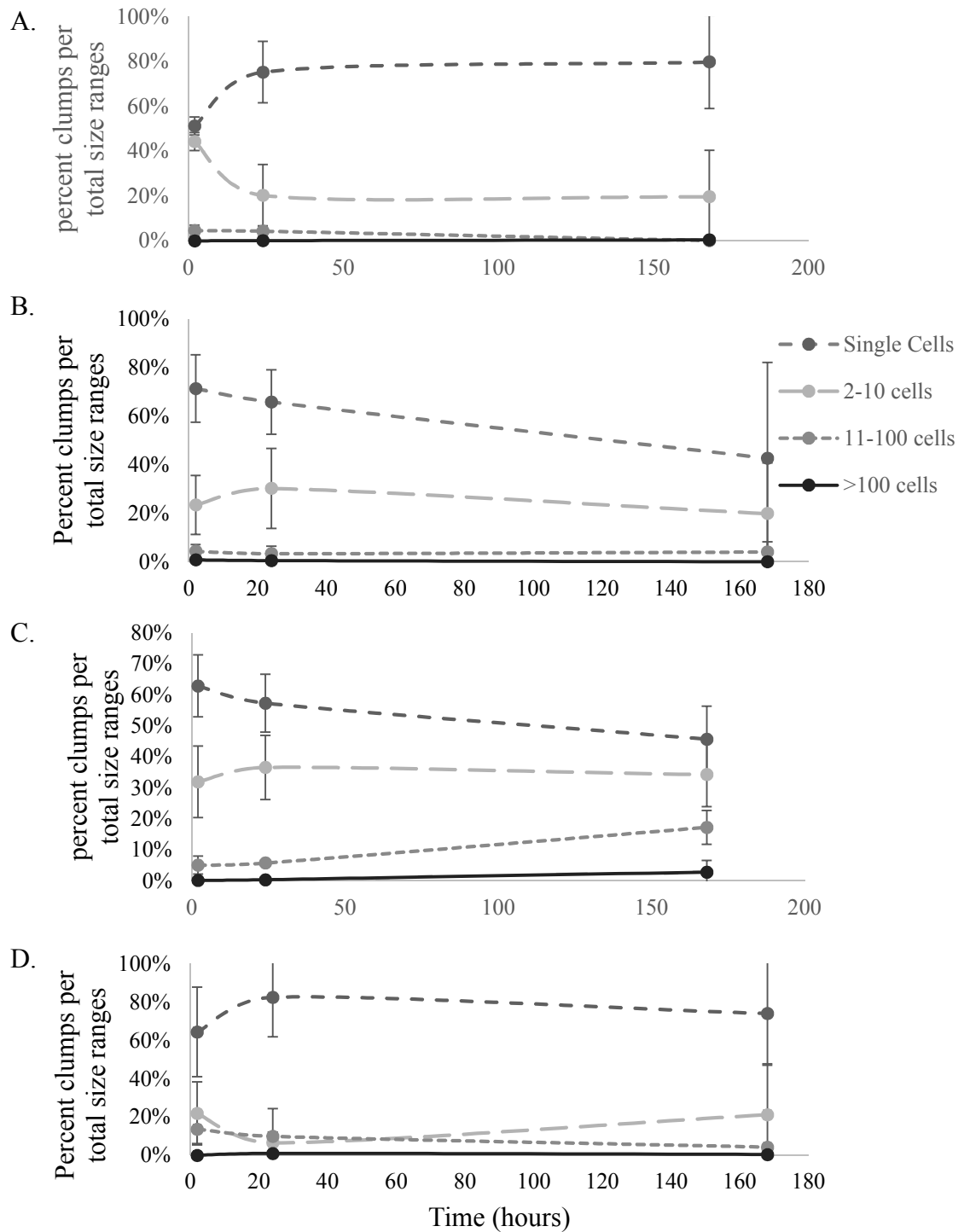


Figure 3.3. Distribution of cell clumping over total counts per time point for each surface: (A) *C. utriculata*, (B) *S. acutus*, (C) clean Teflon nylon, and (D) biofilm Teflon nylon. Root locations were combined for a total mean. Bars represent standard deviation from all experimental replicates.

total events counted for all size ranges. Overall, total clumping increased within the first 24 hours for all larger clumps sizes on each surface. The visualization of clumps above single cells in size is seen to increase, and this is more dramatic in *S. acutus* (23 to 36 events) and clean Teflon nylon experiments (96 to 109 events). The number and variance of single cells was much higher in all experiments as seen through the large standard deviations. This is understandable as single cell attachment is more transient than a formation of larger groups. The total clump counts did decrease from 2 to 168 hours (22.6 average events to 13.3 average events), however with the increase of much larger microcolony formations (average increase of 1 event for >100 cell clump size), an accurate total cell number cannot be identified.

qPCR

The DNA extracts were analyzed via qPCR for relative abundance compared to the non-inoculated controls over time. ANOVA linear fit modeling was performed to verify statistical difference between surface types. For the biotic experiments, location and condition of the root were analyzed, and in the abiotic experiments, the established biofilm difference was compared.

The relative abundance for each experiment over time is shown in Figure 3.4. For all experimental surfaces, *E. coli* O157:H7 population decreased over time, averaging a 2 log reduction from 2.84×10^{12} gene copies/cm² at 2 hours to 8.95×10^{10} gene copies/cm² at 168 hours. The change was significant from the total initially attached cells to the endpoint of one week ($P \leq 0.002$). Between the surfaces, *S. acutus* behaved significantly different from both nylon replicates and *C. utriculata* roots ($P \leq 0.032$). Attachment was

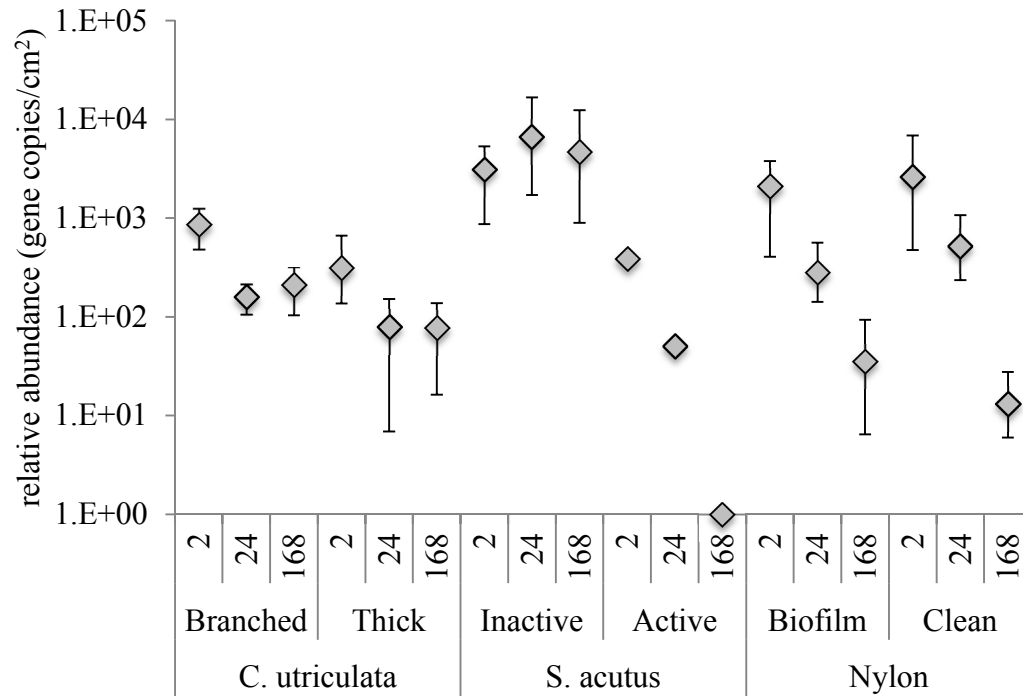


Figure 3.4. Relative abundance of *E. coli* O157:H7 per surface area normalized to background fluorescence. The data is differentiated over time and by root type or nylon condition for each respective surface.

much higher for the *S. acutus* (average 1.68×10^{12} gene copies/cm²) experimental replicates, and continued to be higher over all time points. Nylon replicates and *C. utriculata* were not significantly different ($P \leq 1.00$), indicating lower *E. coli* O157:H7 attachment on those surfaces.

When looking specifically at the interactions of the two root types, some observable differences were shown. Localization was taken into account via division of the roots between the root tips and the basal roots near the stem. There was no significant interaction between *E. coli* O157:H7 to either position along the root. Differences were also determined within root type, specifically *C. utriculata* root variation from branched

lateral root structures to uniform fibrous taproots. *E. coli* O157:H7 showed observable preference toward branched roots over the taproot in this regard, binding with an average $\frac{1}{2}$ log increase from 1.64×10^{11} gene copies/cm² to 5.82×10^{11} gene copies/cm² ($P \leq 0.0005$). In *S. acutus*, living roots could be easily identified and separated from dead roots by color, active roots being red and inactive roots were black. The two types were extracted separately to test advantage of attachment to an active root, but no meaningful differences were observed.

The two abiotic surface experiments were also compared independently from the roots to identify any variation with respect to preexisting biofilm. There was no significant difference in *E. coli* O157:H7 attachment to the nylon string surfaces with or without an established biofilm. A slight interaction between surface and time was shown at the initial attachment to 24 hours in clean nylon over biofilm-colonized nylon (Supplementary Figure S.3.2).

Discussion

Using both epifluorescent microscopy and qPCR, we were able to verify that *E. coli* O157:H7 had high initial attachment to all surface types and was sustained on the surfaces during high flow conditions for a total of one week. Attachment was significantly lower on abiotic nylon controls than the living root tissue on *S. acutus* and *C. utriculata*, which suggests that there is an attachment advantage for *E. coli* O157:H7 on the root tissue. This higher binding supports the hypothesis that there is a beneficial niche for survival or potential growth for bacterial pathogens within an established root

biofilm (Tyler and Triplett 2008). *E. coli* O157:H7 attachment also shows evidence of being favored in root types where exudates, water and oxygen release may be causing a sustainable microniche environment, even within non-ideal conditions for the pathogen. Many plants have been found to foster biofilm growth along the root structures for a variety of reasons (Gruyer et al. 2013) including protection against plant pathogens to nitrogen fixation for uptake. Given the previous research showing benefits for both plants and bacteria growing in symbiosis, it is not surprising that *E. coli* O157:H7 may also find beneficial attachment and persistence with prolonged association on the root tissue. *E. coli* O157:H7 on roots has been seen with vegetables in agricultural settings (Deering et al. 2012). The lower attachment to *C. utriculata* root types provides evidence for a lower tolerance of biofilm invasion on that root surface, making *C. utriculata* the better candidate in TW for pathogen removal.

It was interesting to discover no statistical difference between a clean nylon surface and established biofilm. It was hypothesized the competition with the natural consortia of bacteria in an environment would be good protection against invading cells such as the inoculum of *E. coli* O157:H7. It was assumed that the indigenous biofilm would be less likely to be colonized than the clean surface. However, attachment was seen both microscopically and through molecular techniques. It is unknown how the attachment persists, given prior evidence that *E. coli* O157:H7 does not effectively utilize the nutrients in the simulated wastewater media for growth (VanKempen-Fryling et al. 2015). Although other carbon sources or symbiosis may occur within the established biofilm, that does not explain the prolonged survival on the clean surface, unless

incoming bacteria in the influent play a role. More research is needed to understand this phenomenon. Attachment did vary much more drastically on nylon than on living surfaces. This may be due to the inert characteristic of the surface giving no lasting benefit to prolonged attachment as there is with plant exudates on root surfaces. *E. coli* O157:H7 cells therefore may have sloughed off or died on the nylon surface given the higher range from 2 hours to 168 hours.

The fate of the *E. coli* O157:H7 cells, whether sloughed off into the effluent at a later time point and still viable or inactivated by predation or competition, has yet to be determined. Other studies have indicated these factors play a large role in lowering human pathogen retention in the environment (Boutillier et al. 2009, Jasper et al. 2013), however for strains with a low infection rate such as *E. coli* O157:H7, these may not be enough to completely eradicate subsequent release and infection. If *E. coli* O157:H7 is able to attach to a root for nutrients and protection, it may have prolonged ability of survival in a TW. Further research is needed for optimizing pathogen removal if TW are to be implemented in a broader area for wastewater treatment.

Conclusion

As determined by microscopy and qPCR, *E. coli* O157:H7 attached and persisted on root and nylon surfaces for at least one week. The formation of microcolonies after twenty-four hours was seen and indicated through an increase in clumping of multiple cells over time.

Although no surface was shown to have significantly greater growth or retention microscopically, there was a significant difference in relative abundance via qPCR between the nylon and the two plant species for attachment over time, showing higher attachment on inert surfaces than living tissue. The plant roots showed preferential binding and retention on branched roots where exudates and root elongation occur. No measurable difference occurred between nylon with clean and established biofilm indicating that competition against an existing biofilm did not significantly hinder *E. coli* O157:H7 attachment to the surface.

