Public Health Ecosystem Services and Potential Concerns of Freshwater Wetlands

DISSERTATION

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By
Tsung-Ta (David) Hsu
Graduate Program in Environmental Science

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Dissertation Committee:
Jiyoung Lee, Ph.D., Advisor
William Mitsch, Ph.D.
Karen Mancl, Ph.D.
Warren Dick, Ph.D.
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Abstract

Wetlands provide extensive ecosystem services, such as biodiversity enrichment, flood control, carbon sequestration and water purification. However, the performance of wetlands with respect to treating microbial pathogens is less understood. Since wetlands serve as an interface where human and wildlife come into contact, potential health risks may emerge from human exposure to a variety of zoonotic pathogens that are shed from wildlife. The main goal of this dissertation is to provide an overview of freshwater wetland's ecosystem service and public health implications with regard to bacterial pathogens. The objectives include: (1) to assess ecosystem services of freshwater wetlands in terms of public health, specifically in reducing fecal indicator bacteria and pathogens (Chapter 2); (2) to model freshwater wetlands’ ecosystem service in improving bacterial water quality under climate change (Chapter 3); (3) to evaluate the role of wildlife in pathogen dissemination in freshwater wetlands (Chapter 4); and (4) to investigate antimicrobial resistance in freshwater wetlands (Chapter 5). Study sites included created and restored marshes in Ohio at the Olentangy River Wetlands Research Park (ORWRP), which receive water from an urban/agricultural watershed and the Ottawa National Wildlife Refuge (ONWR), which is a heavily avian species-impacted coastal Lake Erie wetland. Bacterial culture and real-time polymerase chain reaction were conducted to enumerate E. coli as a fecal indicator and determine the levels of bacterial pathogens (Campylobacter, Arcobacter, Shiga toxin-producing E. coli), microbial source
tracking markers (human, ruminant, avian species) as well as antimicrobial resistance genes (\textit{tetQ, sul1}), respectively. US EPA's Stormwater Management Model (SWMM) and load duration curve analysis were employed to estimate wetlands' performance in \textit{E. coli} reductions under climate change scenarios. For an emerging zoonotic pathogen in water, isolation and characterization of antimicrobial resistance patterns of \textit{Arcobacter} were also conducted. The wetlands were found to reduce \textit{E. coli} by 22.3% as water moved across the inflow to outflow of the urban freshwater wetlands. A simple and empirical model projected that freshwater wetlands would be able to decrease \textit{E. coli} loads to meet regulatory requirements for both near (2020-2050) and far (2045-2074) terms despite increasing trends of \textit{E. coli} levels. However, direct investigation of Canada goose feces sampled from freshwater wetlands identified Shiga toxin-producing \textit{E. coli} gene which was linked to a toxin form of high potency and outbreak-causing strains (O157:H7, O165:H25 and O111:H-). \textit{Arcobacter} was isolated from freshwater wetland water and demonstrated multiple antimicrobial resistance. More long-term monitoring of pathogens and surveillance of more wildlife species are necessary to better understand how wetlands mitigate pathogens and what health risks could arise when humans are exposed to wetlands. Nevertheless, findings from this dissertation (regarding both freshwater wetlands' ecosystem service and public health perspective) could help support planning for a "wise use of wetlands."
Dedication

To those who have helped me get this far
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Vita

2007........................................B.S. Biochemistry, National Taiwan University
2009........................................M.S. Microbiology, National Taiwan University
2012 to present .........................Graduate Teaching and Research Associate,
                                      College of Public Health, The Ohio State
                                      University

Publications


Fields of Study

Major Field: Environmental Science
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Chapter 1

Introduction

1.1 Ecosystem services of wetlands

Wetlands provide extensive benefits to both natural environments and humans. According to the Ramsar Convention on Wetlands, wetlands provide a wide array of ecosystem services (IWMI, 2006; Russi et al., 2013; Mitsch and Gosselink, 2015). According to the Millennium Ecosystem Assessment (2005), wetlands provide four types of ecosystem services. Provisioning: wetlands provide food (fish and grain), fuel (wood), medicines from biota, and genetic pools for biodiversity. For example, wetlands contributed 11.2 billion kilograms of inland capture fisheries and 41.7 billion kilograms of inland aquaculture production globally (FAO, 2012). Regulating: wetlands regulate water to help flood control and coastal protection, mitigate global warming by absorbing carbon dioxide, and improve water quality by reducing nutrients and pathogens. In China, wetlands are estimated to help retain 4.6 trillion grams of total nitrogen and 0.6 trillion grams of total phosphorus from water resources (An et al., 2007). Cultural: wetlands bring aesthetic and spiritual values and opportunities for recreational and educational
purposes. In Belize, it was estimated that mangrove and coral reef tourisms contributed to 12-15% to the GDP for 2007 (Cooper et al., 2008). **Supporting:** wetlands accumulate sediment and organic matter, aiding in the process of soil formation. Many nutrient cycles also take place in wetlands, e.g. nitrogen cycling. Supporting ecosystem services is the foundation for other types of services, while having more long-term and indirect effects on people (Millennium Ecosystem Assessment, 2005).

In the era of climate change, it is likely that increasing heavy precipitation and average tropical cyclone maximum wind speed will be observed. It is also possible that heavy rainfall will increase local flooding events (IPCC, 2012). Wetlands could act as a buffer area for flood mitigation and coastal protection (Marios and Mitsch, 2015; Mitsch and Gosselink, 2015). A model proposed by Ogawa and Male (1986) suggested that more than a 100% increase in downstream peak flow would have occurred if upstream wetlands had been removed from the Charles River watershed in Massachusetts. After a tsunami event in India in 2004, coastal areas with mangrove and *Casuarina* plantations were found to have no, to only partial, damage (Danielsen et al., 2005). These examples indicate the protecting roles of either inland or coastal wetlands. The U.S. Army Corps of Engineers estimated an average flood damage cost would be $17 million per year if all wetlands in the Charles River watershed in Massachusetts were lost. Conserving wetlands would be a less expensive option for flood protection than man-made structures, such as dikes and dams (U.S. Army Corps of Engineers, 1972). On the other hand, wetlands could also act as a carbon absorber to help alleviate the problem of elevated carbon dioxide. Mitsch et al. (2013) analyzed various wetlands in the world and concluded that
wetlands are global carbon sinks with a net carbon sequestration rate of 830 Tg \((10^{12} \text{g})\) per year, which averages 118 g-C m\(^{-2}\) year\(^{-1}\).

1.2 Treatment wetlands

Building on wetlands' function as a method for water purification, thousands of "treatment wetlands" have been constructed or utilized to treat various wastewaters across the globe, such as municipal, urban stormwater, mine drainage, and agricultural runoffs (Vymazal, 2011; Mitsch and Gosselink, 2015). Based on hydrology, constructed wetlands are categorized into surface-flow (SF) and subsurface-flow (SSF) constructed wetlands (CW). The SF constructed wetlands are open water systems, whereas no water is exposed in the SSF systems (Rodgers and Castle, 2008). In general, the SF systems are subject to freezing in winter and exposure to contaminants and mosquitos, but have lower maintenance costs over the SSF systems due to reduced clogging (Kadlec, 2009). Compared to other wastewater treatment technologies, the advantages of utilizing treatment wetlands are low costs for construction, operation and maintenance and flexibility in design (Rodgers and Castle, 2008). It has been estimated that wetland systems would only require 20% of the electrical energy of conventional wastewater treatment systems and save more than 60% of total capital and operation expenditures over a 20-year timeframe (Nelson et al., 2001).

Excess loading of nutrients in aquatic ecosystems, e.g. nitrogen and phosphorus, has caused eutrophication, leading to ecological threats and public health problems
(Conley et al., 2009). Mitsch et al. (2001) and Mitsch and Day (2006) proposed ecological restoration of wetlands along the Mississippi-Ohio-Missouri (MOM) Basin to alleviate the hypoxia in the Gulf of Mexico due to an excess amount of nitrogen. In Lake Erie, to abate harmful algal bloom issues, wetlands are suggested as one of the nutrient management practices to intercept phosphorus runoff from agricultural fields (Ohio EPA, 2010; Mitsch, 2014). Wetlands' ability to retain nutrients for water purification depends on the biogeochemistry cycles of each element. In the nitrogen cycles, organic nitrogen is converted to ammonia nitrogen via ammonification. Nitrification transforms ammonia nitrogen to nitrate, whereas nitrate is further reduced to nitrite and nitrogen gas via denitrification. Phosphorus is retained in the wetlands by plant assimilation and sedimentation (Kadlec and Wallace, 2009).

When compared to nutrients, the performance of wetlands on pathogen reduction has been less studied. Specifically, most of these studies used fecal indicator bacteria, such as total coliforms, fecal coliforms, and E. coli as surrogates for enteric pathogens (Sleytr et al., 2007; Chang et al., 2010). In a constructed wetland receiving septic effluent in Florida, the degree of reduction for fecal coliforms and E. coli was 98.93% and 99.92%, respectively (Chang et al., 2010). In a constructed wetland receiving pretreated municipal wastewater in Austria, removal rates (log reductions) were 4.35 for E. coli, 4.31 for total coliforms, and 4.80 for Enterococci (Sleytr et al., 2007). A number of other studies have investigated the fate of specific pathogens in treatment wetlands (Thurston et al., 2001; Mollenda et al., 2008; Song et al., 2008). In Arizona, reduction rates in a constructed wetland receiving secondary sewage effluent were 95.2% for coliphage, 87.8% for Giadia cysts, and 64.2% for Cryptosporidium oocysts (Thurston et al., 2001). In
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Several variables are associated with pathogen reductions in wetlands, including: temperature, dissolved oxygen, solar radiation, hydraulic retention time, turbidity, and presence of waterfowl and plants (McFeters and Stuart, 1972; Curtis et al., 1992; Brix, 1997; Craig et al., 2004; Toet et al., 2005; Kadlec and Wallace, 2009). Die-off rates of coliform bacteria increased significantly from 5°C to 25°C (McFeters and Stuart, 1972). Higher levels of dissolved oxygen (DO) had negative effects on E. coli survival in the presence of light (Curtis et al., 1992); sunlight accounted for 75% of the inactivation of E. coli (Craig et al., 2004). An increase in hydraulic retention time also enhanced sediment deposition and UV inactivation of pathogens (Toet et al., 2005). In opposition to these factors associated with reduction of indicator bacteria, an increase of turbidity resulting from resuspension of sediment, as well as presence of waterfowl, could lead to higher concentrations of indicator pathogen species (Craig et al., 2004 and Kadlec and Wallace, 2009). Plant growth on constructed wetlands showed improvement in pathogen reduction, as the presence of plants reduced the velocity of water and increased the surface area for attachment and die-off of pathogens (Brix, 1997). It was also suggested that more dissolved oxygen was released from plants in a constructed wetland that received tertiary
sewage (Toet et al., 2005). In addition, root systems in wetlands produce antibiotics, inhibiting certain indicator and pathogenic bacteria, such as coliforms, *Salmonella* and *Enterococci* (Brix, 1997).

1.3 Wetland loss and protection

Both human activities and natural events lead to wetland losses and degradation. Human activities include agricultural drainage, stream channelization, filling for development, conversion to aquaculture, and levee construction as well as mining of peat; while natural events consist of erosion, hurricanes, drought, and sea-level rise (Mitsch and Gosselink, 2015). In the early 20th century, federal policy actually encouraged land drainage for agricultural usage and flood control. Various federal programs had been established to help landowners by providing technical assistance and financial incentives (USGS, 1996). From the mid-1950s to the mid-1970s, it was estimated that total loss of freshwater vegetated wetlands was 0.6 million hectares in the U.S., with 80% attributed to agricultural land use (Office of Technology Assessment, 1984). Overall, it has been estimated more than 50% of original wetlands was lost in the conterminous 48 states from 1780s to 1980s. In fact, Ohio and California lost 90% or more of their original wetlands (Dahl, 1990). Development of public awareness of wetlands' ecosystem service, decrease in the profitability of conversion to agricultural fields, and implementation of wetland protection regulations have recently led to reduced wetland loss rates (Heimlich and Melanson, 1995). Average annual wetland loss in the conterminous U.S. has dropped
from 185,300 hectares between 1950's and 1970's, to 5,600 hectares between 2004 and 2009 (Frayer et al., 1983; Dahl, 2011).

Although the US has no specific national wetland law, several federal statutes have been implemented to protect wetlands, such as the Clean Water Act (CWA). The CWA Section 404 requires that anyone who dredges or fills in "waters of the United States," including wetlands, must obtain a permit from the U.S. Army Corps of Engineers (Mitsch and Gosselink, 2015). The Conservation Foundation's National Wetland Policy Forum (1988) recommended the concept that the country's remaining wetlands must achieve no overall net loss in terms of acreage and function. In the early 21th century, two U.S. Supreme Court decisions have limited federal authority, while expanding wetland protection regulations at state and local levels, such as establishing state wetland permitting programs (BenDor et al., 2008). Under the no-net-loss policy, as well as dredge-and-fill programs, various wetland mitigation practices have been conducted to recreate and restore wetland resources to replace those being degraded or lost during development (BenDor et al., 2008; Mitsch and Gosselink, 2015).

1.4 Waterborne outbreaks and pathogens

During 2007-2008, approximately 14,000 cases of waterborne diseases associated with recreational water in the US were identified (CDC, 2011). The predominant disease was acute gastrointestinal illness (AGI), comprising nearly 90% of total cases. The transmission routes consisted of ingestion (60.4%), direct contact (18.7%), and inhalation
(13.4%). Among the disease-causing agents, more than 70% of the cases were caused by microbial pathogens with bacteria etiological agents, causing a significant amount of the illnesses (16.5%), including *Escherichia coli* O157:H7, and *Shigella* spp. In 2009-2010, *Campylobacter jejuni* was identified as another bacterial etiological agent causing waterborne outbreaks (CDC, 2014).

*E. coli* O157:H7 causes more than 70,000 cases of gastrointestinal disease each year in the US (Smith et al., 2009). From 1986-2000, Shiga toxin-producing bacteria, including *E. coli* O157:H7 accounted for nearly 15% of all waterborne outbreaks associated with US recreational waters (Arnone and Walling, 2007). Typical symptoms include abdominal cramps, bloody diarrhea, hemolytic uremic syndrome (HUS), and acute renal failure. *E. coli* O157:H7, as well as other non-O157 serotypes, belong to Shiga toxin-producing *E. coli* (STEC) that cause illness by producing Shiga toxins (Arnone and Walling, 2007). Ruminants are effective reservoirs of STEC and are responsible for dissemination into food animals, dairy products and water supplies, thus threatening human health (Callaway et al., 2013; Farrokh et al., 2013).

In the western world, *Campylobacter* is the most common bacterial agent causing gastroenteritis. From 1997 - 2008, *Campylobacter* were responsible for more than one-third of total waterborne disease cases in the USA (Taylor et al., 2012). The typical symptoms are diarrhea and abdominal cramps (Hunter, 1997). Unlike STEC, the virulence mechanisms, which are associated with colonization of the small intestine and cytotoxin production, are less characterized (Guerry et al., 2007; Asakura et al., 2008).
Broilers and cattle have been identified as the main reservoirs of *Campylobacter* (Atabai and Corry, 1998; EFSA, 2010).

A *Campylobacter*-like emerging enteric pathogen, *Arcobacter*, has been linked to several cases of gastrointestinal illnesses (Fong et al., 2007; Lappi et al., 2013). *A. butzleri* shares similar clinical features with *Campylobacter* and is the fourth most common *Campylobacter*-like species (Vandenberg et al., 2004). The main symptom of *A. butzleri* infection is persistent watery diarrhea (Vandenberg et al., 2004). *Arcobacter* has been detected in a variety of food animals, food-processing environments, food matrices, and water bodies worldwide (Hsu and Lee, 2015).

Due to the pervasive utilization of antimicrobials for human, veterinary and agricultural practices, antimicrobial resistant bacteria and genes have been detected in a wide range of environments, including sewage, wastewater treatment plant outfalls, hospital effluents, urban runoffs, and ponds (Hamelin et al., 2007; Ibekwe et al., 2011; Pruden et al., 2012; Marion et al., 2015; Rodriguez-Mozaz et al., 2015). Out of 131 human *E. coli* O157 isolates recovered from several countries (including the U.S., Brazil, Argentina, Japan, and Saudi Arabia), 27.5% were identified as antimicrobial resistant, with one isolate showing resistance to five antimicrobials (Schroeder et al., 2002). In France, a longitudinal surveillance reported that 58% of *Campylobacter* isolates recovered from human sources were resistant to at least one antimicrobial (Gallay et al., 2007). Infections with resistant pathogens have been known to increase the mortality and morbidity of the diseases and the costs of health care (Williams, 2002). In Missouri, in
late 1989 to early 1990, in a waterborne outbreak, 4 died and 32 were hospitalized; the outbreak was caused by contamination of municipal water with multidrug-resistant *E. coli* O157:H7 (Swerdlow et al., 1992). It has been suggested that antimicrobial resistance (AMR) should be considered from an ecological perspective, because of the complex nature of the emergence and dissemination of the AMR (Salyers et al., 2002) and the need to apply multidisciplinary approaches to address this issue (Cantas et al., 2013).

**1.5 Public health risks from wildlife**

Wildlife carry a wide array of zoonotic pathogens that have the potential to be transmitted to humans, including viruses, bacteria, fungi and protozoa (Hubálek, 2004; Altizer et al., 2011; USEPA, 2011). Potential dissemination of zoonotic disease by migrating birds has been documented, including H5N1 avian influenza, West Nile virus, and Lyme disease as well as several enteric pathogenic bacteria (Abulreesh et al., 2007). Pathogenic *E. coli* serotypes have been isolated from Canada goose feces in urban areas (Kullas et al., 2002) and Shiga-toxin producing *E. coli* have been isolated from deer feces, containing virulent factors (Ishii et al., 2007). Deer feces, associated with strawberry consumption, have been linked to an *E. coli* O157:H7 outbreak, leading to a deadly hemolytic uremic syndrome in Oregon (Laidler et al., 2013).

These pathogenic microorganisms can contaminate water resources, thus increasing public health risks. Transport routes of pathogens include direct defecation from wildlife, mechanic transfer by wildlife (e.g., carried by birds as they wade in water
contaminated by sewage), resuspension of sediments with pathogens, and runoffs from contaminated areas (USEPA, 2011). To better identify potential fecal contamination from wildlife, microbial source tracking (MST) has been utilized for water quality analysis (Lee et al., 2013) and watershed management (Ervin et al., 2014; Wilkes et al. 2014). MST complements the limitation of fecal indicator bacteria in conventional water quality assessments and has been applied in total maximum daily load allocations (TMDLs) to better address watershed contamination issues (Arnone and Walling, 2007; Harwood et al., 2014).

