ABSTRACT

Dissertation Title:	*TEMPORAL DYNAMICS, ANTIMICROBIAL RESISTANCE AND PHYLOGENETIC RELATIONSHIPS OF BACTERIAL TAXA IN IRRIGATION WATER SOURCES AND RELEVANCE TO FOOD SAFETY*
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As climate change continues to stress freshwater resources, we have a pressing need to identify alternative (nontraditional) sources of microbially safe water for irrigation of fresh produce. Unfortunately, open water sources are often contaminated with many known human pathogens such as *E. coli, Salmonella* and *Listeria* and unknown/understudied pathogens such as *Aeromonas* that are associated with foodborne outbreaks. To facilitate the adoption of microbiologically safe irrigation water sources, a comprehensive study on the prevalence and virulence potential of human pathogens and their transferability to fruit and fresh produce vegetables was conducted. The effect of irrigation water types on crop surface microbial community structure, presence of virulence factors and antimicrobial resistance were investigated to evaluate the potential of transfer of pathogenic and antimicrobial resistant bacteria in humans. Initially, the prevalence of indicator bacteria was determined using culture methods and then microbiological water quality profiles (MWQP) was created to identify water sources that complied with the U.S Food Safety Modernization Act water standards. Next, using culture and molecular methods, investigation of the

antimicrobial resistance profile of one known foodborne pathogen *Escherichia coli* retrieved over a two-year period was done. *E. coli* resistance against widely prescribed antibiotics, extended spectrum β-lactams, was determined phenotypically and genetically. The diversity, distribution and potential for pathogenesis of one understudied pathogen, *Aeromonas*, prevalent in a variety of typical or potential irrigation water sources and collected over a one-year period was investigated. The study revealed spatial and temporal patterns in species richness, evenness, virulence gene carriage and attachment behaviors on both biotic and abiotic surfaces, of this bacterial taxon. Finally, the effect of using highly treated reclaimed water and pond water on lettuce surface microbiomes was investigated. The study provided an integrated assessment of the shifts in microbial community that result from using different irrigation water sources for irrigation of lettuce. Understanding the ecology of lettuce associated microbiota can be useful to infer risks of transfer and establishment potential of possible pathogenic strains from irrigation water sources to minimally processed raw consumed fresh produce crops.

TEMPORAL DYNAMICS, ANTIMICROBIAL RESISTANCE AND
PHYLOGENETIC RELATIONSHIPS OF BACTERIAL TAXA IN IRRIGATION
WATER SOURCES AND RELEVANCE TO FOOD SAFETY

by

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Dedication

My PhD thesis is wholeheartedly dedicated to my mother, Shereen Akhter
Who dedicates her life just to see us happy and successful

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Chapter 1: Introduction

Use of microbiologically contaminated agricultural water can enhance the chance of foodborne outbreaks/illnesses due to its ability to become a vehicle of transmission of many known, unknown and under-studied enteric pathogens (Steele & Odumeru, 2004; Uyttendaele et al., 2015). Several multistate foodborne illness/outbreaks via different kinds of food crops, caused by familiar and unfamiliar human pathogens, have been reported (CDC, 2018). Among all fruit and vegetable crop harvests, leafy green vegetable contamination presents a heightened risk because of its popular raw consumption. Fresh produce and fruit often come in direct contact with irrigation water, soil and the environment (Carstens et al., 2019). Moreover, minimal post-harvest handling and treatment, limited preservative and antimicrobial or sanitizing agent applications and only occasional heat treatment (Seymour & Appleton, 2001), make leafy vegetables more vulnerable to pathogen contamination presenting a threat to public health. From 2010 to 2017, 85 multistate foodborne illness outbreaks linked with fresh produce crops were reported, and determined to be caused by only five etiological agents (*E. coli, Salmonella, Listeria monocytogenes, Campylobacter* and hepatitis A) (bacterial pathogens were responsible for 83 outbreaks and two viral pathogens for two outbreaks) resulting in 4,658 illness followed by 1,187 hospitalizations and 55 deaths (Carstens et al., 2019). The average mesophilic microbial load was found to be 10^3-10^9 colony forming units per gram (CFU/g) of fresh produce before post-harvest processing and 10^3-10^6 after post-harvesting processing (Nguyenthe & Carlin, 1994). Another emerging threat that has been

reported is the transfer of antimicrobial resistance genes to fresh produce. Several studies have reported the presence of antimicrobial resistance genes (ARGs) in fresh-cut vegetables and salad (Hölzel et al., 2018; Larrañaga et al., 2018; Thanner et al., 2016; Verraes et al., 2013). Some of these findings suggest that irrigation water as the source of these antimicrobial resistance (Iwu et al., 2020; Li et al., 2014; Silbergeld et al., 2008). Consumption of foods carrying antimicrobial resistant microorganisms can transmit these microbes' transmission (Angulo et al., 2004; Ekwanzala et al., 2018; Ibekwe et al., 2011). There is also evidence that these genes can be transmitted to human pathogens (Baker et al., 2018).

In addition to contributing to foodborne diseases, microbiological quality of irrigation water affects the microbiome of crops. The term "microbiome" means the community of microorganisms living in a certain niche. Plants have their specific microbiome that contributes to their growth (eg. growth promoting bacteria), health, and immunity (Berendsen et al, 2012; Hirsch and Mauchline, 2012). The microbiome is influenced by several factors, including specific environmental conditions, agricultural practices, soil type, plant age, the plant immune system and plant genetic diversity, down to the cultivar level (Berg and Smalla, 2009; Micallef et al., 2009a; Micallef et al., 2009a,b). Physical and environmental change in the community might deter the growth of beneficial microbes for human and nurture the pathogenic one.

The present work focuses on the microbiological quality of surface and recycled water considered as prospective irrigation water. Evaluating the microbial load and characterizing the bacterial risk of surface and recycled water to be used for irrigation of fresh produce crops is crucial, to ensure the suitability of using such water.

As such, my research hypotheses are:

1. Temporal, spatial and physical-chemical factors alter bacterial ecology in surface and reclaimed water in the mid-Atlantic region.

2. Reclaimed and surface waters harbor levels of diverse bacteria that have the potential to be opportunistic pathogens through the carriage of virulence genes, antimicrobial resistance genes and the capacity to attach to surfaces.

3. The microbial quality of irrigation water influences the crop microbiome when it contacts the crop directly.

To test these hypotheses, surface and reclaimed water was collected from different sites in eastern and western Maryland and Delaware. The study first determined the prevalence of bacterial indicators (total coliform), fecal indicators (*Escherichia coli* and enterococci) and *Aeromonas* spp. over a spatial and temporal scale. As a second step, the study characterized both pathogenic and non-pathogenic *E. coli*, by phylogrouping and investigating their antimicrobial resistance gene phenotypes and genotypes. As a next step, this study focused on investigating the diversity and distribution in surface and recycled water of an under-studied human pathogen in the U.S, *Aeromonas*, their virulence potential and possibility of attachment in the lettuce phyllosphere. Finally, the study explored the influence of irrigation water on the Romaine lettuce crop microbiome in response to pond and reclaimed water overhead applications over the entire field growing cycle. Findings from this study provide important insights on the applicability of surface and reclaimed water for irrigation

without the risk of pathogen contamination to fresh produce. In each chapter, a thorough explanation on the background, methodology, results and impacts of the study are discussed.

Chapter 2 provides a comprehensive literature review on the current knowledge on the applicability and difficulties of using alternative sources of irrigation water and emphasizes the knowledge gaps that need to be addressed before adopting alternative irrigation water sources.

Chapter 3 discusses prevalence of *E. coli*, *Enterococcus*, total coliforms (TC) and *Aeromonas* spp. in surface and reclaimed water that may be available for irrigation of fresh produce. A standard membrane filtration method following approved methodology (EPA1600, 1604 and 1605) provided by Environmental Protection Agency (EPA) was employed to determine the contamination level of bacterial agents with subsequent specific aims.

> *Specific Aim 1-1*: Explore the temporal dynamic of *E. coli*, TC, *Enterococcus* and *Aeromonas* spp. in various water types in the mid-Atlantic region over a period of two years.
>
> *Specific Aim 1-2*: Explore the relationship in prevalence among the various bacterial taxa.
>
> *Specific Aim 1-3*: Explore the relationship between bacterial taxa and physical-chemical parameters of water.

In the following parts (**Chapter 4 and Chapter 5**) the possible role of two specific microorganisms on foodborne disease outbreaks are discussed. In addition to high

pathogen loads, other factors including the spread of antimicrobial resistant bacteria play a role in affecting human health. A commensal member, *E. coli* can be a vehicle of antimicrobial resistance genes. This taxon also consists of pathogenic species. Based on the presence of the heme transport, stress related and lipase/esterase genes, *E. coli* is divided into 4 phylogenetic groups: A, B1, B2 and D. Enterotoxigenic strains of *E. coli* are more likely to be groups A and B1 (Clermont et al., 2000). However, members of the other groups B2 and D are also important due to their ability to carry resistance genes against extended spectrum β-lactam antibiotics and their potential to transfer them to pathogenic strains. In **Chapter 4**, the diversity of *E. coli* in surface and reclaimed water and their antimicrobial resistance is assessed by determining and characterizing diversity and antimicrobial resistance of *E. coli* isolated from various water types over a two-year period. Specific aims employed here were:

> *Specific Aim 2-1*: Conduct phylogenetic analysis of *E. coli* isolates from surface and recycled water by Clermont typing.
>
> *Specific Aim 2-2*: Identify extended spectrum β-lactamase (ESBL) producing *E. coli* from all phylogroups.
>
> *Specific Aim 2-3*: Determine the genetic material employed for extended spectrum β-lactamase production in *E. coli* isolated from water samples.

Another major food safety concern is foodborne infection due to unspecified agents or understudied microorganisms. From 2012 to 2015, 19 foodborne outbreaks were reported due to unspecified/understudied etiological agents (CDC, 2018). According

to Scallan et al. (2011), *Aeromonas* can be placed in the "unspecified pathogens" category (Scallan et al., 2011). In **Chapter 5**, the diversity of *Aeromonas* spp. in different water types, their virulence profiles and their attachment capability on both biotic and abiotic surfaces is explored <u>by determining the phylogenetic relationship of *Aeromonas* spp. isolates collected from various water types over a one-year period and determining the virulence potential of *Aeromonas* spp. in different water sources (surface and reclaimed water)</u>. Specific aims employed here were:

> *Specific aim 3-1*: Conduct phylogenetic analysis of specific *Aeromonas* isolates based on nucleotide sequence of *rpoD* gene fragment.
>
> *Specific aim 3-2*: Investigate the virulence potential of isolates of *Aeromonas* spp. based on the presence of virulence genes.
>
> *Specific aim 3-3*: Investigate the attachment capability of *Aeromonas* spp. isolates on an abiotic surface and leaf surfaces.

Finally, the effect of using contaminated surface and reclaimed water for irrigation on the lettuce microbiome is discussed in **Chapter 6**. A metagenomic approach was employed to describe leaf phyllosphere microbiome responses to overhead applications of recycled or pond water for leafy green irrigation. The objective here was to <u>investigate crop microbiota in relation to irrigation with recycled and pond water</u> through 3 specific aims.

> *Specific Aim 4-1*: Compare the crop surface microbiota after irrigation with reclaimed and pond water using shotgun metagenomics sequencing.

Specific Aim 4-2: Determine the distribution and relative abundance of antimicrobial resistance genes in reclaimed and pond water treated crop surface by using metagenomic approaches.

Specific Aim 4-3: Compare the relative abundances of virulence genes of bacterial pathogens on crop surface treated with reclaimed and pond water.

In **Chapter 7**, highlights of the findings of my study are discussed. The chapter also shows how and why microorganisms exhibit diverse characteristics in terms of prevalence, diversity, pathogenicity, attachment and antimicrobial resistance in the mid-Atlantic region in the USA and the broader impact of using certain surface and reclaimed water to irrigate fresh produce crops on food safety management.

Chapter 2: Literature Review

2.1 Water availability for agriculture

Although three quarters of the earth's surface is covered in water, a very small proportion is usable. Approximately 96.5% (oceans, seas, bays and others) of the total earth water storage is unusable due to high salt concentration. The remaining 1.76% is distributed as ice caps, glaciers, ground ice and permanent snow, 1.69% is trapped underground as fresh and saline water and only 0.01% is distributed as surface/other fresh water that is available for use (Gleick, 1993; Levy and Sidel, 2011) (Figure 2.1).
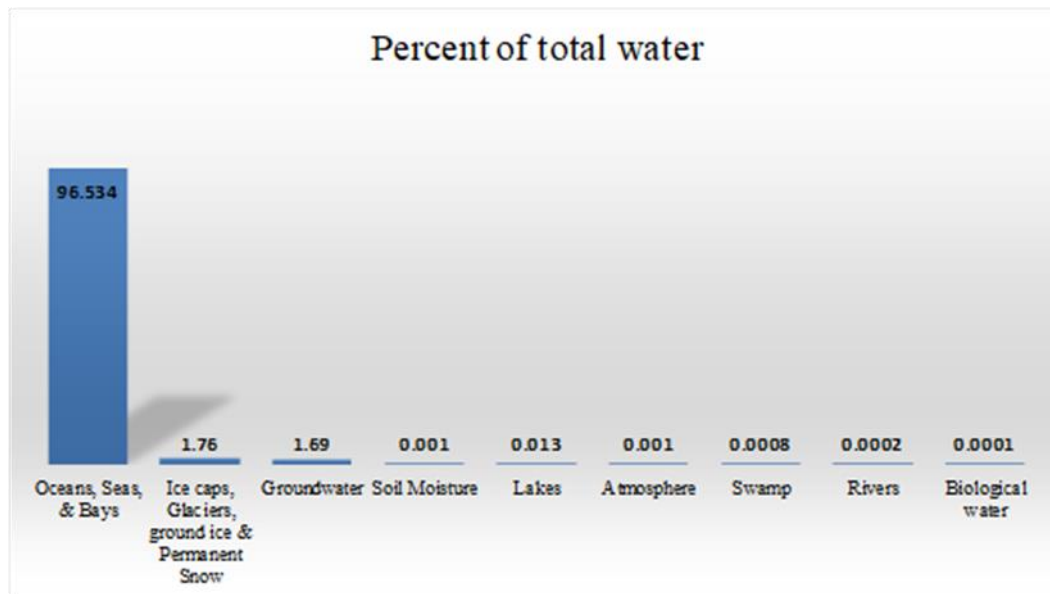
Figure 1.1: Water distribution in different sources (Gleick, 1993; Levy and Sidel, 2011)

Agriculture is the second largest sector which consumes almost 37% of this tiny portion of the freshwater (USGS, 2015). With the population growing at an exponential rate, it is estimated that by 2050, the world's water requirement for

agriculture will increase by 19% even with the improvement of irrigation systems, crop yields and proper water management (United Nations, 2019). The water scarcity problem in the near future may lead to series impacts on global food security. In addition to population growth, climate change is expected to affect the availability of irrigation water through fluctuating rainfall patterns, reduction in soil moisture and changing river flow. These changes may induce climactic variability (United Nations, 2019).

In the USA, the major consumers of ground and surface water are thermoelectric power, agriculture (mostly irrigation), domestic supply, non-agricultural industry and aquaculture. Irrigation comprises 64% of total freshwater withdrawal in the USA when we exclude thermoelectric power from the calculation (Dieter et al., 2018). To supply enormous amounts of water in these sectors, surface and groundwater withdrawals have been gradually increasing from 1950 to 2015. The estimated total water withdrawal (surface and ground) in 1950 was 180 billion gallons per day while in 2010 it had increased by 174 billion gallons per day. However, in 2015 this number was 322 billion gallons per day which is almost 9 percent less than 2010 withdrawal. The major reason for this drop, was only due to the low water requirement in thermoelectric power, therefore, the water requirement for the agriculture has never been dropped (Dieter et al., 2018). For irrigation only, in 2015, 118 billion gallons per day water was withdrawn in the USA which was 2% higher than withdrawal in 2010. (Dieter et al., 2018). However, one of the major factors that significantly affects irrigation water availability is climate change (Malek et al., 2018). In spite of drawing larger percentage of total freshwater for irrigation, many parts of the USA are

experiencing water insufficiency for irrigation due to extreme to moderate drought. The current drought that is concentrated in the West and Southwest is affecting California, Idaho, Montana and Colorado (total water withdrawal 44% of the USA). These states are facing extreme to moderate drought and suffering water insufficiency for irrigation (USGS, 2018). The south-west, mainly California, uses 80% of its total water withdrawal for agriculture (Kearney et al., 2014). In the 20th century, the west part of the USA turned into a major agricultural hub due to having artificial and natural water reservoirs. Now because of extreme drought, U.S. agriculture has been pushed to the eastern part, including the mid-Atlantic side of the country and creating pressure on non-agricultural industry (Walton, 2014).

2.2   Alternative irrigation water: a milestone for sustainable agriculture

Excessive groundwater withdrawal for irrigation will reduce the water availability in aquifers and wells that may lead to changes in the balance in environment and negatively affect the nation's economy. In addition, changes in climate and weather patterns have created a new challenge for not only agriculture, but also different sectors in the country. The global population is increasing drastically, it is expected that in 2050, the population will increase to nearly 10 billion (Worldbank, 2020, United Nations, 2019). To provide food for this extensive population, agricultural production needs to be expanded by 70% (Worldbank, 2020). An alternative source of irrigation water can nurture sustainable food production and can reduce the nation's burden caused by environmental and human factors.

2.2.1 What is alternative irrigation water

Non-traditional water that is not typically used for irrigation of food crops is termed

as "alternative irrigation water". Non-traditional water such as treated wastewater

(also referred as reclaimed water), mildly saline water, produce wash water or

household graywater could reduce the heightened demand on groundwater resources.

In recent years, these sources have become potentially available water sources not

only for the commercial agricultural sectors, but also urban irrigation (Cabrera, 2017).

However, these sources are not considered as microbiologically safe that can be used

without any physical or chemical treatment. Several studies reported that reclaimed

water harbors high number of total coliforms, fecal coliforms and other foodborne

pathogens. Higher levels of *Salmonella, Streptococcus, Listeria, Clostridium,*

Shigella and *Vibrio* spp. were detected in crops irrigated with reclaimed water (Bryan,

1977; Haymaker et al., 2019; Lopez-Galvez et al., 2014; Sharma et al., 2020b). Other

pathogens including helminths, viruses and protozoa were found in recycled water

(RW) (Aertgeerts & Angelakis, 2003; Deng et al., 2019).

2.3   Foodborne outbreaks/illness associated with irrigation water

The Centers for Disease Control and Prevention (CDC) reported that every year,

roughly one in six Americans (48 million people) get sick, 128,000 are hospitalized

and 3,000 die due to consumption of contaminated food (Scallan et al., 2011a, b).

Only 20% of these illnesses (9.8 million in total) are caused by known agents and the

remaining 80% remain undefined and many understudied etiological agents (38.2

million in total) (Scallan et al., 2011a; Scallan et al., 2011b). Research showed that

many of those foodborne outbreaks/illnesses caused by consumption of fresh produce

crops were irrigated with microbiologically contaminated irrigation water (Ackers et al., 1998; Gil et al., 2015; Steele & Odumeru, 2004; Strausbaugh & Herwaldt, 2000; Uyttendaele et al., 2015). Poor-quality irrigation water is a reservoir for many types of microorganisms including bacteria, viruses, protozoa and helminths that may cause foodborne outbreaks/illnesses (Steele & Odumeru, 2004). Risk increases many-fold in the case of raw or minimally processed fruits and vegetables. Fresh produce is considered as a major contributor of foodborne illness, with leafy vegetables ranked as the commodity that is linked to the highest number of foodborne illnesses (22%). During the period 1998-2008, it was reported that among all foodborne illnesses, leafy greens were responsible for 14% of hospitalizations (second most frequent cause in foodborne illness) and 6% of deaths (fifth most frequent cause in foodborne illness) (Painter et al., 2013). Use of contaminated irrigation water and agricultural water in fresh produce production is considered to be a route of crop contamination (Allende and Monaghan, 2015) via contamination in the field and pre- and post-harvesting equipment during the production of fresh produce crops (FDA, 2008). *E. coli* O157:H7 was reported to transmit to lettuce from irrigation water (Solomon et al., 2002; Wachtel et al., 2002). In 2005, a multistate foodborne outbreak from tomato was reported caused by *Salmonella enterica* which was transmitted from irrigation water (Greene et al., 2008). In 2006, another multistate outbreaks of Shiga toxin-producing *E. coli* O157:H7 from leafy greens was reported that was also associated with agricultural well water (CDC, 2018b). *Listeria monocytogenes* was identified in 2014 in the production chain of mung bean sprouts and water used for its irrigation. Pulse Field Gel Electrophoresis (PFGE) and whole genome sequence confirmed the

link between the listeriosis in Illinois and Michigan with 2 deaths and the isolate

collected in the production chain (CDC, 2018a). Two other multistate outbreaks were

reported that were associated with irrigation canal and agricultural water reservoir on

the farm, one of them was spread on July 2018 by Shiga toxin-producing *E. coli*

O157:H7 and associated with Romaine lettuce with over 200 illness, 96

hospitalization and 5 deaths. Another multistate outbreak was disseminated in

December, also caused by Shiga toxin-producing *E. coli* O157:H7 associated with

Romaine, red leaf and green leaf lettuce with 25 hospitalizations. Whole genome

sequencing confirmed the outbreak strain of *E. coli* O157:H7 was found in sediment

within an agricultural water reservoir on the farm located in Santa Barbara county,

California, USA (CDC, 2019).

Due to the risk of transfer of pathogenic microorganisms from agricultural water to

humans via food crops, equipment/facility, transportation system and handling,

microbiological quality monitoring of irrigation water has become a mandatory part

to ensure food safety and to fulfill the specifics in the Food Safety Modernization

Act's Produce Safety Rule (Allende and Monaghan, 2015).

2.4 Microbiological quality of irrigation water: possible risks and regulatory measurements

Irrigation water can be contaminated with many known, unknown and understudied

pathogens such as *Escherichia coli*, *Salmonella* spp. or *Campylobacter*, *Vibrio*,

Aeromonas and other pathogens from various sources. Regardless the source of

irrigation water, major contaminants are assumed to be fecal materials. Research

showed that rivers, reservoir and treated wastewater possess higher risk of microbial

contamination (Adefisoye and Okoh, 2016; Ahmed et al., 2012; Castro-Ibáñez et al., 2015; Ferguson et al., 2012; Ibekwe et al., 2018). Typical risks in irrigation water are:

2.4.1 Known human pathogens:

Approximately 9.8 million foodborne illness that occur every year in the USA are caused by known human pathogens (CDC, 2018a; Scallan et al., 2011b). Several of these incidences are associated with irrigation water (from 2005 to 2018, 5 multistate outbreaks) (CDC, 2018b). Research showed that the most common human pathogen found in irrigation water is *Escherichia coli* although it is a commensal microorganism and used as a fecal indicator in water. Many studies have identified human pathogens such as *E. coli* O157:H7 and *Cyclospora cayetanensis* in both irrigation water and fresh produce (Ackers et al., 1998; Decol et al., 2017; Haymaker et al., 2019; Leaman et al., 2014; Strausbaugh and Herwaldt, 2000). Two other very common human pathogens widespread in irrigation water are *Salmonella* and *Listeria monocytogenes*. Studies found that *Listeria* can transfer from irrigation water to leafy greens (Oliveira et al., 2011). Several other studies identified *Salmonella* and *Listeria monocytogenes* in fresh produce and soil irrigated with contaminated water (Chitarra et al., 2014; Mawak et al., 2009; Sharma et al., 2020a; Weller et al., 2015). From 2009 to 2018, 130 outbreaks caused by *Vibrio* were reported associated with pond, treated/ partially treated wastewater and water reservoirs (CDC, 2018b); therefore, it is assumed that *Vibrio* is another widely known pathogen that can contaminate food crops via irrigation water. Several studies isolated and identified *Vibrio* in current and

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possible agricultural water, fresh vegetables and seafood (Cañigral et al., 2010; Hounmanou et al., 2016; Pianetti et al., 2004).