Acknowledgements

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Supplementary Figures and Tables

Table S.3.1. Properties of the growth media

Nutrient	Chemical Compound	Concentration (g/L)
Organic carbon	$C_{12}H_{22}O_{11}$	0.0997
Iron	$FeCl_3$	0.0004
Magnesium	$MgSO_3 \cdot 7 H_2O$	0.062
Ammonia	NH_4Cl	0.0191
Copper	$CuSO_4$	0.0008
Manganese	$MnSO_4$	0.0078
Zinc	$ZnSO_4$	0.0078
Calcium	$CaCl_2$	0.0019
Phosphorus	K_2HPO_4	0.044
Nitrate	$NaNO_3$	0.1214
Boron	H_3BO_3	0.01
Potassium	KI	0.0019
Molybdenum	Na_2MoO_4	0.004
Primatone	complex	0.111

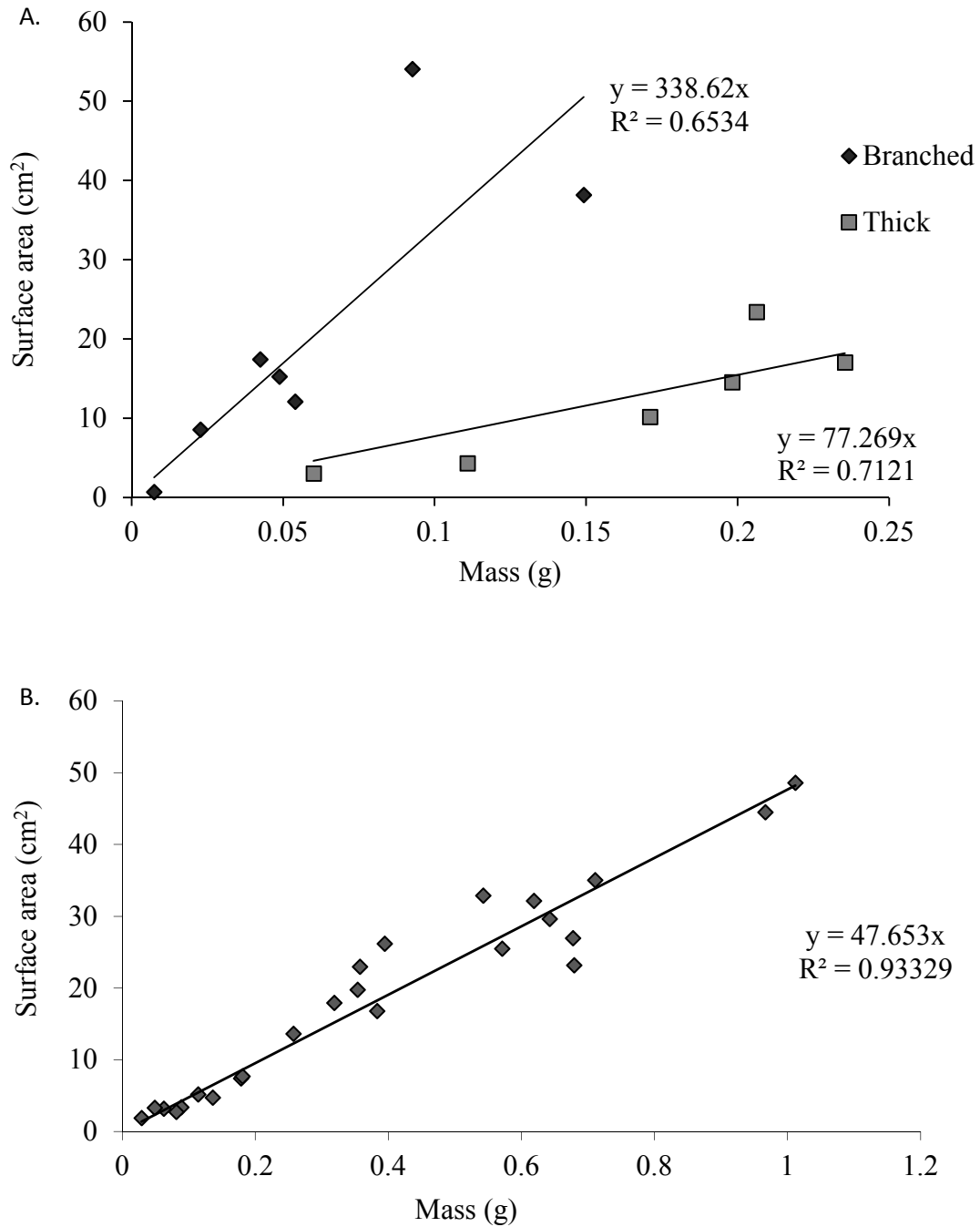


Figure S.3.1. Mass to surface area ratios for (A) *C. utriculata* and (B) *S. acutus* roots. *C. utriculata* is separated by significant difference in root structure between branched lateral roots and thick tap roots.

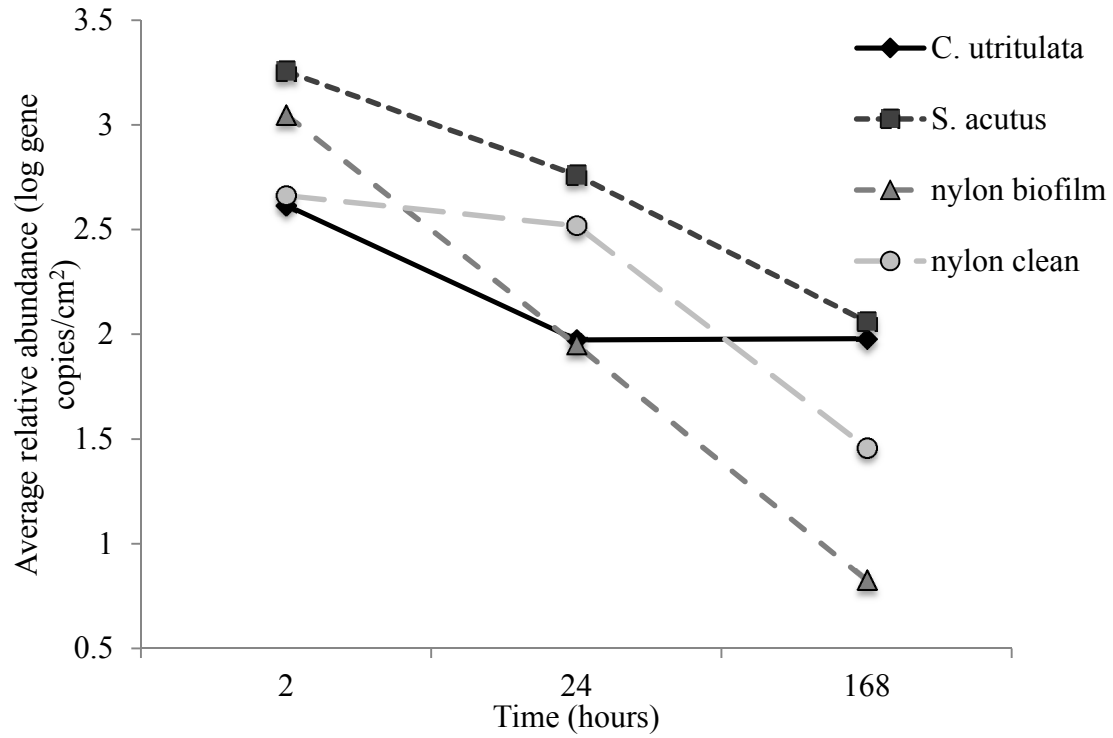


Figure S.3.2. Plot of the means for relative abundance of gene copies per cm² surface area for each surface over time. Nonparallel slopes between data points signify interactive difference between samples.

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CHAPTER FOUR

USING MOLECULAR AND MICROSCOPIC TECHNIQUES TO TRACK THE
WASTEWATER PATHOGEN *ESCHERICHIA COLI* O157:H7
WITHIN MODEL TREATMENT WETLANDS

Contribution of Authors and Co-Authors

Manuscript in Chapter 4

Author: Rachel J. VanKempen-Fryling

Contributions: Experimental design, laboratory experiments, data analysis, and manuscript editing

Co-Author: Anne K. Camper

Contributions: PI advisor on the project, experimental design, and manuscript editing.

Manuscript Information Page

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Abstract

Treatment wetlands (TW) are a promising alternative technique for secondary domestic wastewater treatment and control for nonpoint sources such as agricultural and cattle farms. They provide a safe and effective option that utilizes inexpensive natural processes to reach removal rates similar to standard wastewater treatment plant systems. While TW are advantageous in many ways, it is important to understand their ability to mitigate health risks such as waterborne pathogen contamination. This work focused on tracking a pathogenic strain of *Escherichia coli* O157:H7 transformed with a DsRed fluorescent protein plasmid through model scale batch TW columns. Two plant species, *Carex utriculata* and *Schoenoplectus acutus* were used as model plant species along with an unplanted gravel control in batch fed systems. *E. coli* O157:H7-DsRed abundance was measured over time using epifluorescence microscopy and DNA qPCR on the roots and the gravel partitions of the column. Microscopic visualization of *E. coli* O157:H7 persisted over a three-week time-course with significant microcolony clumping on the roots 1 week post-inoculation before eventual decrease in cell counts. Gravel showed higher relative abundance, which could be indicative of higher competition at the nutrient-rich rhizosphere. This work provided evidence for *E. coli* O157:H7 persistence in TW and showed need for further research into optimal designing for removal.

Introduction

Wetlands are nature's filter. They play this role in natural environments at the deltas of rivers and along edges of riverbanks (Jasper et al. 2013, Kurzbaum et al. 2012). Great engineering builds upon nature, and this natural process was utilized and optimized for wastewater treatment. Treatment wetlands (TW) have been developed and used as a more environmentally and economically favorable alternative treatment option since the 1960s (Brown et al. 2000). Research has been performed to optimize and further understand the effectiveness of TW ever since.

In brief, TW are composed of an impermeable base layer to prevent release of the wastewater into the environment and filled with media ranging from fine soil particulates to gravel of variable or similar grade size and obligate or facultative wetland plant species. There has been extensive research into the role of monoculture and mixed plant species within the wetland. It has been found that species can have a profound effect on the uptake of nutrients from the wastewater, rate of removal, and final concentrations of commonly tracked pollutants such as nitrogen, sulfates, phosphorus, and bacterial pathogens (Ballantine and Tanner 2010, Hensch et al. 2003, Scholz et al. 2010, Taylor et al. 2011).

One aspect of TW that has only recently been investigated is the role and diversity of the microbial population associated with the subsurface matrix of the wetland including plant root surfaces. Depending on the design of the TW, there is substantial surface area in contact with the wastewater within the gravel media and the rhizosphere. The rhizosphere, a term first coined in 1978, is defined as the surrounding soil influenced

by and associated with plant roots and plant-produced material (Bhattacharyya and Jha 2012, Bringhurst et al. 2001). Although an exact distance from the roots is not specified, there is a clear difference in the environment near the roots. Plant roots release exudates including but not limited to amino acids, sugars and small signal molecules into the environment (Bhattacharyya and Jha 2012). Up to 25% of photosynthesis products are allocated to root exudates to increase soil richness (Santos et al. 2014). This provides an essential nutrient source in the nutrient-starved environment of the subsurface soil (Berendsen et al. 2012). Soil within the rhizosphere is one of the most biologically diverse environments in the world, with upwards of 10^{11} cells/gram root and 30,000 unique species, compared to 4000 species in soil unassociated with a root system (Berendsen et al. 2012, Bhattacharyya and Jha 2012, Saharan and Nehra 2011). This rhizosphere associated microbial population has been compared to a secondary genome and innate immune system, much like the human gut microbiome (Berendsen et al. 2012).

As more research is performed on the diverse microbial population associated with roots, more evidence for beneficial symbiosis is continually found. This includes the emergence of a new group of bacteria known as plant growth promoting rhizobacteria (PGPR), defined as a biota of indispensable organisms that stimulate the growth of the host plant (Bhattacharyya and Jha 2012). Plants stimulating these microorganisms on the roots establishes significant increased growth and defense against plant pathogens, both microbial and insect (Berendsen et al. 2012, Bhattacharyya and Jha 2012, Loh et al. 2002, Mayak et al. 2004, Santos et al. 2014). Aggressive colonization by PGPR prevents

attachment or biofilm formation from other species; siderophore production collects Fe from the environment, localizing it to plant roots and limiting other bacterial scavenging; the release of antibiotics offers biocontrol of invading species; and nitrogen fixing bacteria aid uptake of nitrogen into the plant from the roots (Bhattacharyya and Jha 2012, Vymazal 2011).

There is also increasing evidence that plants influence their own rhizosphere population. Microbial populations have specific intercellular signalling processes known as quorum sensing. These small molecules are released in conjunction with increasing cell density for a variety of reasons from gene expression changes to biofilm formation or virulence and antibiotic defense (Miller and Bassler 2001). Plants can mimic these signalling molecules, thus adding to the threshold for signal transduction (Loh et al. 2002) and causing much quicker beneficial changes to the microbial population. They also influence the actual population makeup in the rhizosphere. The communal species population has been shown to change drastically with environmental changes (Faulwetter et al. 2009) even within the same plant species (Berendsen et al. 2012).

PGPR are an important aspect of wastewater treatment in TW as they provide initial defense to the plants against invading microbial cells present in the influent water. There are many bacterial species associated with wastewater, but those of concern and therefore interest in removal are human pathogens such as *Escherichia coli* O157:H7. *E. coli* O157:H7 enters water through fecal contamination due to improper treatment or nonpoint sources (Olsen et al. 2002). It is an enterohemorrhagic strain that causes gastrointestinal disease which in 20% of cases could lead to hemolytic uremic syndrome

resulting in kidney failure (Huffman et al. 2003, Mead and Griffin 1998, Olsen et al. 2002). It is one of the main causes of foodborne disease in the United States with numerous outbreaks and food product recalls every year (Soller et al. 2009).

One of the main reservoirs for *E. coli* O157:H7 is cattle. Contaminated sources include unpasteurized dairy, undercooked meat, and colonized vegetables from the use of contaminated manure (Griffin and Tauxe 1991). The cattle reservoir can also effect water quality as dairy run-off contains a large number of fecal indicator bacteria and pathogenic *E. coli* O157:H7 (Hancock et al. 1998, McGarvey et al. 2005) that enter streams and rivers at nonpoint sources. Using TW along dairy farms has the potential to control the water contamination from these sources (Ibekwe et al. 2002).

The removal of fecal indicator bacteria such as *E. coli* strains from wastewater is commonly documented using plate counts from influent to effluent, but this poses severe limitations. Upon entering a low nutrient environmental system, *E. coli* O157:H7 is known to enter a viable but non culturable (VBNC) state in which plate counts do not detect cells despite molecular techniques verifying that the cells are still viable and active (Barcina and Arana 2009, Dinu and Bach 2011, Liu et al. 2009, Oliver 2010, Sleytr et al. 2007). This potentially misrepresents *E. coli* O157:H7's prolonged survival and release beyond a single measured residence time, and it has been documented to resuscitate from the effluent months post inoculation (Paruch 2011, Paruch 2015).

This research aims to gain better understanding of the rhizobacterial effect on TW's ability to remove human pathogens from wastewater. The objectives are two-fold:

- (1) Determine through microscopy and molecular techniques if *E. coli* O157:H7 survives

in the rhizosphere and gravel matrix of a laboratory scale TW system and (2) identify any localization for increased survival between the rhizosphere or the gravel matrix.