1.6 Rationale

Ecosystem services provided by wetlands have been documented around the world (Mitsch and Gosselink, 2015). With regard to water purification, the ability of wetlands to reduce pathogens, compared to nutrients, has been less studied (Rea et al., 2015). Moreover, even fewer studies have been carried out to investigate the effects of wetlands on specific pathogens. Wetland-adjacent areas usually provide important water resources to humans (e.g. drinking or recreational waters). Given that bacterial pathogens are one of the agents causing waterborne outbreaks, it is important to better understand how wetlands could contribute to mitigating potential health risks as a sustainable alternative in protecting water resources.

Developing public awareness of wetland protection and changing federal policies have led to wetland creation and restoration (Dahl, 2011). As more wetlands are restored
and human population grows, wetlands will be the main interface where humans and wildlife come into contact (Cromie et al., 2012). However, little has been investigated with regard to the potential health risks from the wildlife in wetlands, given that wildlife could be a reservoir for pathogens. A balanced wetland management approach should include both ecosystem service and human health components (Millennium Ecosystem Assessment, 2005; Horwitz and Finlayson, 2011). From a public health perspective, it is critical to better understand how wildlife in wetlands could threaten public health.

1.7 Objectives

The main theme of this dissertation was to provide an overview of the ecosystem services of wetlands related to public health and the implications of fecal indicators and bacterial pathogens in wetlands.

Chapter 2 investigated created freshwater wetlands' ecosystem service in terms of reductions in fecal indicator bacteria and pathogens.

Aim 1: Determine if fecal indicator bacteria / pathogens are reduced across wetlands
Aim 2: Correlate environmental variables and reductions in fecal indicator and pathogens.

Chapter 3 estimated freshwater wetlands' ability in improving microbial water quality under climate change scenarios.

Aim 1: Simulate runoffs in urban watersheds.
Aim 2: Predict E. coli levels in urban watersheds.
Aim 3: Project the effects that created and restored wetlands would have in reducing *E. coli* in an urban watershed.

Chapter 4 assessed the roles of wildlife in pathogen dissemination in freshwater wetlands.
Aim 1: Assess the impact of wetlands on reducing wildlife pathogens
Aim 2: Quantify the amount of pathogens from goose feces in wetlands
Aim 3: Evaluate the diversity of Shiga toxin-producing *E. coli* in goose feces in wetlands.

Chapter 5 characterized antimicrobial resistance (AMR) in freshwater wetlands.
Aim 1: Investigate the distributions and potential sources of AMR genes in wetlands
Aim 2: Characterize the AMR patterns of *Arcobacter* isolates from wetland water.

Chapter 6 provided overall conclusions of this dissertation and implications and suggested future direction to fully understand the public health ecosystem services and public health risks of freshwater wetlands.

1.8 Study sites

This research selected two wetlands, the Olentangy River Wetland Research Park (ORWRP) in central Ohio and the Ottawa National Wildlife Refuge (ONWR) along Lake Erie in northern Ohio, as study sites due to their heavy influence by wildlife and proximity to human contact.
1.8.1 Olentangy River Wetland Research Park

The Olentangy River Wetland Research Park (ORWRP) is located in Columbus, Ohio (40.0204, -83.0186), receiving water from the Olentangy, which is heavily influenced by an adjacent urbanized area and upstream agricultural land. The park includes two 1-hectare experimental wetland basins. Wetland 1 (W1) was planted with native wetland species when first established in 1994, whereas wetland 2 (W2) was unplanted and allowed to colonize naturally. Many studies have documented vegetation and soil development, water quality changes (including physiochemical parameters), turbidity, nitrogen and phosphorus, and carbon and nitrogen dynamics (Mitsch et al., 1998, 2005, 2012, 2014a,b).

1.8.2 Ottawa National Wildlife Refuge

The Ottawa National Wildlife Refuge (ONWR) is at Oak Harbor, Ohio (41.6156, -83.2245), along the southwestern shore of Lake Erie. The ONWR has an area of 2700 hectares and provides protected habitat for important avian species in the Great Lakes region. Study sites were located in two areas, the Ottawa National Wildlife Refuge (ONWR) and the swimming beach of the adjacent Magee Marsh Wildlife Area (MMWA). The west and south sides of the OWNR are surrounded by an agricultural area in northwestern Ohio. Receiving water from nearby agricultural fields, Crank Creek flows through the ONWR and empties into Lake Erie (Rea et al., 2015).
Chapter 2

Fecal Indicator Bacteria / Pathogens Reductions in Freshwater Wetlands

Abstract

Wetlands can provide important ecosystem services, such as reductions in nutrients, and are routinely utilized to treat wastewater and stormwater. However, wetlands' impact on fecal indicator bacteria / pathogens remains less explored. The aims of this study were to determine if: (1) fecal indicator bacteria / pathogens are reduced across wetlands and (2) any correlations exist among environmental variables and fecal indicator bacteria / pathogens reductions. Fate of fecal indicator E. coli and other major and emerging disease-causing bacteria were investigated, including: Campylobacter, Shiga toxin-producing Escherichia coli (STEC), and Arcobacter. Wetland water samples were collected at inflow, midpoint, and outflow sites in two experimental wetlands at the Olentangy River Wetland Research Park at The Ohio State University (OSU) from June 2013 to June 2014. Water samples were concentrated and DNA was extracted for molecular analysis. Bacterial cultivation was conducted to quantify the amount of viable E. coli, while real-time polymerase chain reaction (PCR) was performed to determine the concentrations of pathogens. Nonparametric statistical analysis was performed to
compare the pathogen concentrations among various sites across the wetlands and establish relationships with environmental factors. *E. coli* had the highest occurrence in 99.2% samples, followed by *Arcobacter* (94.9%), STEC (74.6%) and *Campylobacter* (1.7%). Seventy nine percent of outflow samples were above the Ohio EPA water quality standard for primary contact water (298 CFU / 100 mL). Inflow *E. coli* concentrations were significantly associated with precipitation levels, turbidity, and inflow total phosphorus (*p* < 0.05). While significant reductions in turbidity and nutrients were found as the water moved through the wetlands, no significant differences were found in *E. coli* and *Arcobacter* between inflow and outflow. Overall, the reduction in *E. coli* was 22.3% from inflow to outflow. Seasonal analysis found winter had the most significant *E. coli* reductions (42.2%). Correlations among reductions in *E. coli*, *Arcobacter*, total phosphorus, and turbidity by the wetlands were established. This study demonstrated wetlands' ecosystem service in *E. coli* reductions, which could provide a sustainable way to abate bacterial contaminations in urban settings. Long-term surveillance of fecal indicator bacteria / pathogens and wildlife's role in pathogen dissemination are required to better understand the fates of pathogens in wetlands.

### 2.1 Introduction

Wetlands can provide extensive ecosystem services, such as wildlife habitat, biodiversity enhancement, flood prevention, water purification, and recreational and aesthetic values (Horwitz et al., 2012). Well-functioning wetlands provide beneficial
ecosystem service and improve human health status (Horwitz et al., 2012). For more fifty years, wetlands have been constructed worldwide to mimic natural wetlands for water quality improvement (Vymazal, 2011). Wetlands have been constructed to treat wastewater and stormwater (Mitsch and Gosselink, 2015). However, most of the studies used fecal indicator bacteria, such as total coliforms, fecal coliforms, and E. coli as surrogates for pathogens (Thurston et al., 2001; Molleda et al., 2008; Hathaway et al., 2009; Chang et al., 2011). Given the fact that each pathogen leads to different disease outcome when infected (Hunter, 1997; Vandenberg et al., 2004; Brooks et al., 2005), it is important to evaluate the fate of specific pathogens in wetlands.

The objective of this study was to determine if freshwater wetlands reduce levels of waterborne pathogens to improve water quality and protect public health in urban settings. Specific aims included (1) to determine if fecal indicator bacteria / pathogens are reduced across wetlands and (2) to identify any correlations among environmental variables and fecal indicator bacteria / pathogens reductions.

2.2 Materials and Methods

2.2.1 Sample collections and data acquisition

This study was conducted at the Olentangy River Wetland Research Park (ORWRP) in Columbus, Ohio, USA (40.0204, -83.0186). The park includes two 1-hectare experimental wetland basins. Wetland 1 (W1) was planted with native wetland
species when first established in 1994, whereas wetland 2 (W2) was unplanted and allowed to colonize naturally (Mitsch et al., 1998, 2005, 2012, 2014a,b). The water from the Olentangy River is continuously pumped into the wetlands, with rates adjusted based on river discharges. Water samples were collected in duplicate at the inflow, midpoint, and outflow of both wetlands every other week at approximately 11:00 am from June 23, 2013 to June 21, 2014 (Fig. 1) and filtered at lab. Water quality data, including water temperature, pH, dissolved oxygen, water conductivity, and reduction-oxidation potential, were acquired from the OWRRP on-site monitoring system every other week at approximately 11:00 am. Weather parameters, including air temperature, solar radiation and precipitation, were acquired from the Ohio Agricultural Research and Development Center Weather Station in Columbus, Ohio (40.0112, -83.0442). Sanitary sewer overflow data were retrieved from the City of Columbus, Sewer Overflow Discharge Information (http://eapp.columbus.gov/ssocso/). Turbidity, nitrate-nitrogen and total phosphorus concentrations for each water sample were determined in the Environmental Microbiology Laboratory, College of Public Health, at The Ohio State University. The turbidity of the samples was measured with a Hach turbidimeter (Hach, Loveland, Colorado). Nitrate-nitrogen and total phosphorus were measured with the Hach DR/2800 (Hach, Loveland, Colorado). The total phosphorus was determined using USEPA accepted PhosVer® 3 with Acid Persulfate Digestion Method (Hach Method 8190), with a detection range from 0.06 to 3.5 mg/L total phosphorus (Hach, Loveland, Colorado). The nitrate-nitrogen was determined using cadmium reduction method (Hach Method 8192), with a detection range from 0.01 to 0.50 mg/L NO₃⁻-N (Hach, Loveland, Colorado). Detailed protocols were shown at the DR 2800 Spectrophotometer Procedures
Manual (Hach, 2007a,b). Standard solutions (0.40 mg/L nitrate-nitrogen and 3.00 mg/L phosphate) were used to check the accuracy of the test procedures according to the Procedures Manual (Hach, 2007a,b).

2.2.2 Enumeration of *E. coli*

*E. coli* was used as fecal indicator bacteria (FIB) in this study. Collected water samples were filtered through a mixed cellulose ester membrane, with a pore size of 0.45 μm (Millipore, Billerica, MA). For *E. coli* enumeration, the membrane was placed on modified m-TEC agar (BD, San Jose, CA), preincubated at 35°C for 2 h, the plates then incubated at 44.5°C for 18-20 h, and the red / magenta colored *E. coli* colonies counted (USEPA, 2009).

2.2.3 Sample processing and DNA extraction

Water samples (100 mL) were filtered through a mixed cellulose ester membrane for DNA extraction (pore size 0.45 μm, Millipore, Billerica, MA). Total DNA was extracted from the filter membrane using PowerWater DNA Isolation Kits (MoBio, Carlsbad, California), with 100 μL of final eluate. Overall, 118 water DNA were used for molecular analysis.
2.2.4 Quantification of pathogens by real-time polymerase chain reaction (PCR)

The concentrations of pathogens markers (Table 1) were determined by real-time PCR which was conducted with a CFX96 C1000 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). All DNA extracts were diluted to one tenth of their original concentrations to minimize PCR inhibition and were subjected to real-time PCR in duplicate.

2.2.4.1 Real-time PCR analysis of Arcobacter

The PCR components for Arcobacter determination contained 1X of SYBR Green PCR Mastermix (Invitrogen, Grand Island, NY), 500 nM of Arcobacter 23S rRNA forward and reverse primers, 2.0 µL of DNA template (20 µL total volume). Thermal cycling conditions consisted of an initialization step at 95°C for 10 min, followed by 40 cycles of denaturation steps at 95°C for 15 sec, and annealing and extension steps at 60°C for 3 min. Melting curve analysis started with a denaturation step at 95°C for 15 sec, followed by an annealing and extension step at 60°C for 3 min, and ended at 95 for 15 sec, with a temperature increase rate of 1.0°C / min (Bastyns et al., 1995; Gonzalez et al., 2007).
2.2.4.2 Real-time PCR analysis of Campylobacter

The PCR components for *Campylobacter* determination contained 1X TaqMan Mastermix (Invitrogen, Grand Island, NY), 500 nM of *Campylobacter* 16S rRNA forward and reverse primers (Sigma), 250 nM of FAM-labeled probe (Eurofins, Huntsville, Alabama), and 2 μL of DNA template, with 20 μL in total. Thermal cycling conditions consisted of an initialization step at 50°C for 2 min and at 95°C for 10 min, followed by 50 cycles of denaturation step at 95°C for 15 sec, annealing step at 60°C for 30 s and extension step at 72°C for 90 s (Josefsen et al., 2004; Van Dyke et al., 2010).

2.2.4.3 Real-time PCR analysis of Shiga toxin-producing *E. coli*

The PCR components for Shiga toxin-producing *E. coli* determination contained 1X TaqMan Mastermix (Invitrogen, Grand Island, NY), 330 nM of stx2 forward and reverse primers (Sigma), 250 nM of JOE-labeled probe (Eurofins, Huntsville, Alabama), and 2 μL of DNA template (20 μL in total volume). Thermal cycling conditions consisted of an initialization step at 95°C for 5 min, followed by 45 cycles of denaturation step at 95°C for 15 sec, and annealing and extension steps at 60°C for 60 sec (Ibekwe et al., 2002; this study).
2.2.4.4 Generation of standard curves for molecular markers

To establish markers with known copy numbers, each target DNA fragment was amplified using corresponding forward and reverse primers (Table 1) in a MultiGene Gradient Thermal Cycler (Labnet, Edison, NJ). Amplified DNA was examined on agarose gel and purified using QIAquick PCR purification kits (Qiagen, Valencia, CA). The purified DNA was ligated into pGEM-T vector (Promega, Madison, WI) and transformed into E. coli DH5α competent cells by heat shock transformation. The transformed plasmids were extracted using QIAprep Spin miniprep kits (Qiagen, Valencia, CA). DNA concentrations were determined by a Qubit Fluorometer (Life Technologies, Grand Island, NY). Ten-fold serial dilutions of the transformed plasmids were prepared and applied to real-time PCR analysis. Standard curves of Ct values against known gene copy numbers of each genetic marker were established. Samples with detected markers below the detection limits were marked as DNQ (detected-but-not-quantifiable).

2.2.5 Statistical analysis

The following statistical analyses were performed with STATA 12 (StataCorp, College Station, TX). Nonparametric statistical treatments were utilized with relatively small sized samples (< 10). Mann-Whitney tests were applied to test the significance of reductions in turbidity, nutrients, E. coli and Arcobacter between inflow and outflow of
the two wetlands. Mann-Whitney tests were also used to test for significant differences between the two wetlands in terms of reductions from inflow to outflow in turbidity, nutrients, \textit{E. coli}, and \textit{Arcobacter}. Spearman's correlation analysis was performed to identify any significant relationships among \textit{E. coli}, \textit{Arcobacter}, turbidity, phosphorus and precipitation for inflow samples as well as overall reductions. To analyze \textit{Campylobacter} and STEC data, chi-square tests were applied to compare the occurrence across the wetlands, and logistic regressions were used to establish correlations among other bacteria, nutrients and precipitation.

2.3 Results

\subsection*{2.3.1 FIB / Pathogens in urban riverine wetlands}

From June 23, 2013 to June 21, 2014, 21 samples were collected in both experimental wetlands at the Olentangy River Wetland Research Park, yielding a total of 118 inflow, midpoint and outflow samples. Summarized descriptive statistics are shown in Table 2 for environmental variables and bacteria (Table 2). \textit{E. coli} had the highest occurrence of pathogens as a fecal indicator with a presence in 99.2\% of all samples and 33 out of 42 outflow samples (78.6\%) above the Ohio EPA Class A primary contact recreational standard of 298 CFU / 100 mL (USEPA, 2003). During the recreational season (May 1st to Oct. 31st), 24 out of 26 samples (92.3\%) were greater than this standard. A prevalence of \textit{Arcobacter} (94.9) was also detected, with a mean of $2.8 \times 10^4$ gene copies / mL. Lower detection frequencies of Shiga toxin-producing \textit{E. coli} (STEC)
and *Campylobacter* were observed (74.6% and 1.7%, respectively). Levels of STEC and *Campylobacter* were also lower compared to those of *Arcobacter* (mean $1.8 \times 10^2$ and $7.3 \times 10^{-2}$ gene copies / mL, respectively).

### 2.3.2 Correlations among FIB / Pathogens and environmental variables at wetland inflow

Precipitation was positively related to concentrations of *E. coli*, *Arcobacter*, and turbidity (Fig. 3) at inflow. Precipitation within 24hr was significantly correlated with inflow *E. coli* levels ($r = 0.447$, $p < 0.05$); whereas precipitation within 48hr was significantly associated with inflow *E. coli* levels and turbidity ($r = 0.659$ and 0.469, respectively, $p < 0.05$). Precipitation within 72 hr had significant correlations only with *E. coli* ($r = 0.442$, $p < 0.05$). Inflow *E. coli*, turbidity and phosphorus were significantly correlated with each other. Inflow *E. coli* levels were significantly associated with turbidity ($r = 0.787$, $p < 0.05$) and phosphorus ($r = 0.609$, $p < 0.05$) and inflow turbidity was significantly associated with phosphorus ($r = 0.708$). No significant difference ($p > 0.05$) in the inflow *E. coli* between when SSOs occurred within 7 days ($n = 7$) and when SSOs did not occur within 7 days upstream ($n = 14$) was found, indicating that upstream sanitary sewer overflow (SSO) events did not affect inflow *E. coli* concentrations.
2.3.3 Reductions in FIB / Pathogens across urban riverine wetlands

When analyzed in sum, turbidity was significantly reduced from inflow to outflow at the planted wetland (W1) by 45.3% \((p < 0.05)\). The level of reduction from inflow to outflow was not different in the naturally-colonized wetland (W2). The total phosphorus was significantly reduced from inflow to outflow in both wetlands by 28.0% \((p < 0.05)\). Spatial analysis found turbidity was significantly reduced from inflow to midpoint at both wetlands \((p < 0.05)\) (Table 3). Overall reduction in \(E. coli\) was 22.3% from inflow to outflow across both wetlands. No overall reductions in \(Arcobacter\) were observed \((-3.2\%)\). No significant reductions in either \(E. coli\) or \(Arcobacter\) were observed at various points at both wetlands \((p > 0.05)\) (Table 4). No significant reductions in STEC or \(Campylobacter\) were found. No significant differences in reductions in turbidity, nutrients, \(E. coli\) and \(Arcobacter\) were found between the W1 and W2 \((p > 0.05)\), thus, the two wetlands were treated as replicates for seasonal analysis. Seasonal analysis found winter (December 1, 2013 – March 8, 2014) had the most significant \(E. coli\) reductions \((42.2\%, p < 0.05)\) compared with other seasons (Fig. 2).