2.4.2 Unknown/under-studied human pathogens:

A large proportion of foodborne outbreaks were reported to be caused by unknown agents. Every year more than 38 million people are infected with many unknown and understudied foodborne pathogens (Scallan et al., 2011a). Due to the scarcity of relevant information about unknown and understudied foodborne pathogens in the USA, taking precautionary measurement is sometimes impossible. From 2012 to 2015, 19 foodborne outbreaks were reported due to unknown etiological agents (CDC, 2018a). Scallan et al. (2011) shows that every year 1686 deaths occur due to unknown agents as a result of unidentified domestic foodborne transmission. Annually, almost 80% of illness, and 56% of hospitalization were reported due to unspecified/unknown agents (Scallan et al., 2011a). The term "unspecified pathogens" includes known pathogens with insufficient data to categorize them, such as *Aeromonas* spp. (Scallan et al., 2011a, b). The number of foodborne outbreaks and deaths in recent years suggested that this problem is not going away (CDC, 2018b). Elderly people, pregnant women, immune-compromised people and children are thought to be more vulnerable (Akbar and Anal, 2011).

In the literature, reports on population-based incidences of human *Aeromonas* infections are far from being comprehensive. One of the early reports came from King et al. (1992) who stated an annual incidence of 10.6 *Aeromonas* infection cases per million people from California in 1988. Between January 1988 to December

1989, *Aeromonas* spp. was isolated from 1.8% (45 out of 2,480) of patients with acute

gastroenteritis in India (Deodhar et al., 1991). The estimated incidence of *Aeromonas*

bacteremia in England and Wales in 2004 was 1.5 cases per million population (Janda

and Abbott, 2010). In France in 2006 it was 0.66 cases per million inhabitants (Lamy

et al., 2009) and in southern Taiwan during 2008 to 2010 it was 76 cases per million

people (Wu et al., 2014). Cytotoxic strains of *Aeromonas veronii* were isolated from

2.5% (13 out of 514) of chronic diarrheal patients in southern Taiwan (Chen et al.,

2015) and 2% (18 out of 863) of traveler's diarrhea patients in Barcelona (Vila et al.,

2003). In addition to foodborne infections and traveler's diarrhea, *Aeromonas* spp.

was also found to be associated with skin and soft tissue infections among the

survivors of the tsunami in southern Thailand in 2004. At the species level, A.

hydrophila, followed by *A. veronii* were responsible for most of those skin and soft

tissue infections in humans (Hiransuthikul et al., 2005). In addition, *Aeromonas*

species was found to cause true indwelling-device related infections in healthcare

facilities (Hsueh et al., 1998). These reports suggest significant geographic variation

and wide abundance of *Aeromonas* infection cases in different regions throughout the

world during the last three decades. As reports on *Aeromonas* infection in the U.S. are

not well documented, Scallan et al. suggested *Aeromonas* to be one of the potential

etiological agents responsible for outbreaks associated with unspecified disease

agents in the U.S. (Scallan et al., 2011a). Diseases caused by this emerging foodborne

pathogen can be life threatening, therefore researchers considered this bacterium as

infectious as *Salmonella, Campylobacter, Shigella, Vibrio parahaemolyticus,*

Yersinia and enteric-type *Escherichia coli* (Koutsoumanis et al., 2014; Schmidt et al., 2003).

2.4.3 Antimicrobial resistance in pathogenic/non-pathogenic bacteria:

One of the major public health challenges in the area of food safety is the presence of antimicrobial resistant bacteria (AMR) in irrigation water and food. The widespread use of antibiotics as growth promotors and for treatment against bacterial infection in food-producing animals has led to the emergence of AMR in food (Prestinaci et al., 2015) (Figure 2.2). Every year, approximately 0.4 million people get sick because of AMR foodborne infections whereas 26,000 cases are associated with Extended-Spectrum β-Lactamase (ESBL) producing Enterobacteriaceae (CDC, 2013). ESBL-producing bacteria are resistant to most β-lactam antibiotics, including 3rd and 4th generation cephalosporins and sometimes resistant to multiple other classes of antibiotics. *Escherichia coli* is the most common ESBL-producing bacteria, a natural microflora often found in the lower intestines of warm-blooded animals and aquatic systems (Franz et al., 2015).
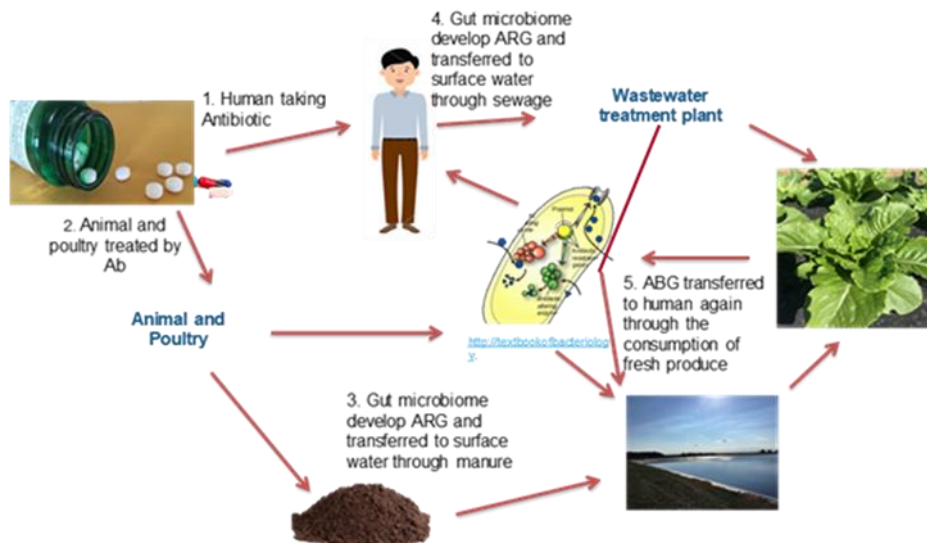
Figure 2.2: Spread of Antibiotic resistant bacteria in the environment (Franz et al., 2015; van Hoek et al., 2015)

Commensal organisms usually do not cause disease. However, like most resistance genes, β-lactamase is encoded as a plasmid mediated gene that can transfer to other pathogenic bacteria through horizontal or vertical gene transfer. Acquiring AMR genes limits treatment options against pathogenic microorganisms. A recent scientific report (2015) published in Nature on the prevalence of ESBL producing *E. coli* from Dutch surface water and wastewater showed that 17.1% of all ESBL producing *E. coli* are predicted to be virulent (Franz et al., 2015). ESBL-producing *E. coli* can spread to fresh produce via irrigation water (van Hoek et al., 2015). Consequent transfer to humans, due to consumption of raw produce can negatively impact consumer health.

2.4.4 FDA standards for irrigation water usage

While many studies found significantly higher bacterial load in both fresh produce and its irrigation water (Castro-Ibáñez et al., 2015; Ceuppens et al., 2014), due to low

prevalence of human pathogens in fresh produce, few researchers are unwilling to

make a direct link between risk of outbreaks/illness from fresh produce and

contaminated irrigation water (Leaman et al., 2014). Considering the widespread

contamination in agriculture water, thermotolerant total coliform, fecal coliform (*E.*

coli), and in some cases *Enterococcus* are used as indicator for fecal contamination in

water (Edberg et al., 2000; EPA, 2004; Leclerc et al., 2001). The standard varies in

different countries based on the outbreak data and regulatory agency that ranged from

100 to 1,000 CFU per 100 ml of water for total coliforms, 100 to 1,000 CFU per 100

ml water for fecal coliforms and 35 CFU of enterococci per 100 ml of water (EPA,

2004; Fremaux et al., 2009; Jones & Shortt, 2005). In recent years, the US Food and

Drug administration (USFDA) recommended a regulation guideline under the Food

Safety Modernization Act, the Produce Safety Rule (FSMA PSR) based on generic *E.*

coli counts, to minimize foodborne outbreaks associated with irrigation water in the

USA. FSMA PSR recommends the construction of a Microbial Water Quality Profile

(MWQP) for any irrigation site using 20 water samples collected over 4 years, from

which the geometric mean (GM) and the standard threshold value (STV) of generic *E.*

coli should be calculated. The GM should not exceed 126 CFU/100 mL of water and

the STV should not exceed 410 CFU/100 ml of water (FSMA, 2011). However, many

studies failed to correlate generic *E. coli* load and the presence of other foodborne

pathogens (*E. coli* O157 or *Salmonella* spp.), therefore building a case to move away

from using *E. coli* as a fecal indicator (Benjamin et al., 2013; Leaman et al., 2014;

Shelton et al., 2011) while others believe *E. coli* is still the best fecal indicator for

water quality checking. However, due to the unpredictable nature of environmental

resources like water, and based on the data available, *E. coli* spp. could be used to indicate fecal contamination in irrigation water (Allende and Monaghan, 2015) but should not be exclusive of investigating other pathogens.

2.5 Effect of alternative irrigation water sources on the crop surface microbiome: reclaimed vs surface water

Tertiary treated wastewater (also referred as reclaimed water) is an excellent alternative water source for irrigation that could reduce the heightened demand on groundwater resources. However, the challenges to use this water on fresh crops cannot be denied. In addition to having a huge contribution to foodborne diseases, contaminated irrigation water might affect microbiomes on the surface of crops. Research shows that microbial community diversity also varies in different parts of the plant. Each plant harbors different microbiome in specific organ especially in phyllo-sphere (leaves) and rhizosphere (roots) (Hirsch and Mauchline, 2012; Ottesen et al., 2013). In most cases, microbiomes are found to have a symbiotic or commensal relationship with their host specially with those live on rhizosphere. However, in some cases, they also found to have antagonistic relationship with their host as plant pathogens are also part of plant-associated microbiota (Berg et al., 2005; Leff and Fierer, 2013; Mendes et al., 2013).

It is important to identify the role of reclaimed water (RW) on the structure of microbial communities and their interaction with the host to evaluate their suitability as agricultural water. Use of reclaimed water might have direct and indirect effects on structural and functional responses of microbial communities of plants. Several studies have been done to identify the effect of RW irrigation on plant associated

microbes. In 2000, Filip et al. reported that higher microbial activity was noticed in plant roots and soils irrigated with RW compared to surface freshwater irrigated crop root and soils (Filip et al., 2000). The paper also reported that RW introduction in crops significantly enhanced the growth of copiotrophic microbial assemblages (organisms that grow in carbon rich environments) whereas, crops irrigated with freshwater enhance the richness of oligotrophic community (microbes grow in low nutrient environment). Authors detected significantly higher microbial biomass in soils irrigated with RW which releases essential organic nutrient for plant growth. A study by Hidri et al. (2010) also found that long-term applications of RW on soil and crop stimulate microbial abundance and change in diversity, measured by Automated Ribosomal Intergenic Spacer Analysis (ARISA) (Hidri et al., 2010). RW irrigation induced a specific community composition which is significantly correlated to the duration of irrigation and seasonal variations. Studies found differences in community structures in plants irrigated with RW and surface fresh water. Nitrosomonas-like strains of ammonia-oxidizing bacteria (AOB) dominated in soil irrigated with treated wastewater whereas Nitrosospira-like strains dominated in soil irrigated with freshwater (Frenk et al., 2014; Ndour et al., 2008; Oved et al., 2001). All studies indicated that treated wastewater-irrigated crops have a certain tendency for higher microbial activity, higher microbial biomass and shifts in community diversity compared to freshwater-irrigated crops.

Other than changing the community structure, RW application also may introduce human pathogens to plant surfaces which might have a negative impact on overall community. RW application on crops could reduce the abundance of plant beneficial

communities and enhances the occurrence of human pathogenic bacteria due to harboring high pathogen load (Berg et al., 2005). Some opportunistic pathogens such as *Salmonella enterica*, *E. coli* O157:H7, *Burkholderia (cenocepacia) cepacia, Pseudomonas aeruginosa, Bacillus cereus* and *Proteus vulgaris* (Berg et al., 2005) were found in the microbial communities on plant surfaces, although they may well be a normal part of plant microbiota. However, some argued that abundant and highly diverse indigenous microbial communities in the phyllosphere provides a strong barrier against the invasion of human pathogens (Cooley et al., 2003) As an example, Cooley et al. mentioned the growth and proliferation of *Salmonella enterica* and *E. coli* O157:H7 are suppressed by plant-associated microbes, *Enterobacter asburiae* on root surfaces. Nevertheless, several human pathogenic bacteria are able to colonize and replicate on the plant surface in the presence of indigenous communities due to their high competitiveness for nutrients (Brandl and Mandrell, 2002; Cooley et al., 2003; Mendes et al., 2013)

In case of the human microbiome, it was reported that single pathogen introductions cause the shift of a whole community including supporting microorganisms (Clemente et al., 2012; Blaser et al., 2013; Ramírez-Puebla et al., 2013). It is assumed that the same effect could be observed in plant microbial communities. RW and freshwater application could have some significant effect on the change of bacterial communities and increased species richness on plant surface. Research confirmed that the phytopathogenic fungus *Rhizoctonia solani* from soil and its antagonistic counterpart biocontrol agent *Bacillus amyloliquefacies* FZB42 shifted the overall community on the lettuce surface, with the enterobacterial fraction being especially

impacted by the plant pathogen (Erlacher et al., 2014). In addition, it was reported that *Enterobacteriaceae* has the tendency to be invasive into the plant cell even though some plant leaves have a wax layer to protect their tissue from microbial colonization and invasion. (Bernstein et al., 2008; Nikaido et al., 2010). This particular information is vital in terms of food safety because post-treatment decontamination processes such as chlorination, ozonation and UV radiation, are reported to be successful against pathogenic microbes on the surface of RW irrigated crops (Hey et al., 2012; Martínez et al., 2011) but not against internalized one. Erlacher et al. (2015) reported that biotic stress (introduction of pathogen or non-pathogenic strains) induces shifts in microbial communities associated with lettuce. They also reported that the strains found in higher abundance after disturbance are mostly opportunistic and fast-growing pathogens, possibly indicating that pathogens have a significantly higher level of fitness to survive in environmental turbulence and in resisting competitive exclusion.

2.6   Metagenomic approaches to assess the risk in irrigation water and crops

Bacterial culture methods are the most traditional techniques to isolate and identify microorganisms present in certain niches. However, due to being time-consuming and unable to identify the astronomically high proportion of non-culturable taxa, culture methods fail to estimate the diversity and abundance of microbes in the environment (Hong et al., 2020; Rappé & Giovannoni, 2003). In addition to culture-based identification techniques, quantitative PCR (qPCR) is convenient for the detection and quantification of microbial community members (Hong et al., 2020). The

shortcomings of qPCR, for example detection of only marker genes or inability to detect unknown gene targets, make next generation sequencing technology more comprehensive. High throughput sequencing such as 16s rRNA gene amplicon sequencing and shotgun metagenomic sequencing, are used to identify the members of a microbial community and provide a clear picture of the microbial diversity in a certain environment. Shotgun metagenomic sequencing provides taxonomic and phylogenetic information as well as functional gene diversity unlike 16s rRNA gene sequencing. Microbial characterization using next generation sequencing adopts a data mining approach that can help with hypothesis building and future hypothesis testing (Wooley et al., 2010). Next generation sequencing can provide information regarding the microbial community structure in different surface and reclaimed water sources and how much these water types can affect the crop microbiome if they are used for irrigation. In addition, the transfer of virulence genes and antimicrobial resistance genes can be deduced from these types of study. Widely used sequencing platforms for whole genome or microbial community analysis are the MiSeq/HiSeq/NextSeq (Illumina, San Diego, CA). Some other platforms for example: PacBio SMRT Sequencing (Pacific Biosciences, Menlo Park, CA) and MinION (Oxford Nanopore Technologies, Oxford, UK) are used for long read genome sequences.

Despite its numerous advantages, metagenomic sequencing has some limitations; this method is unable to identify the viability of cells where the DNA molecules were derived from or to identify functionally active genes. In contrast, metatranscriptomics

(RNA-based sequencing) or metaproteomics (peptide sequencing) are two methods
that can provide the information missing from metagenomic sequencing.

2.7 Diseases transfer from irrigation water to humans: a public health concern

Different risk factors such as microbial load, their virulence, and transferability from
prospective irrigation water in the mid-Atlantic region, to food crops, still need to be
investigated. The infection and virulence strategies exploited by the vast microbial
populations found in water environments, that can transfer to food crops is still far
from being elucidated.

The major implication of using contaminated irrigation water is transmission of
human pathogens to food crops. The most reported diseases that have been
transmitted via irrigation water is gastroenteritis caused by *Salmonella, Clostridium,
Campylobacter, E. coli* O157:H7*, Shigella, Vibrio, Listeria monocytogenes*,
Norovirus, *Giardia* and *Toxoplasma*. However, complications that are associated with
irrigation water other than gastroenteritis are skin infection, sore throat, wound
infection, infection with flesh eating bacteria, pancreatitis, cholecystitis, peritonitis
and head substantial gastrointestinal hemorrhage (Keraita et al., 2008). The post
infection sequelae of these pathogens include hemolytic uremic syndrome (HUS) and
meningitis after *E. coli* infection, Guillain-Barre´ syndrome reactive arthritis, cardiac
problem and uncreative colitis after *Campylobacter* infection and reactive arthritis
after *Salmonella* infection are very important in terms of long term health and
economic burdens. HUS from *E. coli* O157:H7 infections owing to the consumption
of contaminated bagged fresh spinach and lettuce were reported in the USA, in

September 2006 (205 reported infections, including 31 HUS and 3 deaths) and December 2006 (71 reported infections including 8 HUS), respectively (Burger, 2012; CDC, 2018a). Reactive arthritis is mostly associated with *E. coli, Entercoccus spp., Salmonella, Shigella,* and *Campylobacter jejuni* infection (Lahu et al., 2016). Several cases have been reported right after foodborne illness outbreaks in the USA (Townes, 2010).

All these sporadic events or large outbreak associated foodborne illnesses highlight the importance of analyzing the microbial quality of irrigation water and find a transformative solution to mitigate contamination. We should protect irrigation water from fecal contamination and maintain the quality monitoring regularly.

Chapter 3: High prevalence of *E. coli*, *Enterococcus*, coliforms and *Aeromonas* spp. in surface and reclaimed water that may be available for irrigation of fresh produce

3.1   Introduction

Agriculture consumes ~70% of global freshwater withdrawals annually (UNESCO-WWAP, 2012), with much higher proportions used in agriculturally intensive countries. In 2015, irrigation accounted for 42% of freshwater (surface and groundwater) withdrawals for all uses in the USA (Dieter et al., 2018). Increasing population growth and agricultural demands, competing interests for surface water, unsustainable groundwater abstraction, and changing precipitation and drought patterns are placing a strain on water availability for agriculture. As a result, concerns of long-term water scarcity in the USA are growing (Walthall et al., 2012; Ebi & Bowen, 2016), and there is a need to explore alternative water sources for agriculture to reduce dependence on high-quality, environmentally sensitive groundwater sources. In the mid-Atlantic region of the US, such alternatives include tertiary treated wastewater (reclaimed water), pond and river surface water, and recycled vegetable processing wash water. The safe use of these alternative sources could reduce the heightened demand on existing groundwater resources.

Aside from availability, a major reason for using high-quality water, such as groundwater, in agriculture is to assuage food safety concerns in the irrigation of fruit and vegetable crops. Microbiologically contaminated irrigation water has the potential to spread infectious agents to crops (Steele & Odumeru, 2004; Park et al., 2012), and enteric pathogen-contaminated irrigation water has been implicated in

several foodborne illness outbreaks (Greene et al., 2008; Gelting et al., 2011).

Pathogens can survive for extended periods in surface and reclaimed water under

favorable conditions, but bacterial dynamics are complex and water physicochemical

parameters alone do not provide strong predictive potential (Avery et al., 2008;

McEgan et al., 2013). Therefore, assessing the microbial quality of alternative water

sources for the irrigation of fresh produce is a critical step in evaluating the suitability

of those sources (Uyttendaele et al., 2015). The earliest standards to evaluate the

microbial quality of irrigation water made use of total coliforms (TC), a heterogenous

group of bacteria (EPA, 1972). However, due to their inability to reliably indicate the

presence of fecal contamination determined to be the most probable source of

pathogens in water, microorganisms most frequently found in feces were selected

later as more appropriate indicators. These included *Escherichia coli* for drinking

water and Enterococcus spp. for recreational water use (NRC, 2004). The U.S.

Environmental Protection Agency (EPA) recommends the use of Enterococcus as an

indicator of fecal contamination for both freshwater and saltwater (EPA, 2000)

because of their long survival in water. In recent years, the FDA FSMA established

standards in a Produce Safety Rule (PSR; 21 CFR 112) specific to preharvest

agricultural water that will come in direct contact with edible portions of fresh

produce crops during cultivation. The rule requires yearly water testing and the

generation of a rolling microbial water quality profile (MWQP), based on data from at

least 20 samples collected over the most recent 2- to 4-year period. The PSR standard

uses *E. coli* concentrations and sets a geometric mean (GM) not to exceed 126 CFU

and a statistical threshold value (STV) not to exceed 410 CFU of *E. coli* in 100 ml of water.

As part of a U.S. Department of Agriculture-funded center, called CONSERVE (Center of Excellence at the Nexus of Sustainable Water Reuse, Food and Health), with the long-term goal of facilitating the adoption of safe agricultural water reuse, a mid-Atlantic team conducted a longitudinal study of microbial water quality of 11 alternative irrigation water sources. With the impending implementation of the agricultural water provision of the PSR, the microbial quality of these potential irrigation water sources was evaluated. Sites were selected to represent various types of surface and reclaimed water, and four bacterial taxonomic groups (*Escherichia coli*, TC, *Enterococcus* spp., and *Aeromonas* spp.) were enumerated to monitor the bacterial quality of the water source over a 2-year period. *Escherichia coli*, TC, and *Enterococcus* spp. were selected for their role as indicator bacteria. *Aeromonas* spp. were included as an understudied group of potential human pathogens ubiquitously found in water environments. We hypothesized that despite seasonal and geographical variability, surface water from various sources would be microbiologically safe to use for irrigation based on the proposed PSR criteria. We also hypothesized that reclaimed water would meet the PSR criteria and, moreover, exhibit more consistent parameters over time. Seasonal dynamics, relationships between bacterial taxa and physicochemical parameters of water, and compliance with the proposed agricultural water provision of the PSR were explored to assess which of the various water types are appropriate sources for irrigation of fresh crops.

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3.2 Method and Materials

3.2.1 Sampling sites and sample collection

Samples were collected from 11 locations (current and prospective irrigation water sources) in the mid-Atlantic region over a period of 2 years, from September 2016 to October 2018, twice a month from May to October and once a month from November to April. Sites included 3 highly treated reclaimed wastewater effluents (RW) (MA01, MA02, and MA06), five nontidal, freshwater rivers (NF) (MA03, MA04, MA05, MA07, and MA09), one tidal brackish river (TB)(MA08), and two on-farm ponds (PW) (MA10 and MA11). Overall descriptions of the sites are given in Table 1. From each site, 1 liter of water was collected into cleaned and sterile 1-liter polypropylene bottles (Thermo Fisher Scientific, Waltham, MA, USA). For surface water sites, bottles held with a sampling stick (Zenport Industries, Portland, OR, USA) were inverted, submerged 15 to 30 cm below the water surface, and turned sideways until full. For reclaimed water sites, water was collected from spigots close to field release sites (e.g., sprinklers used for groundwater recharge or irrigation of animal feed crops). Water was allowed to run for 1 min prior to collection. Immediately after sample collection, 1 ml of 10% sodium thiosulfate (Alfa Aesar, Heysham, England) solution was added to reclaimed water samples to quench residual hypochlorite added as part of the water reclamation process. For all water types, bottles were immediately transferred to coolers containing ice packs for transport to the laboratory. Samples were processed within 12 h of collection. Physicochemical parameters, i.e., water temperature, dissolve oxygen (DO), conductivity, turbidity, nitrate, chloride, salinity, pH, and oxidation reduction

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potential (ORP), were measured at each sampling site right after the collection of

water samples with an EXO2 or ProDSS mulitparameter water quality sonde/meter

(YSI, Yellow Springs, OH, USA). Precipitation measurements 24 h and 7 days before

sampling were obtained from a weather forecast website

(https://www.wunderground.com/history/), using appropriate locations for each

sampling site.

Table 3.1. Sampling site description and sampling frequency. RW: reclaimed water,

NF: non-tidal (fresh) river water, TB: tidal (brackish) river water, PW: pond water.