Materials and Methods

Formation of TW Laboratory Scale Columns

Two plant species were used in monoculture batch columns for scale models of TW. Western sedge *Carex utriculata* and common bulrush *Schoenoplectus acutus* were found in a previous study to be top performers for common pollutant removal including chemical oxygen demand (COD), nitrogen species, and sulfate (Taylor et al. 2011). Sections of polyvinyl chloride (PVC) pipe (10 cm diameter) were cut to 30 cm lengths, capped on one end, and an effluent channel placed 3 cm above the base (Figure 4.1). The effluent channels were linked with rubber tubing and collected to secondary containment per biohazard level 2 protocol. Forty-five columns were made using gravel grade No. 4 to No. 8 as the media matrix, giving an average grain diameter of approximately 20 mm. Columns were separated into 3 treatments: 15 planted with *C. utriculata*, 15 with *S. acutus*, and 15 unplanted gravel columns. The experimental design for destructive sampling is given in Table 4.1. The plants were grown within the columns for six months prior to the beginning of the experiment in an environmentally controlled greenhouse that simulated seasonal temperature fluctuation from 20°C to 4°C in 4 degree step increments 2 months in length. Columns were inoculated during the spring and fall 8°C cycle temperature to eliminate seasonal variability.

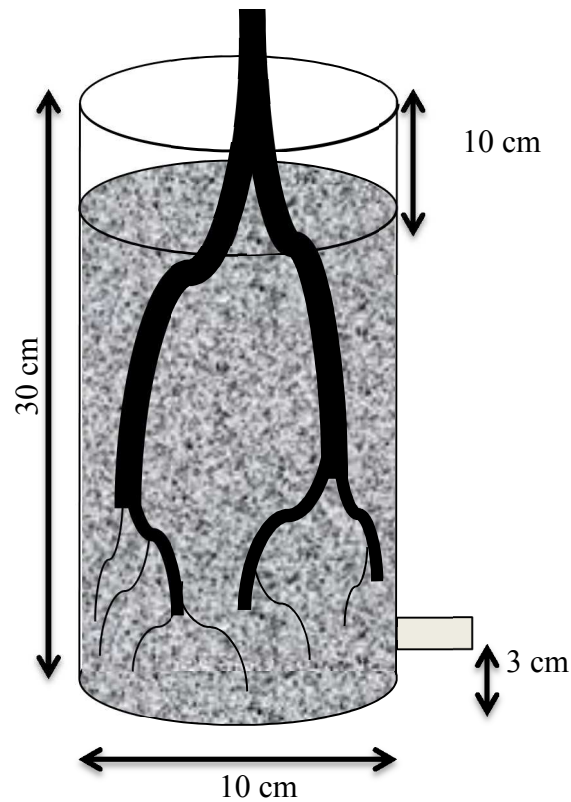


Figure 4.1. Diagram showing column design for vertical batch system. Each column was monoculture of either *C. utriculata*, *S. acutus*, or an unplanted gravel. The gravel media was filled to approximately 10 cm below the top of the column to allow expansion due to root growth.

The columns were fed every three weeks in batch with simulated wastewater described previously (VanKempen-Fryling and Camper 2015b). Columns were run in a batch mode with simulated wastewater media added to the top and effluent leaving the bottom. The wastewater media was not sterilized to allow a natural consortium of bacteria within the inoculum as would be expected in real wastewater. Single uninoculated controls were kept on a separate, isolated effluent channel. Between

subsequent complete water removal and refill on three week intervals, tap water was added as needed to maintain the water level losses from transpiration and evaporation.

Table 4.1. Column replicates for destructive sampling schedule

Time Point	Fall (2013)		Spring (2014)	
	<i>E. coli</i> (+)	Control	<i>E. coli</i> (+)	Control
24 hours	NA	NA	2 <i>C. utriculata</i> 2 <i>S. acutus</i> 2 gravel	2 <i>C. utriculata</i> 2 <i>S. acutus</i> 2 gravel
1 week	2 <i>C. utriculata</i> 2 <i>S. acutus</i> 2 gravel	1 <i>C. utriculata</i> 1 <i>S. acutus</i> 1 gravel	2 <i>C. utriculata</i> 2 <i>S. acutus</i> 2 gravel	NA
2 weeks	NA	NA	2 <i>C. utriculata</i> 2 <i>S. acutus</i> 2 gravel	NA
3 weeks	NA	NA	2 <i>C. utriculata</i> 2 <i>S. acutus</i> 2 gravel	2 <i>C. utriculata</i> 2 <i>S. acutus</i> 2 gravel

NA = Not applicable; no sample was taken for that experimental time point.

Growth of *E. coli* O157:H7

The *E. coli* O157:H7 strain was isolated from a waterborne outbreak in 1993 and transformed with the fluorescent enzyme gene plasmid DsRed to allow constitutive expression under epifluorescent microscopy (Klayman et al. 2009, Pyle et al. 1995, VanKempen-Fryling and Camper 2015). This strain has been used in previous research to show attachment in biofilm conditions when grown in mixed culture (Klayman et al. 2009).

Initial growth of *E. coli* O157:H7-DsRed was in tryptic soy broth (TSB) media custom formulated without casein-derived peptone for maximum fluorescence stability prior to wastewater inoculation (Klayman et al. 2009). The TSB specifications were non-

casein enzymatic digest of protein (24 g/L), NaCl (5 g/L), and K₂HPO₄ (2.5 g/L). 0.25 g/L carbenicillin was added to the initial growth inoculum for plasmid stability and retention. This retention was previously tested and confirmed within wastewater media (VanKempen-Fryling and Camper 2015).

Inoculation into Columns

E. coli O157:H7 was grown planktonically in custom TSB overnight to approximately 10⁸ cells/mL. The liquid volume capacity of each column was measured and an average of 500mL was used to calculate the initial inoculum of simulated wastewater. The overnight culture of *E. coli* O157:H7 was washed in nanopure water and concentrated by centrifugation at 5000 x g for 10 minutes at 5 °C. It was added to the 500mL influent wastewater to give 10⁹ cells/mL for duplicate reactors of each column type. The controls were given 500 mL simulated wastewater without any *E. coli* O157:H7. The 500 mL volumes were added top-down to each column.

Duplicates for each experiment were inoculated. Columns were destructively sampled in duplicate at 1 week in the fall and at 24 hours post treatment addition, and at 1 week, 2 weeks, and 3 weeks in the spring. Uninoculated controls were destructively sampled at the beginning and endpoint of each experimental cycle. Effluent samples were taken from the inoculated column channel weekly to determine bulk water concentration over time.

Destructive Sampling Procedure

Plants were destructively sampled at each time point for molecular analysis of the microbial populations in the rhizosphere. In brief, the column effluent channel was opened and allowed to fully drain into secondary containment. The column was then detached from the effluent tubing and poured into an autoclavable bin, maintaining stratification. Samples were immediately taken with ethanol sterilized forceps of gravel



Figure 4.2. Image of destructive sampling technique. *S. acutus* plant is pictured removed from the batch column in horizontal stratification for sampling of root and gravel while in secondary containment for *E. coli* O157:H7 handling procedure.

and roots in duplicate at the top, middle, and bottom of the column (Figure 4.2). Gravel samples were collected in 50mL conical Falcon tubes and roots were collected in 2mL microcentrifuge tubes. The samples were transferred on ice and frozen. Two root samples per column including controls were also threaded using wire hooks (Aircraft Spruce) into 2 mm capillary glass tubes for microscopy (Wale Apparatus, PA), washed, and viewed immediately following collection. Sampling trays were cleaned and sterilized with bleach between destructive sampling.

Epifluorescent Microscopy

The root excisions within the glass capillary were rinsed with water and viewed under 600X magnification with a water optical focus objective. The samples were viewed under 558 nm wavelength absorption for the DsRed plasmid excitation as previously described (VanKempen-Fryling and Camper 2015). Due to the nature of the capillary tube, only portions of the root could be scanned and viewed, giving representative images along the entire length. Because the photosystem II chlorophyll enzymes within the root naturally fluoresce, the surface of the roots could be viewed under a contrasting condition wavelength at 400 nm, verifying the presence of *E. coli* O157:H7-DsRed. The images were semi-quantitatively analyzed using MetaMorph ® Image Analysis Software to determine number of cells and colony size using an averaged *E. coli* O157:H7 single cell fluorescence taken from 30 single cell images pixel areas.

DNA Extraction and qPCR Analysis

The water samples were thawed and centrifuged at 4816 x g for 20 minutes at 4°C. After the supernatant was discarded, the pelleted sample was resuspended in the remaining liquid and transferred to a PowerBead Tube from the PowerSoil® DNA Isolation Kit (MoBio Laboratories). DNA extraction followed the established protocol except a Fastprep®-24 bead beater (M.P. Biomedicals) was used at 5.5 m/sec for 45 seconds in place of the MO BIO Vortex Adapter. DNA was isolated in 100 µL of DEPC water (Ambion®) and quantified using a Nanodrop® ND-1000 spectrophotometer (Nanodrop). The average values for DNA concentration and purity were recorded (Table 4.2).

Material from the gravel samples was removed through a wash and vortex step. Initially, 10 mL of autoclaved nanopure H₂O was added to the collection tube. The tube was inverted 10 times to remove loose debris and the liquid removed. 10mL autoclaved nanopure H₂O was added and the tube vortexed for 60 seconds. The liquid fraction was removed and centrifuged at 4816 x g for 5 minutes. The supernatant was removed and the pellet resuspended and added to a PowerBead™ Tube for extraction as previously described.

qPCR was used to analyze the presence and prolonged survival of *E. coli* O157:H7 using species specific primers generated to target the Shiga toxin subunit a for the O157:H7 serotype (IDTDNA). Hydrolysis probes were used due to high background fluorescence from interfering plant and humics material. The primer sets and probes are listed in Table 4.3. A 20 µL reaction mixture was prepared using Kapa® Probe Fast

Table 4.2. DNA quantification and purity per sample type

Species	Surface	<i>E. coli</i> present	Time (weeks)	(ng/uL)	Purity (260nm/ 230nm)	Purity (260nm/ 280nm)
<i>C. utriculata</i>	gravel	control	3	22.8	0.8	1.15
<i>C. utriculata</i>	gravel	<i>E. coli</i>	0.14	19.3	0.66	1.09
<i>C. utriculata</i>	gravel	<i>E. coli</i>	1	35	0.9	1.39
<i>C. utriculata</i>	gravel	<i>E. coli</i>	2	27.2	0.76	1.23
<i>C. utriculata</i>	gravel	<i>E. coli</i>	3	16.4	0.61	1.18
<i>C. utriculata</i>	root	control	3	41.2	0.99	1.35
<i>C. utriculata</i>	root	<i>E. coli</i>	0.14	43.7	0.94	1.32
<i>C. utriculata</i>	root	<i>E. coli</i>	1	30.9	0.79	1.36
<i>C. utriculata</i>	root	<i>E. coli</i>	2	42.6	1.06	1.39
<i>C. utriculata</i>	root	<i>E. coli</i>	3	34.5	0.84	1.34
<i>S. acutus</i>	gravel	control	3	26.2	0.78	1.28
<i>S. acutus</i>	gravel	<i>E. coli</i>	0.14	17.4	0.76	1.15
<i>S. acutus</i>	gravel	<i>E. coli</i>	1	23.4	0.87	1.13
<i>S. acutus</i>	gravel	<i>E. coli</i>	2	29.1	0.78	1.32
<i>S. acutus</i>	gravel	<i>E. coli</i>	3	22.1	0.79	1.22
<i>S. acutus</i>	root	control	3	34.9	1.19	1.57
<i>S. acutus</i>	root	<i>E. coli</i>	0.14	36.1	0.86	1.28
<i>S. acutus</i>	root	<i>E. coli</i>	1	41.2	0.83	1.3
<i>S. acutus</i>	root	<i>E. coli</i>	2	35.3	0.95	1.4
<i>S. acutus</i>	root	<i>E. coli</i>	3	36.2	0.87	1.33
Mixed	water	<i>E. coli</i>	0.14	109.8	1.84	2.22
Mixed	water	<i>E. coli</i>	1	28.8	1.75	1.76
Mixed	water	<i>E. coli</i>	2	6.2	1.6	0.91
Mixed	water	<i>E. coli</i>	3	10.2	1.53	1.31
Mixed	water	control	3	10.6	1.86	1.46

Master Mix (Kapa biosystems), 0.5mM primer and probe concentrations and 5 μ L DNA diluted to 1 ng/uL from the extraction concentration. The DNA from the top, middle, and bottom of column was extracted and quantified. Equivalent ratios of DNA material from each location were normalized and combined to prevent bias from any one source. The Rotor-Gene Q PCR machine (Qiagen) was used with the following program: Hold 15

minutes at 95°C, hold 15 minutes at 50°C, cycle 38 times with 30 second melt at 92°C, 30 second annealing at 62°C and 30 second extension at 72°C. The total gene copies were obtained from a standard curve generated by known gene copy number dilution series to C_q . This value was normalized to approximate surface area, determined using correlation curves of measured surface area to mass of collected sample for plants (VanKempen-Fryling and Camper 2015). Gravel specific surface area was estimated to be 11 cm²/g collected using a sphericity approximation of the ratio of the surface area of a sphere to the surface area of a particle (Fang and Daniels 2006). The specific controls for each plant species and unplanted gravel were subtracted to account for background fluorescence.

Table 4.3. Primer sequences for qPCR

Target Gene	Melt(°C)	Sequence
Shiga toxin A 709F	60	GGACAAGACTCTGTTCGTGTAG
Shiga toxin A 854R	60	CCGGACACATAGAAGGAAACTC
Probe 780R	60	AATGCCACGCTTCCCAGAATTGC

Data Analysis

Multifactor analysis of variance (ANOVA) was performed to determine statistical significance between experiments, looking specifically at location within the rhizosphere or gravel matrix, plant species and unplanted gravel, and time. General linear models (GLM) were used to compare interactions using Minitab 17. Statistical significance

between specific factors for each variable were identified by Tukey's *post hoc* multiple comparison test using a 0.05 p-value cut-off.