2.3.4 Correlations among reductions in FIB / Pathogens and environmental variables across urban riverine wetlands

Reductions in \(E. coli\) levels across the wetlands were significantly correlated with inflow \(E. coli\) concentrations, turbidity, and nutrients (Fig. 4). Positive correlations were
shown between reductions in *E. coli* from inflow to outflow and inflow *E. coli* ($r = 0.89$, $p < 0.05$), turbidity ($r = 0.67$, $p < 0.05$), nitrate-nitrogen ($r = 0.43$, $p = 0.05$), total phosphorus ($r = 0.64$, $p < 0.05$). Trends between times after precipitation and reductions in turbidity, nutrients and fecal indicator bacteria were also observed. Positive correlations of noted significance existed between precipitation within 24hr and *E. coli* reduction from inflow to outflow ($r = 0.54$, $p < 0.05$), precipitation within 24hr and phosphorus reduction from inflow to outflow ($r = 0.499$, $p < 0.05$), precipitation within 48hr and *E. coli* reduction from inflow to outflow ($r = 0.603$, $p < 0.05$), precipitation within 48hr and *Arcobacter* reduction from inflow to outflow ($r = 0.388$, $p = 0.082$), precipitation within 48hr and turbidity reduction from inflow to outflow ($r = 0.414$, $p = 0.062$) and precipitation within 48hr and phosphorus reduction from inflow to outflow ($r = 0.402$, $p = 0.064$).

### 2.4 Discussion

#### 2.4.1 Extreme precipitation and urban water quality

In urban areas, extreme precipitation can deteriorate river water quality, resulting in elevated levels of fecal indicator bacteria such as *E. coli*. This study showed that precipitation amounts were a significant factor affecting inflow *E. coli* concentrations from the Olentangy River. Other studies also confirmed the positive relationships between precipitation and *E. coli* concentrations. A model demonstrated a three-fold increase in *E. coli* when precipitation events occurred within 24 hr (Tornevi et al., 2014).
After heavy precipitation events, 10 to 1000-fold increases in fecal indicator bacteria and enteropathogens were found (Tryland et al., 2014). Antecedent precipitation was one of the significant predictor of *E. coli* in suburban watersheds (Chen and Chang, 2014). Similar to turbidity findings in this study, significant associations between *E. coli* and total suspended solids (TSS) were also established. Heavy rainfall events severely degrade water quality through combined sewer overflows (CSO) and sanitary sewer overflows (SSO) in urban areas. It has been reported that significantly higher *E. coli* concentrations were identified following CSO and SSO events than rainfall alone (McLellan et al., 2007). Although multiple SSO sites discharge upstream of the Olentangy River experimental wetlands, there was no significant difference in inflow *E. coli* concentrations whether or not SSO events occurred in this study. Possible explanation is that the sampling frequency in this study might not capture the impact. The sampling frequency in McLellan’s study was 10 surveys in 1 month when more frequent CSO and SSO events were observed. In addition, samples were collected near a harbor in Lake Michigan and its flow rate is expected to be lower compared to the Olentangy River. Thus, weaker dilution effects would maintain higher *E. coli* concentrations after CSO and SSO events in Lake Michigan.

### 2.4.2 Waterborne pathogens found in urban wetlands

Shiga toxin-producing *E. coli*, *Campylobacter* and *Arcobacter* were all detected at the Olentangy River experimental wetlands, with detection rates of 74.6%, 1.7% and
94.9% across all sampling sites, respectively, during the whole sampling period. These pathogens are known for causing foodborne and waterborne outbreaks and thus pose public health risks. From 1986-2000, Shiga toxin-producing bacteria, including *E. coli* O121:H9 and *E. coli* O157:H7, accounted for nearly 15% of all waterborne outbreaks associated with US recreational waters (Arnone and Walling, 2007). From 1997 - 2008, *Campylobacter* were responsible for more than one-third of total waterborne disease cases in the USA (Taylor et al., 2012). More harsh conditions in the surface water, such as temperature variation and UV exposure, may explain why less outbreaks and low detection rates of *Campylobacter* at the Olentangy River experimental wetlands were found in the surface water (Taylor et al., 2012; Whiley et al., 2013). Although not confirmed as a direct etiological agent, *Arcobacter* was linked to a waterborne outbreak in Lake Erie, causing nearly 1500 cases of gastroenteritis (Fong et al., 2007). No cases of waterborne outbreaks have been reported near wetlands that are proximate to populated urban areas. However, it is important to monitor the levels of pathogens and trace the possible sources for management of potential waterborne outbreaks in all bodies of water.

2.4.3 Reductions of fecal indicator bacteria in urban wetlands

The experimental wetlands on the Olentangy River did not provide significant reductions in *E. coli* across the wetlands in a central Ohio urban area. Factors that were correlated with *E. coli* reductions from inflow to outflow in the wetlands included reductions in turbidity, reductions in nutrients and inflow *E. coli*. Past studies have found
varied *E. coli* reduction rates, ranging from 17% to 80%. In an earlier study in the same experimental wetlands in central Ohio, an overall reduction of 17% in *E. coli* from inflow to outflow was reported for 2009 using the Most Probable Number (MPN) method (Young, 2009). Turbidity reductions were significantly correlated with *E. coli* and total phosphorus reductions, indicating that particle settling contributes to removal of *E. coli* and nutrients in wetlands. The *E. coli* reductions at the Olentangy River experimental wetlands were lower than those reported for some stormwater wetlands.

A 64.5% average reduction from influent to effluent samples of *E. coli* numbers was found for two stormwater best management practices (BMP) wetlands in North Carolina, with one wetland showing a significant reduction (*p < 0.05*) (Hathaway et al., 2009). In another constructed stormwater wetland in the North Carolina coastal plain, the reduction was 59% from inlet to outlet samples for *E. coli* (Humphrey et al., 2014). In South Korea, the removal efficiency was 40% - 80% as a part of a series of stormwater treatment facilities (Cheng et al., 2013). In the two Olentangy River experimental wetlands, 78.6% of the samples were above the Ohio EPA Class A primary contact recreational standard (298 CFU / 100 mL) for outflow *E. coli* levels and even a higher percentage of outflow samples (92.3%) were above the standard for recreational seasons (May 1st to Oct. 31st). During wet seasons (when rainfall amount within 48 hours were above 0.48 mm), all the samples had *E. coli* levels above the standard, although significant reductions from inflow to outflow were observed. Other studies also demonstrated high rates of violation of the regulatory standard. It was reported that 50%-90% of the effluent samples were above the EPA full body contact recreational standard.
(126 CFU / 100 mL) in stormwater BMP wetlands in North Carolina (Hathaway et al., 2009).

2.4.4 Study limitations

At the Olentangy River Wetland Research Park, a significant amount of literature has been published on wetland vegetation and soil development, on water quality changes including physiochemical parameters, turbidity, nitrogen and phosphorus, and on carbon and nitrogen dynamics (Mitsch et al., 2012, 2014a,b). However, little research has been conducted regarding water quality changes in terms of E. coli or pathogens, with only one master’s thesis completed (Young, 2009). A major limitation of that study was that only one year of sampling was conducted. In that study at the same experimental wetlands at the Olentangy River Wetland Research Park, similar conclusions have been made that E. coli reductions from inflow to outflow were associated with inflow E. coli as well as turbidity reductions from inflow to outflow. However, E. coli reductions were higher in the spring, which differs from what was observed in this study (winter). Thus, it is important to gather long-term data to clarify the wetlands' response to pathogens of public health concern more comprehensively, especially under conditions of extreme weather event. In addition, wildlife's roles in pathogen dissemination in urban wetlands need to be further explored since wetlands often attract large population of wildlife. Nevertheless, wetlands have shown a potential for providing a sustainable way to improve water quality and protect public health in urban settings.
2.5 Summary

In this chapter, the ecosystem services of urban freshwater wetlands in fecal indicator bacteria / pathogen reductions were determined. Findings included,

(1) Highest occurrence was found in *E. coli* (99.2%), followed by *Arcobacter* (94.9%), STEC (74.6%) and *Campylobacter* (1.7%).

(2) Inflow *E. coli* concentrations were significantly associated with precipitation levels, turbidity, and inflow total phosphorus (*p* < 0.05).

(3) Overall, the reduction of 22.3% in *E. coli* from wetland inflow to outflow was not significant (*p* > 0.05) compared to significant turbidity and nutrients reductions.

(4) Winter caused the most significant reductions in *E. coli* compared with other seasons.

(5) Correlations among reductions in *E. coli*, *Arcobacter*, total phosphorus, turbidity by the wetlands were established.

This study showed wetlands could be used as sustainable ways to alleviate bacterial contaminations in urban settings. More long-term data on dynamics of fecal indicator bacteria / pathogens and wildlife's roles in pathogen transmission are needed to better understand pathogen reductions in wetlands.
Chapter 3

Modeling of E. coli Reductions by Urban Wetlands under Climate Change Conditions

Abstract

Climate models have predicted more intense rainfalls in the future. As urban areas expand, higher impervious areas would likely lead to more surface runoffs. Urban runoff is known to contain pathogenic microorganisms, causing potential health concerns. Wetlands have been successfully employed as one of best management practices (BMP) to abate bacterial contamination. This study developed a simple and empirical model to predict the future response of E. coli in an urban river to precipitation and wetlands' ecosystem service in E. coli reductions under climate change scenarios. Three specific aims include: (1) to model runoff in urban watersheds; (2) to estimate E. coli levels in urban watersheds; and (3) to project wetlands’ ecosystem service in E. coli reductions. US EPA's Stormwater Management Model (SWMM) simulated surface runoff from precipitation. Predicting E. coli levels was performed though linear regression against simulated runoffs. E. coli load duration curves were analyzed to compare E. coli loads
between inflow and outflow under various hydrologic conditions. Future predictions were
categorized into near (2020-2050) and far (2045-2074) terms. In the urban watershed,
increasing trends of monthly mean runoffs were estimated for both near and far terms. *E.
coli* levels were also predicted to be increased for both terms. However, *E. coli* loads
were retained by wetlands under moist hydrologic conditions. This model showed poor
agreement between observed and simulated values, which could result from a short *E.
coli* sampling period and uncertainty. Model performance could also be improved by
considering other aspects of the watershed hydrology (e.g., groundwater and
evapotranspiration). Nevertheless, this model presents simple ways to estimate bacterial
water quality and wetlands' ecosystem service in *E. coli* reduction under climate change.

### 3.1 Introduction

Due to urban development with increased impervious areas, more surface runoff
contributes to deteriorated water quality, posing public health risks to urban residents in
close proximity to urban waters (Arnold and Gibbons, 1996; Gaffield et al., 2003; Arnone
and Walling, 2007). Bacterial, viral and protozoan pathogens all have been detected in
urban runoff (Pitt et al., 1996). Fecal indicator bacteria, such as *E. coli* and total coliforms,
are used as surrogates for the potential existence of these pathogenic microorganisms
(Kadlec and Wallace, 2009). In Long Island Sound, 47.3% of the annual fecal coliform
load was attributed to urban runoff (USEPA, 1994). Rainfall-induced microbial contamination
due to stormwater runoff has been documented (Kleinheinz et al., 2009). At one beach in Wisconsin, samples collected from stormwater outfall during rain events
had significantly higher *E. coli* levels compared to mean seasonal *E. coli* concentration (Kleinheinz et al., 2009).

Climate models suggested an increase in precipitation intensity, especially in mid to high latitudes, including Ohio (Patz et al., 2008; Union of Concerned Scientists, 2009). Under the higher-emission scenario, Cincinnati is expected to increase heavy rainfalls (> 2 inches of rain within 24 hours) by 30% in the following few decades and experience more than twice in frequency of heavy precipitations by the end of the century (Union of Concerned Scientists, 2009). Seasonal analysis demonstrated that more rain will be anticipated in spring, fall, and winter by the end of the century under the higher-emission scenario. However, summer is projected to reduce precipitation by 5% due to elevated evapotranspiration as a result of higher temperature (Union of Concerned Scientists, 2009). More heavy rainfall is expected to bring more surface runoff and wash more bacterial contaminants into waterways, particularly in urban areas with many impervious surfaces (Schiff et al., 2003). Among the 548 reported outbreaks analyzed from 1948 through 1994, 68% were preceded by extreme amounts of precipitation and 20% among the highest amounts of rainfall (Curriero et al., 2001).

Watershed management programs have adopted best management practices (BMP) to treat urban runoff as at a lower cost compared to conventional wastewater treatment plants (Gaffield et al., 2003). Wetlands have been included as one of the BMP to treat fecal indicator bacteria (Hathaway et al., 2009; Humphrey et al., 2014; Leisenring et al., 2014). Two stormwater best management practices (BMP) wetlands in North Carolina showed an average of 64.5% reductions in *E. coli* from influent to effluent.
(Hathaway et al., 2009). Another stormwater BMP wetland in the North Carolina coastal plain showed 59% of reduction from inlet to outlet for *E. coli* (Humphrey et al., 2014). A BMP database summary report demonstrated significant decreases in Enterococcus, *E. coli*, and fecal coliforms in wetland basins from influent to effluent (Leisenring et al., 2014). Hydraulic conditions, plant growth, and physiochemical parameters (temperature, dissolved oxygen, solar radiation) all contributed to pathogen reductions in wetlands (McFeters and Stuart, 1972; Curtis et al., 1992; Brix, 1997; Craig et al., 2004; Toet et al., 2005; Kadlec and Wallace, 2009).

Watershed models are useful when estimating urban runoffs and bacterial loadings from non-point pollution sources (e.g., urban stormwater runoff) because it would be impractical to measure flows and bacterial loadings from all of the watersheds (Ackerman and Schiff, 2001a,b). First developed in 1971, EPA's Stormwater Management Model (SWMM) is a continuous rainfall-runoff simulation model for mainly urban areas (USEPA, 2010). The SWMM divides a whole study area into multiple subcatchments and simulates runoff based on individual subcatchment's response to rainfall (USEPA, 2010). The SWMM has been utilized to model runoff for a number of urban watersheds (Barco et al., 2008; Beling et al., 2011; Liu et al., 2012; Maryland Department of the Environment, n.d.). Bacterial modeling is critical when developing Total Maximum Daily Load (TMDL) to address bacterial impairment of streams (Benham et al., 2006). Hydrological Simulation Program-FORTRAN (HSPF) and Soil and Water Assessment Tool (SWAT) are two commonly used models to simulate the fate and transport of bacteria at a watershed scale (Bicknell et al., 1997; Sadeghi and Arnold, 2002). The load duration curve analysis provides an empirical
presentation of bacterial water quality under various hydrologic conditions at specific stream sites (Benham et al., 2006).

The objective of this study is to evaluate freshwater wetlands' ecosystem service in improving bacterial water quality under climate change scenarios. The three specific aims were: (1) to simulate runoff in urban watersheds; (2) to predict E. coli levels in urban watersheds; and (3) to project the effects that created and restored wetlands would have in reducing E. coli in an urban watershed.

3.2 Methods

3.2.1 Stormwater management model

The Stormwater management model (SWMM) version 5.1 (USEPA, 2015) was used to simulate runoff from rainfall data. The overall Olentangy River watershed was divided into subwatersheds based on HUCs (Hydrologic unit codes). The area and impervious area for each subwatershed were estimated from National Land Cover Database. Delineation of subwatersheds and estimation of impervious area were performed on ArcMap 10.2.2 (ESRI, Redlands, CA) (Table 5). Other parameters for each subwatershed were estimated from survey documents (slope) and SWMM User Manual reference tables (Manning's number and depth of depression storage).

Historical rainfall data were input to the SWMM to estimate runoff volume. Rainfall data were retrieved from the Ohio State University Airport weather station (-
83.0762, 40.0790). Predicted runoff from the SWMM model was compared with field runoff data that were estimated using automated base flow separation and recession analysis (Arnold et al., 1995; Arnold and Allen, 1999). The program separated baseflow from river flow data, which was collected from USGS Worthington station (Site ID: 3226800) and multiplied by the watershed ratio (1.19) between Worthington and Olentangy River experimental wetlands. Calibration and validation periods were from Jun. 23, 2009 to Jun. 22, 2013 and from Jun. 22, 2013 to -Jun. 21, 2014, respectively. Predicted runoff levels by SWMM were compared with the estimated values by automated base flow separation and recession analysis for model evaluation. Evaluation criteria are discussed in 3.2.4.

Future precipitation data were adjusted into the SWMM to predict future runoff patterns. The Stormwater Management Model- Climate Adjustment Tool (SWMM-CAT) estimated future rainfall based on the climate model, World Climate Research Programme (WCRP) Coupled Model Intercomparison Project (CMIP5). Future prediction of runoffs was categorized into near-term (2020-2049) and far-term (2045-2074).

3.2.2 Runoff-E. coli model

A linear calibration curve was generated to predict E. coli from SWMM simulated runoff. Calibration and validation period was from Jun. 22, 2013 to -Jun. 21, 2014. Data were log-transformed to increase normality. Predicted E. coli levels by the calibration
curve were compared with the observed *E. coli* values for model evaluation. Evaluation criteria are discussed in 3.2.4. Future prediction of *E. coli* was categorized into near-term (2020-2049) and far-term (2045-2074).

### 3.2.3 *E. coli* load curve analysis

Reductions of *E. coli* by wetlands were assessed by load curve analysis. Hydrologic condition was expressed as percent exceedance (%), which was shown from 0 to 100%, with 0% as the lowest flow and 100% as the highest flow of the observed values (USEPA, 2007). Based on the percent exceedance, 5 groups of hydrologic conditions were indicated: high flow (0-9%), moist conditions (10-39%), mid-range conditions (40-59%), dry conditions (60-89%), and low flow (90-100%). *E. coli* loads at each hydrologic condition were calculated by representative observed river flow multiplied by observed *E. coli* levels during the sampling period (Jun. 23, 2013 to Jun. 21, 2014). The observed loads were compared with the target load, which was calculated by representative observed river flow at each hydrologic condition multiplied by 298 CFU / 100 mL (Ohio EPA water quality standard for primary contact water), to derive load reduction (%). Wetlands' performance on *E. coli* reduction was expressed as percent improvement between outflow *E. coli* load reduction and inflow *E. coli* load reduction.

Future prediction of inflow *E. coli* loads was calculated by monthly simulated river flow multiplied by modeled *E. coli* levels from Section 3.2.2. Future monthly river flow was simulated from a linear calibration curve of the observed stream flow against
the estimated runoff by automated base flow separation and recession analysis. Prediction of *E. coli* loads was categorized into near-term (2020-2049) and far-term (2045-2074). Prediction of outflow *E. coli* loads at each corresponding hydrologic condition was adjusted with the ratio from Table 6.