Site Code	Water Type	Overall Description
MA01	RW	Influent is treated through activated sludge processing (Sequential Batch Reactor), filtration, UV light and chlorination, then stored in an open-air lagoon before land application. The spray fields are wooded with grass lanes. Samples were collected from a spigot in the irrigation line of sprinkler heads.
MA02	RW	Influent is treated through activated sludge processing (Sequential Batch Reactor), filtration, UV light and chlorination, then stored in an open-air lagoon before land application. Water irrigates agronomic cropland (corn, soybeans) through center pivots. Samples were collected from a spigot at the base of the center pivot.
MA03	NF	Non-tidal freshwater creek, tributary of the Nanticoke River which runs through Delaware and Maryland into the Chesapeake Bay. At sampling site, width was ~3 m and depth ~1 m. Wooded, agronomic cropland adjacent to the creek (~30-50 m). Within 1.6 km downstream from wastewater treatment discharge facility.
MA04	NF	Non-tidal freshwater creek, tributary of the Choptank River which runs through Delaware and Maryland into the Chesapeake Bay. At sampling site, width was ~ 76 m and depth ~ 0.3-0.6 m. Catchment area: marshland/forested. Parts of this creek could be tidal.
MA05	NF	Non-tidal freshwater river, tributary of Patuxent River, a Western Shore tributary of the Chesapeake Bay. At sampling site, width was ~3-4 m and depth ~0.2-0.5 m. Catchment area: forested, with grasses on shoreline.
MA06	RW	Influent is treated through grinding, activated sludge processing and secondary clarification, then stored in an open-air lagoon. It is chlorinated prior to land application on grass. Samples collected from spigot along sprinkler line, between chlorine contact chamber and field application.
MA07	NF	Non-tidal freshwater creek, tributary of the Nanticoke River. At sampling site width was ~10 m and depth ~1 m. Catchment area: flood plain grasses and woodland (hardwoods). Within 4 km downstream from several poultry houses.

MA08	TB	Tidal brackish river flowing into the Chesapeake Bay. At sampling site, width was ~15 m and depth ~2-3 m. Marsh grasses on both sides (~25-50 m wide), then pine woods. Located within 1.5-2.5 km downstream from broiler concentrated animal feeding operations (CAFOs).
MA09	NF	Non-tidal freshwater creek, tributary of the Pocomoke River. At sampling site, width was ~8 m and depth ~1 m. Catchment area: forested and agronomic cropland. Located less than 1.5 km downstream from several poultry houses.
MA10	PW	Collected from the surface of a freshwater pond with a maximum depth of ~3.4 m and a surface area of ~0.26 ha. At sampling site, width was ~20 m and depth ~1 m. Catchment area: agricultural.
MA11	PW	Collected from the surface of a freshwater pond with a maximum depth of ~3 m and a surface area of ~0.40 ha. At sampling site, width was ~52 m and depth ~0.6 m. Catchment area: agricultural.

3.2.2   *Sample processing and bacterial enumeration*

Quantification of bacterial taxa was conducted using standard membrane filtration methods according to EPA method 1604 for *E. coli* and TC (EPA 1604, 2002) and EPA method 1600 for *Enterococcus* (EPA 1600, 2006) on all samples collected. EPA method 1605 was used for *Aeromonas* spp. (EPA 1605, 2001) for a subset of samples collected between September 2016 and September 2017. Serial volumes of each sample (0.1, 1, 10, and 100 ml) were filtered through 0.45µm, 47-mm cellulose ester membrane filters (Pall Corporation, Ann Arbor, MI, USA). Smaller volumes were made up to 10 ml with sterile water before filtration. Filters were placed aseptically onto mI agar plates (Becton, Dickinson and Company [BD], Franklin Lakes, NJ, USA) for quantification of *E. coli*/TC and on mEI agar plates (BD) for *Enterococcus*. For *Aeromonas* spp., filters were transferred to ampicillin dextrin agar (ADA) (Hardy Diagnostics, Santa Maria, CA, USA) supplemented with ampicillin, sodium salt (10 µg/ml; Fisher Scientific, Hampton, NH, USA), and vancomycin (V) (1 µg/ml) (AMRESCO, Solon, OH, USA). All mI and ADA-V plates were incubated for 24 h at 37°C, and mEI plates were incubated at 41°C for 48 h for improved visualization and

differentiation between enterococci and non-enterococci. Blue colonies on mI plates were counted and recorded as *E. coli*. The fluorescent colony count under UV light (365 nm) was added to the nonfluorescent blue colony count to obtain a TC count. All colonies less than or equal to 0.5 mm in diameter (regardless of color) with a blue halo on mEI were recorded as enterococci colonies. Yellow colonies on ADA-V plates were counted and recorded as *Aeromonas* spp. The limit of detection for *E. coli*, TC, Enterococcus spp., and *Aeromonas* was 1 CFU/100 ml. Upto three blue colonies from mI and mEI plates and yellow colonies from ADA plates from each water sample were achieved for downstream analysis.

3.2.3   Calculation of GM and STV

Calculations of geometric mean (GM) and statistical threshold value (STV) were performed on all data points collected during May to October, as described by the Produce Safety Alliance (PSA) (Bihn et al., 2019). For GM, *E. coli* counts per 100 ml of water from the same site were log transformed and then averaged. The STV was calculated by using the formula

$$\log(\text{STV}) = \text{average (log values)} + 1.282 \times \text{SD (log values)} \qquad (I)$$

where average (log values) are the GM values for each site, SD is the standard deviation, and 1.282 is a constant to calculate the STV (90th percentile of the data set). STVs were calculated for each sampling site separately.

3.2.4   Data management and statistical analysis

For statistical analyses of the effect of water type and seasonality on bacterial levels, data were pooled by water type (5 NF, 1 TB, 2 PW, and 3 RW sites) and the year was divided into four groups, defined as spring from 01 March to 30 May, summer from 01 June to 31 August, fall from 01 September to 30 November, and winter from 01 December to 28 February. The whole year was also categorized into vegetable crop-growing (May to October) and nongrowing (November to April) seasons, based on the region's recommended frost dates (https://extension.umd.edu/hgic/topics/when-plant-vegetables-maryland). Vegetable refers specifically to raw agricultural commodities. Although certain vegetable crops can be grown outside these periods, they are unlikely to be irrigated regularly during due to lower temperatures and high moisture levels. A mixed-effect model was used to assess the effect of season or water type on each bacterial taxon. The repeated measurements effects were controlled by using the random effect of site in the model, while season and water type were fixed effects. Tukey's honestly significant difference test with $\alpha= 0.05$ was employed to assess differences within groups. Physicochemical parameters were analyzed for temporal variation by month for each site using analysis of variance. Pearson correlation analysis was applied to explore correlations among bacterial indicators and between indicators and water physicochemical parameters ($\alpha= 0.05$). Linear regression analysis was used to assess the relationship between bacterial counts and rainfall 1 day and 7 days before sampling by site. Statistical analyses were conducted in JMP Pro 14.1 (Cary, NC, USA).

3.3   Results

3.3.1 Bacterial prevalence and differences by water types

The 11 water sites were sampled longitudinally, resulting in 333 water samples. *E. coli* was detected in 288 (86.5%), TC in 327 (98.2%), and *Enterococcus* spp. in 299 (89.8%) of these. From the subset of 133 water samples tested for *Aeromonas* spp., 131 (98.5%) were positive. The highest microbial loads were generally detected in river water (NF and TB, respectively). Higher *E. coli* counts were detected in TB and NF than PW and RW ($P < 0.001$). TC levels were higher in TB than PW ($P < 0.05$), and *Enterococcus* levels were higher in TB, NF, and PW than RW ($P < 0.001$) (Figure 3.1). No water type variations were detected in *Aeromonas* levels. Overall, concentrations of *E. coli* ranged from 0 to 4.4 log CFU/100 ml, TC ranged from 0 to 5.9 log CFU/100 ml, *Enterococcus* ranged from 0 to 5.1 log CFU/100 ml, and *Aeromonas* spp. ranged from 0 to 5.7 log CFU/100 ml (Figure 1). Comparing data between year 1 (September 2016 to August 2017) and year 2 (September 2017 to August 2018) of collection revealed little discrepancy, with only *Enterococcus* species counts differing significantly between years for PW (year 2 counts were 0.94 log CFU/100 ml higher than year 1, $P < 0.001$) and TB (year 1 counts were 0.52 log CFU/100 ml higher than year 2, $P < 0.05$).

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Figure 3.1: Bacterial levels in log CFU/100 ml in various water types for *E. coli* (A), total

coliforms (TC) (B), *Enterococcus* spp. (C), and *Aeromonas* spp. (D). Data for each water type

are pooled from various sites: nontidal fresh river (NF; n = 166 from 5 sites), pond water

(PW; n = 69 from 2 sites), reclaimed water (RW; n = 64 from 3 sites), and tidal brackish

rivers (TB; n = 34 from 1 site). The boxplots show the median and the 25th and 75th

percentiles of the range. The whiskers show lower and higher observations than the 25th and

75th percentiles, respectively. Lowercase letters denote statistically significant differences at

a P value of <0.05 among water types for each taxon.

3.3.2 Effect of season on bacterial dynamics

Seasonal dynamics in bacterial counts were dependent on water type. In NF rivers, seasonal differences were detected in *E. coli*, TC, *Enterococcus*, and *Aeromonas* (Figure 3.2). In this water type, *E. coli* counts were higher in fall than in winter or spring (P<0.001) (Figure 3.2A). Total coliform counts were higher in fall than in spring (P < 0.001) and summer (P < 0.01) (Figure 3.2B), and *Enterococcus* levels were elevated in fall compared to spring (P < 0.01) (Figure 3.2C). *Aeromonas* spp. were detected at higher levels in fall and spring; statistically supported differences were detected between fall and both winter and summer (both P < 0.01) and between spring and summer (P< 0.01). A weaker difference was detected between spring and winter (P < 0.05) (Figure 3.2D).

In PW, seasonal differences were only detected for *E. coli* and TC. As in NF, *E. coli* counts were higher in fall than winter (P < 0.05) and spring (P < 0.01) (Figure 3.2A). *E. coli* concentrations were also higher in summer than spring (P < 0.01). The highest TC counts were found in fall and were different from counts obtained in the spring and summer seasons (P < 0.01) (Figure 3.2B). No seasonal differences in bacterial prevalence were detected in TB or RW (Figure 3.2).
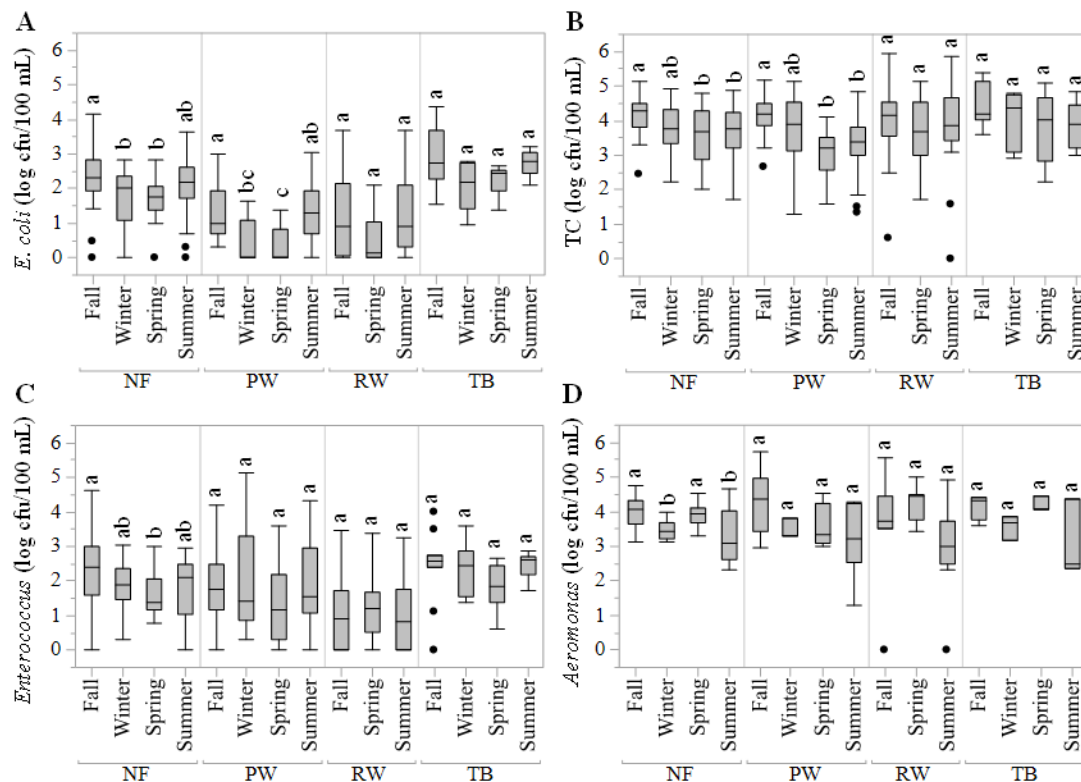
Figure 3.2: Seasonal variation in bacterial counts in log CFU/100 ml for *E. coli* (A), total coliforms (TC) (B), *Enterococcus* spp. (C), and *Aeromonas* spp. (D), enumerated in different water types. Data for each water type are pooled from various sites. The boxplots show the median and the 25th and 75th percentiles of the range. The whiskers show lower and higher observations than the 25th and 75th percentiles, respectively. Lowercase letters denote statistically significant differences at a P value of <0.05 among water types for each taxon.

3.3.3 Differences in bacterial counts between vegetable crop-growing and nongrowing seasons and PSR compliance.

Significant differences were detected in bacterial counts between the vegetable crop-growing and nongrowing season for *E. coli* and TC (Figure 3.3). The divergence was most consistently observed for *E. coli*, with higher population levels retrieved in the crop-growing season than the nongrowing season in NF, PW (both P < 0.001), RW (P

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< 0.01), and TB (P < 0.05) (Figure 3.3A). Total coliform counts in NF (P < 0.01) and

RW (P < 0.05) water samples showed the same trend (Figure 3.3B). No statistically

significant differences in *Enterococcus* and *Aeromonas* species counts were detected

by growing season; however, similar patterns were sometimes discernible. Counts

were higher in the growing season for *Enterococcus* spp. in NF (P = 0.06) and

Aeromonas spp. in PW (P < 0.07) and NF (P < 0.08) (Figure 3.3C and D).

In the crop-growing season, 5 out of 11 sites had *E. coli* GM levels above the FSMA

PSR threshold value of 2.10 log CFU/100 ml (exceeded by 0.01 to 0.61 log CFU/100

ml), whereas 8 out of 11 sites had noncompliant STV values above 2.61 log CFU/100

ml (exceeded by 0.19 to 0.89 log CFU/100 ml) (Figure 3.4A). All sites exceeding the

GM threshold also failed to meet the STV metric. None of the river sites (MA03,

MA04, MA05, MA07, MA08, and MA09) were compliant (Figure 3.4A). Only one

RW (MA02) and both PW (MA10 and MA11) sites met the FSMA PSR GM and

STV generic *E. coli* metric. Applying the FSMA PSR mitigation measure of letting

up to 4 days elapse between irrigation and harvest to allow for bacterial die-off,

stipulated at a decay rate of 0.5 log CFU/day per day, would bring all sites into

compliance within 1 to 2 days (Figure 3.4A). Breaking down *E. coli* counts by

months within the crop-growing season reveals little variation (Figure 3.4B to E).

Fluctuations in *E. coli* densities throughout the growing season were detected only for

NF (Figure 3.4B), while counts in TB and RW remained relatively steady (Figure

3.4C and E, respectively). In NF, *E. coli* counts were significantly lower at the

beginning of the growing season in May (1.84 log CFU/100 ml) and in August (1.96

log CFU/100 ml) than in October (2.71 log CFU/100 ml, P < 0.05) (Figure 3.5A). A

substantial discrepancy of 1 log CFU was also observed between May and October

for PW (0.39 and 1.52 log CFU/100 ml, respectively) and RW (0.63 and 1.62 log

CFU/100 ml, respectively), but these differences were not statistically supported

(Figure 3.4D and E).

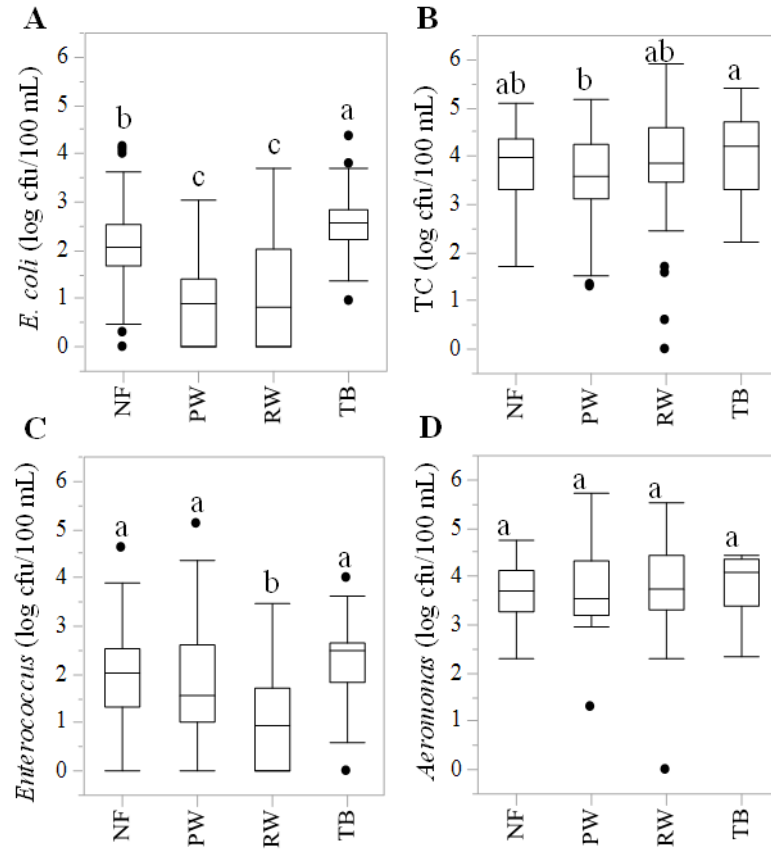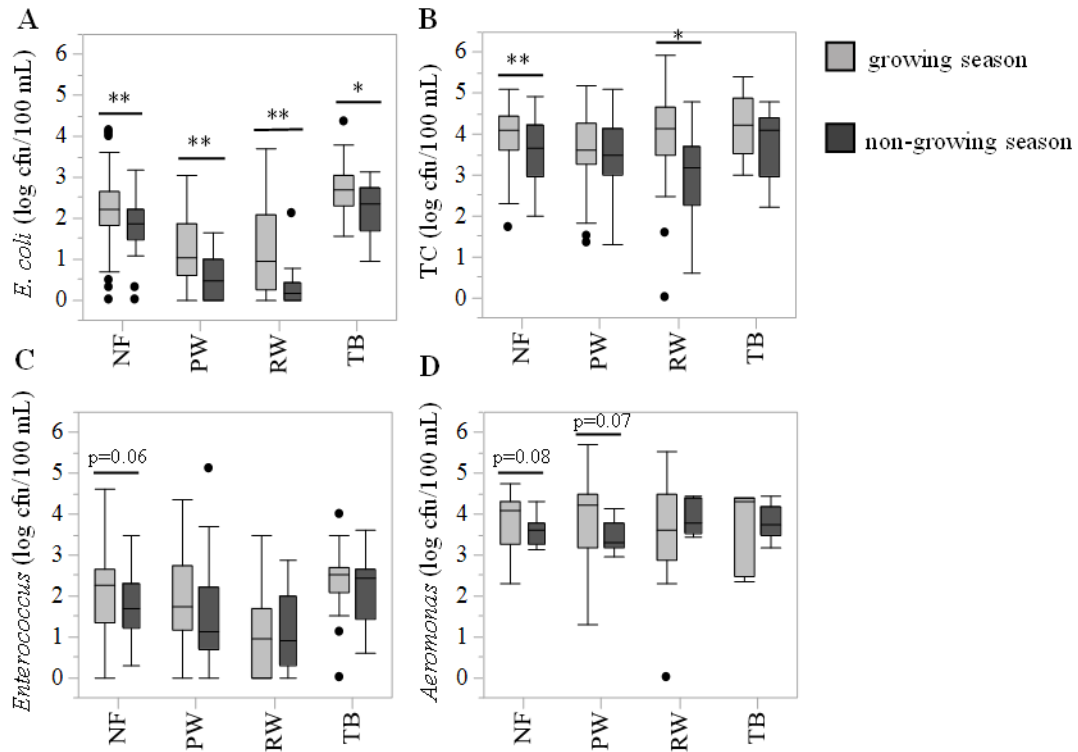**Figure 3.3**: Bacterial counts in log CFU/100 ml for *E. coli* (A), total coliforms (TC) (B),

Enterococcus spp. (C), and *Aeromonas* spp. (D), enumerated in different water types and

categorized by vegetable crop-growing (light gray bars) and nongrowing (dark gray bars)

seasons. Asterisks indicate a significant difference by Student's t test. **, $P < 0.01$; *, $P <$

0.05. The boxplots show the median and the 25th and 75th percentiles of the range. The

whiskers show lower and higher observations than the 25th and 75th percentiles, respectively.
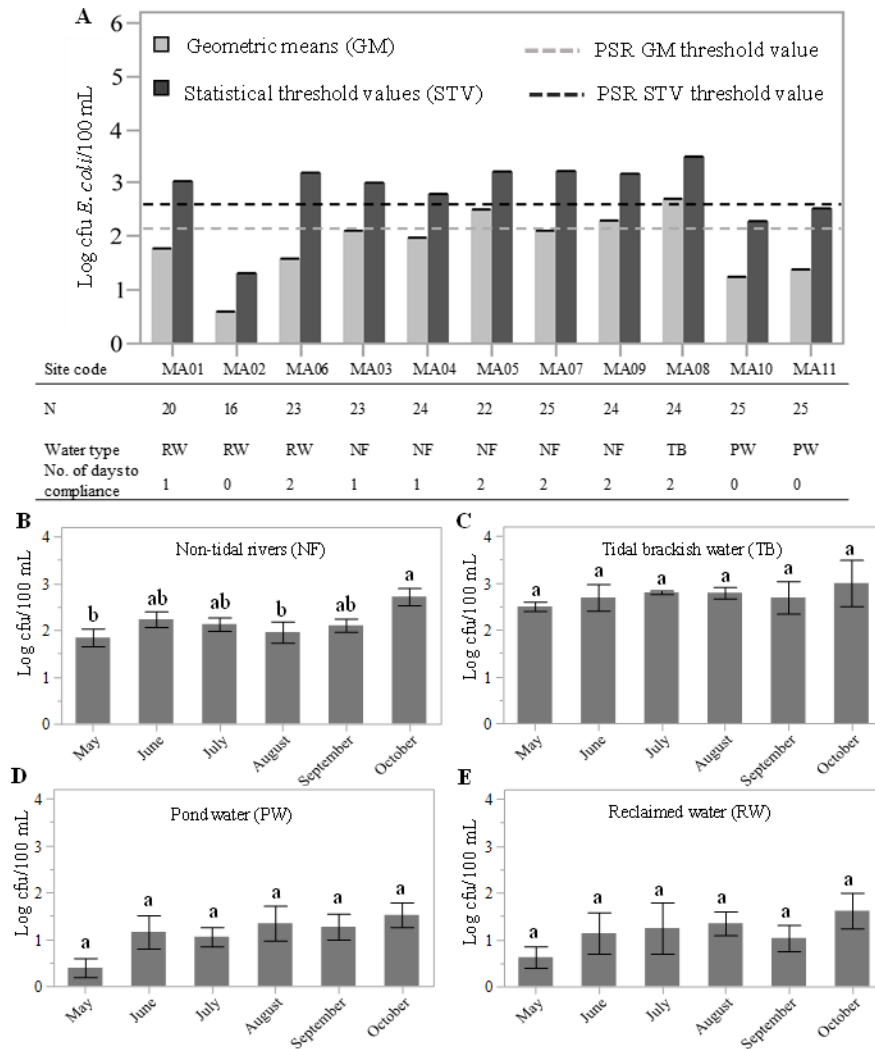
Figure 3.4: (A) Geometric means (GM) and statistical threshold values (STV) of *E. coli* counts in log CFU/100 ml for vegetable crop-growing season months only for 11 sites, including nontidal rivers (NF), tidal rivers (TB), pond water (PW), and reclaimed water (RW) sites, in relation to the Produce Safety Rule (PSR) standards. Light gray bars indicate GM, dark gray bars indicate STV, the light gray dashed line indicates PSR GM threshold value of 2.1 log CFU/100 ml, and the dark gray dashed line indicates PSR STV threshold value of 2.61 log CFU/100 ml. The table in the figure displays delays needed between application of irrigation water and harvest to allow for bacterial die-off, stipulated in the PSR to occur at a rate of 0.5 log CFU/day. (B to E) Month-to-month variation in average *E. coli* counts over

two growing seasons in nontidal rivers (NF) (B), tidal brackish water (TB) (C), pond water (PW) (D), and reclaimed water (RW) (E). Different lowercase letters denote statistical differences at a P value of <0.05 in bacterial counts by month of collection, and error bars denote standard errors.