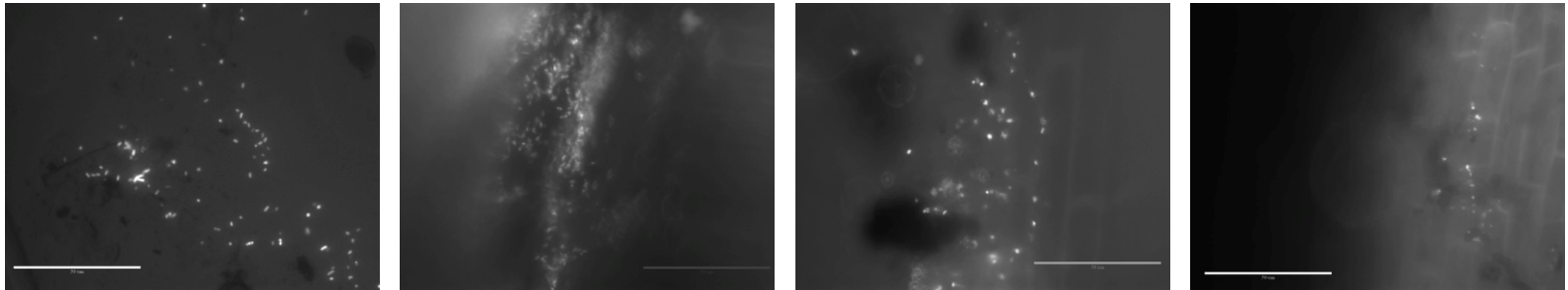
Results

Epifluorescent Microscopy Showing *E. coli* O157:H7 on Roots

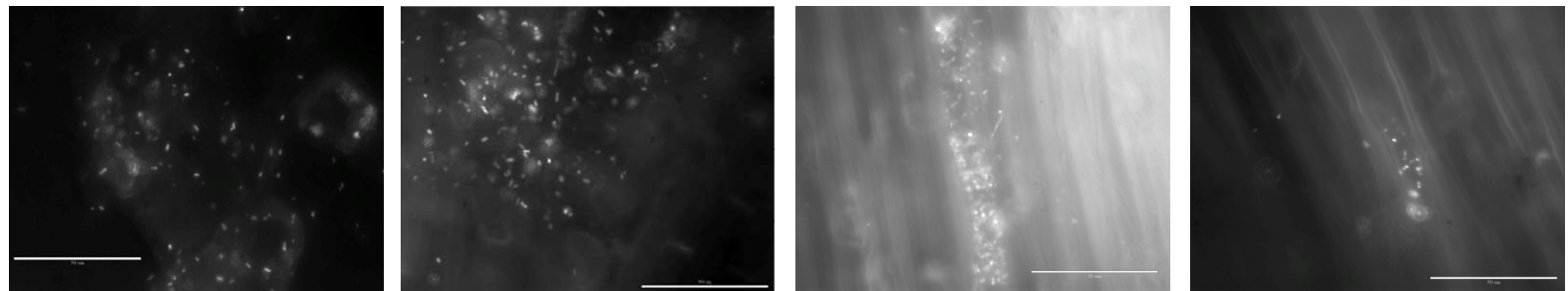
Images were taken for *C. utriculata* and *S. acutus* root samples for each time point. Representative images are displayed in Figure 4.3. Duplicate samples were taken for each column. Due to the limitation of imaging where the root is along the edge of the 2 mm capillary tube, only one side of the root could be scanned along its length. *E. coli* O157:H7 was visible after 3 weeks on roots of both plant species. There was a distinct visual increase in microcolony formation for both plant species from the initial attachment viewed at 24 hours post inoculation to 1 and 2 weeks post inoculation. There was a subsequent decrease at the final time point of 3 weeks where single cells were most frequently viewed.

A minimum of 20 images was taken for each replicate root. The cell clumping in each image was counted and data from the duplicate root samples for each species combined. Using Metamorph ® Image Analysis software and a technique used previously for clumping analysis (Behnke et al. 2011, Wilson et al. 2004), the fluorescent signal for each image was given a threshold and divided by the average cell area for *E. coli* O157:H7 to determine approximate number of cells in each clump. Counts per

C. utriculata



S. acutus



24 hours

1 week

2 weeks

3 weeks

Figure 4.3. Representative images of *C. utriculata* and *S. acutus* over time course within batch greenhouse columns. Scale bar equal to 50 μm .

clump size were reported as a percentage of total counts and graphed over time to show the variation of clump size over the course of the experiment (Figure 4.4). For both species, the larger clump sizes of 11-100 cells and >100 cells increased over time, however the majority of events were single cells, which remained high. Total cell counts decreased over time.

qPCR of Roots and Gravel to Determine Relative Abundance

Values were run in duplicate to show relative abundance for each surface over time (Figure 4.5). There was a significant decrease in relative abundance over time for all extracted types ($P = 0.005$). Gravel and both plant roots being significantly different across comparisons ($P = 0.006$). Significant interaction between time and plant type became more pronounced in the later time points in *C. utriculata* fractions. It gave the largest decrease over the course of the experiment by a 2 log reduction from 4.21×10^6 gene copies/cm² to 4.16×10^4 gene copies/cm² average across root and gravel ($P = 0.002$). Unplanted gravel had the highest overall retained abundance of *E. coli* O157:H7 for each time point with 1.13×10^7 gene copies/cm² after 24 hours decreasing to 2.44×10^5 gene copies/cm² after three weeks, followed closely by *S. acutus* having 4.71×10^6 gene copies/cm² to 2.20×10^5 gene copies/cm² from 24 hours to 3 weeks. Combined *C. utriculata* surface fractions were significantly lower in abundance compared to the unplanted gravel, giving an average of 2.5×10^5 gene copies/cm² compared to 1.75×10^6 gene copies/cm² ($P = 0.005$). *S. acutus* fractions were not statistically different from the other planted or unplanted columns.

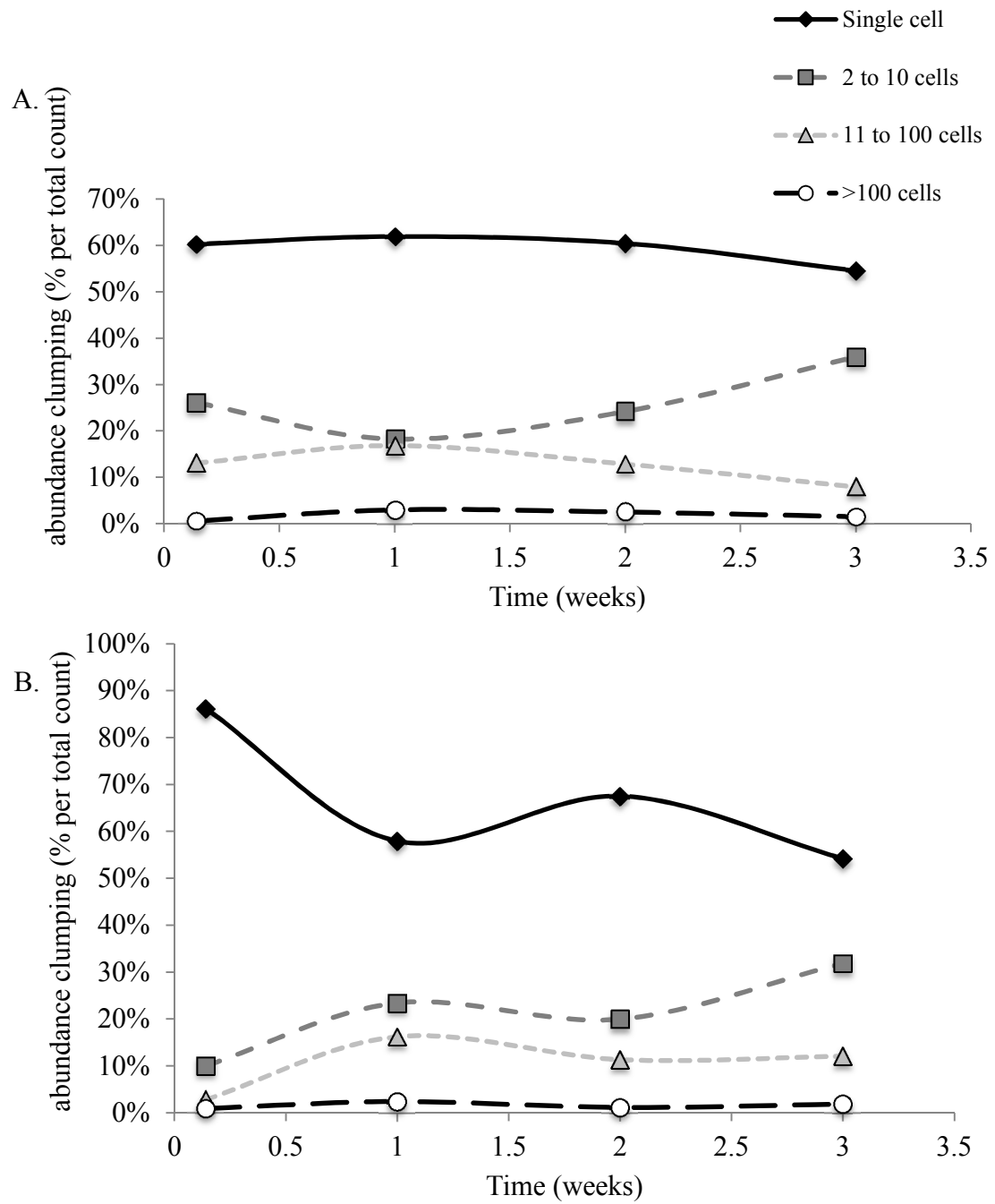


Figure 4.4. Total counts of clump sizes over time for (A) *C. utriculata* and (B) *S. acutus*.

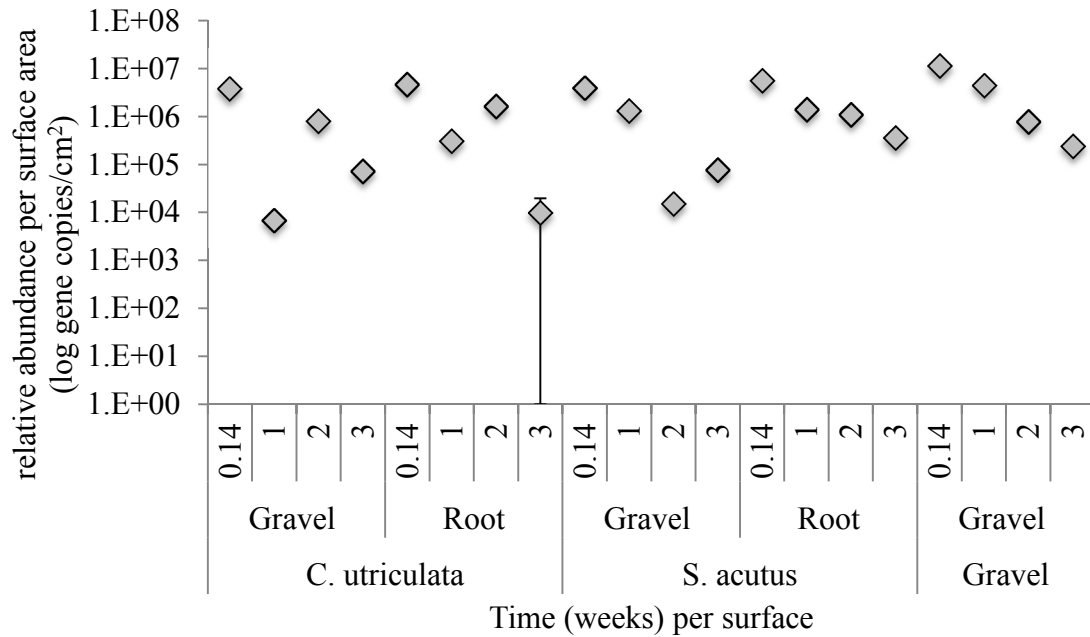


Figure 4.5. Relative abundance of *E. coli* O157:H7 for each location over time. Abundance of *E. coli* O157:H7 was normalized to surface area, obtained through known correlation curves to extracted mass. Error bars represent standard deviation in both experimental and technical replicates.

The data were further analyzed by splitting the results into relevant subgroups. The DNA extractions off the gravel from planted and unplanted columns were separated and analyzed to determine plant effect within the gravel matrix, and the planted columns root and gravel fractions were also partitioned to analyze surface significance within the column. When just the gravel fractions were accounted for, the unplanted column *E. coli* O157:H7 values (1.75×10^6 gene copies/cm²) were significantly higher than either *S. acutus* (2.79×10^5 gene copies/cm²) or *C. utriculata* (1.98×10^5 gene copies/cm²); all column types were significantly different with *C. utriculata* lowest ($P < 0.0005$).

When regarding the planted column fractions, all areas showed significant difference from each other, including strong interactions between plant type, surface and

time. The root portions were higher for *E. coli* O157:H7 than gravel overall with average 6.56×10^5 gene copies/cm² root and 2.35×10^5 gene copies/cm² gravel, however when the plant type interaction was taken into account *C. utriculata* root parsed out with both gravel fractions, giving only *S. acutus* roots significantly higher difference at 1.32×10^6 gene copies/cm² on average ($P < 0.0005$).

Discussion

Using both microscopy and molecular techniques, *E. coli* O157:H7 was found in root and gravel samples of both plant types and the gravel control through the course of a three-week batch system. Although relative abundance as shown through qPCR did decrease from 5.83×10^6 gene copies/cm² average over all 24 hour points to 1.53×10^5 gene copies/cm² at 3 weeks, that is still a substantial detectable level remaining, and imaging of the root surface showed potential growth within the first week that could harbor survivability through the full retention time. Given the low infectious dose of *E. coli* O157:H7, it is important to establish complete reduction to effectively reduce the risk of water contamination leading to further outbreaks.

The microscopy work showed an increase in microcolony and biofilm formation on the root surfaces beginning at week one, and larger clumping continued to develop over time (Figure 4.4). Although overall cell counts did decrease (1857 to 264 counts for *C. utriculata* and 4526 to 323 counts for *S. acutus*) and this was more pronounced on the root surfaces (Figure 4.5), it is currently unclear whether *E. coli* O157:H7 was inactivated or migrated within the plant and up to the stems or leaves from the rhizosphere. Previous

research in agricultural settings have found *E. coli* O157:H7 is capable of utilizing stomata openings or wound damage to migrate inside plant vascular structure (Cooley et al. 2003, Deering et al. 2012a, Saldana et al. 2011). This serves as a protection strategy for the bacteria against surface sterilization and predation as well as a reservoir until potential transfer to a host through ingestion of the plant (Saldana et al. 2011, Tyler and Triplett 2008). Further research is required to determine accurate conclusions of *E. coli* O157:H7 fate within the rhizosphere.