### 3.2.4. Model evaluation

Coefficient of determination ($R^2$), Nash-Sutcliffe efficiency (NSE) and percent bias (PBIAS) were used to evaluate model performance. Coefficient of determination represents the degree of variance in observed data explained by the model, with 1.0 being the perfect model accounting for all the variance in the measured data (Rosner, 2010). Nash-Sutcliffe efficiency compares the residual variance to the measured data variance, with 1.0 being the ideal model (Nash and Sutcliffe, 1970). Model performance was categorized as excellent ($R^2$, NSE $\geq$ 0.90), very good ($R^2$, NSE = 0.75-0.89), good ($R^2$, NSE = 0.50-0.74), fair ($R^2$, NSE = 0.25-0.49), poor ($R^2$, NSE = 0-0.24) and unsatisfactory ($R^2$, NSE < 0.0) (Moriasi et al., 2007; Parajuli et al., 2007; Borel et al., 2012). Percent bias determines the tendency as to whether simulated data are larger or smaller than the observed data (Gupta et al., 1999). Model evaluation for streamflow was categorized as very good (PBIAS $<\pm10$), good ($\pm10 < \text{PBIAS} < \pm15$), satisfactory ($\pm15 < \text{PBIAS} < \pm25$), and unsatisfactory ($\text{PBIAS} \geq \pm25$). Model evaluation for sediment was categorized as very good (PBIAS $<\pm15$), good ($\pm15 < \text{PBIAS} < \pm30$), satisfactory ($\pm30 < \text{PBIAS} < \pm55$), and unsatisfactory ($\text{PBIAS} \geq \pm55$) (Moriasi et al., 2007). Same criteria
were applied to the *E. coli* model because high correlations between turbidity and *E. coli* were found in Chapter 2.

### 3.3 Results

#### 3.3.1 Predicting runoffs in urban watersheds under climate change

The overall Olentangy River watershed was divided into 17 subwatersheds, based on HUCs (Hydrologic unit codes) for the Stormwater Management Model (Fig. 5a). The area and impervious area for each subwatershed are shown in Fig. 5b. Other parameters for each subwatershed are shown in Fig. 5c and Table 5 (FLOW, 2003 and EPA, 2010). Statistical analysis demonstrated that the runoff modeled by SWMM was significantly correlated with the estimated runoff for the calibration period from Jun. 23, 2009 to Jun. 22, 2013 (*r* = 0.3544, *p* < 0.05) (Fig. 6a,b). A poor model performance was observed (*R*^2^ = 0.13, NSE = 0.08), although the percent bias (PBIAS) was satisfactory (-24.88). A significant correlation between the runoff modeled by SWMM and the estimated runoff for the validation period from Jun. 22, 2013 to Jun. 21, 2014 was also found (*r* = 0.3029, *p* < 0.05) (Fig. 7a,b). A poor model performance was seen (*R*^2^ = 0.09, NSE = 0.09), although the percent bias (PBIAS) was satisfactory (23.30). Monthly mean runoff was predicted to be increased by 6.3% and 10.9% for both near (2020-2050) and far (2045-2074) terms, respectively (Fig. 8).
3.3.2 Predicting E. coli levels in urban watersheds under climate change

A simple regression curve was generated to predict *E. coli* from SWMM simulated runoff for the calibration period from Jun. 23, 2013 to Jun. 21, 2014 (Fig. 9a). Predicted *E. coli* levels by the calibration curve were compared with the observed *E. coli* values (Fig. 9b), and statistical analysis demonstrated that both values were significantly correlated (*r* = 0.63, *p* < 0.05). A poor to fair model performance was observed (*R²* = 0.39, NSE = 0.11), although the percent bias (PBIAS) was satisfactory (35.72). Monthly mean *E. coli* levels were predicted to be increased by 7.4% and 12.7% for both near (2020-2050) and far (2045-2074, respectively (Fig. 10). Predicted monthly *E. coli* levels were above the Ohio EPA Standard (298 CFU / 100 mL), except for February in current and far-term.

3.3.3 Assessing E. coli reductions in wetlands under climate change conditions

An empirical *E. coli* load duration curve analysis was conducted to estimate the *E. coli* load under difference hydrologic conditions (Table 6). The load reduction indicated how much *E. coli* reduction was needed to meet the Ohio EPA water quality standard for primary contact water (298 CFU / 100 mL). For the sampling period (Jun. 23, 2013 to Jun. 21, 2014), an overall improvement of 57.4% in *E. coli* load reduction by wetlands was observed. With regard to various hydrologic conditions, moist and dry conditions demonstrated positive improvement, whereas high flow, mid-range and low flow conditions showed negative improvement, indicating *E. coli* loads actually increased at
these specific hydrologic conditions. For both near-term (2020-2049) and far-term (2045-2074), monthly mean $E. \ coli$ loads in the wetland inflows were predicted to exceed the target loads. However, wetlands were projected to reduce $E. \ coli$ loads year-round, with mean loads in January, February, March, August, September, November and December below the target loads (Fig. 11). The results demonstrated effective ecosystem service in terms of $E. \ coli$ reductions provided by urban wetlands. Based on the average inflow and outflow $E. \ coli$ concentrations (2471 and 1344 CFU / 100 mL, respectively), mean flow rate (1.7 cubic meter per minute) under the moist hydrologic condition during the sampling period, and approximate area of the two Olentangy River experimental wetlands (2 ha), about 11,000 ha of wetlands in the Olentangy River watershed would need to be constructed or restored to achieve the $E. \ coli$ reductions as predicted by the load curve analysis.

### 3.4 Discussion

#### 3.4.1 Increasing surface runoff under climate change conditions

In this study, a simple and empirical model was developed to simulate surface runoff and $E. \ coli$ levels in an urban watershed under climate change. General increasing trends of surface runoffs and $E. \ coli$ levels were predicted. In the urban coastal Great Lakes area, it has been predicted that more high runoff events, while a less total storm runoff, would be observed for 2050 under either best or worst cases of global circulation model (GCM) predictions. Both annual mean total suspended solid (TSS) and fecal
coli form bacteria would also be increased (McLellan et al., 2013). In Australia, another study predicted similar results from 2021-2050: reduced average annual rainfall and runoff but higher extreme daily precipitation (Chiew et al., 2003). In New York City, increased flow exceedances, annual maximal peak discharges, and runoff volume were predicted under climate change using SWMM (Zahmatkesh et al., 2014). More extreme rainfall events would be expected to bring higher urban runoff and potentially overwhelm drinking water and wastewater treatment systems (Patz et al., 2008). More than 400,000 cases of acute gastrointestinal illness (AGI) were attributed to a drinking water treatment plant that was overwhelmed by a high turbidity load after a period of heavy precipitation in Milwaukee, Wisconsin, in 1993 (MacKenzie et al., 1994). In 2008, a waterborne outbreak with *Shigella sonnei*, *Cryptosporidium*, and *Giardia* infections was attributed to a large volume of storm water and diluted sewage released into a lake after heavy rainfall and flooding events in Illinois (CDC, 2011).

### 3.4.2. Wetlands and pathogen reduction

Reductions in indicator bacteria by wetlands have been documented (Hathaway et al., 2009; Humphrey et al., 2014; Leisenring et al., 2014). Other model studies demonstrated that *E. coli* effluent concentrations could be predicted by *E. coli* influent concentrations, solar radiation using the adaptive neuro-fuzzy inference systems (ANFIS) (Hamaamin et al., 2014), by initial *E. coli* concentrations and physical parameters of wetlands such as length, width, depth, and Manning’s roughness coefficient using the water quality analysis and simulation program (WASP) (Boutilier et al., 2011).
According to the *E. coli* load curve analysis, decreasing monthly mean *E. coli* loads from the inflow to outflow of the Olentangy River experimental wetlands were predicted for both near (2020-2049) and far-terms (2045-2074). Based on the current mean *E. coli* concentrations and flow conditions of the experimental wetlands, 11,000 ha of wetlands in the Olentangy River watershed would be required to be constructed or restored to achieve the *E. coli* reductions as predicted by the load curve analysis. The estimated area (11,000 ha) consists of about 8% of the whole Olentangy River watershed (131,000 ha). Research has shown that a larger wetland surface area to watershed area ratio of a wetland is more effective at nutrient and *E. coli* reductions (Line et al., 2008; Humphrey et al., 2014). Humphrey and others (2014) demonstrated a 7% of wetland surface area to drainage area contributed to nearly 60% of *E. coli* attenuation for a Best Mangement Practice (BMP) stormwater wetland. However, it is expected that less area of wetlands would be needed if they are placed in well-planned locations, i.e., near combined and sanitary sewer overflow outfalls, to intercept high level of bacterial contaminants and maximize treatment efficiency. It is also anticipated that less area would be required to meet the target loads based on Ohio EPA water quality standard for primary contact water (298 CFU / 100 mL) due to the difference in flows between the Olentangy River and the experimental wetlands if the wetlands were regarded as wastewater treatment facilities (Mancl, personal communication).

### 3.4.3. Model limitations
Lower model performance could be attributed to the assumptions and uncertainty associated with this study. Several assumptions were made throughout this modeling study. The SWMM model considered surface runoff as the only contribution to river flow, neglecting groundwater and evapotranspiration. However, other components also play significant roles in the water cycle. In fact, climate models for urban coastal Great Lakes area predicted an increase in potential evapotranspiration (PET) due to elevated air temperatures (McLellan et al., 2013). The current SWMM model did not consider the Delaware Dam and a number of low-head dams along the Olentangy River. These dams largely control the response of flows to rain events (Durand, personal communication) and thus influenced estimation of the surface runoff derived from automated base flow separation and recession analysis (Arnold et al., 1995; Arnold and Allen, 1999). In addition, the SWMM model also assumed that one-year bacterial data could be extrapolated to evaluate *E. coli* levels in response to surface runoff and wetlands' performance in *E. coli* reductions under future climate change scenarios. However, as urban sprawl in the Olentangy River watershed progresses, more impervious area is expected to increase surface runoff (Arnold and James, 1996) and produce different *E. coli* responses compared to what has been derived from the one-year *E. coli* data. Future climate change may lead to negative wetlands impact (e.g. wetland loss due to increased PET) (Mitsch and Gosselink, 2015) and thus may alter the status of wetlands' ecosystem service in pathogen reduction. Moreover, intrinsic uncertainty associated with field sample collection and examination have been documented, including streamflow measurement, sample collection, sample preservation / storage, and laboratory analysis (Harmel et al., 2006). Point measurements of rain gauges are typically associated with up
to 5% error of precipitation data (Shirmohammadi et al., 2006). These assumptions and uncertainty should be considered for future model design to build a more accurate model in this urban watershed. Bacterial models might have the highest errors and least confidence compared to the models with regard to sediments, nutrients, and surface hydrology (Novotny, 2003). Yet, this model presents simple ways to evaluate microbial water quality, how wetlands could contribute to *E. coli* reduction under climate change scenarios, and serve as a foundation to help future model design.

### 3.5 Summary

In this study, a simple, empirical model was developed to simulate surface runoff, *E. coli* levels in an urban watershed, and assess freshwater wetlands' ecosystem service in *E. coli* reductions under climate change. Findings included:

1. Monthly mean runoff in an urban watershed were predicted to be increased by 6.3% and 10.9% for both near (2020-2050) and far (2045-2074) terms, respectively;
2. Monthly mean *E. coli* levels in an urban watershed were predicted to be increased by 7.4% and 12.7% for both near (2020-2050) and far (2045-2074, respectively; and
3. Monthly mean *E. coli* loads in the wetland inflows were predicted to exceed the target loads for both near (2020-2050) and far (2045-2074) terms. However, when 11,000 ha of wetlands were projected be restored or created in strategic location in the watershed, to reduce *E. coli* loads under moist hydrologic conditions, with mean loads in January,
February, March, August, September, November and December compliant with each corresponding target load.

Poor agreement between observed and simulated values could be due to the short *E. coli* sampling period (1 year) and uncertainty. The rainfall-runoff-*E. coli* model could also be improved by considering other aspects of the watershed (e.g. groundwater and evapotranspiration). Nevertheless, this model presents a simple way to evaluate microbial water quality and how wetlands could contribute to *E. coli* reduction under climate change.
Chapter 4

Wildlife's Roles in Pathogen Dissemination in Freshwater Wetlands

Abstract

Various pathogenic microorganisms have been detected in wildlife. Wetlands are the interface where human and wildlife are continuously coming into contact; however, potential health risks from wildlife when humans are exposed to wetlands are less characterized. The objectives of this study were to (1) assess wildlife's impacts across wetlands and (2) evaluate the diversity of Shiga toxin gene detected in goose feces in wetlands. Water and Canada goose fecal samples were collected at the Olentangy River Wetland Research Park (ORWRP) and the Ottawa National Wildlife Refuge (ONWR) in Ohio, respectively. Microbial source tracking (human, avian and ruminant) as well as pathogen-specific markers (Campylobacter, Arcobacter, Shiga-toxin producing E. coli) were performed using real-time polymerase chain reaction (PCR) to evaluate wildlife's impacts. Denaturing gradient gel electrophoresis and genetic clustering analysis were conducted to assess the diversity of Shiga toxin gene (stx2) found in goose feces in wetlands. Microbial source tracking (MST) analysis demonstrated that human fecal markers decreased, while increasing trends of avian fecal contaminations were seen
from inflows to outflows at the ORWRP. Both avian and ruminant markers were correlated with *E. coli* and *Arcobacter* concentrations. The *stx*2 was detected in 93.2% of the goose feces collected at the ORWRP and 20.8% of those at the ONWR. Diversity analysis showed that the *stx*2 detected in goose feces collected at ONWR were associated with a more potent toxin type (*stx*2a) and various outbreak-causing strains (O157:H7, O165:H25 and O111:H-). This chapter clearly indicates public health risks when exposure to wetlands due to wildlife activity. Long-term monitoring and more rigorous surveillance of pathogenic microorganisms, in conjunction with additional wildlife management practices, are suggested to reduce health risks while maximize wetlands' ecosystem service in pathogen reductions.

### 4.1 Introduction

As an attractive habitat for wildlife, it is important to investigate the impact of wildlife and its public health risks in wetlands due to their ability to disseminate zoonotic pathogens, including avian influenza, Lyme diseases and West Nile virus (Abulreesh et al., 2007; Cromie et al., 2012). A variety of enteric pathogens have been isolated from wildlife, such as Shiga toxin-producing *E. coli* in Canada geese and deer (Kulas et al., 2002; Ishii et al., 2007) and *Campylobacter* in waterfowl (Fallacara et al., 2004). An *E. coli* O157:H7 outbreak in Oregon in 2011 was also attributed to strawberry consumption contaminated with deer feces, causing deadly hemolytic uremic syndrome (HUS) (Laidler et al., 2013).
Shiga toxin-producing *E. coli* (STEC) accounted for more than 110,000 cases of gastrointestinal illness in the U.S. each year (Mead et al. 1999). Shiga toxins are the main virulent factors of STEC, which are composed of two toxin types: Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2). It has been shown that Stx2 has higher toxicity and genetic variance than Stx1, with the Stx2a and Stx2d having higher potency than Stx2b and Stx2c (Fuller et al. 2011). Based on the diversity of the coding sequences, a subtyping method for stx2 has been developed that targets partial sequences of the stxAB2 operon. It was shown that Stx2 subtypes Stx2a and Stx2c are the primarily causes of HUS and bloody diarrhea among patients (Persson et al. 2007).

The most common bacteria-causing gastroenteritis is *Campylobacter* in the western world (Taylor et al., 2012). The virulence mechanisms include cytotoxin production and bacterial colonization of small intestine, but are not as well characterized as STEC (Guerry et al., 2007; Asakura et al., 2008). Close to *Campylobacter*, *Arcobacter butzleri* is the fourth most similar species with regard to clinical features (Vandenberg et al., 2004). The genus *Arcobacter* was detected in a wide range of environment (Hsu and Lee, 2015) and associated with foodborne and waterborne outbreaks (Fong et al., 2007; Lappi et al., 2013).

Microbial source tracking (MST) has been utilized to identify potential fecal contamination sources, including wildlife (Ervin et al., 2014; Wilkes et al. 2014). MST complements the limitation of using fecal indicator bacteria when the sources of contamination are difficult to track (Harwood et al., 2014). MST has been applied in the EPA's Total Maximum Daily Load (TMDL) programs to address bacterial impairment in
watersheds (Arnone and Walling, 2007). Various molecular markers have been developed and applied to identify fecal contaminations from wildlife, including an avian fecal marker targeting unclassified Helicobacter spp. (Green et al., 2012) and a ruminant marker fecal marker targeting ruminant-specific Bacteroidales (Mieszkin et al., 2009).

To better understand wildlife's roles in pathogen dissemination in freshwater wetlands, the aims of this study were to (1) assess wildlife's impacts across wetlands and (2) evaluate the diversity of Shiga toxin gene detected in goose feces in wetlands.

4.2 Materials and Methods

4.2.1 Site description and sample collection

Two sites were included in this study, the Olentangy River Wetlands Research Park (ORWRP) and the Ottawa National Wildlife Refuge (ONWR). The ORWRP includes two 1-hectare experimental wetland basins. Wetland 1 (W1) was planted with native wetland species when first established in 1994, whereas wetland 2 (W2) was unplanted and allowed to colonize naturally (Mitsch et al., 1998, 2005, 2012, 2014a,b). Water from the Olentangy River is continuously pumped into the wetlands, with rates adjusted based on river discharges. Water samples were collected in duplicate at the inflow, midpoint, and outflow of both wetlands every other week at approximately 11:00 am from June 23, 2013 to June 21, 2014 (Fig. 1). Goose fecal samples were collected on boardwalks at both wetlands every other week at approximately 11:00 am. Overall, 118
water and 44 goose fecal samples were subject to analysis. Samples collected from the ORWRP were used to assess wildlife's impacts across wetlands.

The ONWR provides protected habitat for avian species, including Canada geese. Study sites were located in two such areas, the ONWR and the swimming beach of the adjacent Magee Marsh Wildlife Area (MMWA), along the southwestern shore of Lake Erie at Oak Harbor, Ohio, USA. The west and south sides of the ONWR are surrounded by an agricultural area in northwestern Ohio. Sample collection was performed by Dr. Chris Rea (Rea et al., 2015). Water samples were collected at four locations along Crane Creek in the ONWR and at the MMWA swimming beach from May to December, 2012. Canada goose feces were randomly collected where fresh feces were identified throughout the ONWR. Both water and fecal samples were placed on ice and transported to the laboratory for further analysis. Overall, 72 water and 77 goose fecal samples were analysed. Samples collected from the ONWR were used to evaluate the diversity of Shiga toxin found in goose feces in wetlands.