3.3.4 Relationships between bacterial taxa by season and water type

E. coli, TC, and *Enterococcus* spp. were positively correlated with each other in all seasons and water type, with some exceptions (Figure 3.5). *E. coli* counts were positively correlated with TC in winter and fall (r = 0.51, P < 0.001) and summer (r = 0.38, P < 0.001) but weakly associated in spring (r = 0.22, P <0.05) (Figure 3.5A). Likewise, all correlations between *E. coli* and *Enterococcus* levels were positive in all seasons (P < 0.01) but strongest in fall and summer (r = 0.59 and 0.51, respectively, P < 0.001) (Figure 3.5B). Significant positive relationships were also apparent between TC and Enterococcus counts in all seasons (P < 0.01) (Figure 3.5C).

The strongest correlation between *E. coli* and TC levels was detected in TB (r = 0.62, P < 0.001), followed by RW (r = 0.51; P < 0.001), NF, and PW (both P < 0.01) (Figure 3.5D).

E. coli counts were positively correlated with *Enterococcus* counts in all water types, with NF exhibiting the strongest correlation (r = 0.60; P < 0.001), followed by TB and RW (P < 0.001) (Figure 3.5E). Correlations between TC and *Enterococcus* counts were detected in NF (r = 0.50; P < 0.001), RW, and TB (both r = 0.42; P < 0.001) (Fig. 3.5F). Associations between enterococci and *E. coli*/TC in PW exhibited a weaker relationship (both r = 0.27; P < 0.05) (Figure 3.5E and F).

Significant positive relationships were observed between bacterial indicators and *Aeromonas* counts but not in the winter months. Although *Aeromonas* was positively correlated with *E. coli* only in the fall (r = 0.42, P < 0.01) (Figure 3.6A), a strong positive relationship was found with TC in summer (r = 0.70, P < 0.001), fall, and spring (r = 0.5, P < 0.01) (Figure 3.6B). *Aeromonas* and *Enterococcus* spp. were only associated in the fall (r = 0.50, P < 0.001) and summer (r = 0.46, P < 0.01) (Figure 3.6C).

A correlation was detected between *Aeromonas* and *E. coli* in RW and NF (r = 0.44 and 0.26, respectively, both P < 0.05), while no association was seen in TB and PW (Figure 3.6D). On the other hand, the relationship between *Aeromonas* and TC was discernible in all water types (r = 0.6; P < 0.05) (Figure 3.6E). *Enterococcus* and *Aeromonas* were most positively associated in PW and RW (r = 0.56 and 0.53, respectively, P < 0.01) and less strongly in NF (r = 0.41, P < 0.001) (Figure 3.6F).

Figure 3.5: Relationships between bacterial indicator counts for *E. coli*, total coliforms (TC), and *Enterococcus* spp. in log CFU/100 ml by season (A to C) and water type (D to F). R^2 values indicate goodness of fit of the line using Pearson correlation analysis. NF denotes nontidal river, PW denotes pond water, RW denotes reclaimed water, and TB denotes tidal river.

Figure 3.6: Relationships between *Aeromonas* species counts and bacterial indicator counts (*E. coli*, total coliforms [TC], and *Enterococcus* spp.) in log CFU/100 ml by season (A to C) and water type (D to F). R^2 values indicate goodness of fit of the line using Pearson correlation analysis and are given in each panel. NF denotes nontidal river, PW denotes pond water, RW denotes reclaimed water, and TB denotes tidal river.

3.3.5 Physicochemical parameters of water and relationship with bacterial counts

Counts of the four bacterial taxa displayed significant correlations with various parameters in a water type-dependent manner (Figure 3.7). Variations in physico-chemical levels measured during the study period, grouped by water type, are compiled in Figure S1. Temporal variation was observed in water temperature at all sites ($P \leq 0.001$), with highest mean temperatures recorded in the RW samples MA02 (21.9°C) and MA01 (21.5°C) and PW sample MA11 (20.1°C). These means were all statistically different from the mean water temperature recorded at the NF site MA05 (14.3°C) (Figure S1). However, temperature was only positively correlated with *E. coli* and TC in PW and RW, respectively ($P < 0.05$) (Fig. 3.7).

	Non-tidal River				Tidal River				Pond Water				Reclaimed Water			
	Ec	TC	Ent	Ar	Ec	TC	Ent	Ar	Ec	TC	Ent	Ar	Ec	TC	Ent	Ar
Temp									0.3				0.2	0.3		
pH									-0.3							
Turbidity	0.2		0.2				0.3					0.5				
%DO	-0.3	-0.3	-0.3													
Conduct							-0.3						0.3			
ORP	0.2		0.2										-0.3	-0.3		
Nitrate	-0.2		-0.2									0.8				
Chloride							-0.6		-0.3		-0.5				0.4	
Salinity							-0.4					-0.5	0.3			

Figure 3.7: Relationship between bacterial counts and physicochemical parameters in different water types. EC indicates *E. coli*, TC indicates total coliforms, Ent indicates *Enterococcus* spp., and Aer indicates *Aeromonas* spp. Values in each box indicate Pearson correlation coefficient Iat a P value of 0.05. Pink indicates positive and blue indicates negative association between two variables (bacterial counts and physicochemical parameter). The intensity of the box color increases with increasing value of r.

The tidal river MA08, the only brackish site, showed seasonal variation in turbidity (P < 0.01), with highest measurements recorded from January to March. A positive relationship (r = 0.34, P = 0.06) was detected between *Enterococcus* spp. and turbidity in this water type and in NF (0.21, P < 0.05). MA08 (TB) had the highest nitrate (mean, 18.1 mg/liter) and chloride levels, conductivity, and salinity (mean, 7.3 PSU) (all P < 0.001) of all the water types. Only *Enterococcus* spp. exhibited any significant associations with these parameters in TB, where negative relationships were observed with conductivity, chloride levels, and salinity (P < 0.05) (Fig. 3.7). *Aeromonas* spp. were highly associated with turbidity (r = 0.47, P < 0.05) and nitrate (r = 0.76, P < 0.05) in PW and negatively associated with salinity (r =0.52, P< 0.05). Interestingly, salinity and conductivity were positively correlated with *E. coli* in RW (P< 0.01). pH was higher in PW (7.9) and RW (7.7) than in NF (7.0) and TB (6.8) (P < 0.001).

Pooling data from all sites revealed a positive relationship between *E. coli* and conductivity (r =0.25, P < 0.001), nitrate (r = 0.14, P < 0.05), chloride (r = 0.17, P < 0.01), and salinity (r = 0.25, P < 0.001) and negative relationships between E. coli and percent dissolved oxygen (DO) (r = 0.23, P < 0.001) and pH (r = 0.41, P < 0.001) (Table S1). A positive association between enterococci and turbidity (r = 0.15, P <0.01) and a negative association between enterococci and pH (r =0.25, P < 0.01) were also detected.

3.4 Discussion and Conclusions

Increasing demands for fresh crops and concerns for food safety, coupled with variability in climate that is disrupting surface and groundwater availability, are pressing the need to identify alternative sources of agricultural water. Characterizing the microbiological quality of alternative sources of water for irrigation of fresh crops is critical for the integrity of the crop for human consumption. Understanding the spatiotemporal population dynamics of bacteria used for proposed irrigation water standards (*E. coli*) and other common bacterial indicators (*Enterococcus*) will assist growers in implementing new U.S. standards. Although the mid-Atlantic region of the USA is not considered a water insecure geographical area, subregions within the mid-Atlantic are prone to water availability concerns. Fluctuations in rainfall and periods of drought, depletion of aquifers, and coastal saltwater intrusion have raised the need to identify microbiologically safe alternative sources of irrigation water to expand this precious resource. Farmers in the mid-Atlantic are concerned about water availability and are interested in tapping into alternative sources of irrigation water (Suri et al., 2019). This study characterized the microbial quality of typical rivers, ponds, and reclaimed water sources found in the mid-Atlantic region. We found that fresh and brackish river surface water had higher bacterial indicator counts than reclaimed and pond water. We also detected seasonal dynamics specific to water type and a difference in *E. coli* counts in all water types between growing and nongrowing seasons. The strength of this study lies in the longitudinal approach taken to sample and analyze a variety of water types at a high sampling frequency. As a result, this

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study allows for the detection of patterns and dynamics that are otherwise indiscernible from more limited sampling scopes.

Approximately 86% of our water samples (n =333) tested positive for *E. coli*. This incidence is higher than the 78% (n =255) reported for irrigation water samples collected from farms along the central California coast (Benjamin et al., 2013) and the 59% (n = 120) of samples from greenhouses and open-field farms in Belgium (Holvoet et al., 2014). The latter study also reported that 37% of samples were positive for *Enterococcus*, which differed from the 90% prevalence we found in our study. An extensive study conducted in southern Ontario, Canada, which included 501 irrigation water samples from 17 farms, found that 81% of samples had fewer than 20 CFU *Enterococcus* per 100 ml of water (Steele et al., 2005). In this report, 83% of samples met the Canadian Council of Ministers of the Environment (CCME) *E. coli* standards for irrigation water (100 CFU/100 ml). Using these same data, 11 of 20 ponds met the British Columbia criteria that required testing the same water source at least five times over at least 30 days (Steele & Odumeru, 2004.). In the USA, the FSMA PSR water standards are also based on a microbial water quality profile, i.e., the geometric mean and statistical threshold value of 20 samples collected over 2 to 4 years (PSR; 21 CFR 112). Although this sampling frequency may be deemed low in terms of determining microbial quality, collecting five to 10 samples per crop-growing season for all water sources used for fresh crops on a single farm is considered a financial and time burden by growers (Wall et al., 2019). Regardless, available data remain too scant to support any given sampling schedule applicable nationally. Considering the PSR criteria using the data from this study, when growing

season data were analyzed, only 3 out of 11 sites were acceptable for irrigation

without the need for any mitigation. Irrigation water supplied from these sites would

require implementation of die-off times or be treated by chemical disinfection or

filtration. Also noteworthy was the correlation between rainfall and *E. coli* counts,

and although growers are unlikely to irrigate after heavy rainfall, they should be

advised to avoid collecting water samples for microbiological testing immediately

following rain to avoid skewing their MWQP.

The low compliance rates noted above also signal the importance of making better

links between standards based on generic bacterial counts and actual food safety risk.

Havelaar et al. found a correlation between *E. coli* levels and *Salmonella* presence in

Florida ponds but noted a higher variability in *E. coli* than is accommodated for by

the PSR criterion for STV (Havelaar et al., 2017). Our data support that finding;

considering only the GM criterion, 6 of 11 sites in this study would have met the PSR

standard, as opposed to 3 of 11 not meeting the combined GM and STV criterion.

Moreover, two of the NF rivers sites (MA03 and MA07) exceeded the GM metric

only slightly, at 2.11 log CFU/100 ml, but were well over the STV metric at 3.01 and

3.23 log CFU/100 ml, respectively. This means that MA03 would require 1 day and

MA07 2 days of bacterial die-off delay between irrigation and harvest, despite

coming close to meeting the GM standard. GM and STV calculations were based on

crop-growing season dates only, as recommended by the PSR. This requirement was

supported by our data, with *E. coli* counts being significantly higher in the crop-

growing season than the nongrowing season in all water types tested. Finally, when

bacterial counts were influenced by season, counts were higher in the fall (September

to November). This was the case for *E. coli*, TC, *Enterococcus*, and *Aeromonas* in nontidal freshwater rivers. Despite these observations, we detected little variation in *E. coli* counts between June and September, months with the highest irrigation activity. Understanding the seasonal dynamics of *E. coli* levels in various water types and locations would inform the development of MWQPs in the mid-Atlantic region. In the mid-Atlantic, the use of surface water, including pond water, declined between 2010 and 2013 from 48.5% (n =130) to 23% (n =183), while water testing increased from 11.5% in 2010 to 32% in 2013 (Marine et al, 2017). Likelihood of water testing was associated with farm scale, with 22% (n =18) of small farms versus 54% (n = 13) of large farms reported testing their water in a survey of mid-Atlantic leafy greens and tomato growers (Lichtenberg et al., 2016). In a more recent study (2016 to 2018) of 263 mid-Atlantic growers, only 30% reported using surface water compared to 59% using groundwater (Suri et al., 2019). The decline in surface water use and increase in water testing indicate the growing unease around microbial safety concerns of using surface water for irrigation. However, this water type was the only one that consistently met the proposed PSR standards. Similarly, the study conducted in Florida that analyzed 6 ponds also reported that all ponds met the GM and STV criteria of the PSR, based on 90 samples per pond (Havelaar et al., 2017). The majority of farms in the mid-Atlantic region possess natural or man-made ponds, frequently recharged by underground springs or rainfall. Although farmer surveys along the east coast of the USA have reported use of surface water for irrigation (Marine et al., 2016; Bihn et al., 2013), none of these differentiated among pond, rivers, and other types of surface water. The discrepancy in microbial quality between

pond and river water revealed in this study emphasizes the need for this distinction. Future investigations in farmer practices should explore the various types of surface water of which farmers may be availing themselves. Lower *E. coli* levels in pond water than river water may influence the use of specific surface water sources for irrigation of crops.

The reuse of reclaimed water for irrigation purposes varies by state in the USA and ranges from agricultural use for animal feed and human food crops to use on landscapes (https://www.epa.gov/waterreuse). The level of treatment also varies by treatment plant, and state guidelines determine reuse of water based on treatment class. More extensive water reuse would benefit communities and economies, and the U.S. Environment Protection Agency (EPA) has released an Action Plan (https://www.epa.gov/waterreuse/water-reuse-action-plan) to accelerate the adoption of this re-source. Knowledge of the microbial safety of reclaimed water under various treatment processes and from different regions is crucial to ensure its adequacy for use on fresh crops. In this study, reclaimed water met the GM standards but exceeded the STV criteria in the PSR. Interestingly, we still detected a difference in *E. coli* levels between crop-growing and non-crop-growing seasons in this water source. Investigating relationships between temporal fluctuations in indicator bacteria and presence of food-borne pathogens (Sharma et al., 2020) will reveal important food safety parameters for the various water types.

In the present study, we also quantify and report levels of *Aeromonas* spp. for irrigation water in the USA. Although gastroenteritis caused by *Aeromonas* is not a reportable foodborne illness, this genus is an emerging foodborne pathogen and of

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increasing importance for food safety (Pal, 2018; Daskalov, 2006). This organism has

caused several outbreaks around the world (Zhang et al., 2012; Mendes-Marques CL

et al., 2012; Da Silva et al., 2017; Dixit et al., 2011; Tsheten et al., 2016). It is a well-

known inhabitant of water environments and can attach to plant surfaces (Elhariry et

al., 2011). It has also been detected on market fruit and vegetables (McMahon &

Wilson,2011; Kaur et al., 2017). Hence, the potential for *Aeromonas* transfer from

irrigation water to crops exists. Our study provides novel and relevant data for this

emerging foodborne pathogen on the prevalence and lack of seasonal influence across

surface water types, as well as persistence in treated reclaimed water. These types of

environ-mental data, in combination with assessment of food safety risk associated

with this genus, aid in determining the potential need for inclusion of *Aeromonas*

assessment in agricultural water quality management.

Much research has been conducted to investigate the relationships among indicator

bacteria, enteric pathogens, and physicochemical parameters in water environments

(McEgan et al., 2013; Benjamin et al., 2013; Steele et al., 2005; Ortega et al., 2009;

Economou et al., 2013; Wilkes et al., 2009). The membrane filtration method that we

used for bacterial enumeration was not expected to recover all aerobic heterotrophic

bacteria in our samples (Reverdy et al., 1984) or to correlate strongly with pathogens

(McEgan et al., 2013; Ortega et al., 2009). However, the method was appropriate to

detect indicator bacteria for the purpose of assessing temporal shifts in

microbiological quality and to compare water types. Moreover, both TC and *E. coli*

showed strong correlations with *Enterococcus* and *Aeromonas* spp., genera that may

include human pathogens. We found strong correlations between indicator bacteria in

all water types tested but less predictive use of physicochemical parameters. Other

related but useful measurements, such as biological oxygen demand and total dis-

solved solids, could have aided interpretation of our data and could be included in

future studies. In some cases, we observed bias from having a skewed distribution of

data, such as was the case with *E. coli* and salinity. Most samples had salinity of 0.1

PSU, but for the RW sites the average salinity was 0.35 PSU, supporting a positive

correlation between *E. coli* and salinity. This association held up when all data were

pooled to include MA08, a brackish site with average salinity of 7.3 PSU (Table S3).

Aeromonas exhibited the strongest association with TC and the weakest with *E. coli*.

This is attributed to the psychotropic nature of *Aeromonas*, which may align well with

some members of the diverse TC group but weakly with *E. coli*, which was positively

associated with temperature in PW. Considering the strong associations we detected

between indicators but less so with *Aeromonas*, in combination with the lack of

seasonal effect on *Aeromonas*, it appears that *Aeromonas* quantification would not be

of predictive use for water quality assessment but may be important based on the risk

it poses as a potential foodborne pathogen.

Our study provides a baseline for temporal microbial data for water sources that could

be used for irrigation. The data support the use of pond water, a widely available

source of surface water on farms in the mid-Atlantic, for irrigation of crops. We

found that bacterial counts in reclaimed water, a resource whose use on fresh food

crops varies by state in the USA, were among the lowest we detected. This water type

always met the *E. coli* GM standard in the PSR, although STV thresholds were

exceeded. We detected the most seasonality and the highest microbial loads in river

water, which emerged as the water type that would require the most frequent

mitigation. However, based on the PSR standards alone, even this water source could

be brought to compliance within 2 days of implementing the die-off delay

recommended in the PSR.

Chapter 4: Determining and characterizing diversity and antimicrobial resistance of *E. coli* isolated from various water types over two years.

4.1   Introduction

Agriculture water is one of the major reservoirs of many known and unknown foodborne pathogens (Uyttendaele et al., 2015) that is evidenced by mounting raw vegetable associated foodborne illnesses traced back to irrigation water in recent years (CDC, 2018). Previous studies confirmed that potential and on-farm agriculture water sources such as fresh and tidal river water, treated wastewater and pond water are often contaminated with fecal coliforms and enteric pathogens (Haymaker et al., 2019; Sharma et al., 2020; Solaiman et al., 2020). Therefore, microbiological quality assurance of prospective and on-farm irrigation water is a critical step to minimize crop contamination and to ensure food safety.

The World Health Organization (WHO) and the US FDA recommend *Escherichia coli* as a microbial water quality indicator for fecal contamination over other heterotrophic bacteria because of its generic nature and natural inhabitance in mammalian intestines (Ashbolt et al., 2001; FSMA, 2011). To prevent foodborne disease outbreaks caused by irrigation water, the US FDA recently recommended standards for agricultural water in the FSMA Produce Safety Rule (PSR; 21 CFR 112) based on the presence of generic *E. coli* (FSMA PSR, 2011). Although most strains of *E. coli* are commensal in the human gut, some are pathogenic and able to

cause a variety of illnesses including gastrointestinal and extraintestinal diseases. Six well-characterized *E. coli* pathogenic groups known for causing gastroenteritis are enterohaemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EaggEC), enterotoxigenic *E. coli* (ETEC) and diffusively adherent *E. coli* (DAEC) (Kaper et al., 2004). Strains that are responsible for extraintestinal diseases causes urinary tract infections (UTI), septicemia, neonatal meningitis and avian infections (Orskov & Orskov, 1992). *E. coli* is responsible for 80-90% of global UTIs (Basu et al., 2013; Ejrnaes, 2011; Lee et al., 2010) and 15-40% of fatalities in neonatal meningitis (Kaper et al., 2004). Due to the extensive diversity in genetic substructure, Clermont et al. (2000) classified this bacterium into 4 different phylogroups, A, B1, B2 and D, based on the presence of three virulence genes, heme transport (*chuA*), stress related gene (*yjaA*) and lipase/esterase (*tspE4C2*) (Clermont et al., 2000). Later it was reported that phylogroup members share distinct phenotypic and genotypic characteristics. Group A consists of mostly generic/commensal strains and some Shiga-toxin producing *E. coli* while group B1 consists of generic/commensal and entero-pathogenic strains. Group B2 mostly comprises infectious extra-intestinal strains and group D consists of infectious extra-intestinal strains to a lesser extent and some entero-pathogenic strains (Bingen et al., 1998; Boyd and Hartl, 1998; Clermont et al., 2000; Johnson and Stell, 2000; Picard et al., 1999). This classification separates intestinal, extraintestinal and commensal strains that differ in host, niche colonization, mechanism of disease and antimicrobial resistance (Starčič Erjavec et al., 2017).

Antimicrobial resistance (AMR) in *E. coli* is a public health concern. AMR among both commensal and pathogenic *E. coli* against several broad and extended spectrum antibiotics is a major contributor of infection and enhanced morbidity and mortality (Llor and Bjerrum, 2014). The widespread use of antibiotics in agriculture as growth promotors and therapeutic agents against bacterial infection in food-producing animals, has led to the emergence of AMR in food (Prestinaci et al., 2015). Every year, approximately 0.4 million people are sickened by AMR foodborne infectious bacteria, with 26,000 cases being associated with Extended Spectrum β-Lactamase (ESBL) producing Enterobacteriaceae (CDC, 2013). ESBL-producing bacteria are resistant to most β-lactam antibiotics, including 3rd and 4th generation cephalosporins and sometimes resistant to multiple other classes of antibiotics. *E. coli* is one of the most common ESBL-producing commensal bacteria in the lower intestines of homeothermic animals (Franz et al., 2015). Acquiring AMR genes might limit treatment options against pathogenic microorganisms. About 17% of all ESBL-producing *E. coli* are predicted to be virulent (Franz et al., 2015). ESBL-producing *E. coli* can spread to fresh produce via irrigation water (van Hoek et al., 2015). Consumption of minimally processed raw produce and consequent transfer to humans can negatively impact consumer health (Ye et al., 2017). Therefore, regardless of the phylogenetic class of *E. coli*, antimicrobial susceptibility surveillance especially against extended spectrum antibiotics requires evaluation of actual public health risk (Hassan et al., 2011; Santo et al., 2007).

In a previous study, we conducted a longitudinal assessment of the microbial water quality of eleven alternative irrigation water sources to groundwater (Solaiman et al., 2020). The study sites comprised of surface (irrigation ponds and rivers) and reclaimed (treated wastewater) water which were sampled over 330 times during a two-year period and from which 724 *E. coli* isolates were isolated and archived for further characterization. We hypothesized that the distribution of *E. coli* phylogroups and ESBL-producing strains would not vary by water type (surface river or pond water and reclaimed water). Our goal was to determine whether the risk of introducing pathogenic and ESBL-producing *E. coli* to fresh produce crops via agricultural water was water type dependent. We hence phylogrouped the *E. coli* isolates and subsequently assessed extended spectrum β-lactam resistance phenotypically and genotypically.