With the added benefit of nutrients and plant protection in the rhizosphere (Vymazal 2011), it was surprising to find higher relative abundance levels of *E. coli* O157:H7 within the gravel matrix (Figure 4.5). This could be due to a level of biocontrol by the PGPR limiting the *E. coli* O157:H7 establishment to the already existing biofilm on the root surface (Bhattacharyya and Jha 2012, Jasper et al. 2013). Although this difference was significant in this work, it has been shown that pathogen removal in wetlands is a highly variable process (Gruyer et al. 2013). *E. coli* O157:H7 is more susceptible to inactivation and predation within the gravel matrix over the added protection of plant tissue (Boutilier et al. 2009). Between competition with established biofilm (Gruyer et al. 2013), predation by nematodes, protozoa, rotifers, and copepods (Jasper et al. 2013), and photoinactivation (Boutilier et al. 2011), there are numerous sources of pathogen removal that are highly variable and subject to environmental factors that make tracking *E. coli* O157:H7 consistently difficult.

Despite the difficulties, it is important to better understand *E. coli* O157:H7 behavior within TW in order to better optimize removal and increase water quality while

retaining the economical and environmental benefits of using TW over traditional wastewater treatment.

Conclusions

E. coli O157:H7 was successfully identified through microscopy and qPCR in all treated samples. Microcolony formation was visualized one week post-inoculation and larger clusters continued to form through the three week time course. Relative abundance of *E. coli* O157:H7 was tracked and was at higher levels in the gravel matrix than on the root surfaces, the most pronounced difference being that *C. utriculata* roots had the lowest abundance. Future work is needed to determine whether the fate of *E. coli* O157:H7 is inactivation or harbored within plant tissues and the best method for complete removal.

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CHAPTER FIVE

OVERALL CONCLUSIONS

The goal of this thesis was to track the wastewater pathogen *E.coli* O157:H7 through epifluorescent microscopy and qPCR molecular analysis in model treatment wetlands in order to more accurately determine survivability within the rhizosphere and gravel matrix. One of the major health concerns in TW for use as wastewater remediation is pathogen contamination. *E. coli* O157:H7 is one of the most common waterborne pathogens causing outbreaks in the United States and improper removal from wastewater and subsequent release into the environment is a cause for concern (Benami et al. 2015).

A main reason for the experimental design was the limitations of current methods relying on water sampling and plate counts for determining fecal indicator removal (Reinoso et al. 2008, Sleytr et al. 2007). Although direct microscopy counts would need methods such as direct fluorescence *in-situ* hybridization (FISH) probes (Haffar and Gilbride 2010) which may not be a plausible option in real wastewater systems, the technique provided useful evidence into *E. coli* O157:H7 attachment behavior. Using *E. coli* O157:H7-DsRed, there was confirmation of initial attachment 2 hours post-inoculation in 300 mL chemostat hydroponic reactors. This attachment persisted even under relatively high washout conditions (100 min HRT) for one week. Interestingly, due to the direct visualization on the roots through a capillary as opposed to other methods that utilize disruption and filtration (Lothigius et al. 2010, Morato et al. 2014,

Tietz et al. 2007), *E. coli* O157:H7 microcolony formation was observed after 24 hours and some clumping sustained to one week. It is unknown whether the formation was due to cell proliferation or preferential binding from the inoculum, but a clear clustering was observed increasing within the first 24 hours, although the number of counts did decrease overall after one week.

This phenomenon was also seen in the greenhouse samples when root extractions were viewed under epifluorescent microscopy. Even in this much higher complexity environment with roots and gravel and an increase in predation by eukaryotic rotifers, protozoa, copepods and nematodes (Boutilier et al. 2011, Jasper et al. 2013), significant biofilm formation after a week post-inoculation was observed and continued through the three week sampling period. Total counted cells did decrease, but it has yet to be determined whether this is from inactivation or from *E. coli* O157:H7 migration into the root. Similar pathogen bacteria have been found to taxis toward plant roots (Tyler and Triplett 2008) and *E. coli* O157:H7 specifically have been observed to use open wounds or fissures at lateral root junctures (Cooley et al. 2003, Sharma et al. 2009) to enter the plant vascular system.

This observation could be an explanation for the higher *E. coli* O157:H7 population found on the branched type roots compared to the thicker taproots in *C. utriculata* in the hydroponic reactor series (Chapter 3). The lateral roots may provide enhanced opportunity to enter the root system and subsequently migrate into the stem and leaves of the plant where cells are protected from predation by eukaryotes or antibiotics produced by the PGPR (Bhattacharyya and Jha 2012, Boutilier et al. 2009). Higher cell

populations on lateral branched roots despite lower populations on *C. utriculata* overall could be indicative of movement into the plant vascular and out of the root systems entirely. Further research using *E. coli* O157:H7 qPCR copy number from samples of plant stems and leaves could provide useful information into the plants being a reservoir source. The *S. acutus* root structure of thick, well protected rhizomes with single descending taproots may have proven to be less susceptible to *E. coli* O157:H7 invasion, given the cells successfully attached to the surface but were still detectable at higher levels via qPCR both hydroponically and in greenhouse columns.

qPCR as an experimental technique provided promising evidence for *E. coli* O157:H7 detection in any sample source. Two qPCR variations were performed in this study: SYBR green intercalating dsDNA dye, and fluorogenic hydrolysis probes. One important conclusion from this study was the necessity for control samples. There is a large amount of PCR and DNA-binding inhibitors in environmental samples that cause a high background fluorescence and could lead to false positives (Miller et al. 1999). Intercalating dsDNA dyes are especially susceptible to reactivity due to the nonspecific binding. If qPCR is to be a future course for pathogen detection, hydrolysis probes that bind specifically to the bacterial sequence provide a much clearer signal for detection (Chapter 4). The disadvantage of qPCR as a method for pathogen detection is the requirement of primer sets to be specific to the DNA of the pathogen of interest. Research is being performed to alleviate this limitation using multiplex qPCR reactions that use multiple primer sets in a single reaction to detect many pathogens or serotypes at once (Morin et al. 2004, Muller et al. 2007). The requirement for pathogen-specific

primers is still required, but the time of detection is decreased substantially and as the science continues to develop, the cost should decrease as well.

The decision to use a pathogenic strain of *E. coli* over a laboratory model organism, such as *E. coli* K-12, was made in order to be as accurate to a real system as possible. *E. coli* O157:H7 serotype has significantly different binding characteristics over the nonpathogenic model organism commonly used (Jeter and Matthysse 2005, Macarisin et al. 2012, Saldana et al. 2011, Sharma et al. 2009). Many of the attachment proteins used in binding (curli, pilus, type 3 secretion system) are located on the pathogenicity island with the verotoxins and contribute to the cell's pathogenicity (Saldana et al. 2011).

Despite the evidence in support of root colonization, this research found significant attachment still occurring on nonliving surfaces as well. In the hydroponic reactors, *E. coli* O157:H7 relative abundance on nylon strings both clean and with established biofilm was at the same proportion as *C. utriculata*. In the greenhouse column experiments, *E. coli* O157:H7 quantification was higher in the unplanted gravel normalized to surface area than the planted columns, and attachment to gravel within the planted columns was detectable although in lower proportion than on roots. One possible explanation is the higher competition of the root biofilm and biocontrol from the existing microbes in the rhizosphere could prohibit the *E. coli* O157:H7 from gaining the benefits of exudate and oxygen release. Given the larger microbial population around the roots (Saharan and Nehra 2011) as well as the root involvement in drawing beneficial microbial communities to the rhizosphere (Badri et al. 2009, Collins et al. 2004),

rhizobacteria may limit invasion and sustained colonization compared to other surfaces. The presence of a biofilm community did not make a significant difference whether it was pre-established in the case of the nylon strings, however this could also be attributed to a plant-microbe interaction. Plants can mimic quorum sensing in bacteria and thus increase biocontrol of invading microbial cells (Berendsen et al. 2012, Loh et al. 2002, Vymazal 2011). Without the nutrient availability from root exudates or influence from plant signaling molecules for higher protection, the biofilm community by itself does not appear to offer any significant competitive hindrance. It was surprising to note the colonized and clean nylon replicates did not differ over the course of the experiment given the evidence showing *E. coli* O157:H7 does not utilize the simulated wastewater media for growth well (Appendix A). There may be multiple carbon sources in complex environmental samples within the gravel matrix of the greenhouse columns from other microbial metabolisms and decomposing plant material, but in the hydroponic reactors under constant flow, the only nutrient source was the simulated wastewater, yet *E. coli* O157:H7 persisted on nylon strings, although the drop from 2 hours to 168 hours was more drastic with a 2 log reduction than on root systems giving a 1 log reduction.

There is substantial evidence for the benefit of macrophytes for bacterial removal in TW (Brisson and Chazarenc 2009, Gruyer et al. 2013, Vymazal 2011). This research also supports these findings, given the lower concentrations in the planted columns than in the gravel only. There is still a large reservoir of *E. coli* O157:H7 that are not affected by the rhizosphere in the gravel matrix that are more likely to detach and contaminate the effluent water than those strongly bound to the plant surface using physical protein

attachment through curli and flagella expression (Macarisin et al. 2012, Saldana et al. 2011). Bacterial internalization into the plants could prove to be a successful mechanism for pathogen removal. Although considered a negative trait for agricultural crops (Deering et al. 2012a, Patel et al. 2010), for obligate wetland species with no downstream use that could cause subsequent *E. coli* O157:H7 release, it may be an ideal reservoir. Consequently, attempts to use plants with downstream applications such as edible plants for wastewater treatment (Zheng et al. 2010), while in theory a good combination of resources, may end up contaminating the entire crop internally. Increasing plant rhizosphere density to increase the removal rate and lower the reservoir of *E. coli* O157:H7 in the gravel matrix could be a good strategy, however there is an optimal density before pollutant removal suffers (Ibekwe et al. 2007). It may be more beneficial to choose plants with higher removal capacities at lower density, such as *C. utriculata* or other laterally branched species that could provide avenues into the plant vascular system and contain the pathogen.

With the understanding that *E. coli* O157:H7 and other pathogens can invade plant tissue (Deering et al. 2012e), it is important to control the use of the planted wetland species. Given the possibility of using TW to control nonpoint source contamination of water systems, such as along cattle farms (Hancock et al. 1998, Hogan et al. 2012, McGarvey et al. 2005), keeping the wetlands separate from grazing cattle could help decrease the *E. coli* O157:H7 reservoir in cattle. Dairy and meat products from infected cattle serves as another main cause of infection outbreaks in the United States (Griffin and Tauxe 1991, Kaper et al. 2004). *E. coli* O157:H7 has been hypothesized to enter

plant tissue as a means of consumption and transfer to a mammalian host (Tyler and Triplett 2008), whether human or bovine. In order to control *E. coli* O157:H7 infection and outbreak, all sources of contamination must be considered and reduced.

Future Directions

This study was a good preliminary analysis of *E. coli* O157:H7 initial attachment and persistence within TW rhizosphere and the column as a whole, as well as the localization abundance within each partition; however, many questions remained to be answered. Although presence of *E. coli* O157:H7 decreased significantly more within the rhizosphere than in the gravel matrix, it is still unknown if this was due to inactivation, internalization, or detachment and release. A more in depth analysis into each of these possibilities is required in order to best determine the optimal course for *E. coli* O157:H7 reduction and removal within TW.

With evidence that *E. coli* O157:H7, as well as other human pathogens, has the potential to be classified as a plant growth promoting rhizobacteria (PGPR) (Tyler and Triplett 2008), it would be an interesting and applicable study to perform a metagenomic analysis of the rhizosphere in a study and track the population shifts previous to and post addition of a human pathogen to the media. This analysis could provide useful evidence into the natural population of a rhizosphere, as well as understanding what beneficial microbial populations are present and should be selected for, and if *E. coli* O157:H7 is incorporated into the numbers or decreased from competition. Some common soil bacterial strains have been shown to inhibit *E. coli* O157:H7 attachment and binding to

plant surfaces plant (Cooley et al. 2003, Tyler and Triplett 2008), so determining if those species are present could help indicate the overall effectiveness of the TW for *E. coli* O157:H7 removal.

While qPCR was beneficial in determining presence/absence within the system, it would be very beneficial to know the metabolic activity of *E. coli* O157:H7 throughout the time course. Monitoring RNA would show whether *E. coli* O157:H7 was still active, and whether it was growing or in a senescent stage. More targeted RNA or microarray analyses could provide evidence to determine if *E. coli* O157:H7 was binding to plant tissue using external proteins such as T3SS, curli, pili, or flagella, and if *E. coli* O157:H7 continued to produce verotoxins such as the Shiga toxins that cause virulence, or if prolonged environmental exposure decreased its virulence, adapting it into nonpathogenic naturalized strains already present and detected in natural water and sediments (Ishii et al. 2006, Pachepsky and Shelton 2011, Winfield and Groisman 2003).

TW are a promising technique for wastewater remediation. However, like all new and innovative technology, there is still vast room for continued research, improvement, and refinement.

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APPENDICES

APPENDIX A

SURVIVAL CURVES OF *ESCHERICHIA COLI* O157:H7 IN
NANOPURE WATER AND STERILE WASTEWATER

Methods

Preparation of Media

In order to prepare sterilized simulated wastewater media, the chemical components had to be autoclaved separately or precipitation occurred. The complete list of the ingredients and concentrations for two solutions are in Table B.1. The solutions were prepared and sterilized at 2X concentration to the final desired media composition. After sterilization, equal amounts of Solution 1 and Solution 2 were mixed prior to *E. coli* O157:H7-DsRed inoculation as previously described (Chapter 2).

Nanopure water was collected and autoclaved to ensure complete sterilization. It was used as a control for *E. coli* O157:H7-DsRed survival and plasmid retention.

Table A.1. Nutrient ingredients in chemical formula and concentration for preparation of sterilized media.