4.2.2 DNA extraction

Total DNA of the water and goose fecal samples was extracted using MO BIO PowerWater DNA Isolation kits (Cat. No. 14900) and MO BIO PowerSoil DNA Isolation kits (Cat. No. 12888), respectively, per manufacturer’s instruction (MO BIO Laboratories, Inc., Carlsbad, CA, USA). The final eluate for each of the samples was 100 μL. The
concentrations of the DNA extracts were determined using a Qubit Fluorometer (Life Technologies, Grand Island, NY, USA). All DNA extracts were stored at -20°C.

4.2.3 Real-time PCR analysis of human MST marker

The PCR components for human MST marker (HF183, Table 7) determination contained 1X TaqMan Mastermix (Invitrogen, Grand Island, NY), 500 nM of HF183 forward and reverse primers (Sigma), 250 nM of FAM-labeled probe (Eurofins, Huntsville, Alabama), and 2 μL of DNA template in a total volume of 20 μL. Thermal cycling conditions consisted of an initialization step at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles of denaturation steps at 95°C for 15 sec, and annealing and extension steps at 60°C for 1 min (Bernhard et al., 2000; Converse et al., 2009; Haugland et al., 2010).

4.2.4 Real-time PCR analysis of avian MST marker

The PCR components for avian MST marker (GFD, Table 7) determination contained 1X SYBR Mastermix (Invitrogen, Grand Island, NY), 100 nM of GFD forward and reverse primers (Sigma), and 2 μL of DNA template, with a total volume of 20 μL. Thermal cycling conditions consisted of an initialization step at 95°C for 10 min, followed by 50 cycles of denaturation steps at 95°C for 15 sec, and annealing and extension steps at 60°C for 1 min. Melting curve analysis started with denaturation steps
at 95°C for 15 sec, followed by an annealing and extension step at 60°C for 1 min, and ended at 95°C for 15 sec, with an temperature increase rate of 0.3°C / min (Green et al., 2012).

4.2.5 Real-time PCR analysis of ruminant MST marker

The PCR components for ruminant MST marker (Rum2Bac, Table 7) determination contained 1X TaqMan Mastermix (Invitrogen, Grand Island, NY), 200 nM of Rum2Bac forward and reverse primers (Sigma), 200 nM of FAM-labeled probe (Eurofins, Huntsville, Alabama), and 2 μL of DNA template in a total volume of 20 μL. Thermal cycling conditions consisted of an initialization step at 95°C for 10 min, followed by 50 cycles of denaturation steps at 95°C for 15 sec, and annealing and extension steps at 60°C for 1 min (Mieszkin et al., 2009).

4.2.6 Real-time PCR analysis of Campylobacter marker

The PCR components for Campylobacter determination (Table 7) contained 1X TaqMan Mastermix (Invitrogen, Grand Island, NY), 500 nM of Campylobacter 16S rRNA forward and reverse primers (Sigma), 250 nM of FAM-labeled probe (Eurofins, Huntsville, Alabama), and 2 μL of DNA template, with 20 μL in total. Thermal cycling conditions consisted of an initialization step at 50°C for 2 min and at 95°C for 10 min, followed by 50 cycles of denaturation steps at 95°C for 15 sec, annealing steps at 60°C
for 30 sec and extension steps at 72°C for 90 sec (Josefsen et al., 2004; Van Dyke et al., 2010).

4.2.7 Real-time PCR analysis of Arcobacter marker

The PCR components for *Arcobacter* (Table 7) determination contained 1X of SYBR Green PCR Mastermix (Invitrogen, Grand Island, NY), 500 nM of *Arcobacter* 23S rRNA forward and reverse primers, 2.0 µL of DNA template (20 µL total volume). Thermal cycling conditions consisted of an initialization step at 95°C for 10 min, followed by 40 cycles of denaturation steps at 95°C for 15 sec, and annealing and extension steps at 60°C for 3 min. Melting curve analysis started with a denaturation step at 95°C for 15 sec, followed by an annealing and extension step at 60°C for 3 min, and ended at 95°C for 15 sec, with a temperature increase rate of 1.0°C / min (Bastyns et al., 1995; Gonzalez et al., 2007).

4.2.8 Detection of STEC marker (*stx2*)

The presence of *stx2* gene in each of the goose fecal samples was determined by PCR. The total volume of each PCR mixture was 20 µL, containing 2 µL DNA extract, 500 nM *stx2F* (forward) and *stx2R* (reverse) primers (Table 7), 2 µL Taq buffer (ThermoScientific Inc., Waltham, MA, USA), dNTP (ThermoScientific Inc., Waltham, MA, USA), magnesium chloride (ThermoScientific Inc., Waltham, MA, USA), 0.1 µL
Taq polymerase (ThermoScientific Inc., Waltham, MA, USA). Thermal cycling included an initialization at 94°C for 10 minutes, followed by 35 cycles of denaturalization steps at 94°C for 30 seconds, annealing steps at 50°C for 30 seconds, and extension steps at 72°C for 30 seconds. The final extension was 10 minutes at 72°C. The PCR products were examined by electrophoresis on 1.0% agarose gels. The presence of stx2 in each of the water samples was determined by real-time PCR using a StepOne Real-Time PCR system (Life Technologies, Grand Island, NY, USA). The total volume of the PCR mixture was 20 μL, containing 2 μL 1:10 diluted DNA extract, 333 nM stx2F (forward), stx2R2 (reverse) primers and 250 nM stx2P probe (Table 7), 10 μL TaqMan Universal PCR Mastermix (Applied Biosystems, Foster City, CA, USA). Thermal cycling included an initialization at 95°C for 5 minutes, followed by 45 cycles of denaturalization steps at 95°C for 15 seconds and annealing / extension steps at 60°C for 1 minute. Positive signal of amplification was considered presence of stx2 (Ibekwe et al. 2002).

4.2.9 Denaturing-gradient gel electrophoresis (DGGE)

Amplification of stx2 was conducted by seminested PCR. The total volume of the primary PCR mixture was 20 μL, containing 1 μL DNA extract, 500 nM stx2FD1 (forward) and stx2RD (reverse) primers (Table 7), 2 μL Taq buffer, 250 nM dNTP, 0.5U Taq polymerase. Thermal cycling included an initialization at 94°C for 5 minutes, followed by 35 cycles of denaturalization steps at 94°C for 30 seconds, annealing steps at 62°C for 30 seconds, and extension steps at 72°C for 30 seconds. The final extension was
10 minutes at 72 °C. The total volume of the secondary PCR mixture was 30 μL, containing 0.5 μL of the primary PCR products, 500 nM stx2FD (forward) and stx2RDG (reverse, with GC-clamp) primers (Table 7), 3 μL Taq buffer, 250 nM dNTP, 0.5 U Taq polymerase. Thermal cycling included an initialization step at 94°C for 5 minutes, followed by 35 cycles of denaturalization steps at 94°C for 30 seconds, annealing steps at 63°C for 30 seconds, and extension steps at 72°C for 30 seconds. The final extension was 10 minutes at 72 °C. The PCR products were resolved at 60°C using an INGENYphorU system (Ingeny, Leiden, The Netherlands) with a 8% polyacrylamide gel (37.5:1) with a 10% - 35% denaturant gradient at 80V for 1 hour and then at 160V for 15 hours. Analysis of gel images were conducted using a AlphaImager HP System (Proteinsimple, Santa Clara, California, USA) and BioNumerics (Applied Maths Inc., Austin, TX) as described previously (Yu and Morrison, 2004).

4.2.10 Sequence analysis of DGGE bands

Selected DGGE bands were excised under UV transillumination. The DNA was extracted from the gel by placing each gel slice into 30 μL of nuclease-free water and holding at 4°C for 24 hours. Two μL of each DNA extract was used in PCR to re-amplify stx2 gene for cloning. The PCR was performed as mentioned above in producing the PCR products for DGGE analysis except an annealing temperature at 58°C and elongation for 40 sec during the 35 cycles. The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) and ligated into the pGEM-T vector
(Promega Co., Madison, WI, USA). The ligation products were transformed into *E. coli* DH5α competent cells at 42°C for 45 seconds. The *stx2*-positive colonies were identified using blue-white selection and confirmed using PCR as mentioned above. The plasmids containing *stx2* fragments were extracted from overnight LB broth cultures containing ampicillin using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA) and used as the templates for Sanger sequencing. Sequence alignment and genetic clustering analysis were performed with MEGA5 (Tamura et al. 2011). Reference sequences (Scheutz et al. 2012) of *stx2* variants and of *stx2a* of different STEC serotypes (Table 8) were included in the analysis.

### 4.2.11 Statistical analysis

Statistical analyses were conducted using STATA 12 (StataCorp, College Station, TX). Spearman's correlation analysis was performed to identify any significant relationships among *E. coli* and *Arcobacter*. Logistic regressions were used to establish correlations among other bacteria and MST markers for binary data.
4.3 Results

4.3.1 Wildlife’s impacts across wetlands

In the Olentangy River Wetland Research Park (ORWRP), various MST markers were found in the water samples collected at the two Olentangy River experimental wetlands throughout the study period, including human (HF183), avian (GFD) and ruminant (Rum2Bac) markers, with mean occurrences of 16.9%, 44.9% and 38.1% for all the water samples collected across the two Olentangy River experimental wetlands, respectively. GFD had the highest average concentration of $6.0 \times 10^4$ gene copies / mL, followed by a mean concentration of $5.7 \times 10^3$ gene copies / mL for HF183 and a mean concentration of $8.8 \times 10^2$ gene copies / mL for Rum2Bac (Table 9). Spatial analysis showed a decrease in human fecal contamination across both the planted (W1) and the naturally-colonized wetlands (W2) (Fig. 12). However, general increasing trends of avian fecal contaminations were observed with a more significant increase from the inflow to midpoint at the W1 and from the midpoint to outflow at the W2, respectively. Ruminant fecal contamination was reduced from inflow to midpoint, but increased again from midpoint to outflow at both wetlands.

Data analysis from a study of fecal indicator bacteria / pathogens reductions in wetlands (Chapter 2) showed correlations among *E. coli*, *Arcobacter* and microbial source tracking (MST) markers (Table 10). Seasonal analysis found positive correlations between the levels of avian markers and *E. coli* net change from inflow to outflow (Fig. 13). No significant associations were found among STEC, *Campylobacter*, HF183, GFD
and Rum2Bac. Direct analysis of goose feces in the ORWRP showed the presence of STEC and Arcobacter, with occurrences of 93.2% (41/44) and 4.5% (2/44), respectively; the avian marker GFD was detected in 70% of the fecal samples (31/44). However, no Campylobacter was observed (Table 9).

In the Ottawa National Wildlife Refuge (ONWR), direct analysis of goose feces in the found 20.8% (16/77) of the goose fecal samples were detected with Shiga toxin gene (stx2). Of the fecal samples, November and December had the highest detection rate of stx2 (100% and 80%, respectively). Neither Campylobacter nor Arcobacter was detected in goose feces. The occurrence of GFD in ONWR goose fecal samples were 75.3% (58/77).

4.3.2 Diversity of Shiga toxin detected in goose feces in wetlands

The stx2-positive fecal DNA extracts were subject to DGGE analysis to evaluate the diversity of stx2 variants and STEC serotypes. The DGGE data show a low diversity of stx2 variants among different goose fecal samples, indicating a similar distribution among the goose individual at different times of the year. In addition, multiple DGGE bands from single DNA extracts indicated that some of geese carried multiple stx2 variants (Fig. 14). Seventeen DGGE bands were randomly selected, excised and subject to sequencing to further identify the stx2 variants. Fifteen sequences with high quality were aligned and compared with the reference sequences of stx2 variants (Table 8). The genetic clustering analysis demonstrated that all of the fifteen stx2 sequences from goose
fecal samples belonged to one single genetic cluster and were most similar to the \textit{stx2a} variant (Fig. 15). The fifteen sequences were also compared the references \textit{stx2a} sequences of different STEC serotypes (Table 8). The results indicated that all of the fifteen sequences had a close relationship with those of \textit{E. coli} serotype O157:H7, O165:H25 and O111:H- (Fig. 16).

4.4 Discussion

4.4.1 Importance of wildlife activity

MST markers targeting wildlife sources (avian and ruminant) were all associated with \textit{E. coli} and \textit{Arcobacter} concentrations for all wetland samples and seem to affect \textit{E. coli} reductions from inflow to outflow in the Olentangy River experimental wetlands. Spatial analysis showed that significant increase in avian markers from inflow to midpoint at the planted wetland (W1) and from midpoint to outflow at the naturally-colonized wetland (W2), where numbers of Canada geese (\textit{Branta canadensis}) and other water birds were constantly seen throughout the sampling period. In 2013, more open water area was observed in the south portion of the W2 (Mitsch et al., 2014), which could allow more geese and other avian species to stay in the area and thus result in substantial increase in the prevalence of avain MST marker from midpoint to outflow of the W2. High prevalence of Shiga toxin-producing bacteria and an emerging enteropathogen, \textit{Arcobacter}, were identified in goose feces directly collected at the Olentangy River experimental wetlands. Various pathogenic \textit{E. coli} serotypes have been
isolated from Canada goose feces, including enterotoxigenic (ETEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), and enteroagglomerative (EAEC) in urban areas (Kullas et al., 2002). A significantly lower prevalence was reported during winter (December-February) compared with the rest of the seasons. In addition to Canada geese, other wild birds have been shown to harbor enteric pathogens, including Campylobacter, Salmonella, and toxin-producing E. coli (Abulreesh et al., 2007). Although reduced from inflow to midpoint in both wetlands, the ruminant markers again increased from midpoint to outflow in both wetlands. More white-tailed deer (Odocoileus virginianus) activity was often observed at the south parts of both wetlands (midpoint to outflow). Enteropathogenic and Shiga-toxin producing E. coli have been isolated from deer feces, consisting of various virulent factors (Ishii et al., 2007). Deer feces, associated with strawberry consumption, have been linked to an E. coli O157:H7 outbreak, leading to deadly hemolytic uremic syndrome (Laidler et al., 2013). At the Olentangy River experimental wetlands, higher wildlife fecal contamination seems to trade off wetlands' performance of pathogen reductions by loading E. coli and pathogens through their defecation. Thus, further wildlife management practice is suggested to minimize potential health risks to nearby urban residents. In two impoundments where goose management was applied, total and fecal coliforms levels were three times lower compared to the unmanaged site. The goose management practice included using pyrotechnics, lasers, trained dogs and egg oiling (Swallow et al., 2010).
4.4.2 Shiga toxin in goose feces in wetlands

The goose fecal samples harboring stx2 were studied for the diversity of the stx2 and potential associations with outbreak-causing Shiga toxin-producing E. coli strains. DGGE and genetic clustering analysis demonstrated all the stx2 had high similarity with stx2a variant. Stx2a was reported to be more potent than Stx2b and Stx2c in vitro and in vivo (Fuller et al. 2011). A cohort study also found that Stx2a was associated with severe disease including hemolytic uremic syndrome and bloody diarrhea (Persson et al. 2007). The results also indicated stx2a variants had a close relationship with those of E. coli serotype O157:H7, O165:H25 and O111:H- (Fig. 4). The serotype O157:H7 is the main etiological agent causing foodborne STEC infection in the US (Mead et al. 1999). Although O165:H25 is not a major serotype of concern, its isolates have been associated with hemolytic uremic syndrome and bloody diarrhea (Brooks et al. 2005), while E. coli O111:H- has been isolated from clinical stool samples and linked to several outbreaks in Ohio (1990), South Australia (1995), and Oklahoma (2008) (CDC 1995; Banatvala et al. 1996; Piercefield et al. 2010). Kullas and others (2002) surveyed E. coli serogroups from urban Canada goose feces and reported that 6% of the isolates were serotyped as enterohemorrhagic strains (EHEC), which produce Shiga toxins as virulent agents, although the Shiga toxin genes were not detected for the isolates (Kullas et al., 2002). Occurrence of genetic materials doesn't necessarily equate the presence of viable bacteria or toxin, however, the stx2 were associated with high potent toxin variant and several outbreak-causing serotypes. Given the fact that low infection doses ranging from 1-100 CFU were able to cause O111:H- and O157:H7 infections (Griffin et al. 1994 and Paton...
et al. 1996), further rigorous investigations, e.g. bacterial isolation, are suggested to elucidate potential public health risks in Lake Erie recreational areas and the role of Canada geese as reservoirs of Shiga toxin-producing *Escherichia coli* and dissemination of this pathogenic bacteria.

4.5 Summary

In this study, pathogens source were investigated and characterized in freshwater wetlands. Findings included,

(1) Increasing trends of avian fecal contaminations were observed from wetland inflows to outflows at the Olentangy River Wetland Research Park.

(2) Shiga toxin-producing *E. coli* gene (*stx2*) was detected in Canada goose feces at 93.2% at the Olentangy River Wetland Research Park and 20.8% at the Ottawa National Wildlife Refuge, respectively.

(3) Shiga toxin-producing *E. coli* gene (*stx2*) detected in goose feces collected at the Ottawa National Wildlife Refuge had high similarity with a more potent toxin type (*stx2a*) and various outbreak-causing serotypes (O157:H7, O165:H25 and O111:H-).

Long-term monitoring of bacterial and other pathogens (e.g., protozoa) and more rigorous investigations, e.g. bacterial isolation, are necessary to understand potential health risks when humans are exposed to wetlands. Balanced wildlife management is
suggested to reduce health risks while maximizing the wetlands’ ecosystem service in pathogen reductions.
Chapter 5

Antimicrobial Resistance in Freshwater Wetlands

Abstract

Spread of antimicrobial resistant bacteria and genes have become a global concern due to the pervasive use of antimicrobials in human and animals. As a result, antimicrobial resistant (AMR) bacteria and genes have been detected in a wide range of environment, including wetlands which may be acquired from adjacent urban areas and wildlife in the wetlands. The aims of this study were to: (1) investigate the distributions and potential sources of AMR genes in urban wetlands and (2) isolate and characterize the AMR patterns of *Arcobacter* from urban wetlands, which is an emerging zoonotic pathogen. Water and Canada goose fecal samples were collected in two urban wetlands. Levels of the antimicrobial resistant genes, *tetQ* and *sul1*, were determined by real-time PCR. *Arcobacter* spp. were isolated on selective media and applied to 16S rRNA sequencing for species identification. AMR was characterized by minimum inhibitory concentrations. Concentrations of *tetQ* and *sul1* in the urban wetlands ranged from undetected to 3x10^6 and 1.5x10^3 gene copies / mL; however, no significant increase or decrease occurred in either *tetQ* or *sul1*. AMR genes, *tetQ* and *sul1*, were detected in
Canada goose feces with a prevalence of 4.5% (2/44) and 13.6% (6/44). *A. butzleri* and *A. cryaerophilus* were isolated from the urban wetland water samples and both species demonstrated similar rates of resistance to all the tested antimicrobials (except for tetracycline) and multiple antimicrobial resistance to at least three antimicrobials. This study demonstrated a high occurrence of the AMR genes and *Arcobacter* in urban wetland water, which possibly resulted from both urban impact and wildlife activities. This study is the first to report isolation of *Arcobacter* from environmental surface water in North America and the first to characterize AMR patterns in *Arcobacter* in urban wetland.