4.2 Methods and Materials

4.2.1 *DNA Extraction and Identification of E. coli by PCR*

Up to three blue *E. coli* colonies from mI plates were picked for further identification. In total, 724 presumptive *E. coli* isolates, recovered using the selective culture technique (using MacConkey agar), were confirmed through PCR screening. For PCR screening, *E. coli* colonies from Non-selective agar (Tryptic Soy Agar) were resuspended in 7.5% w/v sterile chelex solution for DNA isolation using a rapid heat lysis method (Micallef et al., 2012). A species-specific polymerase chain reaction (PCR) was employed by amplifying the β-glucuronidase gene, *uidA*, to confirm identification of *E. coli* isolates (Jinneman et al., 2003). Amplification was performed

in 1X PCR buffer (New England Biolabs (NEB), Ipswich, MA, USA), 1.5 mM

MgCl₂ (NEB), 0.2 mM dNTPs (NEB), 0.4 µM 16s rRNA gene primers (internal

control) and *uidA* primers (Integrated DNA Technologies (IDT), Coralville, Iowa,

USA) (Table 1), 5 Units Taq DNA polymerase (NEB) and ~20 ng of template DNA.

The standard cycling conditions consisted of an initial single cycle at 95 °C for 30 s,

followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s,

elongation at 72 °C for 30 s and a final single cycle at 72°C for 5 min. *E. coli*

CFT073-UPEC was used as a positive control.

Table 4.1. List of primers used for identification, classification and resistance genes in
E. coli

Gene	Size (bp)	Primer	Sequences (5'-3')	References
uidA	192	Forward	CAGTCTGGATCGCGAAAA	Jinneman et
		Reverse	ACCAGACGTTGCCCACATA	al., 2005
16S rRNA	357	Forward	AGAGTTTGATCCTGGCTCAG	Zhou et al.,
		Reverse	TGTCTCAGTTCCAGTGTGACT	1997
chuA	279	Forward	GACGAACCAACGGTCAGGAT	
		Reverse	TGCCGCCAGTACCAAAGACA	Clermont et al, 2000
yjaA	211	Forward	TGAAGTGTCAGGAGACGCTG	
		Reverse	ATGGAGAATGCGTTCCTCAAC	
TspE4C2	152	Forward	GAGTAATGTCGGGGCATTCA	
		Reverse	CGCGCCAACAAAGTATTACG	

bla_{CTX-MU}	593	Forward	ATGTGCAGYACCAGTAARGT	Pagani, O. et
		Reverse	TGGGTRAARTARGTSACCAGA	al. 2003.
bla_{TEM}	964	Forward	GCGGAACCCCTATTT G	
		Reverse	ACCAATGCTTAATCAGTGAG	
bla_{SHV}	854	Forward	TTCGCCTGTGTATTATCTCCCTG	
		Reverse	TTAGCGTTGCCAGTGYTCG	(Dierikx et al.,
bla_{CMY-1}	915	Forward	GTGGTGGATGCCAGCATCC	2010)
		Reverse	GGTCGAGCCGGTCTTGTTGAA	
bla_{CMY-2}	758	Forward	GCACTTAGCCACCTATACGGCAG	
		Reverse	GCTTTTCAAGAATGCGCCAGG	
bla_{OXA-1}	820	Forward	ATGAAAAACACAATACATATCAAC TTCGC	(Olesen et al., 2004)
		Reverse	GTGTGTTTAGAATGGTGATCGCAT	
bla_{OXA-2}	602	Forward	ACGATAGTTGTGGCAGACGAAC	(Dierikx et al.,
		Reverse	ATYCTGTTTGGCGTATCRATATTC	2010)

4.2.2 Clermont E. coli phylogrouping

A triplex PCR was used for grouping of *E. coli* isolates (confirmed with *uidA* gene),
by amplification of the three virulence genes: heme transport gene (*chuA*), stress
related gene (*yjaA*) and lipase/esterase gene fragment (*TspE4C2*) adapted from to the
method of Clermont (Clermont et al., 2000). The multiplex amplification was
performed in 1X PCR buffer (NEB), 1.5 mM $MgCl_2$ (NEB), 0.2 mM dNTPs (NEB),
0.4 µM *chuA, yjaA* and *TspE4C2* primer pairs (Table 4.1) (IDT) and 5 units Taq

DNA polymerase (NEB). Approximately ~20 ng of template DNA was used for the reaction. The standard cycling conditions consists of an initial single cycle at 95 °C for 1 min, followed by 30 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 15 s, elongation at 72 °C for 30 s, followed by a final extension at 72°C for 5 min. Amplified products were separated by electrophoresis on 2 % agarose gel (Lonza). *E. coli* CFT073-UPEC was used as a positive control.

4.2.3 Phenotypic determination of ESBL resistance in E. coli

Each *E. coli* isolate was inoculated on TSA (Becton, Dickinson and Company (BD), Franklin, NJ, USA) and then sub-cultured in Mueller-Hinton broth (MHB) (BD) and incubated at 37°C with shaking for 3 hours. The young cultures were spread plated onto pre-prepared Mueller-Hinton agar plates (MHA) (BD). Five β-lactam antibiotic disks including two 2nd generation, cefoxitin (30 µg) and cefuroxime (30 µg), and three 3rd generation, ceftriaxone (30 µg), cefotaxime (30 µg) and ceftazidime (30 µg), were placed on MHA plates. Plates were incubated at 37°C for 18-20 h (CLSI, 2020). Data were interpreted from the diameter of the inhibition zone according to CLSI, 2020 (CLSI, 2020).

4.2.4 Genotypic evaluation of ESBL resistance in E. coli

Three sets of multiplex PCRs were employed to find the β-lactam antibiotic resistance genes *bla_{TEM}, bla_{SHV}, bla_{CTX-MU}, bla_{CMY-1}, bla_{CMY-2}, bla_{OXA-1}, bla_{OXA-2}*. Primer pairs used for detected of these genes are listed in Table 1. DNA was extracted from 724 isolates using the QIAGEN Dneasy ultraclean microbial DNA extraction kit

(QIAGEN, Hilden, Germany). Multiplex PCR was done with 1X PCR buffer (NEB), 1.5 mM MgCl$_2$ (NEB), 0.2 mM dNTPs (NEB), and 0.3 µM of each primer (IDT). Standard cycling condition for amplification were performed using 3 protocols. Protocol I included an initial step (95°C for 15 min) followed by 30 cycles of amplification, 95°C for 60 s, 55°C for 40 s and 72°C for 60 s, followed by a final step at 72°C for 3 mins for *bla*$_{TEM}$-*bla*$_{SHV}$-16s rRNA. For protocol II, standard cycling conditions started with an initial step at 95°C for 15 s min, followed by 30 cycles of amplification comprised of 95°C for 60 s, 60°C for 40 s, 72°C for 60 s, with a final step at 72°C for 3 mins for *bla*$_{CMY-1}$-*bla*$_{CMY-2}$-*bla*$_{OXA-1}$-*bla*$_{OXA-2}$-16s rRNA. For protocol III, standard cycling conditions started with an initial step at 95°C for 1 min followed by 30 cycles of amplification comprised of 95°C for 15 s, 52°C for 15 s, 72°C for 10 s for *bla*$_{CTX-MU}$-16s rRNA. PCR products were electrophoresed on 2% agarose (Lonza, Rockland, ME, USA) gels to determine presence or absence of target genes.

4.2.5 Data Management and Statistical analysis

To avoid clonality, 488 *E. coli* isolates were selected for data analysis based on unique combinations of phylogroup assignment, antimicrobial resistance profiles and resistance gene carriage. Data were pooled by water type (5 NF, 1 TB, 2 PW and 3 RW) and season (from March 01 to May 30 as spring, from June 01 to August 31 as summer, from September 01 to November 30 as fall, from December 01 to February 28 as winter) as in Solaiman et al. (2020). Effect of variables was assessed using the χ-squared test with α=0.05. Multinomial logistic regression was used to assess the effect of season or water type on *E. coli* phylogroup distribution. Dendrograms were

constructed using hierarchical cluster analysis (HCA) from a similarity matrix, based

on the presence/absence of ESBL-resistance genes among four *E. coli* Clermont

groups. Multiple correspondence analysis (MCA) was conducted using group

classification, presence/absence of resistance genes, season and water type to assess

the associations among these categorical variables. The adjusted inertia explaining the

variability in each dimension on the MCA plots were calculated according to

Greenacre (1984). Statistical and MCA analysis, and graphical representation were

performed in R studio v. 4.0. HCA analysis were performed in JMP Pro v. 14.1.0.

4.3 Results

4.3.1 Phylogenetic classification of uidA+ E. coli

All confirmed *E. coli* (n=488) isolates were classified into 4 phylogroups: A, B1, B2

and D; 69 isolates were classified as group A (14.1%), 193 isolates as group B1

(39.9%), 104 isolates as group B2 (23.1%) and 122 isolates as group D (25.0%). The

most prevalent phylogroup retrieved from all water types was B1 and the least

prevalent group was A (Figure 4.1). Phylogroup distribution varied by sampling site

even for the same water type. All 3 reclaimed water sites contained mostly group B1

and D. A more uniform distribution of groups was observed in pond water sampling

sites, MA10 and MA11. Our only tidal brackish water site, MA08 contained a higher

percentage of group A amd B2 isolates compared with all other sites.
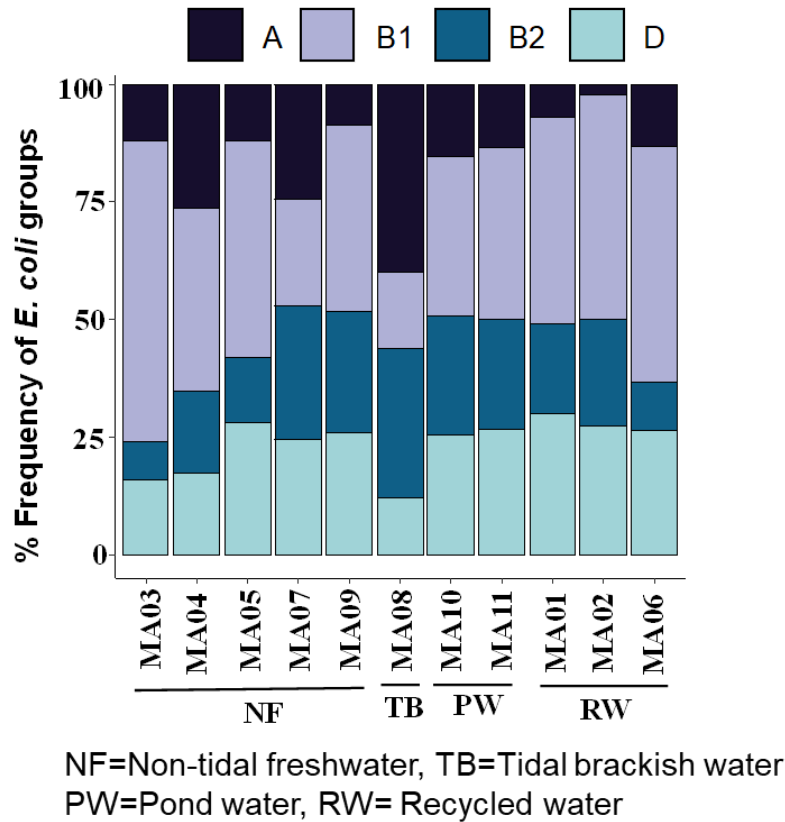
Figure 4.1. Distribution of different phylogenetic groups of *E. coli* in surface and reclaimed water sites. Each column showing the % frequency of different *E. coli* types in 11 sampling sites. Non-tidal freshwater (NF) includes sites MA03, MA04, MA05, MA07, and MA09; tidal brackish water (TB) includes only MA08; on-fam pond water (PW) includes MA10 and MA11; reclaimed water (RW) includes MA01, MA02 and MA06.

When all isolates were pooled together and divided into 4 groups to assess the effect of water type and seasonal variation on phylogroup distribution, The distribution was significantly affected by water types (Pearson's χ^2 (n=488, df=9) = 17.022, p<0.05). Group A was more likely to be detected in reclaimed water and less likely in pond water. B1 was more likely to be found in pond water compared with other water types.

The likelihood of detecting B2 was lower in pond and D was lower in reclaimed

water (Figure 4.2A). Seasonal variation exerted little effect on the distribution

(Pearson's Chi-squared test: $\chi^2 = 4.9$, df = 9, p>0.05) (Figure 4.2B).
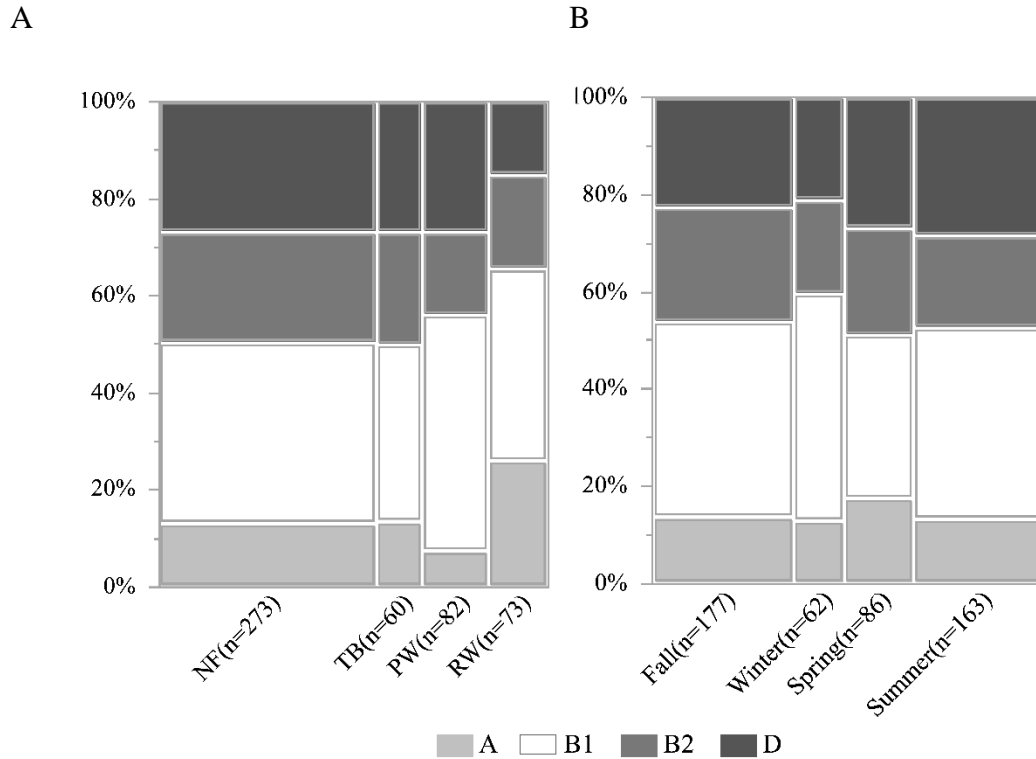
A   B

Figure 4.2. Distribution of different types (Clermont) of *E. coli* in 4 different water types (A)

and seasons (B). Each column of both figures showing the percent frequency of isolates of

different phylogenetic group retrieved from 4 water types (NF, TB, PW and RW) and in 4

seasons (Fall, Winter, Spring and Summer). The vertical length of X axis of each rectangle is

proportional to the percentage of isolates for each water types (A) and seasons (B).

4.3.2 Phenotypic resistance against β-lactam antibiotics

The vast majority of *E. coli* isolates exhibited susceptibility to all β-lactam antibiotics

tested (Table 4.2). Only 16 (3.3%) isolates exhibited resistance to single or multiple

β-lactam antibiotics; 6 of them were group A, 4 were group B1, 4 were B2 and 2 were

D. Out of 16, 10 (62.5%) were retrieved from non-tidal fresh river water, 1 (6.3%)

from tidal brackish river water, 1 (6.3%) from on-farm ponds and 4 (25.0%) from

reclaimed water (Figure 4.3A).

Table 4.2: Antimicrobial resistance of *E. coli* isolates recovered from surface and
reclaimed water

	% Resistant (n)	% Intermediate (n)	% Sensitive (n)
Ceftazidime (CAZ)	0.7 (5)	0 (0)	99.3 (719)
Ceftriaxone (CRO)	1 (7)	0.1 (1)	98.9 (716)
Cefotaxime (CTX)	1.1 (8)	0.7 (5)	98.2 (711)
Cefuroxime (CXM)	1.1 (8)	1 (7)	97.9 (709)
Cefoxitin (FOX)	2.1 (15)	0.1 (1)	97.8 (708)

Resistance against individual antibiotic appeared to be significantly impacted by

season; ceftazidime (CAZ) (Pearson's χ^2 (n=488, df=3) = 12.583, p<0.01) resistance

showed to have seasonal effect. The most resistant isolates were collected in fall

(43.8%) and summer (31.3%) (n=7 and n=5, respectively), while the least resistant

(n=1) (6.2%) were collected in spring. The remaining 3 (18.8%) isolates were

collected in winter (Figure 4.3B).
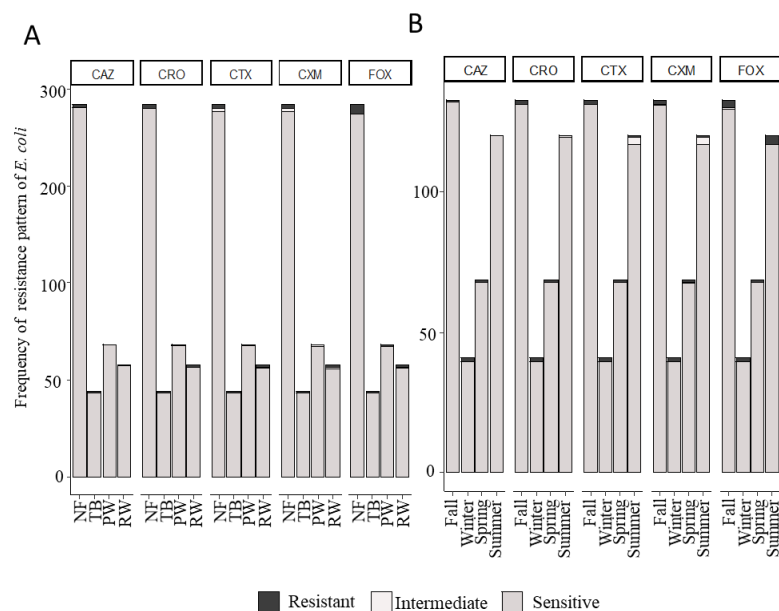
Figure 4.3. Distribution of β-lactam antibiotic resistant *E. coli* in different water types (non-tidal freshwater river (NF), tidal brackish water river (TB), pond water (PW) and reclaimed water (RW)) (A) and seasons (Fall, Winter, Spring and Summer) (B). Each column in both figures showing the number of sensitive, intermediate and resistant *E. coli* against five β-lactam antibiotics. CAZ denotes Ceftazidime, CRO denotes Ceftriaxone, CTX denotes Cefotaxime, CXM denotes Cefuroxime, FOX denotes Cefoxitin.

Group A resistant isolates were retrieved from non-tidal freshwater (n=4) and reclaimed water (n=2) in only summer and fall; isolates belonged to group B2 from retrieved from non-tidal freshwater (n=2) and reclaimed water (n=2) in summer (n=1), fall (n=2) and spring (n=1); B1 from non-tidal freshwater (n=3) and pond water (n=1) in summer (n=1), fall (n=2) and winter (n=1); D from non-tidal freshwater (n=1) and brackish water (n=1) in winter (n=2). No water type effect was noticed on resistance against individual antibiotics.

Out of 6 resistant isolates from group A, six exhibited resistance against only cefoxitin and one exhibited resistance against cefoxitin and cefotaxime. Out of four, one B1 isolate showed resistant to all antibiotics, another showed resistant to all but ceftazidime, two other showed resistant to cefoxitin. From four, one B2 isolate showed resistant to all antibiotics, one showed resistant to all but cefoxitin, one showed resistant to all but cefoxitin and ceftazidime, the fourth one showed resistance to only cefoxitin. Both D isolates showed resistant to all five β-lactam antibiotics (Figure 4.4).
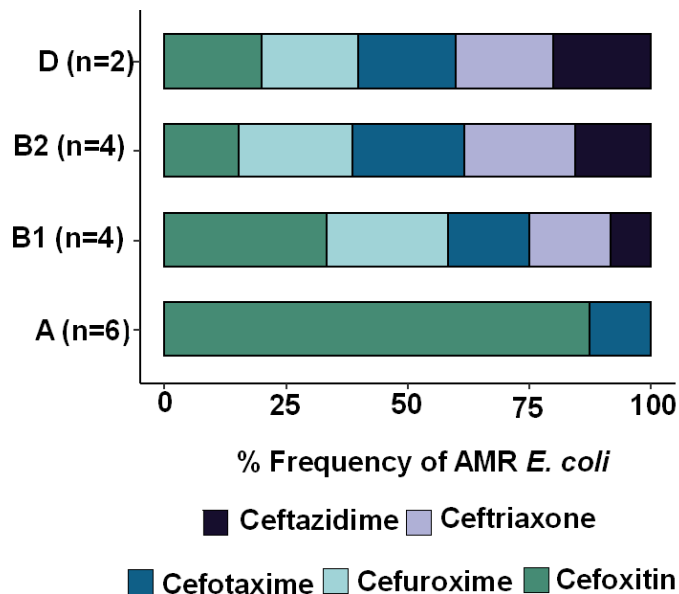
Figure 4.4. Different phylogenetic groups of *E. coli* showing resistance against individual β-lactam antibiotics. n= number of total isolates that were resistant to individual antibiotic.

4.3.3 β-lactam resistance gene profile

All 17 isolates were screened for seven β-lactam resistance genes: $bla_{TEM}, bla_{SHV}, bla_{CTX-MU}, bla_{CMY-1}, bla_{CMY-2}, bla_{OXA-1}, bla_{OXA-2}$. Eight (50%) from Groups A, B1, B2

and D harbored two or more AMR genes while one Group B1 isolate had none of the seven resistance genes. The most prevalent gene among the isolates was bla_{CMY-2} (11/16, 68.8%) and the least prevalent genes were bla_{OXA-1}, bla_{OXA-2} and bla_{SHV} (1/16, 6.2% for all). One B2 isolate collected from reclaimed water in fall was found to carry 5 AMR genes: bla_{TEM}, bla_{SHV}, bla_{CTX-MU}, bla_{CMY-1}, and bla_{OXA-1}. All (6/6, 100%) isolates from group A were found to carry bla_{CMY-2} and the majority of group A isolates (4/6, 67.7%) carried bla_{TEM}. Only group B2 and D isolates (extraintestinal group) carried the bla_{CTX-MU} gene.

4.3.4 Relationship among phenotypic and genotypic traits and environmental variables

An MCA plot using 16 isolates was plotted to evaluate the association between different variables: resistance pattern, gene patterns, phylogenic groups, seasons, and water types (Figure 4.5). The most contributing factors to the dimension 1 were presence of bla_{CTX-MU}, resistance to CRO (cro-r) and CXM (cxm-r), sensitivity to FOX (fox-s); while presence of bla_{SHV} and bla_{OXA-1}, winter, group D and winter season contributed most to the dimension 2. The MCA plot supported the association among the presence of bla_{TEM} and group B1 (n=3). The plot showed group A and summer was closely positioned to the absence of bla_{CTX-MU}, and presence of bla_{CMY-2}, supported the data that none of isolates from group A harboured bla_{CTX-MU} and found mostly in summer (3/6, 50%). Although the presence of bla_{TEM} located closely to the group B1, only one (25%) isolate carried bla_{TEM} in addition to bla_{CMY-1} and bla_{CMY-2}.