SOLUTION 1		
Nutrient	Chemical	Concentration (mg/L)
Carbon	C ₁₂ H ₂₂ O ₁₁	99.7
Iron	FeCl ₃	0.4
Magnesium	MgSO ₃ 7 H ₂ O	62.0
Ammonia	NH ₄ Cl	19.1
Copper	CuSO ₄	0.8
Manganese	MnSO ₄	7.8
Zinc	ZnSO ₄	7.8
Calcium	CaCl ₂	1.9
SOLUTION 2		
Nutrient	Chemical	Concentration (mg/L)
Phosphorus	K ₂ HPO ₄	44.0
Nitrate	NaNO ₃	121.4
Boron	H ₃ BO ₃	10.0
Potassium	KI	1.9
Molybdenum	Na ₂ MoO ₄	4.0
Primatone	Complex protein	111.1

E. coli O157:H7-DsRed Survival Assay

An overnight culture of *E. coli* O157:H7 was prepared using custom TSB media previously describes (Chapter 2). 1 mL was spun down and washed in sterile nanopure water prior to inoculation into 100mL flasks with 50 mL of sterile wastewater or sterile nanopure water. The flasks were left in planktonic batch culture on the bench top. Sampling was performed daily to track survival in depleting and nutrient starved conditions. The flask was shaken to fully resuspend cells prior to sampling. *E. coli* O157:H7 samples were enumerated with plate counting, epifluorescent microscopy and qPCR.

Plate Counts

E. coli O157:H7 was verified using the drop plate method (Herigstad et al. 2001) on standard Luria Broth (LB) nutrient agar. Replicates were also enumerated on R2A agar plates. A ten-fold serial dilution was performed on 1 mL from each flask. Five replicate 10 μ L drops were place on one quarter of a plate for the four final dilution concentrations estimated to produce countable results. The five drops were averaged and back calculated to determine CFU/mL.

Epifluorescent Microscopy

1 mL from the experimental flask was removed and sonicated to disrupt any cell clumping that may have occurred. The sample was diluted according to estimated original cell concentration either ten or hundred fold. The sample was added to a black 25 mm 0.2 μ m pore size polycarbonate filter through vacuum filtration and positioned on

a glass slide. Slides were viewed with a 100X magnification oil objective. At least 20 images were taken in a randomized pattern of five rotations along the x-plane before moving one rotation in the y-plane and repeating five rotations in the opposite direction in the x-plane until the appropriate statistical sampling was reached for approximately 300 cells captured.

DNA Extraction and qPCR

300 μ L of original experimental flask culture was extracted and added to PowerSoil Bead Tube® from the Powersoil DNA extraction kit (Qiagen) as previously described (Chapter 2).

DNA was isolated in DEPC water for storage and diluted ten-fold prior to qPCR analysis. Primers and qPCR run protocol were used as described previously (Table 2.1; Chapter 2).

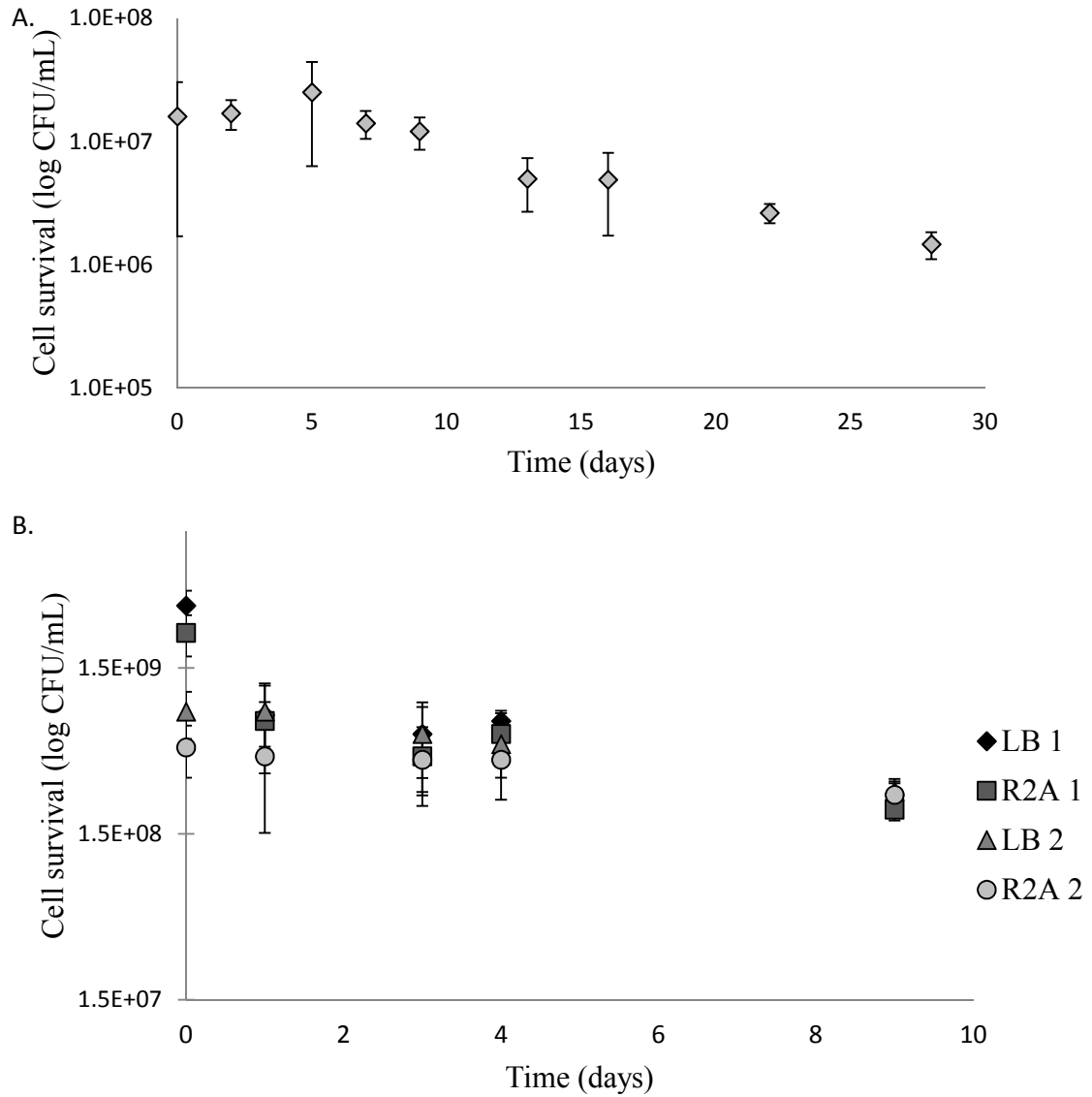
Results

Figure A.1. *E. coli* O157:H7 survival was tracked in sterile nanopure water (A) and sterilized simulated wastewater media (B). Values are given in CFU/mL as enumerated by plate counting. R2A plates were used for nanopure water and R2A and LB agar plates for simulated wastewater plate counts. Error bars represent standard deviation between technical and biological replicates. (N = 10: 2 biological, 5 technical)

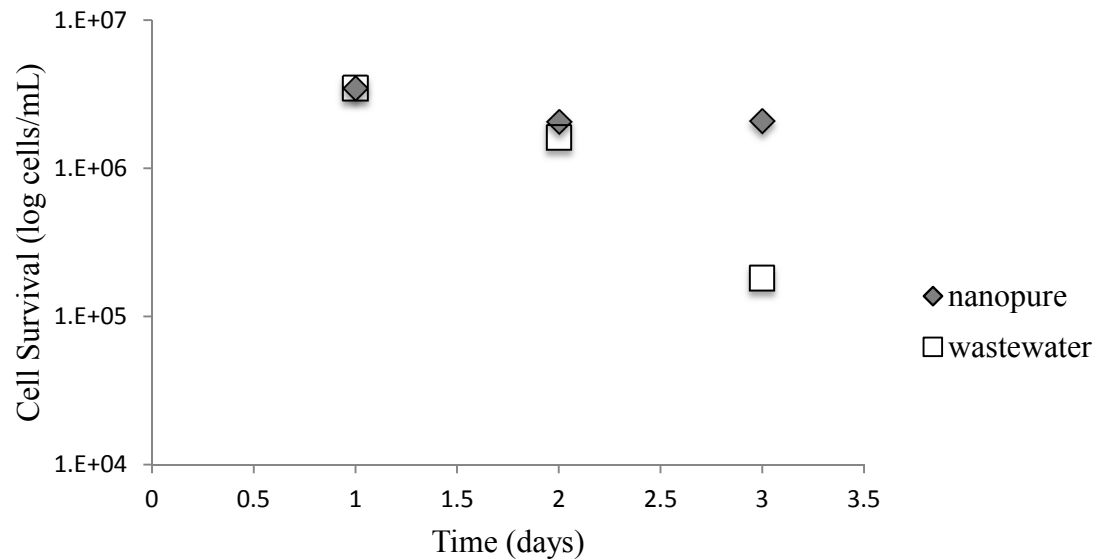


Figure A.2. *E. coli* O157:H7 survival tracked through epifluorescence microscopy. Cell counts were enumerated through calculation of from the area of one picture

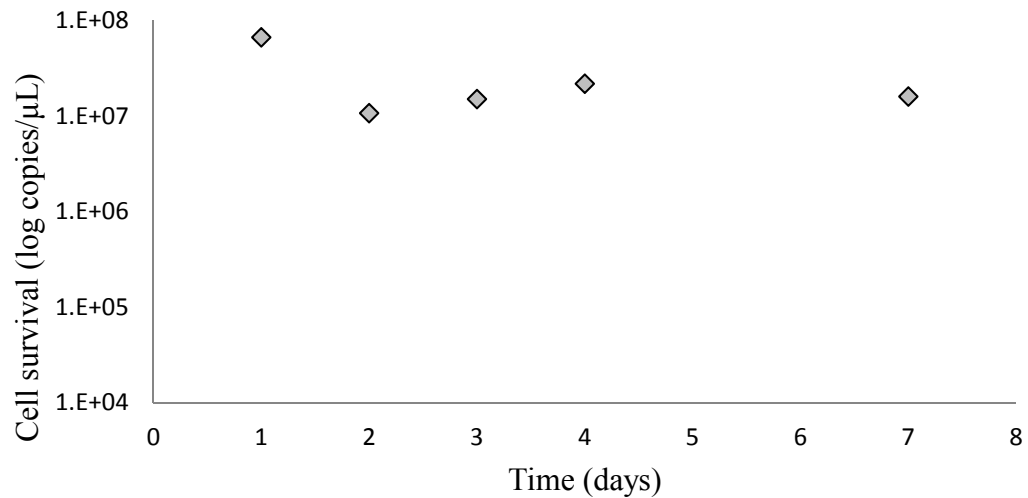


Figure A.3. qPCR verification of *E. coli* O157:H7 survival in nutrient starved sterile nanopure water. Cell survival is determined by the number of gene copies per qPCR sample.

Reference

Herigstad, B., Hamilton, M. and Heersink, J. (2001) How to optimize the drop plate method for enumerating bacteria. *Journal of Microbiological Methods* 44, 121–129.

APPENDIX B

STATISTICAL BASIS FOR EXPERIMENTAL DESIGN
OF GREENHOUSE COLUMNS

Stat consultation with Rachel VanKempen-Fryling

AEP, 11/9/11

We talked about experimental design.

Response: from qPCR, copies/gram of soil collected from a column in which some plant is growing

Factors of interest:

- plant type, 4 levels
- *E. coli*, +/-
- time, 3 levels
- PMA, +/- (possibly not included, if it is, each sample would be subsampled twice, one for PMA, one for non-PMA)

Some variance estimates

To interpret the horizontal axis in the power plots below, one needs an estimate of the standard deviation of the response. Jennifer Faulwetter's thesis (2010) contained variance estimates for qPCR data in copies/gram normalized to 16S, which may be helpful,

sample	id	sd
water	W1	0.64
roots	R2	0.19
roots	R3	0.32-.36
roots	R4	0.59
gravel	G5	0.22
gravel	G6	0.16

Model with plant and *E. coli*:

The model can be fit with only 2 pots per plant/ecoli combo:

- df for plant: 3
- df for ecoli: 1
- df for interaction: 3
- With 2 pots per plant/ecoli combo, $2*4*2=16$ reps, total df = 15, df error = $15 - 7 = 8$.
- With 3 pots per plant/ecoli combo, $3*4*2=24$ reps, total df = 23, df error = $23 - 7 = 16$

Power:

- With 2 pots per combo, the power curve is:

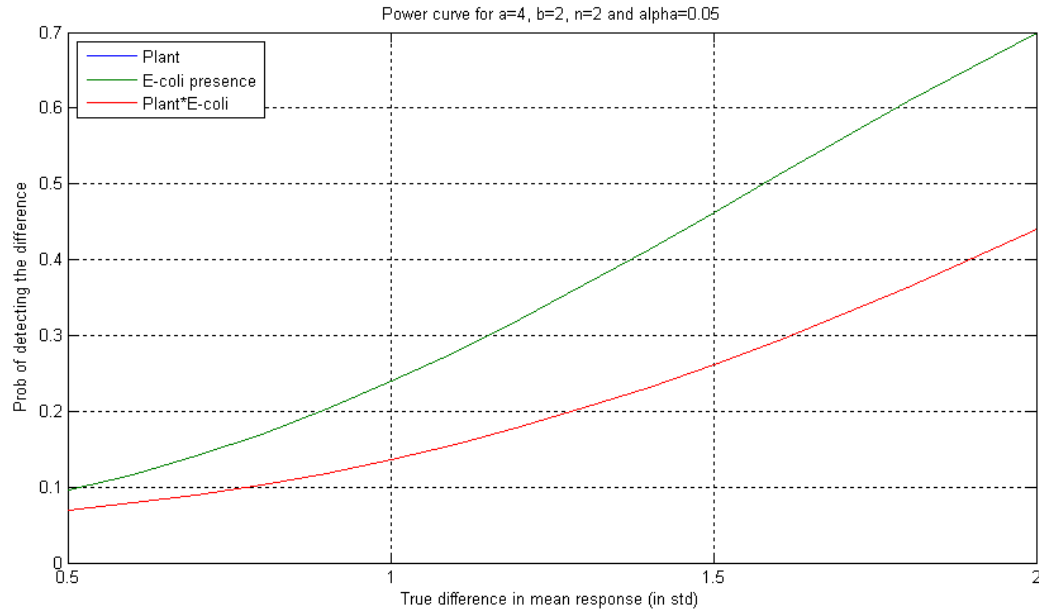


Figure B.1. Power curve analysis for experimental design in duplicate for plant and *E. coli* O157:H7 as variables.