**5.1 Introduction**

Due to the pervasive use of antimicrobials in human and animals, antimicrobial resistant (AMR) bacteria and genes have been detected in a wide range of environments (Cantas et al., 2013). Infection with resistant pathogens has been shown to increase the mortality and morbidity of diseases and costs of health care (Williams, 2002). In addition, AMR must be considered from an ecological perspective because of its complex nature of emergence and dissemination (Salyers et al., 2002), and multidisciplinary approaches are needed to address the problem (Cantas et al., 2013).

Urban areas contribute significant amounts of antimicrobials to the environment, such as wastewater treatment plants effluents, where they may exert selection pressure favoring AMR bacteria (Watkinson et al., 2009). AMR has been detected in a variety of urban water bodies, including sewage, outfalls from wastewater treatment plants,
wastewater lagoons, hospital effluents, urban runoff, and ponds (Hamelin et al., 2007; Ibekwe et al., 2011; Pruden et al., 2012; Marion et al., 2015; Rodriguez-Mozaz et al., 2015). As water moves through wetlands, valuable services are provided by their ecosystems, including abatement of AMR genes (Chen and Zhang, 2013; Nólvak et al., 2013; Chen et al., 2015). At the same time, wetlands also attract wildlife, which have been shown to harbor AMR (Cole et al., 2005; Middleton and Ambrose, 2005; Kozak et al., 2009; Smith et al., 2014). Whether urban wetlands serve as reservoirs or sinks of AMR remains to be determined.

As an emerging enteric pathogen, various resistant Arcobacter have been reported in clinical stool samples, food animals, and food products (Houf et al., 2004; Vandenberg et al., 2006; Son et al., 2007; Shah et al., 2011; Mandisodza et al., 2012; Rahimi et al., 2014; Yesilmen et al., 2014). However, the major AMR pattern of Arcobacter in water remains to be explored. In a previous study, we detected a high prevalence of Arcobacter (94.9%) in samples from urban wetlands (Chapter 2). Because Arcobacter has been linked to a waterborne disease outbreak (Fong et al., 2007) and water serves as an important vehicle for transmission of AMR, it is important to characterize the resistance pattern of Arcobacter in the urban wetland waters.

The aims of this study were to: (1) investigate the distributions and potential sources of AMR genes in urban wetlands and (2) characterize the AMR patterns of Arcobacter isolates from urban wetland waters.
5.2 Materials and Methods

5.2.1. Sampling sites and sample collection

This study was conducted at two experimental wetlands in the Olentangy River Wetland Research Park (ORWRP) in Columbus, Ohio, USA (40.0204, -83.0186). Wetland 1 (W1) was planted with native wetland species when first established in 1994, whereas wetland 2 (W2) remained unplanted and allowed to colonize naturally (Mitsch et al., 1998, 2005, 2012, 2014a,b). The wetlands receive water from the Olentangy River, which is heavily influenced by an adjacent urban watershed. Water quality in the wetlands was also greatly impacted by fecal contamination from wildlife (Chapter 4).

For determination of *E. coli* and genetic markers, one liter of water samples were collected in duplicate, every other week, at the inflow, midpoint, and outflow from both W1 and W2 from June 2013 to June 2014. Goose feces were collected on boardwalks at both wetlands. For isolation and AMR pattern characterization of *Arcobacter*, water samples of 50 mL were collected at the outflow of the W1 from June to August 2014.

5.2.2. *E. coli* enumeration

*E. coli* concentration was used as an indicator of fecal contamination in this study. Water samples were filtered through a mixed cellulose ester membrane (pore size 0.45 μm, Millipore, Billerica, MA). The membrane was placed on modified m-TEC agar (BD, San
Jose, CA), followed by incubation at 35°C for 2h and then at 44.5°C for 18-20h. After incubation, the red / magenta colonies were counted as *E. coli* (USEPA, 2009).

### 5.2.3. DNA extraction

Water samples (100 mL) were filtered through a mixed cellulose ester membrane for DNA extraction (pore size 0.45μm, Millipore, Billerica, MA). Total DNA was extracted from the filter membrane using PowerWater DNA Isolation Kits (MoBio, Carlsbad, California), with 100 μL as final eluate. Total fecal DNA was extracted from goose feces using PowerSoil DNA Isolation Kits (MoBio, Carlsbad, California), according to the modified user manuals. One hundred milligrams of feces was used for DNA extraction with 100 μL as the final eluate volume. Overall, DNA from 118 water and 44 fecal samples were subject to molecular analysis.

### 5.2.4. Determination of molecular markers

Quantification of genetic markers, including AMR genes (tetracycline (*tetQ*) (Nikolich et al., 1994) and sulfonamide (*sulI*) (Pei et al., 2006)) and microbial source tracking (MST) markers (human (HF183) (Bernhard et al., 2000; Converse et al., 2009; Haugland et al., 2010), ruminant (Rum2Bac) (Mieszkin et al., 2009) and avian (GFD) (Green et al., 2012)) was conducted using a CFX96 C1000 Touch™ Real-Time PCR.
Detection System (Bio-Rad, Hercules, CA). All DNA extracts were diluted to one tenth of their original concentrations to minimize PCR inhibition and were subjected to real-time PCR in duplicate.

The primers, probes, and thermal profiles for each assay are shown in Table 11 and Table 12. For SYBR assays, including tetQ, sulI and GFD, additional melting curves were required to check the specificity of amplification (Table 12). The PCR mixture consisted of 10 µL of 1X TaqMan or SYBR Green PCR Mastermix, corresponding concentrations of primers or probes (Table 12) and 2 µL of diluted DNA extracts, with a total volume of 20 µL.

To establish markers with known copy numbers, each target DNA fragment was amplified using corresponding forward and reverse primers (Table 11) in a MultiGene Gradient Thermal Cycler (Labnet, Edison, NJ). Amplified DNA was examined on agarose gel and purified using QIAquick PCR purification kits (Qiagen, Valencia, CA). The purified DNA was ligated into pGEM-T vector (Promega, Madison, WI) and transformed into E. coli DH5α competent cells by heat shock transformation. The transformed plasmids were extracted using QIAprep Spin miniprep kits (Qiagen, Valencia, CA). DNA concentrations were determined by a Qubit Fluorometer (Life Technologies, Grand Island, NY). Ten-fold serial dilutions of the transformed plasmids were prepared and applied to real-time PCR analysis. Standard curves of Ct values against known gene copy numbers of each genetic marker were established. Samples with detected markers below the detection limits were marked as DNQ (detected-but-not-quantifiable).
5.2.5 Isolation of Arcobacter spp.

Collected water samples were diluted 10-fold and filtered through a mixed cellulose ester membrane (pore size 0.45μm, Millipore, Billerica, MA). The filtered membrane was placed on Arcobacter Enrichment Broth agar supplemented with cefoperazone, amphotericin B and teicoplanin (CAT) antibiotics (Oxoid, Hampshire, UK) and incubated at 30°C in ambient air for 24 hours. White or grey round colonies were selected as potential isolates and confirmed using polymerase chain reaction (PCR) with 16S rRNA primers (Harmon and Wesley, 1996; Lee et al., 2012). The PCR mixture contained 1X PCR buffer, 0.75 mM magnesium chloride, 100 μM of dNTP, 200 nM of forward (Arco1: AGAGATTRGCCTGTATTGTAT, R = A or G) and reverse primers (Arco 2: AGAGATTGGCCTGTATTGTAT), 0.5 U of Taq DNA polymerase and a touch of colony as DNA template, with a total volume of 25 μL. Detailed PCR procedures were describe in Lee et al., 2012. The PCR products were examined on 1.5% agarose gels stained with ethidium bromide. The PCR products with successful amplification were purified using a QIA quick PCR purification kit (Qiagen, Valencia, CA, USA) and sent for sequencing using the ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA) at the Plant-Microbe Genomics Facility of The Ohio State University (https://pmgf.osu.edu/). The sequences were compared with the GenBank databases using the BLAST program to determine the Arcobacter species. Phylogenetic analysis for all Arcobacter isolates was performed with MEGA5 (Tamura et al., 2011). Reference
sequences were *A. butzleri* ATCC 49616 (NR118509) and *A. cryaerophilus* CCUG 12018 (EF064151). *Pseudomonas aeruginosa* PAO1 (NC002516) was selected as an outgroup. The *Arcobacter* isolates were preserved in 15% of sterile glycerol at -80°C.

### 5.2.6 Characterization of AMR of *Arcobacter* isolates

Sixteen *A. butzleri* and six *A. cryaerophilus* isolates were selected for AMR characterization using minimum inhibitory concentration (MIC) methods. The antimicrobials tested included aminoglycosides (gentamycin), ketolides (telithromycin), macrolides (azithromycin and erythromycin), quinolones (ciprofloxacin and nalidixic acid), lincosamides (clindamycin), phenicols (florfenicol), and tetracyclines (tetracycline). The characterization was performed using Sensititre™ *Campylobacter* MIC Plate, based on the modified user manual (TREK Diagnostic systems, Cleveland, OH, USA). The NARMS *Campylobacter* MIC Plate was used, as a close relationship exists with *Arcobacter*. Isolates were preincubated on Mueller-Hinton agar at 37°C in ambient air for 24 hours. *A. butzleri* (ATCC® 49616™) was used as a quality control strain. The lowest concentration of antimicrobials that showed no *Arcobacter* growth was recorded as the MIC.
5.2.7 *Statistical analysis*

Statistical analysis was conducted using STATA 13 (College Station, Texas, USA). Data were log-transformed to normalize values. Analysis of variance (ANOVA) was used to evaluate the significant difference of tetQ or sull by sampling locations. Spearman's Rank Order correlation was applied to determine the relationship between tetQ or sull and other FIB (E. coli), pathogen (Arcobacter), and MST markers (HF183, Rum2Bac and GFD). Chi-square test was used to examine the significant difference of the resistance patterns between A. butzleri and A. cryaerophilus.

5.2.8 *Nucleotide sequence accession numbers*

The 16S rRNA sequences of Arcobacter isolates were submitted to GenBank. The accession numbers were from KT149772 to KT149793.

5.3 *Results*

5.3.1 *Distributions and potential sources AMR genes in urban wetlands*

Both AMR genes, tetQ and sull, were detected at a high prevalence across the two urban wetlands. For tetQ, the occurrence for inflow, midpoint, and outflow at Wetland 1 was 72.7%, 38.9% and 50.0%, respectively, while the occurrence for inflow, midpoint, and outflow at Wetland 2 was 77.2%, 38.9% and 53.3%; sull was detected in
all inflow and midpoint samples and in 95.5% of outflow samples in both wetlands. Concentrations of \textit{tetQ} and \textit{sull} in the urban wetlands ranged from undetected to $3 \times 10^6$ and $1.5 \times 10^3$ gene copies / mL (Fig. 17). No significant differences in the concentrations of \textit{tetQ} or \textit{sull} were found among various sampling sites ($p > 0.05$). AMR genes, \textit{tetQ} and \textit{sull}, were also detected in Canada goose feces, with a prevalence of 4.5\% (2/44) and 13.6\% (6/44).

Correlations among AMR genes and other FIB, pathogen, and MST markers were established (Table 13). Significant associations between \textit{tetQ} and \textit{sull} were established ($r = 0.2829$, $p < 0.05$). Both \textit{tetQ} and \textit{sull} were significantly associated with \textit{E. coli}, \textit{Arcobacter}, and human-associated fecal marker HF183 ($p < 0.05$. The amount of precipitation within 48 hour had a significantly positive correlation with \textit{sull} at inflow ($p < 0.05$).

5.3.2 Isolation and characterization of AMR patterns of the \textit{Arcobacter} spp.

Forty nine \textit{Arcobacter} spp. were isolated from the Olentangy River experimental wetlands from June to August, 2014. Of of the 49 isolates, 38 were \textit{A. butzleri} (78\%), whereas 11 were \textit{A. cryaerophilus} (22\%). Sixteen \textit{A. butzleri} and six \textit{A. cryaerophilus} were selected for AMR characterization based on the number of isolates for each sampling date. The genetic clustering analysis showed that 15 out of the 16 (93.8\%) \textit{A. butzleri} isolates belonged to a single genetic cluster similar to \textit{A. butzleri} ATCC 49616. All \textit{A. cryaerophilus} isolates belonged to a single genetic lineage similar to \textit{A. cryaerophilus} CCUG 12018 (Fig. 18).
Both species showed resistance to all nine of the tested antibiotics (Fig. 19). All 
*A. butzleri* and *A. cryaerophilus* isolates were resistant to azithromycin and clindamycin 
(100%). Both species demonstrated similar rates of resistance to all the tested 
antimicrobials, except for tetracycline (*p* < 0.05). With regard to multi-drug resistance, 
both species showed resistance to at least three antimicrobials (Fig. 20). One *A. butzleri* 
isolate expressed resistance to all nine of the antibiotics.

5.4 Discussion

5.4.1 AMR genes in urban surface water

In this study, median *sulf* level at the wetland inflow of about 2x10^2 copies / mL 
was observed. AMR genes have been reported to be present extensively in urban 
environments, including outfalls from wastewater treatment plants (WWTP), sewage, 
wastewater lagoons, ponds, urban runoffs and hospital effluents (Hamelin et al., 2007; 
Ibekwe et al., 2011; Pruden et al., 2012; Marion et al., 2015; Rodriguez-Mozaz et al., 
2015). In an urban site, likely affected by municipal wastewater effluent, 27% of *E. coli* 
isolates carried at least one AMR gene, including *sulf* (Hamelin et al., 2007). Linear 
models indicated that riverine *sulf* levels were associated with upstream WWTP (R^2 = 
0.34, *p* < 0.001) (Pruden et al., 2012). In an urban watershed, a high percentage of *E. coli* 
isolates with multiple AMR was identified and found to be associated with urban runoff 
(Ibekwe et al., 2011). They also reported that the prevalence of tetracycline resistance
genes, *tetC*, was approximately 40% of both sediment and surface water samples. An increasing trend of *tetQ* levels in an urban lake was identified as more bathers were fully exposed in the water (p = 0.01) (Marion et al., 2015). Rodriguez-Mozaz and others (2015) reported about *sul1* concentrations of approximate $10^3$ copies / mL for the samples collected downstream of a WWTP outfall, and $10^5$ copies / mL for those collected in the hospital effluent (Rodriguez-Mozaz et al., 2015). The discrepancy between this and other studies could be attributed to sampling locations, which were not directly under the influence of hospital effluents or WWTP. Nevertheless, prevalence of AMR genes in the wetland inflows was still impacted by adjacent urban runoffs.

### 5.4.2 Distribution of AMR genes in wetlands

This study found no significant difference in the levels of *tetQ* and *sul1* among various sampling location. However, wetlands have shown to have capacity to reduce AMR genes (Chen and Zhang, 2013; Nõlvak et al., 2013; Chen et al., 2015). In a constructed wetland receiving rural domestic wastewaters in China, significant reductions in AMR genes were observed, such as tetracycline and sulfonamide resistance genes (including *sul1*), with the removal rates ranging from 83% to 100% (Chen et al., 2015). In other constructed wetland that was fed with secondary wastewater effluents in China, it was reported that tetracycline resistance genes were removed by 1.3-2.1 orders of magnitude and sulfonamide resistance gene (*sul1*) by 1.5 orders of magnitude (Chen and Zhang, 2013). Horizontal subsurface flow wetland mesocosms in Estonia, which were constructed to treat municipal wastewater, demonstrated high frequencies of reductions in
tetracycline and sulfonamide resistance genes (including \textit{sulI}), although the removal rates varied throughout the study period (Nölvak et al., 2013). However, a constructed wetland in Canada, receiving rural wastewater from a secondary wastewater lagoon, showed no significant removal of AMR genes (including \textit{tetQ} and \textit{sulI}) (Anderson et al., 2013). Several possible explanations can account for different findings. The studies reporting significant reductions had a stable wastewater supply and higher AMR genes at wetland inflows (\textit{sulI} > 10^4 \text{ copies / mL}), whereas the Olentangy River wetlands receive river water impacted by intermittent urban runoffs and lower concentrations of AMR genes (\textit{tetQ} and \textit{sulI}: \sim 10^2 \text{ copies / mL}). AMR genes have been detected from wetland sediments that are heavily impacted by urban runoffs (Cummings et al., 2011); thus, wetland sediments could be a potential source of AMR genes. Urban wetlands attract wildlife that harbor AMR (see Discussion 4.3). In sum, these factors could compromise the roles of urban wetlands in alleviating AMR genes.

\textbf{5.4.3 Wildlife are potential carriers of AMR genes}

The study demonstrated that 4.5\% (2/44) and 13.6\% (6/44) of Canada goose feces contained \textit{tetQ} and \textit{sulI}. In the previous study, high levels of fecal contamination were reported from avian and ruminant sources in the Olentangy River experimental wetlands (Chapter 2). Other studies have shown that wildlife are potential carriers of AMR bacteria and genes, including small wild mammals (Kozak et al., 2009), gulls and deer (Smith et al., 2014), and Canada geese (Cole et al., 2005; Middleton and Ambrose, 2005). It was reported that small wild mammals, living on swine farms, harbored tetracycline-resistant
*E. coli* at rates higher than those living in natural areas, suggesting that human and agricultural activities may increase AMR in small wild mammals (Kozak et al., 2009). Higher rates (87%) of resistant *E. coli* were isolated from herring gull feces collected from a suburb of Dublin, Ireland. In comparison, resistant *E. coli* (31%) were isolated from hybrid deer feces from a National Park in Ireland (Smith et al., 2014). It is noteworthy that only the deer contained resistant pathogenic *E. coli* strains. Among the *E. coli*, isolated from Canada goose feces collected in agricultural fields, more than 95% were resistant to penicillin G, ampicillin, cephalothin, and sulfathiazole; whereas, among the *Enterococcus* isolates, it was observed that a higher percentage were resistant to cephalothin, streptomycin, and sulfathiazole (Middleton and Ambrose, 2005). *E. coli* isolated from Canada goose feces, collected near a swine housing facility, demonstrated resistance to a wide range of antimicrobials and presence of AMR genes, including *sul1* (12%, n =25) (Cole et al., 2005). Although the *sul1* resistance rate in this study was lower than that reported by Cole and others (2005), it still showed the Canada geese could be a reservoir and may aid in the transmission of AMR genes. Deer are another source of AMR in the study area, and their feces have been reported to harbor resistance genes (Smith et al., 2014). Future investigations that focus on characterization of resistance genes in deer feces may be useful to more fully recognize their contribution of AMR into this urban wetland.
5.4.4 Resistant Arcobacter in urban surface water

This study is the first to report isolation of *Arcobacter* from environmental surface water in North America and the first to characterize AMR patterns in *Arcobacter* in urban wetland water. A high percentage of resistance to clindamycin (100%) and phenicol (72.7%, florfenicol) was demonstrated. Various resistant *Arcobacter* have been reported in clinical stool samples (Houf et al., 2004; Vandenbargh et al., 2006; Mandisodza et al., 2012), poultry (Houf et al., 2004; Son et al., 2007; Rahimi et al., 2014), cattle (Shah et al., 2011), and dairy products (Yesilmen et al., 2014). *Arcobacter* has been detected from various water sources, including ground water (Rice et al., 1999), recreational water (Lee et al., 2012) and drinking water treatment plants (Jacob et al., 1998). However, little has been reported regarding resistant *Arcobacter* in water. Fera et al. (2003) tested for AMR in *Arcobacter* isolated from brackish water and found that the isolates exhibited a high degree of resistance to chloramphenicol, clindamycin, trimethoprim, and vancomycin. González et al. (2011) isolated *Arcobacter butzleri* from sewage samples in Spain and reported that 23.3% (n = 60) were resistant to both ciprofloxacin and levofloxacin. Otth et al. (2004) conducted a study on AMR in *Arcobacter* isolates from river water, but combined the resistance rate with isolates from other food sources. In this study, a high percentage of resistance to clindamycin and phenicol was detected as also reported by Fera and others (2003). However, a lower percentage of resistance to ciprofloxacin (9.2%) was found in this study than that reported by González and others (2011). Given the fact that ciprofloxacin is used as a first-line drug to treat infections with *Campylobacteraceae*, including *Arcobacter* (Houf et al., 2004), it is understandable that

80
resistant *Arcobacter* would reduce the efficacy of ciprofloxacin treatment. *Arcobacter* has been linked to a waterborne disease outbreak (Fong et al., 2007), thus, it is important to monitor *Arcobacter* and its AMR patterns in environmental waters, particularly in recreational waters and drinking water sources.