Presence of *bla$_{CMY-2}$* also positioned closely to the water type NF, supported the data that 10 out 11 isolates retrieved from NF, were carrying *bla$_{CMY-2}$* (91.0%).
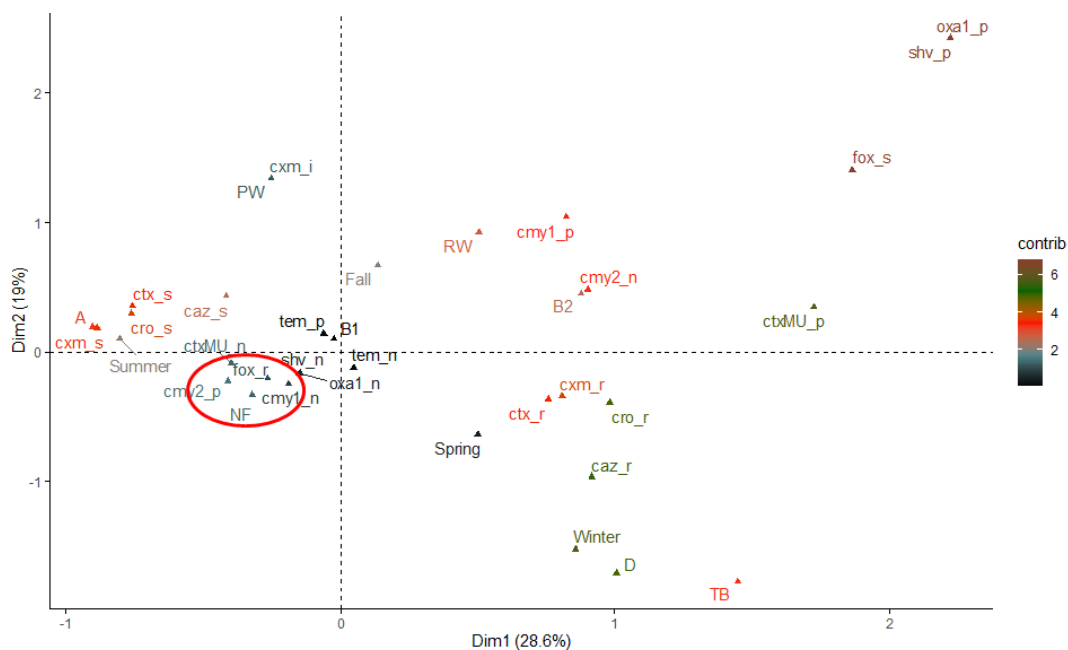
Figure 4.5. Multiple Correspondence Analysis (MCA) plot showing the association between phylogenetic groups of *E. coli* (number of isolates=16), seasons, water types, phenotype and harboring resistance genes. The adjusted inertia explains the variability in each dimension on the MCA plots, calculated according to Greenacre (1984). Presence and absence of each gene, *bla$_{TEM}$, bla$_{SHV}$, bla$_{CTX-MU}$, bla$_{CMY-1}$, bla$_{CMY-2}$, bla$_{OXA-1}$, bla$_{OXA-2}$* were denoted as tem_p, tem_n, shv_p, shv_n, ctxM_p, ctxM_n, cmy1_p, cmy1_n, cmy2_p, cmy2_n, oxa1_p, oxa1_n, oxa2_p and oxa_n respectively. s and r at the end of antibiotic name denote sensitivity or resistant to that antibiotic. Color changes with the change of contribution to both dimensions.

4.3.5 Interaction between β-lactam resistant profile, resistance gene, and phylo-groups

The similarity among the *E. coli* isolates across all water samples were plotted based on their genotypic and phenotypic profile. All 16 β-lactam resistant *E. coli* isolates

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employed to generate hierarchical cluster analysis based on the sensitivity against ceftazidime, ceftriaxone, cefotaxime, cefuroxime, cefoxitin (Figure 4.6A) and on the presence or absence of 7 different resistance genes (Figure 4.6B). Two clusters, a and b, consisting of 9 and 7 isolates respectively showing distance ranged from 1 to 2.65.
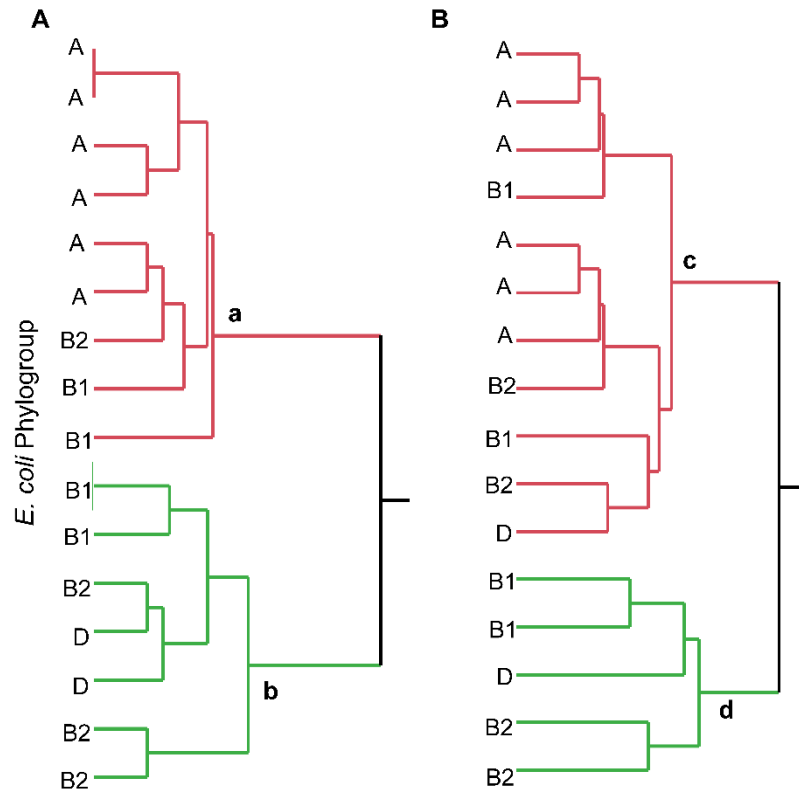
Figure 4.6. Dendrogram produced by hierarchical cluster analysis (HCA) based on (A) genotype, resistance gene profile resistance profile of each *E. coli* isolates from different phylogenetic group (B) phenotype, resistance profile against ceftazidime, ceftriaxone, cefotaxime, cefuroxime, cefoxitin from different phylogenetic group and showing relatedness of isolates across samples.

4.4 Discussion

Demand for alternative irrigation water sources is a pressing need for sustainable food production in the USA. Contamination in these water types with pathogenic and antimicrobial resistant *E. coli* will increase the potentiality of transferring pathogen to human through fresh produce crops. Moreover, due to horizontal and vertical gene transfer capability, the dissemination of antimicrobial resistance gene in different human pathogens might limit the treatment in patient with critical infection. Thus, an extensive research needed to be done to identify pathogenic and antimicrobial resistant *E. coli* in prospective irrigation water sources that can be transferred to human. This study aimed to detect potential pathogenic *E. coli*, assess the extended spectrum β-lactam resistance profile, and identify resistance gene carriage in *E. coli* retrieved from surface and reclaimed water sources. Our study found all four *E. coli* phylogroups in all water types and in all season. Isolates were found resistant to the multiple extended spectrum β-lactam antibiotics except group A which varied depending on phylogroups. All group A isolates showed resistance to only second-generation cephalosporin (cefoxitin) except one. All isolates belonged to group B2 and D were resistant to multiple antibiotics including both second and third generation cephalosporin. Most frequent resistance gene was found in all phylogroups is *bla$_{CMY-2}$*.

Most frequent group from all water type and season, was B1 (39.6%), commonly found in environmental samples and consists of mainly commensal and some diarrheagenic strains (Gordon et al., 2008; Walk et al., 2007). The least frequent group was A, approximately 14% also found in environmental samples and mostly

consists of commensal (Clermont et al., 2000; Le Gall et al., 2007). A different observation was noticed in a study from different farms in Nuevo Leon and Coahuila states in Mexico, isolates were collected from irrigation water hose and workers. Most frequent groups were A (86%) and least frequent was B2 (0.9%) (Corzo-Ariyama et al., 2019) while our study found ~21% isolates belonged to phylogroup B2. Similar to our study, 56% B1 isolates and 22.3% group A isolates were retrieved from irrigation water from household farms in North of Portugal (Araújo et al., 2017). Another study from irrigation water, animal bodies, and human and animal (pets and zoo) feces in the Mid-Atlantic region, found that most isolates were belonged to group B1 (31%) (Higgins et al., 2007). It appeared that geographical variation affects phylogroup distribution pattern that also confirmed by Gordon and Cowling (2003). However, apart from the dissimilar proportion in phylogroups distribution, it is evidenced that B1 and/or A are the two most predominant groups in all environmental studies. Approximately 21 and 25% of our isolates belonged to phylogroup B2 and D respectively that are represented as extra-intestinal *E. coli* group (Johnson and Russo, 2002). Studies in Europe and in South America showed that strains classified as phylotypes A and B1 have the potential to cause intestinal such as enterotoxigenic, enterohemorrhagic, enteroinvasive and sometimes, enteroaggregative infections that are widespread in water and food, also have the potential to spread diseases (Mosquito et al., 2015; Valverde et al., 2009). Enterotoxigenic *E. coli* (ETEC) and enteroinvasive *E. coli* (EIEC) strains were identified from fresh produce crops indicates that these isolates might came from irrigation water (Araújo et al., 2017). Moreover, human pathogens were found to survive on fresh produce surface for

extended time period (Solaiman and Micallef, Submitted) due to availability of nutrient and moisture (De Roever, 1998) which indicates transfer of human pathogen from irrigation water through fresh produce.

Majority of *E. coli* isolates exhibited susceptibility against both second and third generation cephalosporin. Only 3.3% (16/488) isolates recovered from 4.8% water samples (16/333) showed resistance to cefoxitin, cefotaxime, cefuroxime, ceftazidime and ceftriaxone, while most of them were identified as phylogroup A (10/16). Resistance against cefoxitin was observed more frequently in group A and against multiple antibiotics in other groups. Similar findings were recorded in two studies in Portugal with 3.6% β-lactam resistant *E. coli* retrieval from irrigation water and 5.3% retrieval from fresh vegetables in household farms (Araújo et al., 2017). All these reports differed from a study in Switzerland that found 22% β-lactam resistant *E. coli*, recovered from irrigation water in a fresh produce farm (Gekenidis et al., 2018). However, research have shown that this proportion is much higher in clinical samples. Almost 65-73% of clinical *E. coli* isolates showed resistance to β-lactam antibiotics in Ethiopia (Gashe et al., 2018). A study conducted in the USA found higher cefoxitin resistant clinical isolates (17.2% A, 11% B1, 2.7% B2 and 6.6% D) (Johnson et al., 2012) compared with 14.5% A , 2.1% B1, 3.9% B2 and 1.6% D environmental isolates in our study. These findings indicate the higher dissemination of resistance genes in human intestinal microbiota than in environment strains. Winokur et al. (2001) reported that graphical variation affects the distribution of ESBL producing *E. coli*. Enterobacteriaceae retrieved in Western Pacific (~30-75%) and Latin America (~41-72%) showed higher resistance to extended spectrum beta-

lactam antibiotics (cefepime, cefoxitin, ceftazidime and ceftriaxone) than in USA (~6-62%) and Canada (~6-20%). Misuse or overuse of antibiotics in certain geographical location might drives the microbial population to become more resistant against different antimicrobial agents.

In our study bla_{CMY-2}, encoding AmpC beta-lactamases was found predominantly, almost in 69% resistant isolates while bla_{OXA-1}, bla_{OXA-2} and bla_{SHV} were least predominant, only in 6.3% resistant *E. coli* isolates. All group A isolates (6/6) carried bla_{CMY-2} while 50% of rest of the phylogroups (B1: 2/4, B2: 2/4, D: 1/2) carried this gene. This is in contrast to the detection of bla_{CMY-2} in 36% A, 25% B1, 4% B2 and 36% D isolates in Canada (Jamborova et al., 2018) and the zero prevalence in fresh produce farm in North Portugal (Araújo et al., 2017). Our study also found the prevalence of bla_{CTX-MU} in only group B2 and D and predominance of AmpC gene bla_{CMY-1} and bla_{CMY-2} in phylogroups A and B1. The findings concurred by the studies that showed bla_{CMY} and/or bla_{TEM}, were the most widespread gene in phylogroup A and B1 while bla_{CTX-MU} was in phylogroup B2 and D (Reich et al., 2013; van Hoek et al., 2015). However, prior groups showed less pathogenicity and exchange of antimicrobial resistance gene in the community (Logue et al., 2017).

Chapter 5: Phylogenetic relationship of *Aeromonas* spp. isolates collected from various water types (surface and recycled water) over a one-year period and determining their virulence potential.

5.1 Introduction

Climate change and population growth, excessive groundwater discharge and escalating water demands from the agricultural and non-agricultural sectors, necessitate exploration of alternative irrigation water sources for sustainable food production. In the mid-Atlantic region of the USA, tertiary treated wastewater (reclaimed water), surface water from rivers, creeks, ponds and recycled vegetable processing wash water have potential as irrigation water sources for fruit and vegetable crops that demand strict agricultural standards to ensure microbiological safety of food crops. Unfortunately, these water types often harbor coliforms, fecal coliforms, enterococci and human pathogens that can limit their use in agriculture (Haymaker et al., 2019; Sharma et al., 2020; Solaiman et al., 2020). Microbiologically contaminated irrigation water is frequently reported as the cause of many foodborne and waterborne outbreaks (Alegbeleye et al., 2018; Carstens et al., 2019; Jongman and Korsten, 2018; McDaniel and Jadeja, 2019). Therefore, in order to mitigate potential public health risks that come from using alternative irrigation water in the mid-Atlantic region, extensive research on both known and understudied bacterial pathogens residing in these water sources is required.

Aeromonas, a gram negative opportunistic bacterial pathogen, is a natural inhabitant of water environments (Cui et al., 2017) and an emerging food safety concern that remains understudied (Fernández-Bravo and Figueras, 2020). While both psychrophilic and mesophilic species are considered pathogenic, mesophilic strains of *Aeromonas* spp. are of major concern due to their ability to cause a variety of intestinal, extra-intestinal and systemic infections in humans (Beaz-Hidalgo et al., 2013; Dallaire-Dufresne et al., 2014; Janda, 1991). In addition to water environments, pathogenic species of *Aeromonas* have been isolated from several foods, including fruit and vegetables (Chang et al., 2008; McMahon and Wilson, 2001; Nagar et al., 2011; Szabo et al., 2000; Xanthopoulos et al., 2010). and patients with diarrheal disease (Igbinosa et al., 2012). Chang et al. (2008) found that *A. caviae* followed by *A. hydrophila* were the most frequently isolated species from foods suspected to have been linked to food poisoning incidents. The emerging public health concern and food safety risk of *Aeromonas* spp. are due to their ability to produce different cytotoxins and enterotoxins and the ability to survive and grow under refrigerated conditions, and in high pH and saline environments (Kirov, 1997; Majeed et al., 1990). It has been postulated that the food safety risk of *Aeromonas* should be given more attention, on par with other notorious pathogens such as *Salmonella* and *Campylobacter*, following the finding from foodborne illness outbreak analyses that infective doses as low as 10^3 CFUs could cause gastroenteritis in humans (Teunis and Figueras, 2016).

Transmission, infection and disease development by *Aeromonas* spp. from aquatic environments to humans directly or via food depend on several factors including

contamination level of the water, transfer potential from water to host or food, microbial persistence on the food surface, attachment potential and virulence (Steele and Odumeru, 2004) . Bacterial flagella, surface poly- and lipopolysaccharides, glucans, cytotoxins, hemolysins/aerolysin, secretory metabolites and extracellular enzymes are considered the major virulence factors of *Aeromonas* (Tomás, 2012). Flagellar genes play a crucial role in attachment to both biotic and abiotic surfaces, while enterotoxin genes are prominent determinants for the success of infection and are frequently associated with gastroenteritis (Santiago et al., 1999; Sen and Rodgers, 2004; Von Graevenitz, 2007).

Data on *Aeromonas* spp. in irrigation water used for fresh fruit and vegetable crops is not available. This study aimed to address the knowledge gap on *Aeromonas* risk from irrigation water use in fresh produce agriculture by investigating the abundance, diversity and distribution of *Aeromonas* isolated from various irrigation water sources, including surface and recycled water, in the mid-Atlantic region. Isolates were obtained from a two-year longitudinal study as part of a U.S. Department of Agriculture funded Centre of Excellence (COE) called CONSERVE: A Center of Excellence at the Nexus of Sustainable Water Reuse, Food and Health. CONSERVE has a long-term goal of facilitating the adoption of safe reuse of water in agriculture of fresh fruit and vegetables. As such, virulence gene patterns of the *Aeromonas* spp. isolates and their attachment and transfer potential from irrigation water to abiotic and lettuce leaf surfaces, were also investigated.

5.2. Materials and Methods

5.2.1 Isolation and identification of Aeromonas spp.

Aeromonas spp. were isolated by standard membrane filtration and incubation on

Ampicillin Dextrin Agar (Criterion, Hardy Diagnostics, Santa Maria, CA, USA)

supplemented with ampicillin, sodium salt (10 µg/mL) (Fisher Scientific, Hampton,

NH, USA) and vancomycin (10 µg/mL) (Amresco, Randor, PA, USA) (ADA-V)

from objective 1. Up to three yellow colonies from ADA-V plates were picked for

further identification. In total, 342 *Aeromonas* isolates, recovered using the selective

culture technique, were confirmed through PCR screening.

Each colony was cultured on Tryptic Soy Agar (TSA) (Oxoid, Lenexa, KS, USA)

supplemented with ampicillin and vancomycin (TSA-VA). DNA was extracted from

single *Aeromonas* colonies by rapid heat lysis (Micallef et al., 2012). Aliquots of 2.5

µl DNA suspensions were used as templates for genus-specific PCR amplification of

the gyrB gene encoding the DNA gyrase β-subunit. DNA was mixed in a 22.5 µl PCR

reactions containing 1X PCR buffer (New England Biolabs (NEB), Ipswich, MA,

USA), 1.5 mM $MgCl_2$ (NEB), 0.2 mM dNTPs (VWR, Radnor, PA, USA), 0.3 µM

each of forward and reverse primers (gyrB and 16S rRNA as internal control)

(Integrated DNA Technologies (IDT), Coralville, Iowa, USA) (Table 2) and 0.24 µl

of 5 Units of Taq DNA Polymerase (NEB). Amplification reactions were carried out

with an initial denaturation step of 95°C for 15 min, followed by 35 cycles of

denaturation at 94°C for 30 s, annealing at 59°C for 20 s and extension at 72°C for 45

s, with a final extension of 7 min. PCR products were then electrophoresed in 1%

agarose (Lonza, Rockland, ME, USA). *Aeromonas hydrophila* (Chester) Stanier

ATCC 7966, *Aeromonas veronii* Hickman-Brenner et al. ATCC 35624 and

Aeromonas caviae (ex Eddy) *Popoffii* ATCC 15468 were used as reference strains

throughout this study.

5.2.2 PCR amplification and sequencing of RNA polymerase σ70

factor, rpoD

For all *Aeromonas* isolates, an ~800 bp fragment of the *rpoD* gene was amplified by

PCR and sequenced according to Martinez-Murcia et al. (2011) with some

modification. PCR amplification was conducted in 50 µl reactions consisting of 1X

PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 20 mM $(NH_4)2SO_4$ (Becton, Dickinson

and Company (BD), Sparks, MD, USA), 0.2 µM each of forward and reverse primers

and 0.24 µl of 5 Units of Taq DNA Polymerase (NEB). Reaction cycles involved in

this study, were initial denaturation at 95°C for 3 min, followed by 35 cycles of

amplification with denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and

extension at 72°C for 30 s and a final extension step at 72°C for 3 min. Amplicons

were confirmed through gel electrophoresis on 1% agarose (Lonza). Forward end

rpoD (rpoD 4F or *rpoD 70F*) sequencing (700 to 800 bp reads) was conducted at

Eurofins Genomics (Louisville, KY, USA) using industry standard Sanger

sequencing. Sequence chromatograms were checked both manually and using BioEdit

v7.0.5 (Ibis Therapeutics, Carlsbad, CA, USA) to remove any ambiguity and to

identify insertions, deletions, sequencing errors or the presence of stop codon.

5.2.3 Detection of virulence-related genes in Aeromonas spp.

Virulence genes encoding enterotoxins, aerolysin and flagella were evaluated in

Aeromonas spp. isolates and are listed in Table 5.1. DNA was extracted using the

QIAGEN DNeasy kit (QIAGEN, Hilden, Germany) following the manufacturer

protocol. Multiplex PCR were optimized in two protocols. Protocol I was designed

for three sets of target genes, *act-alt-*16S rRNA, whereas protocol II targeted the

*aerA-flaA-alt-*16S rRNA genes. PCR amplification was performed as described above

but with 0.3 mM dNTPs (VWR) and 0.4 µM each of primer (IDT). For each PCR

reaction, 2.5 µl template DNA (~20 ng/µl) was used. For protocol I, standard cycling

conditions for amplification included an initial step (95°C for 15 min) followed by 35

cycles of denaturation at 95°C for 15 s, annealing at 69°C for 30 s and extension at

72°C for 30 s, followed by a final step at 72°C for 10 min. For protocol II, standard

cycling conditions started with an initial step at 95°C for 10 min followed by 40

cycles of amplification comprising of denaturation at 95°C for 30 s, annealing at

55°C for 30 s and extension at 72°C for 45 s, with a final step at 72°C for 10 min.

PCR products were electrophoresed on 1% agarose (Lonza) gels to determine

presence or absence of target genes.

Table 5.1: Primer pairs used for the detection of *Aeromonas* spp. virulence genes.

Function/protein	Gene	Size (bp)	Primer	Sequences (5´-3´)	Reference
DNA gyrase β-subunit	*gyrB*	192	Forward	GAAGGCCAAGTCGGCCGCCAG	Robertson et al., 2014
			Reverse	TCTTGGCATCGCCCGGGTTTTC	
Housekeeping	16S rRNA	352	Forward	AGAGTTTGATCCTGGCTCAG	Motta et al., 2007
			Reverse	CTGCTGCCTCCCGTAGG	
Aerolysin/haemolysin	*aerA*	309	Forward	CAAGAACAAGTTCAAGTGGCCA	Wang et al., 2003
			Reverse	ACGAAGGTGTGGTTCCAGT	
Cytotoxic Heat-labile Enterotoxin	*act*	232	Forward	GAGAAGGTGACCACCAAGAACA	
			Reverse	AACTGACATCGGCCTTGAACTC	
Cytotonic Heat-labile Enterotoxin	*alt*	361	Forward	TGCTGGGCCTGCGTCTGGCGG	Kingombe et al., 1999
			Reverse	AGGAACTCGTTGACGAAGCAGG	
Cytotonic Heat-labile Enterotoxin	*ast*	536	Forward	GACTTCAATCGCTTCCTCAACG	
			Reverse	AGGAACTCGTTGACGAAGCAGG	
Polar flagella	*flaA*	608	Forward	TCC AAC CGT YTG ACC TC	Sen and Rodgers,2004
			Reverse	GMY TGG TTG CGR ATG GT	
RNA polymerase σ^{70} (D) factor	*rpo*D	700	Forward	ACGACTGACCCGGTACGCATGTA	Yamamoto et al., 2000
			Forward	GAAGGCGAAATCGACATCGC	
		800	Reverse	ATAGAAATAACCAGACGTAAGTT	Martinez et al., 2011
			Reverse	ATGCTCATGCGRCGGTTGAT	

5.2.4 In vitro bacterial attachment to a polystyrene surface

A loopful of fresh culture of *Aeromonas* isolates on TSA-VA were resuspended into

Tryptic Soy Broth (BD) supplemented with ampicillin and vancomycin (TSB-VA).

Sterile 96-well polystyrene plates were filled with 200 µL of culture suspension and

incubated at 37°C for 72 h, in replicates of 3 per isolate. Uninoculated TSB-VA

served as a negative control. After incubation, planktonic cells were removed by

inverting the plate and washing three times with sterile water. The remaining attached

cells were fixed by adding 200 µL methanol and incubated at room temperature for

20 min, next, methanol was removed, and the plate air-dried. The remaining attached

cells were stained with 2% (w/v) crystal violet (TCI, Toshima, Kita-ku, Tokyo,

Japan) for 5 min, washed with sterile water and air-dried. The cells were then

suspended in 150 ml of 33% (v/v) glacial acetic acid (Sigma-Aldrich, St. Louis, MO,

USA) to bring the crystal violet adhering to attached cells back into solution. The

optical density (OD) of each well was measured at 570 nm in a microplate reader

(SYNERGY HTX Multi-Mode, BioTek Instruments Inc, Winooski, VT, USA) and

interpreted as previously described (Stepanovic et al., 2000).

5.2.5 Aeromonas attachment to the lettuce leaf surface

Lettuce cultivation: Romaine lettuce cultivar 'Sparx' seeds were planted in the

greenhouse. After germination, seedlings were transplanted and kept at a temperature

of 20°C during day for 13 h and 18°C at night for 11 h. Seven-week-old plants were

used for inoculation.