- With 3 pots per combo

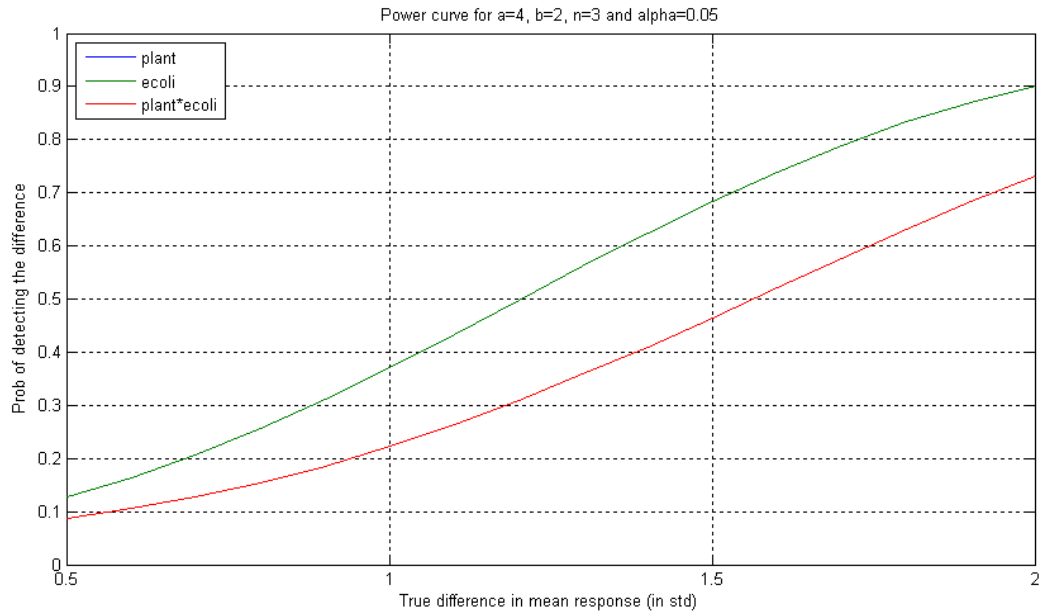


Figure B.2. Power curve analysis for experimental design in triplicate for plant and *E. coli* O157:H7 as variables.

Model with plant and *E. coli* and time:

The model can be fit with only 2 pots per plant/ecoli combo:

- df for plant (P): 3 (2)
- df for ecoli (E): 1 (1)
- df for time (T) 2 (1)
- df for interaction P*E 3 (2)
- df for interaction P*T 6 (2)
- df for interaction E*T 2 (1)
- With 2 pots per plant/ecoli/time combo, $2*4*2*4=64$ reps, total df = 63, df error = $63 - 17 = 46$.
 - o $(2*3*2*2 = 24, df = 23, df\ error = 23 - 9 = 14)$
- With 1 pot per plant/ecoli/time combo, $1*4*2*4=32$ reps, total df = 31, df error = $31 - 17 = 14$

Power:

- With 1 pot per plant/ecoli/time, the power curve is:

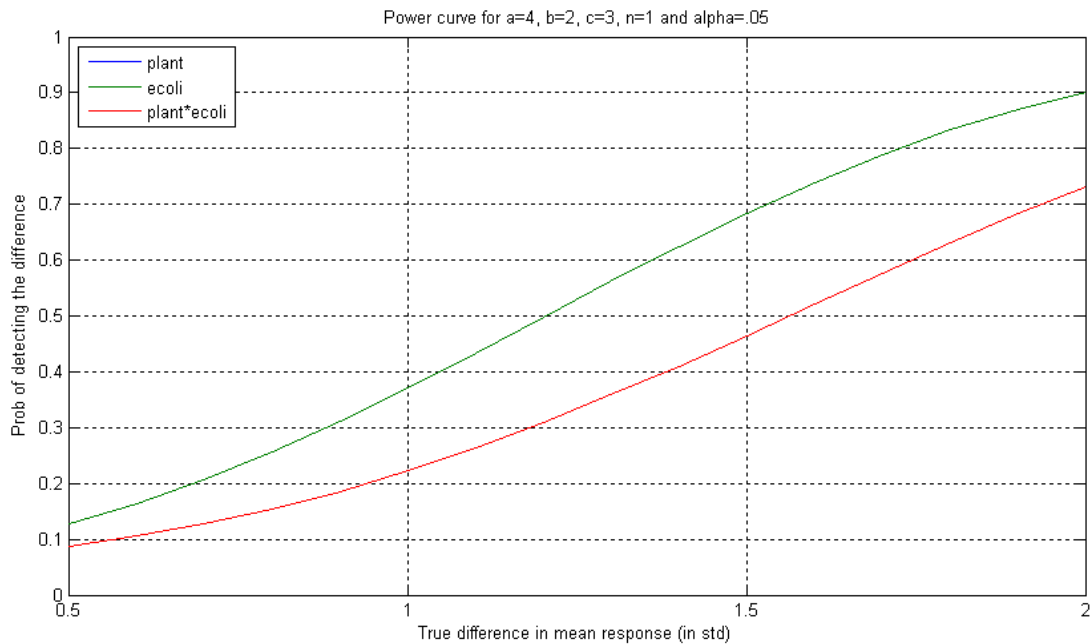


Figure B.3. Power curve analysis for experimental design in singlet for plant and *E. coli* O157:H7, and time as variables.

- With 2 pot per plant/ecoli/time, the power curve i

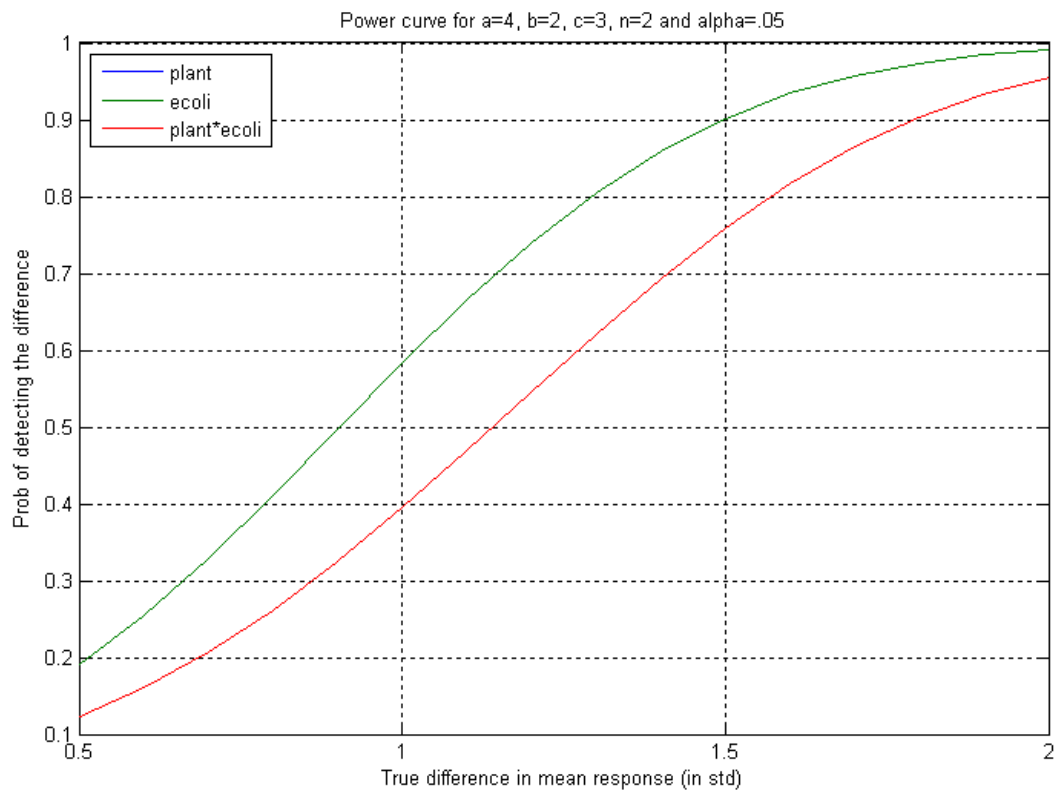


Figure B.4. Power curve analysis for experimental design in duplicate for plant and *E. coli* O157:H7, and time as variables.

Reference

Faulwetter, J.L. (2010) Analysis of microbial biofilm community composition within constructed wetlands, Montana State University, Bozeman, Montana.

APPENDIX C

CLEANUP STRATEGIES FOR QPCR UNINOCULATED
CONTROL SIGNAL REDUCTION

Methods

Determination of Potential Contamination

The first step to analyze the high signal intensity in uninoculated controls was determining potential sources of contamination. Reactions were set up with new DEPC water, primer stocks, and mastermix. DEPC water (Ambion) was replaced with a previously unopened container for all subsequent experiments. New primer stocks in JMS2 and specific to the DsRed plasmid were obtained. All DNA extractions were performed on a fresh PowerSoil® kit (MoBio). Two mastermixes were tested: Kapa® Fast SYBRgreen mix (Kapa Biosystems) and GoTaq® SYBRgreen mix (Life Technologies).

Sterilized Plant and Pure Culture Alternate Bacterial Species DNA Tests

The possibility of plant or biofilm DNA interference was hypothesized. Fresh greenhouse samples were taken of *C. utriculata* and *S. acutus* from the stock planted cells. Roots and shoots were harvested in duplicate, one of which was surface sterilized with bleach to reduce/remove any native microbial population interference to test plant DNA only.

Another determinative test performed was nonspecific binding from the primers. A pure culture *Staphylococcus aureus*, a non-Shiga toxin producing bacteria, was obtained and DNA extraction using the PowerSoil® kit (MoBio) performed on the pure culture as well as the greenhouse samples.

DNA Clean Up Kits

Two kits to remove any inhibitors from the DNA extractions were tested. *OneStep*[™] PCR Inhibitor Removal Kit (Zymo Research) and PowerClean® Pro DNA Clean-Up Kit (MoBio Laboratories, Inc.) were used on extraction examples for *E. coli* O157:H7 positive and uninoculated controls to determine their effect on signal intensity. The protocols were followed as described by the companies.

Dilution Runs

E. coli O157:H7 positive and uninoculated control DNA samples were run in tenfold dilution series to check for inhibition. Dilutions were performed on the original DNA extracts, the planted samples, and the samples run through the PCR clean up kits.

Results and Discussion

The first qPCR checked the contamination in primer sets, mastermix, and DNA extraction kits (Figure D.1.). Using previously determined standard curves for each primer set checked against the *E. coli* positive samples, the extraction kits were not significantly different with either primer set. The plant DNA did cause a higher copy number (values were similar to the controls), providing evidence the plant DNA may be contributing to the interference. The DEPC water was not found to be causing reactivity due to the low non template control (NTC). The control samples, specifically from gravel and water sampling, were still reactive.

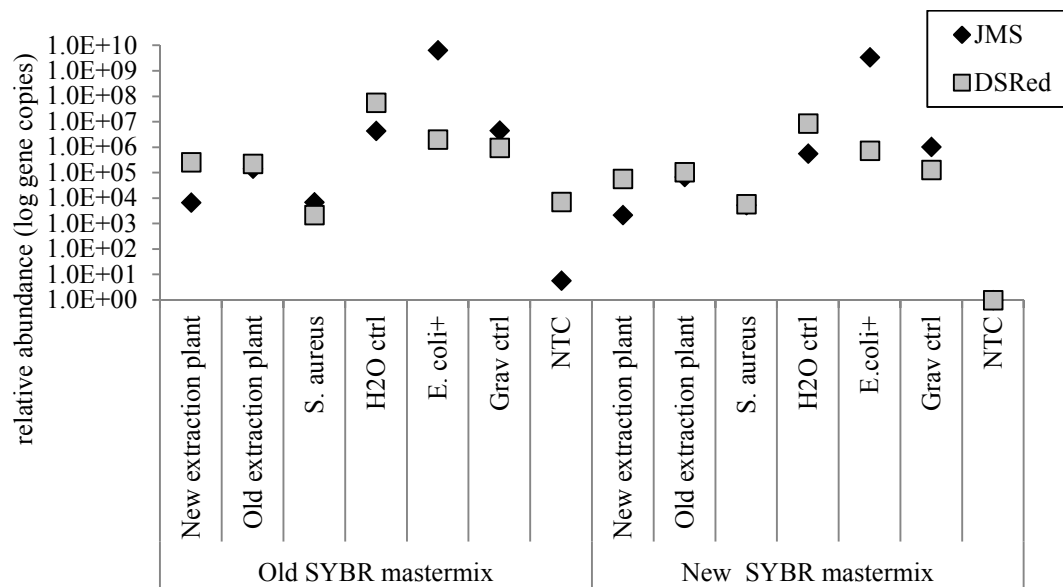


Figure C.1. qPCR reactions to show relative abundance in *E. coli* O157:H7-DsRed copy number according to two different primer sets targeting *StxA* (JMS) and the DsRed plasmid insertion (DsRed). Previously used and new SYBR mastermixes were used to test plant, *S. aureus*, and uninoculated control DNA against an *E. coli* O157:H7 positive control.

The next test further examined plant DNA through testing each plant from the hydroponic reactors (Reactors) discussed in Chapter 3, surfaced sterilized greenhouse samples (Sterile), and nonsterile greenhouse roots and shoots (GH). The results were varied between primer sets; both reactions using DsRed yielded opposite results, showing the plasmid is not stable enough to yield reproducible results and was discarded to continue with JMS2 primers (Figure D.2.).