### 5.5 Summary

In this study, distributions and potential sources AMR genes in urban freshwater wetlands were investigated and AMR patterns of the *Arcobacter* isolates from urban wetland water were characterized. Findings included,

1. Neither increase nor reduction in AMR genes were found across the urban wetlands.
2. AMR genes were detected in Canada goose feces, with a prevalence of 4.5% (tetracycline resistance) and 13.6% (sulfonamide resistance).
3. Emerging enteric pathogens, *Arcobacter* spp., were isolated from urban wetland water.
4. All *A. butzleri* and *A. cryaerophilus* isolates showed high resistance rates to various antimicrobials and multiple resistance.
5. Human impact and wildlife activities in urban wetland settings could contribute to high prevalence of AMR genes and *Arcobacter* spp. 

Given the *Arcobacter* is an emerging enteric pathogen, it is important to monitor this pathogen and its antimicrobial resistance patterns in the environmental waters of high human contact.
Chapter 6

Conclusions

This dissertation provided an overview of wetland's ecosystem service and public health implications with regard to fecal indicator bacteria and bacterial pathogens. Wetlands retained 22.3% of \textit{E. coli} input from an adjacent river that is heavily influenced by urban runoff as water moved across wetland inflow to outflow (Chapter 2). A simple and empirical model estimated that wetlands would be able to reduce \textit{E. coli} loads to meet regulatory criteria for both near (2020-2049) and far (2045-2074) terms in spite of increasing \textit{E. coli} levels (Chapter 3). On the other hand, fecal contaminations from avian and ruminant sources were identified in the studied wetlands. Direct analysis of Canada goose feces collected in wetlands found presence of Shiga toxin-producing \textit{E. coli} gene (\textit{stx2}) that were associated with a high potent toxin form (\textit{stx2a}) and outbreak-causing serotypes (O157:H7, O165:H25 and O111:H-) (Chapter 4). Antimicrobial resistance (AMR) genes (tetracycline and sulfonamide) were detected in wetland waters. An emerging pathogen, \textit{Arcobacter}, was also isolated from wetland water and demonstrated multiple antimicrobial resistance (Chapter 5).
Federal policy and public awareness are now focusing on wetland preservation and restoration. In this dissertation, ecosystem service was shown in terms of *E. coli* reductions in current, as well as near-future and far-future terms under climate change scenarios, demonstrating that wetlands could be sustainable alternatives to mitigate microbial contamination. However, bacterial pathogens were also detected either directly in wetland water or in wildlife feces collected from wetlands, which could compromise wetland's performance in reduction of microbial loadings and pose potential public health risks when human and wildlife come into contact in this interface (Cromie et al., 2012). This illustrates a holistic view and suggests that an integrated approach for stakeholders are required to plan as McInnes (2013) states "wise use of wetlands."

Several suggestions have been made throughout this dissertation. First of all, more long-term surveillance of bacterial and other pathogens (e.g. virus and protozoa) is necessary to provide a comprehensive picture of wetland's ecosystem service with regard to microbial contamination. Surveying more wildlife species will present data to show what other potential health risks may emerge when humans are exposed to wetlands. Lastly, more rigorous investigation, e.g. direct isolation of pathogens, would better characterize the risks of infection. Collectively, these approaches will enhance the knowledge of wetland's ecosystem services and public health implications.
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Caprioli, A., Tozzoli, R., Morabito, S., Strockbine, N.A., Melton-Celsa, A.R., Sanchez,
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245–53.

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R.Dharmasri, C., Sexton, A., Arabi, M., Wolfe, M.L., Frankenberger, J., Graff, C.,
Agricultural and Biological Engineers 49, 1033–1049.

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during the filtration process in constructed wetlands. Science of the Total Environment
380, 173-180.

frequency with commonly used microbial indicators of recreational water quality.

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Table 1. Primers and probes used in Chapter 2 for real-time polymerase chain reaction (PCR) analyses of water flowing through experimental wetlands at the Olentangy River Wetland Research Park. Water samples were collected at inflow, middle and outflow points from June 23, 2013 to June 21, 2014.

<table>
<thead>
<tr>
<th>Genetic marker</th>
<th>Primer/Probe</th>
<th>Sequence</th>
<th>Target</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arco</td>
<td>ArcoI</td>
<td>GTCGTGCAAGAAAAAGCCA</td>
<td><em>Arcobacter</em> spp. 23S rRNA</td>
<td>Bastyns et al., 1995; Gonzalez et al., 2007</td>
</tr>
<tr>
<td></td>
<td>ArcoII</td>
<td>TTCGCTTGCGCTGACAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Campy</td>
<td>CampyF</td>
<td>CTGCTTAACACAAGTTGAGTAGG</td>
<td><em>Campylobacter</em> spp. 16S rRNA</td>
<td>Josefsen et al., 2004; Van Dyke et al., 2010</td>
</tr>
<tr>
<td></td>
<td>CampyR</td>
<td>TTCCTTAGGTCACGTCAGAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CampyP</td>
<td>[FAM]-CGCTCGGAAAGTGTCATCCTCC-[BHQ1]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>stx2</td>
<td>stx2F</td>
<td>ATTAACCACACCCACCG</td>
<td>Shiga toxin-2</td>
<td>Ibekwe et al., 2002; This study</td>
</tr>
<tr>
<td></td>
<td>stx2R2</td>
<td>GGTACAAAACCGCCTGATAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>stx2P</td>
<td>[JOE]-CAGTTATTTTGCTGGATATACGAGGGCTTG-[BHQ2]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Descriptive statistics of environmental variables, nutrients, *E. coli*, and pathogens for the water samples collected at inflow, middle and outflow points of the two experimental wetlands from June 23, 2013 to June 21, 2014.

<table>
<thead>
<tr>
<th>Variable</th>
<th>N</th>
<th>Min.</th>
<th>Max.</th>
<th>Mean</th>
<th>Median</th>
<th>Occurrence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>21</td>
<td>0.3</td>
<td>29.9</td>
<td>15.9</td>
<td>18.2</td>
<td>NA^e</td>
</tr>
<tr>
<td>Solar radiation (Langley^a)</td>
<td>21</td>
<td>0.3</td>
<td>6.3</td>
<td>3.5</td>
<td>4.0</td>
<td>NA^e</td>
</tr>
<tr>
<td>Precipitation within 24 hr (mm)</td>
<td>21</td>
<td>0.0</td>
<td>23.9</td>
<td>2.7</td>
<td>0.0</td>
<td>NA^e</td>
</tr>
<tr>
<td>Precipitation within 48 hr (mm)</td>
<td>21</td>
<td>0.0</td>
<td>46.5</td>
<td>10.7</td>
<td>3.6</td>
<td>NA^e</td>
</tr>
<tr>
<td>Precipitation within 72 hr (mm)</td>
<td>21</td>
<td>0.0</td>
<td>76.7</td>
<td>16.4</td>
<td>11.2</td>
<td>NA^e</td>
</tr>
<tr>
<td>Flow (m^3/min)</td>
<td>21</td>
<td>0.6</td>
<td>2.7</td>
<td>1.3</td>
<td>1.4</td>
<td>NA^e</td>
</tr>
<tr>
<td>Turbidity (NTU^b)</td>
<td>118</td>
<td>3.7</td>
<td>303.5</td>
<td>41.8</td>
<td>26.2</td>
<td>NA^e</td>
</tr>
<tr>
<td>Nitrate-N (mg/L)</td>
<td>118</td>
<td>ND^d</td>
<td>1.0</td>
<td>0.1</td>
<td>0.0</td>
<td>NA^e</td>
</tr>
<tr>
<td>Total phosphorus (mg/L)</td>
<td>118</td>
<td>ND^d</td>
<td>1.2</td>
<td>0.5</td>
<td>0.4</td>
<td>NA^e</td>
</tr>
<tr>
<td><em>E. coli</em> (CFU^c / 100 mL)</td>
<td>118</td>
<td>ND^d</td>
<td>4,600</td>
<td>920</td>
<td>560</td>
<td>99.2</td>
</tr>
<tr>
<td><em>Campylobacter</em> (gene copies / mL)</td>
<td>118</td>
<td>ND^d</td>
<td>4.7</td>
<td>0.073</td>
<td>ND</td>
<td>1.7</td>
</tr>
<tr>
<td><em>Arcobacter</em> (gene copies / mL)</td>
<td>118</td>
<td>ND^d</td>
<td>240,000</td>
<td>28,000</td>
<td>16,000</td>
<td>94.9</td>
</tr>
<tr>
<td>STEC (gene copies / mL)</td>
<td>118</td>
<td>ND^d</td>
<td>3,200</td>
<td>180</td>
<td>36</td>
<td>74.6</td>
</tr>
</tbody>
</table>

^aOne Langley is equal to 41,480 joules per square meter

^bNTU, nephelometric turbidity units

^cCFU, colony-forming unit

^dND, not detected

^eNA, not applicable
Table 3. P values of paired t-tests among turbidity, nutrients and fecal indicator/pathogens between midpoint and inflow, between outflow and midpoint, and between outflow and inflow water samples collected at the two experimental wetlands from June 23, 2013 to June 21, 2014.

<table>
<thead>
<tr>
<th></th>
<th>Planted wetland (W1)</th>
<th>Naturally-colonized wetland (W2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I-M (^b) M-O (^c) I-O (^d)</td>
<td>I-M (^b) M-O (^c) I-O (^d)</td>
</tr>
<tr>
<td>Turbidity</td>
<td>0.031 0.163 0.039</td>
<td>0.004 0.071 0.463</td>
</tr>
<tr>
<td>Nitrate-Nitrogen</td>
<td>0.657 0.224 0.464</td>
<td>0.049 0.053 0.373</td>
</tr>
<tr>
<td>Total Phosphorus</td>
<td>0.088 0.033 0.006</td>
<td>0.047 0.058 0.004</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>0.210 0.962 0.357</td>
<td>0.868 0.868 0.931</td>
</tr>
<tr>
<td><em>Arcobacter</em></td>
<td>0.906 0.906 0.903</td>
<td>0.554 0.435 0.476</td>
</tr>
</tbody>
</table>

\(^a\)P values of less than 0.05 are shown in bold

\(^b\)Compared between midpoint and inflow samples

\(^c\)Compared between outflow and midpoint samples

\(^d\)Compared between outflow and inflow samples
Table 4. Correlation coefficients among *E. coli* and *Arcobacter* for all the water samples collected at inflow, middle and outflow points of the two experimental wetlands from June 23, 2013 to June, 21, 2014.

<table>
<thead>
<tr>
<th>Fecal indicator/pathogen</th>
<th><em>E. coli</em></th>
<th><em>Arcobacter</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>1.000</td>
<td>0.265*</td>
</tr>
<tr>
<td><em>Arcobacter</em></td>
<td>0.265*</td>
<td>1.000</td>
</tr>
</tbody>
</table>

*p < 0.05*
Table 5. Selected parameters for each subwatershed of the Olentangy River watershed in Stormwater Management Model (SWMM). Watershed IDs are arbitrarily assigned to each subwatershed. Subcatchment area and imperviousness were estimated using ArcMap 10.2.2 (ESRI, Redlands, California, USA).

<table>
<thead>
<tr>
<th>ID</th>
<th>Area (hectare)</th>
<th>Imperviousness (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12830</td>
<td>26.6</td>
</tr>
<tr>
<td>2</td>
<td>5284</td>
<td>15.3</td>
</tr>
<tr>
<td>3</td>
<td>10917</td>
<td>17.6</td>
</tr>
<tr>
<td>4</td>
<td>5624</td>
<td>19.1</td>
</tr>
<tr>
<td>5</td>
<td>7742</td>
<td>15.9</td>
</tr>
<tr>
<td>6</td>
<td>16274</td>
<td>19.8</td>
</tr>
<tr>
<td>7</td>
<td>5601</td>
<td>15.9</td>
</tr>
<tr>
<td>8</td>
<td>5919</td>
<td>16.6</td>
</tr>
<tr>
<td>9</td>
<td>7464</td>
<td>35.1</td>
</tr>
<tr>
<td>10</td>
<td>6224</td>
<td>15.9</td>
</tr>
<tr>
<td>11</td>
<td>4379</td>
<td>32.7</td>
</tr>
<tr>
<td>12</td>
<td>7713</td>
<td>19.8</td>
</tr>
<tr>
<td>13</td>
<td>3885</td>
<td>28.4</td>
</tr>
<tr>
<td>14</td>
<td>11367</td>
<td>33.2</td>
</tr>
<tr>
<td>15</td>
<td>12667</td>
<td>31.5</td>
</tr>
<tr>
<td>16</td>
<td>7939</td>
<td>38.7</td>
</tr>
<tr>
<td>17</td>
<td>8288</td>
<td>49.7</td>
</tr>
</tbody>
</table>
Table 6. *E. coli* load reductions under five hydrologic conditions. *E. coli* loads for each hydrologic condition were calculated by multiplying representative *E. coli* concentration (at 90th percentile) by flow (at 50th percentile). *E. coli* load reductions indicates that the percentage of load reduction was needed to meet the Ohio EPA water quality standard for primary contact water (298 CFU / 100 mL). Difference between the After-Wetland and Before-Wetland load reductions demonstrate *E. coli* increases/reductions by the Olentangy River experimental wetlands. A positive value water show *E. coli* reductions by the wetlands. Percent exceeded represents the percent of time that the flow have been met or exceeded during the analysis period (from June 23, 2013 to June, 21, 2014). A lower percent exceeded indicates a high flow condition.

<table>
<thead>
<tr>
<th>Percent Exceeded (%)</th>
<th>Hydrologic Condition Class</th>
<th>Before-Wetland Load Reduction</th>
<th>After-Wetland Load Reduction</th>
<th>Increases/Reductions by Wetlands</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-0.1</td>
<td>High Flow</td>
<td>10.0%</td>
<td>97.6%</td>
<td>-87.6%</td>
</tr>
<tr>
<td>0.1-0.39</td>
<td>Moist Conditions</td>
<td>1361.6%</td>
<td>587.5%</td>
<td>774.1%</td>
</tr>
<tr>
<td>0.4-0.59</td>
<td>Mid-Range Conditions</td>
<td>77.2%</td>
<td>211.9%</td>
<td>-134.7%</td>
</tr>
<tr>
<td>0.6-0.89</td>
<td>Dry Conditions</td>
<td>523.8%</td>
<td>403.7%</td>
<td>120.1%</td>
</tr>
<tr>
<td>0.9-1.0</td>
<td>Low Flow</td>
<td>-58.1%</td>
<td>128.8%</td>
<td>-186.9%</td>
</tr>
<tr>
<td>0-1.0</td>
<td>Entire Curve</td>
<td>1090.3%</td>
<td>516.1%</td>
<td>574.2%</td>
</tr>
</tbody>
</table>
Table 7. Primers and probes used in Chapter 4 for real-time polymerase chain reaction (PCR) analyses of water and goose fecal samples collected at the Olentangy River experimental wetlands from June 23, 2013 to June 21, 2014 and the Ottawa National Wildlife Refuge (ONWR) from May to December, 2012.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers/Probes</th>
<th>Sequences</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HF183</td>
<td>HF183F</td>
<td>ATCATGAGTTCACATGTCCG</td>
<td>Bernhard et al., 2000;</td>
</tr>
<tr>
<td></td>
<td>HF183R</td>
<td>CGTAGGAGTTGGACCGTG</td>
<td>Converse et al., 2009;</td>
</tr>
<tr>
<td></td>
<td>HF183P</td>
<td>[FAM]-CTGAGAGGAAGGTCCCCACATGGGA-[MGB]</td>
<td>Haugland et al., 2010</td>
</tr>
<tr>
<td>GFD</td>
<td>GFDF</td>
<td>TCGCGTGAACACCTAGGG</td>
<td>Green et al., 2012</td>
</tr>
<tr>
<td></td>
<td>GFDR</td>
<td>GCCGTCTTTTGACATCCA</td>
<td>Mieszkin et al., 2009</td>
</tr>
<tr>
<td>Rum2Bac</td>
<td>R2BF</td>
<td>ACAGCCCGCGATGGATACTGGAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R2BR</td>
<td>CAATCGGAGTTCTTCGAT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R2BP</td>
<td>[FAM]-ATGAGGTGGATGGAATTCGATGGTGT-[BHQ1]</td>
<td></td>
</tr>
<tr>
<td>Arco</td>
<td>Arcol</td>
<td>GTCGTGCAAAGAAAGCCA</td>
<td>Gonzalez et al., 2007</td>
</tr>
<tr>
<td></td>
<td>ArcoII</td>
<td>TTCGCTGGCCTGACAT</td>
<td>Josefsen et al., 2004;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Van Dyke et al., 2010</td>
</tr>
<tr>
<td>Campy</td>
<td>CampyF</td>
<td>CTGCTTAACACAAAGGTGAGTAGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CampyR</td>
<td>TTCCTTACGCTACGTTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CampyP</td>
<td>[FAM]-CGCTCCGAAAAGGTGATCCTCAGC-[BHQ1]</td>
<td></td>
</tr>
<tr>
<td>stx2</td>
<td>stx2F</td>
<td>ATTAACCAACACCCACCG</td>
<td>Ibekwe et al. 2002</td>
</tr>
<tr>
<td></td>
<td>stx2R</td>
<td>GTCATGAAACACGTTGCA</td>
<td>Ibekwe et al. 2002</td>
</tr>
<tr>
<td></td>
<td>stx2R2</td>
<td>GGTCAAAAACGCTGATTAG</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>stx2P</td>
<td>CAGTTATTTTTGCTGAGATAGGAGGGCTTGG</td>
<td>Ibekwe et al. 2002</td>
</tr>
<tr>
<td></td>
<td>stx2FD1</td>
<td>GGCACTGCTGAAACCTGCGTCTCTG</td>
<td>Persson et al. 2007</td>
</tr>
<tr>
<td></td>
<td>stx2FD</td>
<td>ACCAGAATGTACGATAACTGGCGA</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>stx2RD</td>
<td>ATTAAACTGCTTCGCAAATCC</td>
<td>Persson et al. 2007</td>
</tr>
<tr>
<td></td>
<td>stx2RDG</td>
<td>GCCCG-GATTAACACTGCTTCGCAAATCC</td>
<td>This study</td>
</tr>
</tbody>
</table>

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Table 8. Reference sequences of *stx*2 variants and STEC serotypes used in Chapter 4 (Scheutz et al. 2012).