Inoculum preparation and inoculation: 0.5 MacFarland standard bacterial suspensions were prepared from overnight *Aeromonas* cultures (108 cells/mL). A 100 µL aliquot of this suspension was then inoculated onto the adaxial surface of the third and fourth leaves of 7-week-old lettuce plants in triplicate. Plants inoculated with water only were considered as negative control. Plants were processed 0, 24 and 120 hours post inoculation (hpi; 3 plants each). Using sterile scissors, inoculated leaves were cut and placed in WhirlPak bags (Nasco, Baltimore, MD, USA) for bacterial retrieval and quantification. For retrieval of loosely attached bacterial cells, 30 mL 0.1% peptone water (BD Difco, Franklin Lakes, NJ, USA) were added to the bag and placed in the shaker for 5 min. Then, 100 µL of the leaf surface washes were plated on TSA supplemented with ampicillin and vancomycin (TSA-AV) as previously described and incubated at 37°C for 24 h. In order to recover strongly associated cells, the suspension was discarded, and leaves were washed with sterile water twice to remove all loosely attached cells, then smashed using a pestle and diluted into 30 ml 0.1% peptone water. The bags were placed on the shaker for 5 min for homogenization and plated on TSA-AV and incubated for 24 h at 37°C.

5.2.6 Sequencing data analysis

Reference strains were selected from NCBI BLASTn comparisons to classify 227 isolates for building phylogenetic trees based on isolates grouped by water type from which they originated and season of sample collection. Alignments were carried out in MEGA v. 7.0 (Pennsylvania State University, PA, USA) using built-in ClustalW (Kumar et al., 2016) with 1,000 iterations in a Neighbor Joining clustering method.

Every single sequence was aligned with a reference organism (Type strain or lab strain from database), then all sequences were aligned together. All ambiguous characters or sequences (whole sequence) were excluded from the final alignment file. Amplicon sequences have been deposited at NCBI GenBank under accession number MW122064 - MW122082 and MW122083 - MW122290.

Isolates were placed into different categories based on water type and season. Kimura's 2-parameter model along with 1,000 bootstrap replications were used to determine the genetic distance between isolates (Kimura, 1980) and evolutionary tree construction was performed by the Maximum Composite Likelihood model with MEGA v. 7.0 (Kumar et al., 2016). Trees were graphically displayed and annotated by iTOL v 4.2.3 (Letunic and Bork, 2016). Species diversity was calculated using the Margalef diversity index (Margalef, 1958):

$$D = \frac{S - 1}{\ln N} \tag{II}$$

Where S is the number of species detected in each water type, and N is the total number of isolates in that water sample.

5.2.7 Data management and statistical analysis

Isolates with unique phylogenetic classification and virulence gene profiles were included in data analysis. To check seasonal and water type effects on bacterial attachment to a polystyrene surface, data were pooled by water type (5 non-tidal freshwater river/creeks (NF), 1 tidal brackish river (TB), 2 ponds water (PW), 3 highly treated reclaimed water (RW) sites) and season. Season was defined as spring from March 01 to May 30, summer from June 01 to August 31, fall from September

01 to November 30, and winter from December 01 to February 28. The Pearson's

Chi-square test was conducted to assess the effects of season and water type on the

presence of virulence genes and on attachment to a polystyrene surface. Bacterial

attachment and persistence capability on a lettuce surface was assessed using mixed

effect model analysis. The repeated measurements effects were controlled using the

random effect of replication and fixed effects of attachment strength and inoculation

time. Tukey's Honestly Significant Differences test with α=0.05 was used to assess

significant differences between bacterial loads at three time points. These statistical

analyses were performed in Rstudio v3.6.3. Multiple correspondence analysis (MCA)

was conducted using virulence gene presence/absence data and water type, season,

attachment and classification data. MCA is a method for visualizing associations

among categorical variables. The adjusted inertia explaining the variability in each

dimension on the MCA plots were calculated according to Greenacre (1984). MCA

were performed in JMP Pro 14.1.0.

5.3. Results

5.3.1 Phylogenetic classification of Aeromonas isolates

A total of 331 non-clonal isolates were confirmed to be *Aeromonas* spp.; 172

recovered from non-tidal fresh river water, 27 from tidal brackish river water, 69

from pond water and 63 from reclaimed water. A subset of 227 *Aeromonas* isolates,

representative of water samples taken from all sources at all sampling dates, were

randomly selected in order to classify them phylogenetically based on water type and

seasonality. Sequence alignments were generated from *rpoD* sequence fragments of

702 bp. In total, 20 different *Aeromonas* species were identified in all water samples:

A. veronii, A. hydrophila, A. jandaei, A. bestiarum, A. dhakensis, A. popoffii, A. piscicola, A. enterica, A. allosaccharophila, A. salmonicida, A. culicicola, A. encheleia, A. eucrenophila, A. aquatica, A. tecta, A. caviae, A. media, A. rivipollensis, A. bivalvium and *A. punctata* (Figure 5.1). Phylogenetic analyses showed that the most frequently encountered classification (making up ~63% of isolates) clustered with three major human pathogenic species *A. veronii, A. hydrophila* and *A. jandaei*. The next two most prevalent classifications, *A. bestiarum* and *A. piscicola* both known to cause disease in fish, together represented 11% of isolates. Two other species known to cause human disease, *A. caviae* and *A. dhakensis* were also detected but at lower frequencies (~2%) (Figure 5.1).
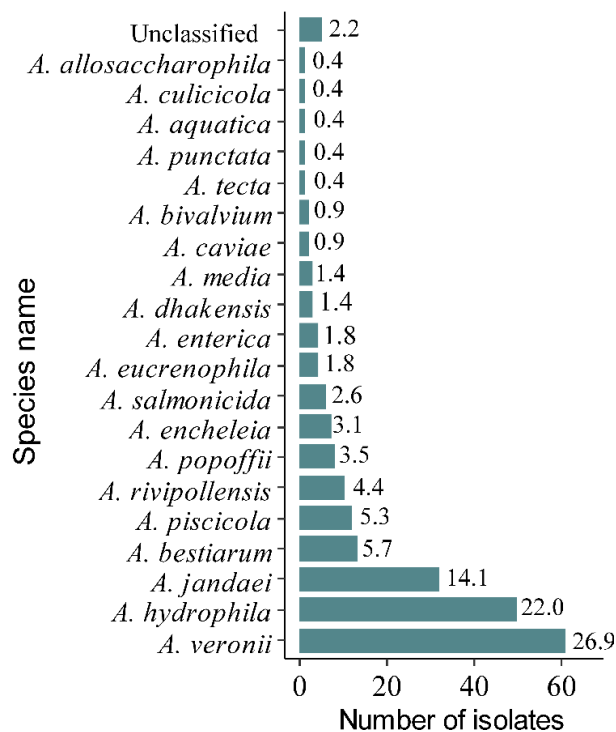
Figure 5.1. Frequency distribution of *Aeromonas* species (n=227) isolated from water collected at 11 sites including tidal and freshwater rivers, ponds and reclaimed water. The x-

axis denotes the total number of isolates and percentages are given alongside the bars for each species.

5.3.2. Diversity of Aeromonas species in different water types

Phylogenetic trees were generated with sequences grouped by water type. The phylogenetic tree constructed from non-tidal freshwater river/creek isolates collected from 5 sites was built using 277 similar and 425 variable nucleotide positions (Figure 5.2A). Pairwise distances between nucleotide sequences of NF isolates ranged from 0.002 to 0.577. A total of 119 sequences clustered with 14 representative species, where Margalef diversity index was calculated as 2.72 (Table 5.2). Out of 119 isolates, 38 (31.9%) clustered with the reference strain *Aeromonas hydrophila* ATCC 7966 and 30 (25.2%) clustered with the reference strain *Aeromonas veronii* ATCC 35624. Three isolates remained unclassified (Figure 5.2A; Table 5.2).

The phylogenetic tree generated using sequences from tidal brackish river water isolates was built with 493 similar and 209 variable nucleotide positions. Pairwise distances between nucleotide sequences ranged from 0.003 to 0.172. Only one tidal brackish water source was sampled, yielding 20 isolates used in this analysis. The 20 sequences clustered with 10 *Aeromonas* species with a Margalef diversity index of 3.00, the highest measure obtained for any water type (Figure 5.2B; Table 5.2). One isolate form TB remained unidentified.

Phylogenetic analysis for pond water isolates was carried out with 35 sequences having 443 similar and 259 variable nucleotide positions. Pairwise distances between nucleotide sequences of PW isolates ranged from 0.001 to 0.234. These sequences clustered with 7 different species giving a Margalef diversity index value of 1.69, the

lowest measure obtained for any water type. Eighteen out of 35 isolates clustered with

A. jandaei (51.4%) and the rest clustered with six other species (Figure 5.2C; Table

5.2).

The phylogenetic tree generated with isolates recovered from reclaimed water sites

was constructed with 408 similar and 294 variable nucleotide positions on the *rpoD*

gene. Pairwise distances between nucleotide sequences ranged from 0.01 to 0.23.

Nine representative species were identified, and the Margalef diversity index value

was calculated at 2.02. Altogether 25 out of 53 sequences (47.2%) clustered with *A.*

veronii, 7 with *A. hydrophila* and 7 with *A. jandaei*. One isolate remained unclassified

(Figure 5.2D; Table 5.2).

A
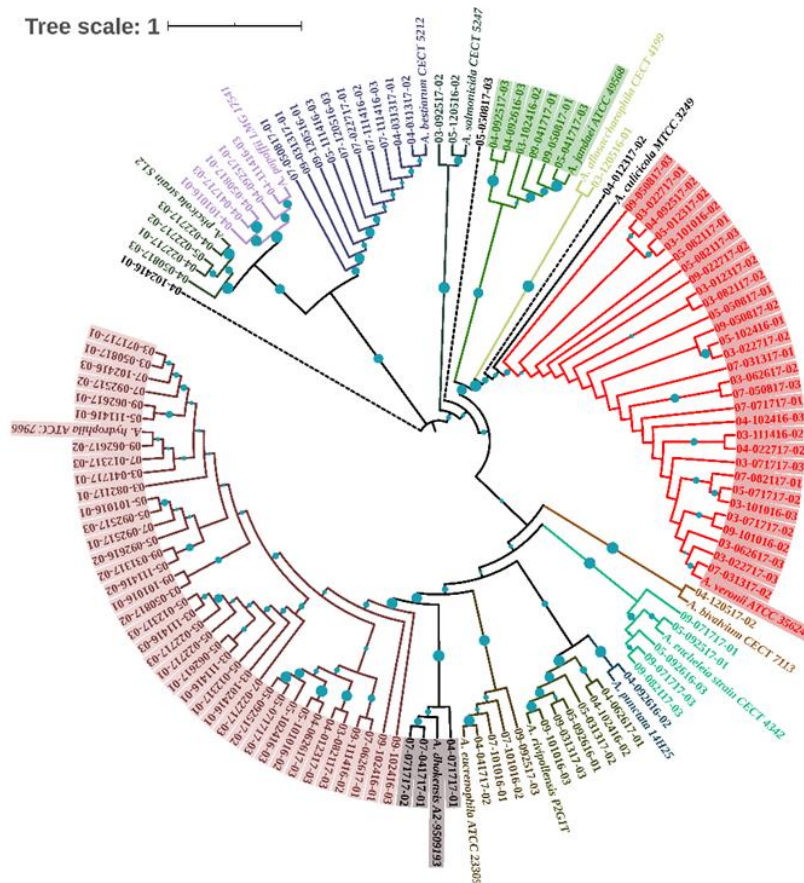
B
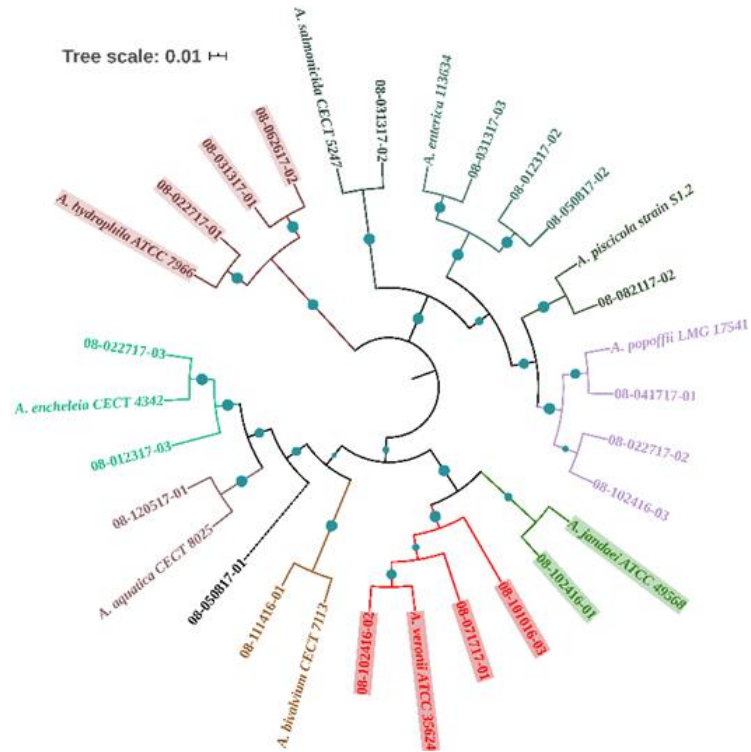
C
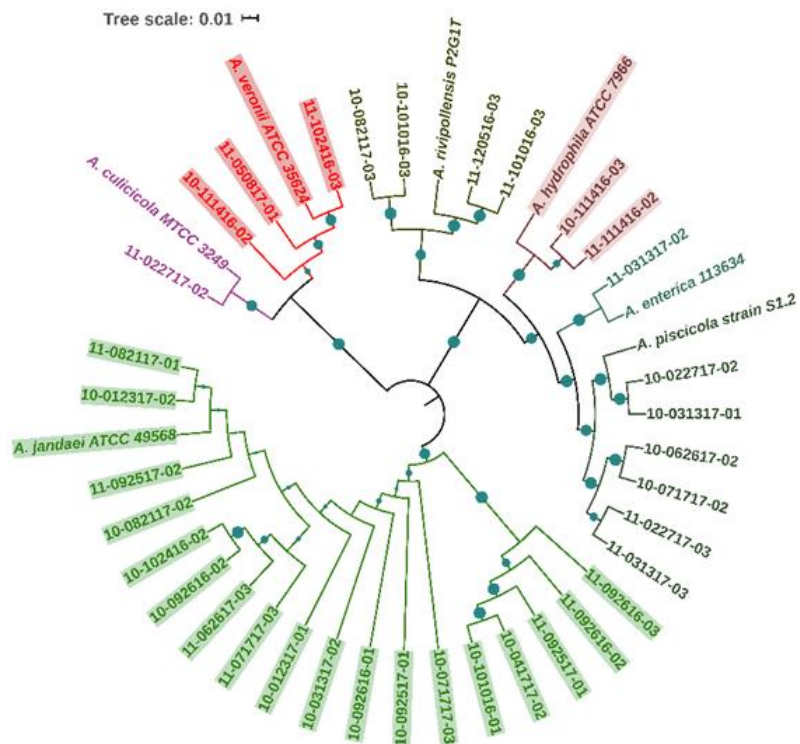
D
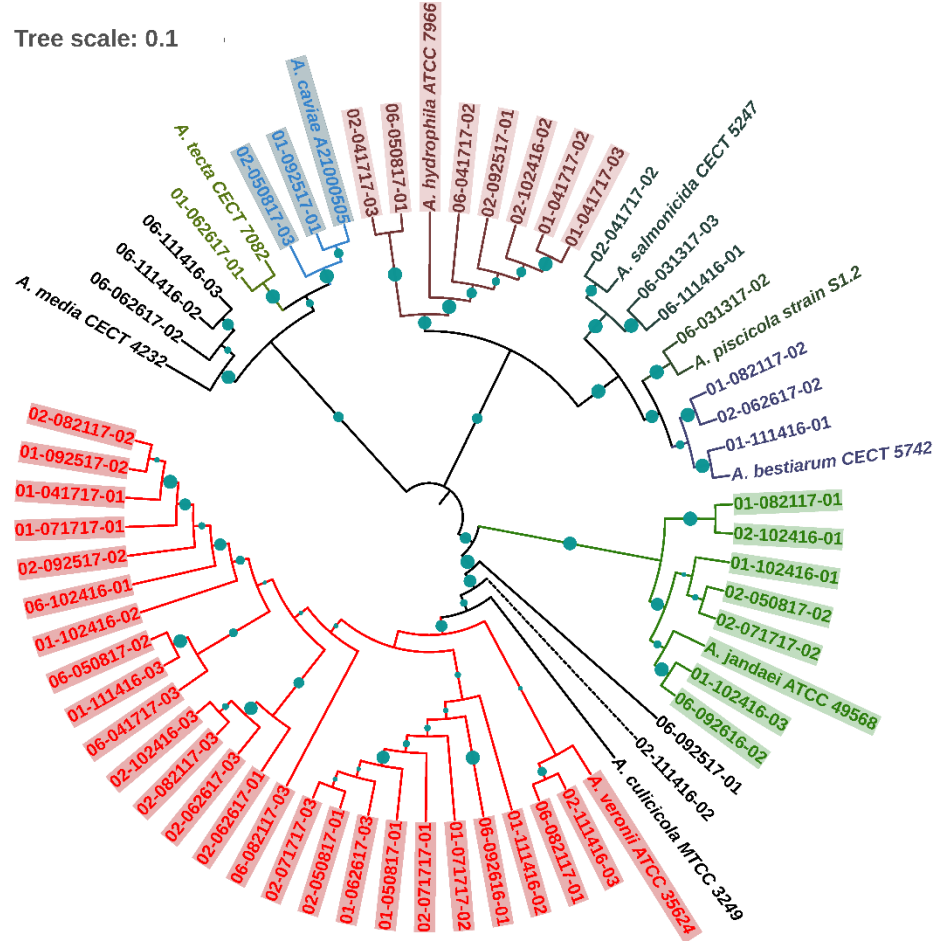
Figure 5.2. Phylogenetic relationship among representative isolates of *Aeromonas* spp. collected from different water sources with potential for use as irrigation water; The trees were constructed from isolates collected from (A) non-tidal fresh rivers (NF; n=119 from 5 sites), (B) tidal brackish rivers (TB; n=20 from 1 site), (C) pond water (PW; n=35 from 2 sites) and (D) reclaimed water (RW; n=54 from 3 sites). Analyses were done using a maximum composite likelihood model. Isolate codes (denoted as site-date-isolate number) were aligned to reference strains from NCBI BLASTn. Each colored node in the circular tree represents specific species. Blue dots in different sizes represent bootstrap values: small, medium and large dots indicate >50% to <70%, ≥70% to <90% and ≥90% values,

respectively. Probable pathogenic species were marked with background color on each leaf. Unidentified isolates were denoted using a dashed line on that specific node.

The human pathogens *A. hydrophila* and *A. veronii* ranked highest in frequency in non-tidal fresh river, tidal brackish river and reclaimed water (Table 5.2).

Table 5.2: *Aeromonas* species distribution and diversity measures by water type and season.

	N°· of Sites	N°· of Isolates	Species Richness	Margalef Diversity Index	Most Frequent Classification (%)
Water Type					
Non-tidal fresh	5	119	14	2.7	*A. hydrophila* (32) *A. veronii* (25)
Tidal brackish	1	20	10	3.0	*A. enterica* (15) *A. hydrophila* (15) *A. popoffii* (15) *A. veronii* (15)
Pond	2	35	7	1.7	*A. jandaei* (51) *A. piscicola* (17)
Reclaimed	3	53	9	2.0	*A. veronii* (48) *A. hydrophila* (13)

					A. jandaei (13)
Season					
Fall	11	86	13	2.7	*A. hydrophila* (22)
					A. veronii (21)
					A. jandaei (17)
Winter	08	35	15	3.9	*A. hydrophila* (8)
					A. veronii (7)
Spring	11	53	12	2.8	*A. hydrophila* (12)
					A. veronii (10)
Summer	11	53	10	2.3	*A. veronii* (22)
					A. hydrophila (11)

A. veronii and *A. hydrophila* were found in all water sources and prevalent at all sites. *A. jandaei* and the fish pathogen *A. piscicola* ranked first and second, respectively, in pond water but were also detected in all other water sources (Figure 5.3). *A. rivipollensis* was also prevalent at both pond water sites. Four species, including the pathogen *A. dhakensis* and probable pathogen *A. punctata* were only found in non-tidal fresh river water. Three species, including the pathogen *A. caviae* were detected only in reclaimed water. Three other probable pathogens were detected sporadically;

A. popoffii was only detected in NF and TB, *A. enterica* in PW and TB, and *A. tecta* in RW. Fish pathogens *A. eucrenophila* and *A. allosaccharophila* were prevalent in NF, *A. bestiarum* in both NF and RW, *A. salmonicida* in NF, RW and TB, and *A. encheleia* in NF and TB (Figure 5.3).
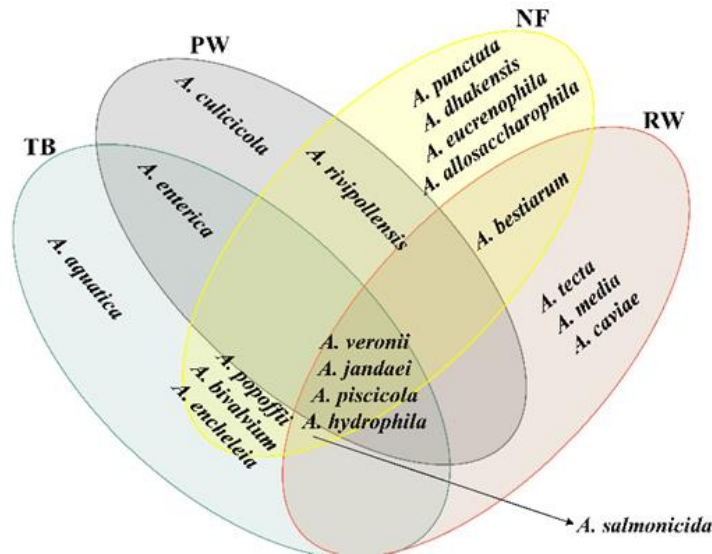
Figure 5.3: Venn diagram showing *Aeromonas* species detection by water type, non-tidal fresh rivers (NF; yellow balloon), tidal brackish rivers (TB, green balloon), pond water (PW, grey balloon) and tertiary treated reclaimed water (RW, red balloon). Four species, *A. veronii, A. jandaei, A. piscicola and A. hydrophila*, were detected in all water types.

5.3.3. Seasonal variability in Aeromonas species detected

Phylogenetic analysis also revealed seasonal variation in *Aeromonas* spp. distribution (Figure 5.4). The 86 isolates collected in the fall clustered with 13 different species giving a Margalef Diversity Index of 2.69 (Table 5.2). Sequences from 36 isolates recovered in the winter clustered with 14 different classifications. Species diversity was found to be higher in winter than other seasons, with a Margalef Diversity Index of 3.62. In spring, 52 isolates clustered with 12 different classifications giving a

Margalef Diversity Index value of 2.77. Sequences from the 53 isolates retrieved in summer clustered with 10 different species yielding a Margalef Diversity Index of 2.27. The majority of *A. veronii* isolates were retrieved in fall (33.9%) and summer (35.3%) (Figure 5.4). *A. hydrophila,* and *A. jandaei* exhibited a different trend, mostly isolated in the fall (44.0% and 53.1%, respectively). *A. caviae* was only isolated in fall (50.0%) and spring (50.0%) while *A. dhakensis* was most prevalent in summer (67%). All other species were distributed uniformly across seasons (Figure 5.4).
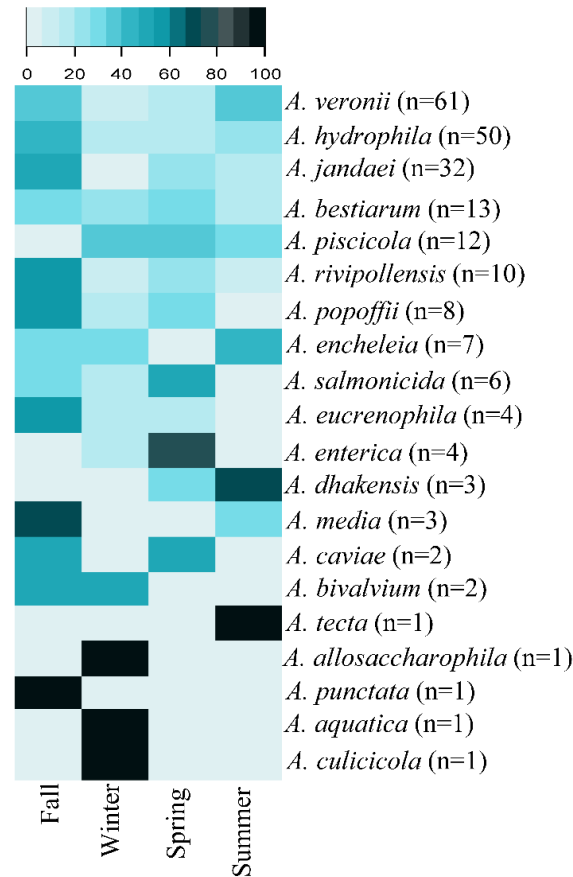
Figure 5.4: Heat map showing the seasonal variation in distribution of pathogenic *Aeromonas* species. Color key shows percent of each species in a specific water type. Color gradation from blue to black denotes frequency increase.