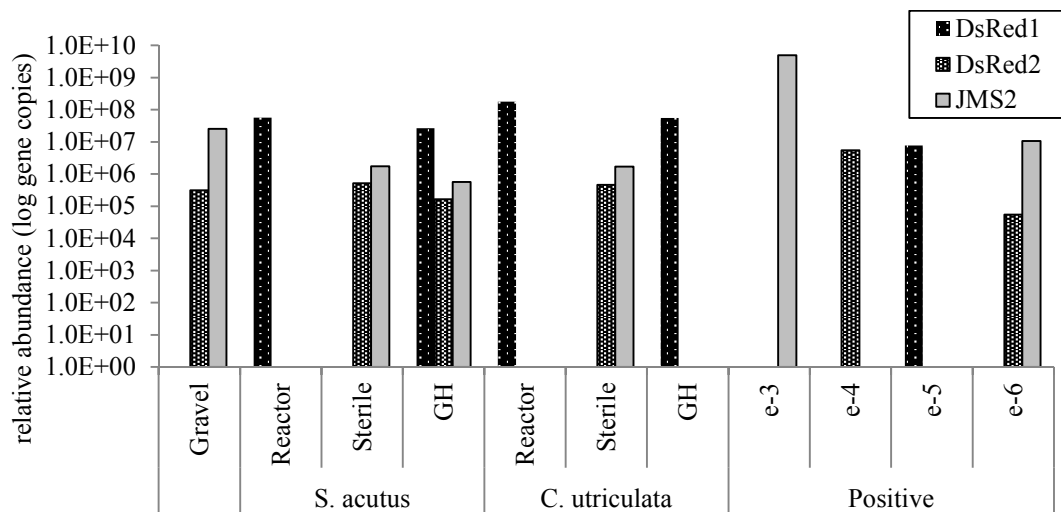


Figure C.2. qPCR showing relative abundance using two primer sets: DsRed and JMS. Three experimental plant types were tested against *E. coli* O157:H7 positive samples from the standard curve and a gravel control sample. Samples were taken from reactor controls (Reactor), greenhouse and surface sterilized (Sterile), and nonsterile greenhouse samples (GH). Two DsRed primer sets were tested.

Dilutions were also tested to determine inhibition (Figure D.3.). The test was performed using the two DNA extraction samples, from the old kit (old) and the new kit (new). Full strength DNA was significantly reduced response in all samples, showing inhibitors are suppressing the signal. Two serial dilutions at one tenth and one hundredth strength gave similar signals due to the inhibitors also becoming diluted. Dilutions down

to 1 ng/ μ L DNA was determined to lower the inhibition concentration for all experimental results.

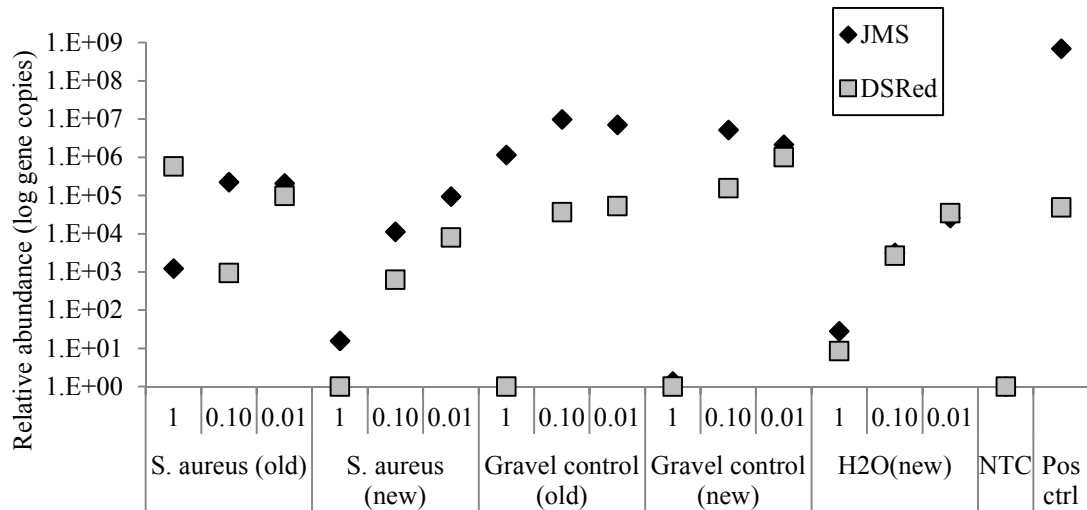


Figure C.3. qPCR relative abundance dilution control test. Samples from two extraction kits, one used previously (old) and one just opened (new) were tested for *S. aureus* to determine nonspecific binding to genes other than *StxA* or DsRed, and controls in gravel and water.

The final test checked two post DNA extraction clean up kits along with a new mastermix (Figure D.4.). Kapa® SYBR green mastermix performed much higher signals over all DNA samples and gave more consistency and was chosen over GoTaq® SYBR green mastermix for further experimental analysis.

When using the DNA clean up kits, the signal was reduced in the control samples. However, the signal was also greatly reduced below detectable limit in the positive samples in the case of PowerClean® (PC) and GoTaq® mastermix, or no difference as seen as with OneStep® (Zymo) using Kapa®.

After all the tests and checks were performed and analyzed, it was concluded that Kapa® Fast mastermix gave the best signal intensity and to rely on dilution solely for inhibitor control as the follow up inhibitor removal kits did not significantly affect the signal intensity between treated and untreated controls.

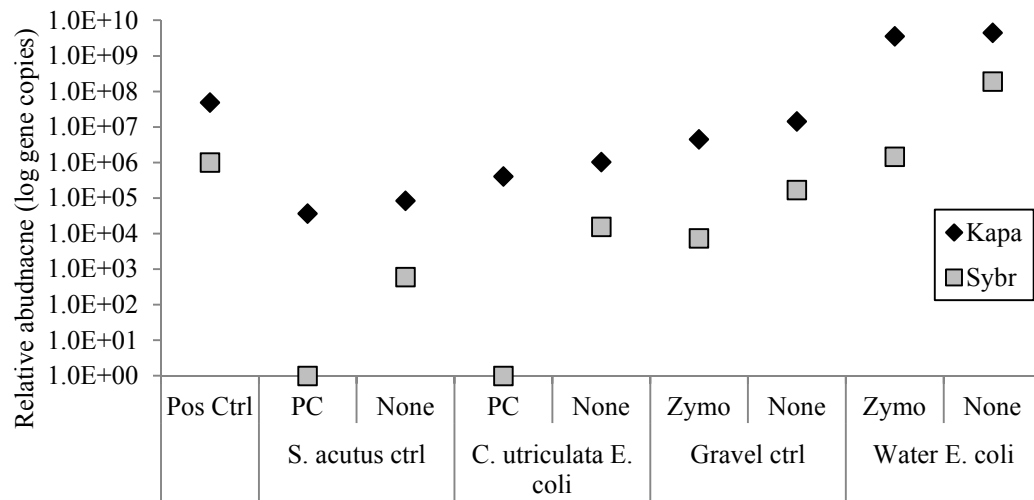


Figure C.4. qPCR relative abundance showing two different mastermixes (Kapa=Kapa® Fast and Sybr=GoTaq® SYBR green) and two PCR inhibitor removal kits (PC=PowerClean® and Zymo=Onestep™) both treated and untreated (None). Uninoculated controls were tested for *S. acutus* and gravel, and *E. coli* O157:H7 positive samples were tested with a positive standard (Pos Ctrl), *C. utriticulata*, and an inoculated water sample.

APPENDIX D

CONFOCAL IMAGES FOR *C. UTRICULATA* AND
S. ACUTUS INITIAL COLONIZATION

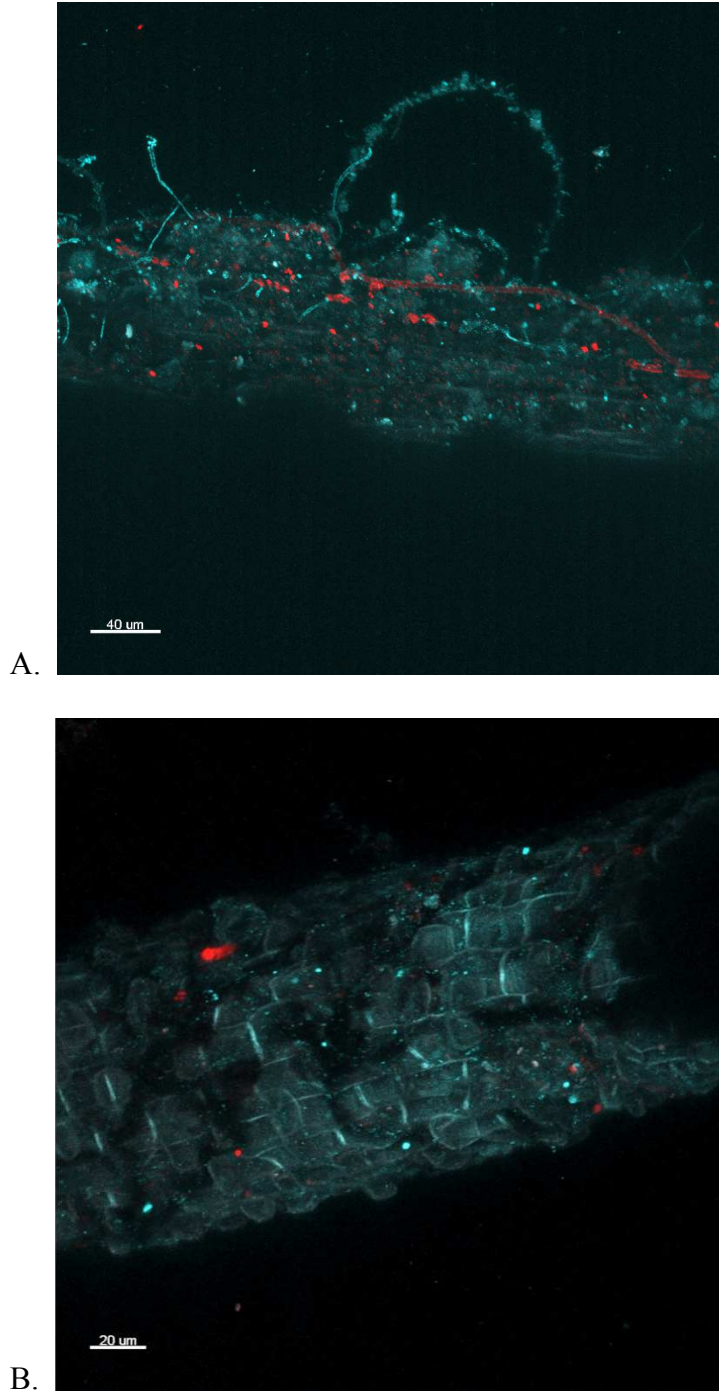


Figure D.1. *C. utriculata* stained with DAPI viewed under confocal microscopy for presence of *E. coli* O157:H7-DsRed inoculation on root surface after 24 hours (A) and 1 week (B). Scale bars are 40 μm at 24 hours and 20μm at 1 week.

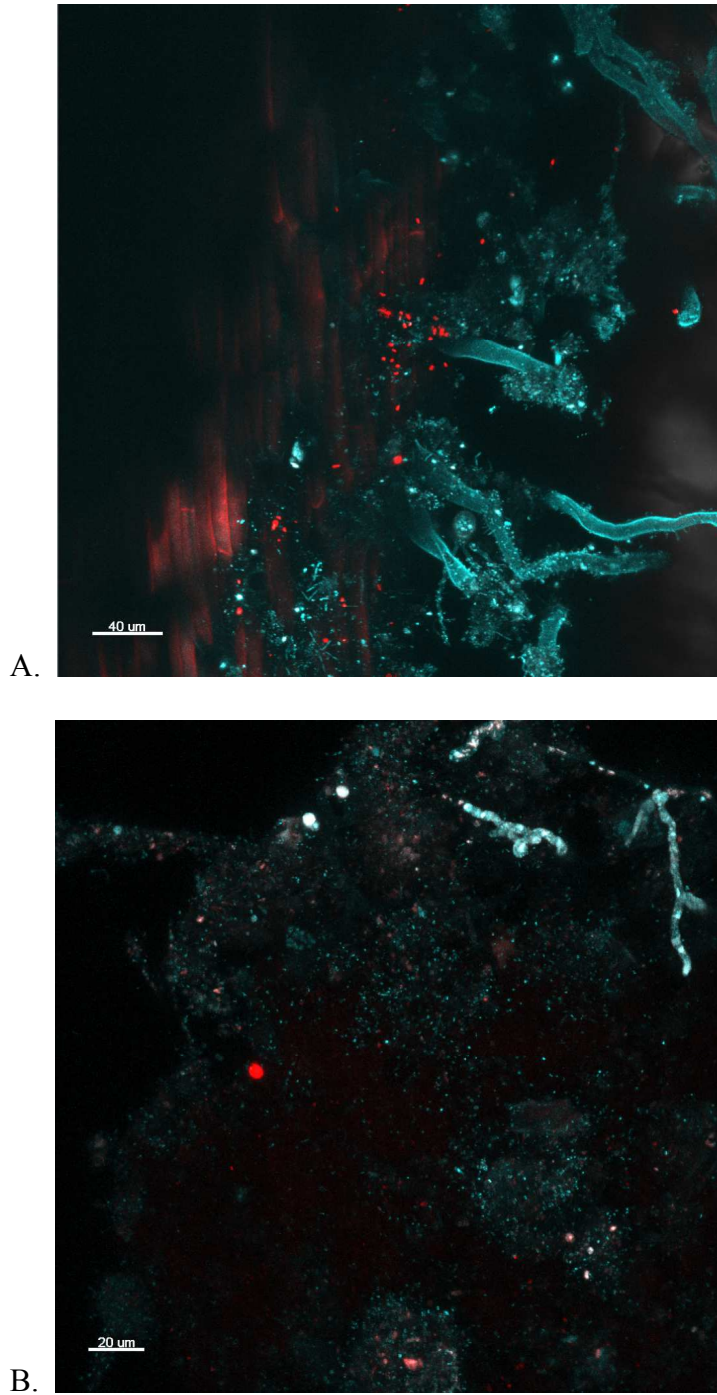


Figure D.2. *S. acutus* stained with DAPI and viewed under confocal microscopy for presence of *E. coli* O157:H7-DsRed inoculation on root surface after 24 hours (A) and 1 week (B). Scale bars are 40 μ m at 24 hours and 20 μ m at 1 week.