<table>
<thead>
<tr>
<th><em>stx</em>2 variants</th>
<th>Source Serotypes</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>stx</em>2a</td>
<td>O48:H21</td>
<td>Z37725</td>
</tr>
<tr>
<td></td>
<td>O157:H7</td>
<td>X07865</td>
</tr>
<tr>
<td></td>
<td>O165:H25</td>
<td>AY633471</td>
</tr>
<tr>
<td></td>
<td>O111:H-</td>
<td>EF441609</td>
</tr>
<tr>
<td></td>
<td>O26:H11</td>
<td>AJ272135</td>
</tr>
<tr>
<td></td>
<td>O178:H19</td>
<td>FM998856</td>
</tr>
<tr>
<td></td>
<td>O22:H8</td>
<td>AY443054</td>
</tr>
<tr>
<td></td>
<td>O113:H21</td>
<td>EF441618</td>
</tr>
<tr>
<td></td>
<td>O48:H21</td>
<td>Z37725</td>
</tr>
<tr>
<td></td>
<td>O101:H10</td>
<td>AY443052</td>
</tr>
<tr>
<td></td>
<td>O104:H21</td>
<td>EF441619</td>
</tr>
<tr>
<td></td>
<td>O8:H19</td>
<td>AY633459</td>
</tr>
<tr>
<td><em>stx</em>2b</td>
<td>O118:H12</td>
<td>AF043627</td>
</tr>
<tr>
<td><em>stx</em>2c</td>
<td>O174:H21</td>
<td>L11079</td>
</tr>
<tr>
<td><em>stx</em>2d</td>
<td>O73:H18</td>
<td>DQ059012</td>
</tr>
<tr>
<td><em>stx</em>2e</td>
<td>O139:K12:H1</td>
<td>M21534</td>
</tr>
<tr>
<td><em>stx</em>2f</td>
<td>O128:H2</td>
<td>AJ010730</td>
</tr>
<tr>
<td><em>stx</em>2g</td>
<td>O2:H25</td>
<td>AY286000</td>
</tr>
</tbody>
</table>
Table 9. Descriptive statistics of microbial source tracking (MST) markers for all the water samples collected at inflow, middle and outflow points of the two experimental wetlands from June 23, 2013 to June, 21, 2014.

<table>
<thead>
<tr>
<th>Environmental variables</th>
<th>N</th>
<th>Min.</th>
<th>Max.</th>
<th>Mean</th>
<th>Median</th>
<th>Occurrence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HF183 (gene copies / mL)</td>
<td>118</td>
<td>ND(^a)</td>
<td>175,000</td>
<td>5,700</td>
<td>ND</td>
<td>16.9</td>
</tr>
<tr>
<td>GFD (gene copies / mL)</td>
<td>118</td>
<td>ND(^a)</td>
<td>3,360,000</td>
<td>60,000</td>
<td>ND</td>
<td>44.9</td>
</tr>
<tr>
<td>Rum2Bac (gene copies / mL)</td>
<td>118</td>
<td>ND(^a)</td>
<td>30,000</td>
<td>880</td>
<td>ND</td>
<td>38.1</td>
</tr>
</tbody>
</table>

\(^a\)ND, not detected
Table 10. Correlation analysis among *E. coli*, *Arcobacter*, and microbial source tracking (MST) markers for all the water samples collected at inflow, middle and outflow points of the two experimental wetlands from June 23, 2013 to June 21, 2014.

<table>
<thead>
<tr>
<th>Fecal indicator/pathogen</th>
<th><em>E. coli</em>&lt;sup&gt;a&lt;/sup&gt;</th>
<th><em>Arcobacter</em>&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HF183&lt;sup&gt;b&lt;/sup&gt;</th>
<th>GFD&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Rum2Bac&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>1.000</td>
<td>0.265***</td>
<td>**</td>
<td>*</td>
<td>***</td>
</tr>
<tr>
<td><em>Arcobacter</em></td>
<td>0.265***</td>
<td>1.000</td>
<td>***</td>
<td>**</td>
<td>***</td>
</tr>
</tbody>
</table>

* *p < 0.15
** *p < 0.10
*** *p < 0.05
Table 11. Primers and probes used in Chapter 5 for real-time polymerase chain reaction (PCR) analyses of water and goose fecal samples collected at the Olentangy River experimental wetlands from June 23, 2013 to June 21, 2014.

<table>
<thead>
<tr>
<th>Genetic markers</th>
<th>Primer/Probe</th>
<th>Sequence</th>
<th>Concentrations</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arco</td>
<td>Arco1</td>
<td>AGAGATTRGCCTGTATTGTAT, R = A or G</td>
<td>200 nM</td>
<td>Harmon and Wesley, 1996</td>
</tr>
<tr>
<td></td>
<td>Arco2</td>
<td>AGAGATTGGCCTGTATTGTAT</td>
<td>200 nM</td>
<td>Lee et al., 2012</td>
</tr>
<tr>
<td>tetQ</td>
<td>tetQF</td>
<td>CATGGATCAGCAATGTCAATATCGG</td>
<td>200 nM</td>
<td>Nikolich et al., 1994</td>
</tr>
<tr>
<td></td>
<td>tetQR</td>
<td>CCTGGATCCACAATGTATTCAGAGCGG</td>
<td>200 nM</td>
<td></td>
</tr>
<tr>
<td>sul1</td>
<td>sull-FW</td>
<td>CGCACCCGAAAACATCGCTGCAC</td>
<td>500 nM</td>
<td>Pei et al., 2006</td>
</tr>
<tr>
<td></td>
<td>sull-RV</td>
<td>TGAAGTTCCGCAGAAGGCTCG</td>
<td>500 nM</td>
<td></td>
</tr>
<tr>
<td>HF183</td>
<td>Hf183F</td>
<td>ATCATGAGTTCACATGTCCG</td>
<td>500 nM</td>
<td>Bernhard et al., 2000;</td>
</tr>
<tr>
<td></td>
<td>Hf183R</td>
<td>CGTAGGAGTTTGGACCGTGT</td>
<td>500 nM</td>
<td>Converse et al., 2009;</td>
</tr>
<tr>
<td></td>
<td>Hf183P</td>
<td>[FAM]-CTGAGAGGAAGTCCCCACATTGGA-[MGB]</td>
<td>250 nM</td>
<td>Haugland et al., 2010</td>
</tr>
<tr>
<td>GFD</td>
<td>GFDF</td>
<td>TCGGCTGAGCAGCTCTAGGG</td>
<td>100 nM</td>
<td>Green et al., 2012</td>
</tr>
<tr>
<td></td>
<td>GFDR</td>
<td>GCGCTCCTTGTACATCCA</td>
<td>100 nM</td>
<td></td>
</tr>
<tr>
<td>Rum2Bac</td>
<td>R2BF</td>
<td>ACAGCCCGCGATTGATACTGGTAA</td>
<td>200 nM</td>
<td>Mieszkin et al., 2009</td>
</tr>
<tr>
<td></td>
<td>R2BR</td>
<td>CAATCGGAGTTCTCTGTGAT</td>
<td>200 nM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R2BP</td>
<td>[FAM]-ATGAGGTGGATGGAATTCGTGGTGT-[BHQ1]</td>
<td>200 nM</td>
<td></td>
</tr>
</tbody>
</table>
Table 12. Real-time PCR programs used in Chapter 5 to determine levels of each molecular marker for the water and goose fecal samples collected at the Olentangy River experimental wetlands from June 23, 2013 to June, 21, 2014.

<table>
<thead>
<tr>
<th>Genetic markers</th>
<th>Assay type</th>
<th>Initial stage</th>
<th>Amplification stage</th>
<th>No. of cycles</th>
<th>Melt curve stage</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>tetQ</td>
<td>SYBR Green</td>
<td>95°C, 10 min</td>
<td>95°C, 30 sec &gt; 62°C, 1 min</td>
<td>40</td>
<td>95°C, 30 sec &gt; 62°C, 1 min &gt; Temp. increased by 0.3 °C/sec &gt; 95°C, 30 sec</td>
<td>Nikolich et al., 1994</td>
</tr>
<tr>
<td>sul1</td>
<td>SYBR Green</td>
<td>95°C, 10 min</td>
<td>95°C, 15 sec &gt; 66°C, 30 sec</td>
<td>50</td>
<td>95°C, 15 sec &gt; 66°C, 30 sec &gt; Temp. increased by 1 °C/sec &gt; 95°C, 15 sec</td>
<td>Pei et al., 2006</td>
</tr>
<tr>
<td>HF183</td>
<td>TaqMan</td>
<td>50°C, 2 min &gt; 95°C, 10 min</td>
<td>95°C, 15 sec &gt; 60°C, 1 min</td>
<td>40</td>
<td>None</td>
<td>Bernhard et al., 2000; Converse et al., 2009; Haugland et al., 2010</td>
</tr>
<tr>
<td>GFD</td>
<td>SYBR Green</td>
<td>95°C, 10 min</td>
<td>95°C, 15 sec &gt; 60°C, 1 min</td>
<td>50</td>
<td>95°C, 15 sec &gt; 60°C, 30 sec &gt; Temp. increased by 0.3 °C/sec &gt; 95°C, 15 sec</td>
<td>Green et al., 2012</td>
</tr>
<tr>
<td>Rum2Bac</td>
<td>TaqMan</td>
<td>95°C, 10 min</td>
<td>95°C, 15 sec &gt; 60°C, 1 min</td>
<td>50</td>
<td>None</td>
<td>Mieszkin et al., 2009</td>
</tr>
</tbody>
</table>
Table 13. Spearman coefficient among *tetQ*, *sul1*, *E. coli*, *Arcobacter*, HF183 for all the water samples collected throughout the two experimental wetlands from June 23, 2013 to June, 21, 2014.

<table>
<thead>
<tr>
<th>AMR genes</th>
<th>E. coli</th>
<th>Arcobacter</th>
<th>HF183</th>
</tr>
</thead>
<tbody>
<tr>
<td>tetQ</td>
<td>0.2419*</td>
<td>0.8641*</td>
<td>0.8859*</td>
</tr>
<tr>
<td>sul1</td>
<td>0.2227*</td>
<td>0.2053*</td>
<td>0.2044*</td>
</tr>
</tbody>
</table>

*, p < 0.05
Fig. 1. Sampling sites at both experimental wetlands at the Olentangy River Wetland Research Park used in this study (Reproduced from Mitsch et al., 2012, permission obtained from the author and publisher: Oxford University Press).
Fig. 2. Overall and seasonal variation of *E. coli* net change from inflow to outflow in the Olentangy River experimental wetlands from June 23, 2013 to June, 21, 2014. Both planted (W1) and naturally colonized (W2) wetlands were treated as replicates. Only winter showed significant reductions in *E. coli* from inflow to outflow ($p < 0.05$). The overall *E. coli* net change was -22.3% from inflow to outflow.
Fig. 3. Spearman correlations (a) between precipitation within 24 hr and inflow E. coli, (b) between precipitation within 48 hr and inflow E. coli, (c) between precipitation within 72 hr and inflow E. coli, (d) between inflow turbidity and inflow E. coli, (e) between inflow total phosphorus and inflow E. coli and (f) between inflow turbidity and inflow total phosphorus for the water samples collected from June 23, 2013 to June, 21, 2014.
(Fig. 3: Continued)

(c) $r = 0.44, \quad p < 0.05$

(d) $r = 0.79, \quad p < 0.05$
(Fig. 3: Continued)

(e)

\[
E. \text{ coli (CFU/100mL)} \quad r = 0.61, \quad p < 0.05
\]

(f)

\[
\text{Total phosphorus (mg/L)} \quad r = 0.77, \quad p < 0.05
\]
Fig. 4. Spearman correlations between *E. coli* reductions from inflow to outflow in both wetlands and variables, including (a) inflow *E. coli*, (b) turbidity reduction, (c) nitrate-nitrogen reduction, and (d) total phosphorus reduction from inflow to outflow in both wetlands for the water samples collected from June 23, 2013 to June 21, 2014.

(Continued)
(Fig. 4: Continued)

(c)

![Graph](image)

\[ r = 0.43, p = 0.05 \]

(d)

![Graph](image)

\[ r = 0.64, p < 0.05 \]
Fig. 5. The Olentangy River watershed in Central Ohio. (a) The overall watershed consists of 17 subwatersheds. (b) The Olentangy River watershed with impervious area (dark-grey color). (c) The Olentangy River watershed with SWMM ID for each subwatershed.
Fig. 6. Rainfall-runoff calibration by SWMM. (a) Comparison between SWMM-simulated runoff and estimated runoff. (b) Significant correlation between SWMM-simulated runoff and estimated runoff ($r = 0.3544$, $p < 0.05$). Model calibration period was from Jun. 23, 2009 to Jun. 22, 2013.
Fig. 7. Rainfall-runoff validation by SWMM. (a) Comparison between SWMM-simulated runoff and estimated runoff. (b) Significant correlation between SWMM-simulated runoff and estimated runoff ($r = 0.3029$, $p < 0.05$). Model validation period was from Jun. 22, 2013 to Jun. 21, 2014.
Fig. 8. Surface runoff in the Olentangy River watershed in now (2009-2014), near-term (2020-2049), and far-term (2045-2074) simulated conditions.
Fig. 9. (a) Predicting *E. coli* concentrations in the Olentangy River watershed from SWMM simulated surface runoff. (b) Comparison between the observed *E. coli* and simulated *E. coli* by the calibration curve from data in (a) (*r* = 0.63, *p* < 0.05).
Fig. 10. Predicted *E. coli* in the Olentangy River watershed without wetlands added in now (2013-2014), near-term (2020-2049), and far-term (2045-2074). The red line denotes the Ohio EPA water quality standard for primary contact water (298 CFU / 100 mL).
Fig. 11. Predicted *E. coli* loads for the Olentangy River experimental wetlands inflow and outflow for each month in (a) near term (2020-2049) and (b) far term (2045-2074). Target loads were calculated based on Ohio EPA water quality standard for primary contact water (298 CFU / 100 mL).
Fig. 12. Dynamics of microbial source tracking (MST) markers for the water samples collected from inflow to outflow across (a) the planted experimental wetland (W1) and (b) the naturally-colonized experimental wetland (W2) at the Olentangy River Wetland Research Park in Columbus, Ohio, from June 23, 2013 to June, 21, 2014. Prevalence was defined as the percentage of sample detected with MST markers divided by total samples. Avian, ruminant and human MST markers were used to assess the impact of each particular source. Sample numbers were 21, 17, 21 for inflow, midpoint, and outflow sites, respectively. The error bars represent standard deviation.
Fig. 13. Comparison between *E. coli* net changes for the water samples collected from inflow to outflow in the Olentangy River experimental wetlands and prevalence of microbial source tracking (MST) markers of midpoint and outflow samples from June 23, 2013 to June, 21, 2014. The blue bars show *E. coli* net change. The red and green lines represent avian and ruminant MST markers, respectively.
Fig. 14. DGGE result of stx2 fragments from the goose fecal samples collected at the Ottawa National Wildlife Refuge (ONWR) from May to December, 2012. Goose fecal sample IDs are listed at the top, while DGGE band IDs are provided adjacent to bands. These bands were randomly selected for excision and DNA sequencing. The stx2 amplified from E. coli O157:H7 was used as positive control (PC). M: DNA marker.
Fig. 15. A dendrogram showing the sequenced stx2 fragments from the DGGE bands (Fig. 14) and the reference stx2 variants (Scheutz et al. 2012). Length of the scare bar shows 0.01 changes per nucleotide position.
Fig. 16. A dendrogram showing the sequenced *stx2* fragments from the DGGE bands (Fig. 14) and the reference *stx2a* of STEC serotypes (Scheutz et al. 2012). Length of the scare bar shows 0.01 changes per nucleotide position.
Fig. 17. Distribution of concentrations of AMR genes for the water samples collected across the two Olentangy River experimental wetlands from June 23, 2013 to June, 21, 2014. (a) *tetQ*. (b) *sul1*. Samples with no AMR genes detected were assigned 1 gene copy /mL. Samples with AMR genes detected-but-not-quantifiable were assigned half limit-of-quantification for data analysis.
Fig. 18. Phylogenetic analysis of the partial 16S rRNA gene sequences of *Arcobacter* isolates from the Olentangy River experimental wetland (W1). *Arcobacter butzleri* strain ATCC 49616 (NR118509) and *Arcobacter cryaerophilus* strain CCUG 12018 (EF064151) were used as reference groups. *Pseudomonas aeruginosa* PAO1 (NC002516) was used as an outgroup. Only bootstrap values of more than 50% are shown. Length of the scale bar shows 0.01 changes per nucleotide position. Accession numbers for each 16S rRNA sequence show in the parenthesis.
Fig. 19. Percentage of resistant *Arcobacter cryaerophilus* (n=6) and *butzleri* (n=16) resistant to antimicrobials. GEN, gentamycin. TEL, telithromycin. AZI, azithromycin. ERY, erythromycin. CIP, ciprofloxacin. NAL, nalidixic acid. CLI, clindamycin. FLO, florfenicol. TET, tetracycline. *A. butzleri* had a significantly higher percentage of resistance to tetracycline (*p < 0.05*).
Fig. 20. Percentage of *Arcobacter* isolates that expressed multiple resistance to three or more antimicrobials.