5.3.4. Virulence gene patterns in different Aeromonas species

All 331 isolates were screened for five virulence genes, the enterotoxin genes *ast, alt, act*, the hemolysin gene *aerA* and the flagellar gene *flaA* (Table 5.1). Of these 331, 319 (~96%) isolates harbored at least one of the virulence genes, 68 harbored only 1 gene, 12 harbored none, and 2 harbored all 5 genes (Fig. 5.5). The *flaA* gene was the most prevalent gene, with 200/331 (~60%) isolates carrying it. The next most prevalent gene was *alt*, detected in 181/331 (~55%) isolates. The genes *act, ast* and *aerA* were detected in 136 (~41%), 126 (~38%) and 116 (~35%) isolates, respectively Table 5.3.
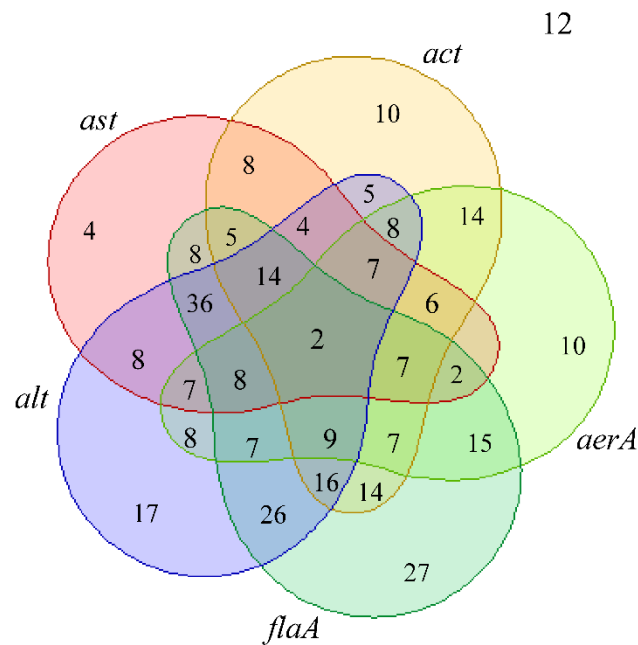
Figure 5.5: Venn diagram showing the distribution of virulence genes in 331 *Aeromonas* spp. isolates. Five colored balloons represent the genes *ast* (red), *alt* (purple), *aerA* (light green), *act* (yellow) and *flaA* genes (dark green). Numerical values in shared areas represent the number of isolates harboring all the genes in overlapping balloons.

Phylogenetic classification of 227/331 isolates showed that those carrying all three enterotoxin genes were classified as *A. popoffii* (n=1) and *A. eucrenophila* (n=1). Among the 50 isolates classified as *A. hydrophila*, the genes *alt* (60%) and *flaA* (60%) were the most frequently detected (Table 5.3). The most common gene pattern was *ast + alt + flaA* (11 isolates), while18 isolates carried two enterotoxin genes (1 *ast + act*, 13 *ast + alt*, 4 *act + alt*) and 5 carried all three enterotoxin genes along with *aerA* or the *flaA* gene. Among 61 probable *A. veronii* isolates, the most frequently detected genes were *flaA* (69%), *alt* (57%) and *ast* (51%). Several *A. veronii* isolates harbored gene combinations. The gene pattern *ast + alt + flaA* (8 isolates) was also common, while 27 isolates carried two enterotoxin genes (7 *ast + act*, 16 *ast + alt*, 4 *alt + act*) and 6 carried all three enterotoxin genes along with *aerA* or *flaA* genes. Among 32 probable *A. jandaei* isolates, *alt* (69%) and *flaA* (63%) were also the most prevalent genes. Common gene combinations included 13 isolates carrying two enterotoxin genes (2 *ast + act*, 5 *ast + alt*, 5 *alt + act*) and 3 harboring all three enterotoxin genes along with or without *flaA*. A high prevalence of *flaA* carriage was detected in *A. piscicola* (83%) and *A. bestiarum* (62%), followed by *aerA* and *alt* (in *A. piscicola* only). Only one probable *A. dhakensis* isolate collected in the summer harbored a combination of *ast + alt*. Another 2 isolates carried *ast* and *act* separately. Likewise, only one probable *A. caviae* isolate carried *ast + act*, the second isolate harbored only the *act* gene.

Table 5.3: Detection of virulence genes in phylogenetically classified isolates.

Classification	N	ast		act		aerA		flaA		alt	
		+	-	+	-	+	-	+	-	+	-
A. allosaccharophila	**1**		1		1	1		1			1
A. aquatica	**1**		1		1		1	1		1	
A. bestiarum	**13**	2	11	5	8	6	7	8	5	3	10
A. bivalvium	**2**		2	1	1	1	1	1	1		2
A. caviae	**2**	1	1	2		1	1	1	1		2
A. culicicola	**1**		1	1		1		1		1	
A. dhakensis	**3**	2	1	1	2		3	2	1	1	2
A. encheleia	**7**	3	4	5	2	1	6	5	2	6	1
A. enterica	**4**		4	1	3	2	2	1	3		4
A. eucrenophila	**4**	3	1	2	2	1	3	4		2	2
A. hydrophila	**50**	21	29	20	30	14	36	30	20	30	20
A. jandaei	**32**	12	20	13	19	11	21	20	12	22	10
A. media	**3**	1	2	1	2	1	2	1	2	2	1
A. piscicola	**12**	4	8	4	8	6	6	10	2	6	6
A. popoffii	**8**	2	6	3	5	6	2	6	2	5	3
A. punctata	**1**	1			1		1	1		1	
A. rivipollensis	**10**	5	5	3	7	2	8	6	4	6	4
A. salmonicida	**6**	6		6		4	2	4	2	3	3
A. tecta	**1**	1		1		1			1		1
A. veronii	**61**	31	29	28	34	26	36	43	19	36	26
n		131		139		120		209		188	

An MCA plot of 199 classified *Aeromonas* isolates, including all classifications with

n≥6, was plotted to assess the association between virulence gene detection in isolates

and assigned species (Figure 5.6A). The plot corroborated the associations between

gene patterns and species assignments. The genes contributing most to inertia were

presence or absence of *alt*, and presence of *aerA* and *ast* for dimension 1, and

presence or absence of *act* and absence of *flaA* for dimension 2. The MCA plot

supported the association between *A. hydrophila* (n=50) and the presence of *flaA* and

alt genes, both detected in 60% of isolates, as well as *ast* and *act* (Figure 5.6A). The

position of *A. veronii* (n=61) on the plot reflected the high prevalence of the genes

flaA, alt, ast and *act* in that species. *A. jandaei* (n=32) was positioned closest to *flaA+*

and *alt+* on the plot, driven by approximately two-thirds of isolates found to harbor

those genes, and influenced by the absence of *aerA*. The subsequent, most prevalent

classifications, *A. bestiarum* (n=13) and *A. piscicola* (n=12) were divergent from the

other species (Figure 6A), attributed to the higher number of gene patterns identified

in relation to spp. richness. Gene patterns:spp. richness ratios were 9:13 (0.7) and

8:12 (0.7) for *A. bestiarum* and *A. piscicola*, respectively, compared to e.g. *A. veronii*

(26:61; 0.4) and *A. hydrophila* (22:50; 0.4).
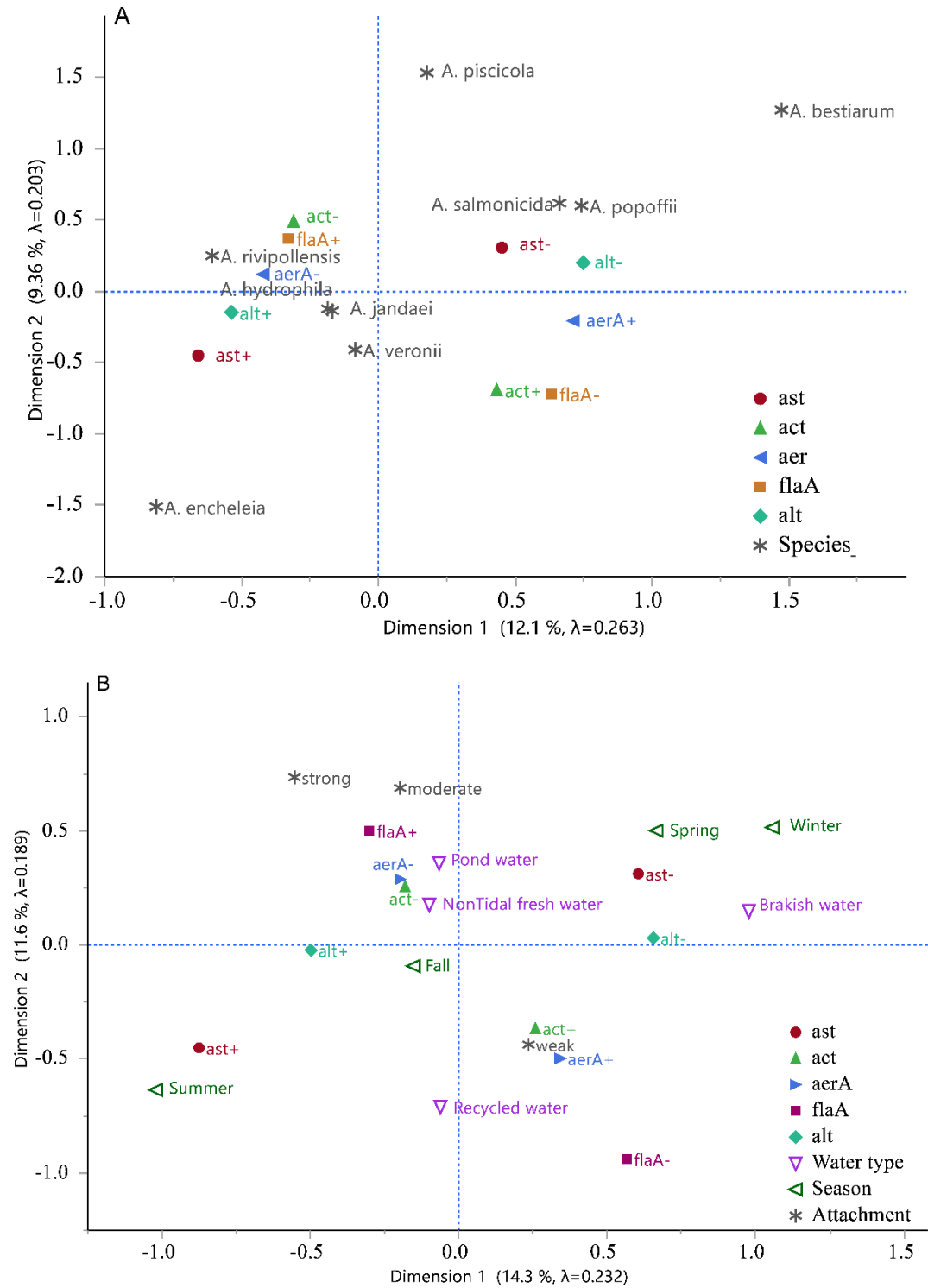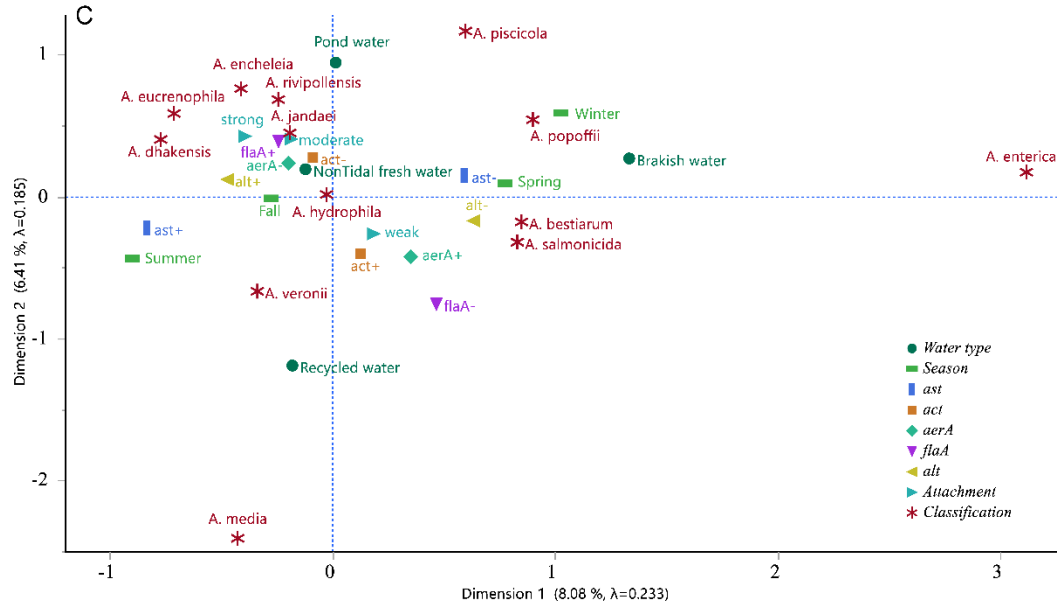
Figure 5.6: Multiple Correspondence Analysis (MCA) plot showing the association between assigned species, harbored virulence genes and attachment to polystyrene surface. MCA plot (A) displays the association between virulence gene profiles and *Aeromonas* species, number of each species ≥6, (n=199), (B) displays the association between virulence genes and attachment in various seasons and water types (n=331) and (C) shows the association among assigned species, harbored virulence genes and attachment to polystyrene in various seasons and water types (n=213). The adjusted inertia explains the variability in each dimension on the MCA plots, calculated according to Greenacre (1984).

Biofilm production for each isolate was interpreted according to the scheme in Table

S3. A large number of isolates in our study were not able to or only weakly able to

attach to an abiotic (polystyrene) surface. Out of 331 isolates tested, 211 (63.7%)

formed weak biofilms on polystyrene while 70 (21.1%) isolates were strong biofilm

producers. An additional 50 (15.1%) were classified as moderately capable of

attaching to the polystyrene surface.

Isolates collected from different water types exhibited variability in their attachment

capability (Figure 5.7A). Non-tidal freshwater isolates and reclaimed water isolates

had the highest proportion of strong biofilm formers, at 41/172 (23.8%) and 16/63

(25.4%), respectively. The other non-tidal freshwater isolates were characterized as

weak (105/172; 61.0%) and moderate (26/172; 15.1%) biofilm formers. Similarly, a

larger proportion of reclaimed water isolates were determined to be weakly (39/63;

63.1%) compared to moderately (8/63; 12.3%) able to attach to polystyrene. None of

the 30 tidal brackish water isolates attached strongly to the polystyrene and these

isolates were grouped as weak (22 isolates; 73.3%) and moderate (5 isolates; 16.7%)

biofilm formers. From the 69 pond water isolates, 46 (64.8%) were categorized as

weak, 11 (15.5%) as moderate and 13 (19.7%) as strong biofilm formers. The

apparent effect of water type on bacterial attachment to a polystyrene surface was not

statistically significant (Pearson's χ^2 (df=6, N=331) = 9.08, p=0.16) (Figure 5.7A).

Our data indicated that season of isolate collection exerted a stronger influence on

attachment capability than water type. A significant seasonal variation was detected

(Pearson's χ^2 (df=6, N=331) = 15.71, p<0.05); isolates collected in spring exhibited a stronger attachment capability to an abiotic surface than in other seasons (Figure 5.7B). From the 79 isolates recovered in the spring (median water temperature 17.7°C), 26 (32.9%) attached strongly to the abiotic surface, while 43 (54.4%) were categorized as weakly and 10 (12.6%) moderately attached. Out of 121 isolates collected in the fall (median water temperature 15.2°C), 81 (67.2%) were categorized as weak, 19 (15.7%) as moderate and 21 (17.3%) as strong biofilm formers. From 80 isolates retrieved in the summer (median water temperature 26.1°C), 46 (57.5%) attached weakly, 16 (20.0%) moderately and 18 (22.5%) strongly to the polystyrene surface. From the 51 isolates collected in the winter (median water temperature 8.0°C), 41 (80.4%) were grouped as weak, 5 as moderate (9.8%) and 5 (9.8%) as strong biofilm formers (Figure 5.7B).
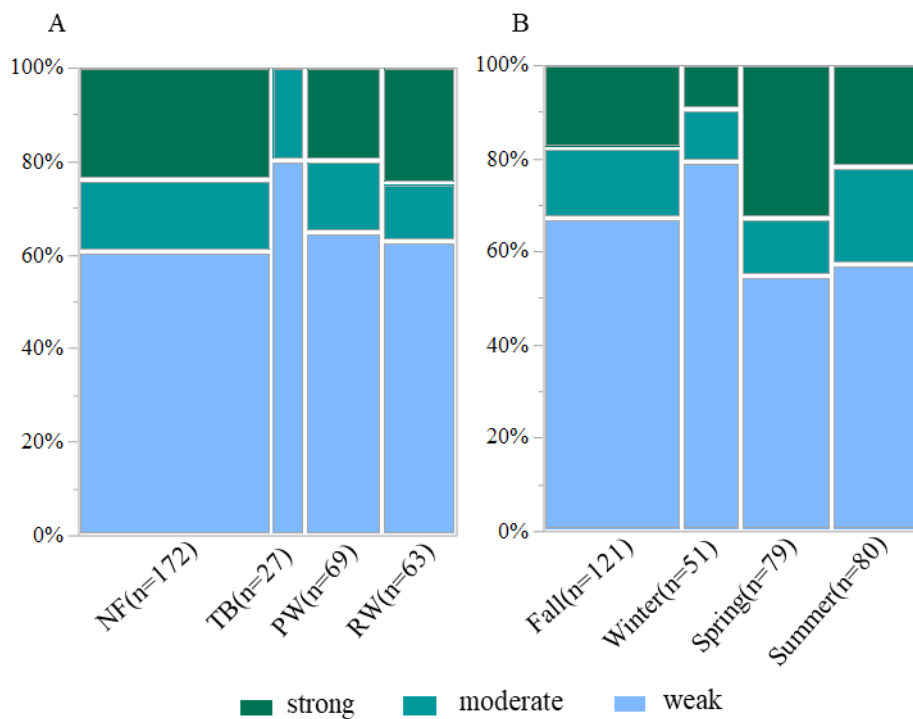
Figure 5.7: Mosaic plots showing attachment capability of *Aeromonas* spp. (N=331) onto polystyrene surface (biofilm formation) in (A) different water types and (B) different seasons. The y axis shows the proportion of isolates forming a biofilm. The vertical length of the x axis of each rectangle is proportional to the percentage of isolates for each water types (non-tidal fresh river, NF=52%, tidal brackish river, TB=8%, pond water, PW=21% and reclaimed water, RW=19%) and seasons (fall=36%, winter=15%, spring=23%, summer=24%). n denotes the number of isolates.

5.3.6. Interaction between virulence genes, season, water type and attachment capability

Out of 70 isolates from different water types collected in different seasons that were capable of strong attachment to an abiotic surface, 44 were assigned a phylogenetic classification and comprised of *A. veronii* (n=11), *A. jandaei* (n=7), *A. hydrophila* (n=8), *A. bestiarum* (n=3), *A. piscicola* (n=3), *A. encheleia* (n=2), *A. salmonicida* (n=2) and 1 isolate each of *A. caviae*, *A. enterica*, *A. eucrenophila*, *A. media*, *A. popoffii*, *A. punctata* and *A. rivipollensis*. The detection of the genes *ast* (Pearson's χ^2 (df=3, N=331) = 8.21, p<0.05) and *flaA* (Pearson's χ^2 (df=3, N=331) = 11.72, p<0.01) differed by water type, with *ast* less likely to be detected in tidal brackish river and pond water and *flaA* more likely to be detected in non-tidal fresh river water. The detection of the genes *ast* (Pearson's χ^2 (df=3, N=331) = 105.97, p<0.001) and *alt* (Pearson's χ^2 (df=3, N=331) = 25.09, p<0.001) differed by season. The gene *ast* was more likely to be detected in the summer and less likely to be detected in the winter and spring, while *alt* was more likely to be detected in the summer and less likely in the winter. All 5 strongly attaching isolates (*A. hydrophila* (n=2), *A. encheleia* (n=2) and *A. popoffii* (n=1)) that carried all enterotoxin genes (*ast, alt* and *act*) were collected from non-tidal fresh river water, in summer (n=4) and fall (n=1).

The MCA plot exploring the associations between virulence genes, attachment, season and water type for all isolates (N=331), revealed a strong association between summer and the gene *ast* (Figure 5.6B). For the MCA plot in Figure 5.6B, presence or absence of *ast* and the summer season were the strongest contributors to inertia for dimension 1, while the presence of the gene *flaA* and strong attachment contributed

most inertia to dimension 2. The presence of *flaA* was strongly associated with moderate and strong attachment while the presence of genes *aerA* and act were associated with weak attachment capability (Figure 6B and 6C). Out of 44 strongly and 40 moderately attaching isolates only 2 (5%) and 6 (15%) were *flaA* negative. On the other hand, the presence or absence of *flaA* was equivalent among weakly attaching isolates.

Strong attachment associated most closely with *A. encheleia* (5/7 were *flaA+)* (Figure 5.6C). Strongly attaching isolates classified as *A. veronii* and carrying enterotoxin genes were collected from RW (n=4) and NF (n=3), in fall (n=4) and summer (n=3); associations evident in the MCA plot of 213 classified isolates (Figure 5.6C). Brackish water isolates associated with spring and winter collection, and the absence of the *ast* and *alt* genes, with *A. popoffi, A. bestiarum* and *A. salmonicida* relating to these factors (Figure 5.6C). *A. jandaei, A. rivipollensis* and *A. piscicola* associated with pond water, with the former 2 also relating to strong/moderate attachment and *flaA* (Figure 5.6C). None of the isolates of the probable enteric pathogens *A. caviae, A. dhakensis, A. enterica* and *A. popoffii* were categorized as strong biofilm producers and enterotoxin gene carriers simultaneously. Altogether, 13 isolates were detected as probable enteric pathogens that harbored more than one enterotoxin gene and had the capability of strongly attaching to an abiotic surface. Seven of these isolates were selected to assess the attachment to lettuce leaves based on water type, virulence gene pattern and attachment to polystyrene surface (Table 5.4).

Table 5.4: List of the isolates exhibiting strong attachment to an abiotic surface. NF=non-tidal fresh river, PW=pond water; RW=reclaimed water. Isolates 1-7 were selected for evaluating attachment to and persistence on biotic surface (lettuce leaves).

Isolate Nº.	Classification	Season of Collection	Water Type	Virulence genes	Attachment to polystyrene	Attachment to lettuce
1	*A. hydrophila*	Summer	NF	*ast, alt, flaA*	Strong	Yes
2	*A. jandaei*	Summer	PW	*ast, alt, flaA*	Strong	Yes
3	*A. hydrophila*	Fall	NF	*ast, alt, flaA*	Strong	Yes
4	*A. hydrophila*	Summer	NF	*ast, alt, act, flaA*	Strong	Yes
5	*A. veronii*	Summer	RW	*alt, act, aerA, flaA*	Strong	Yes
6	*A. veronii*	Fall	RW	*alt, act, flaA*	Strong	Yes
7	*A. veronii*	Summer	RW	*ast, alt, aerA, flaA*	Strong	Yes
8	*A. hydrophila*	Summer	NF	*ast, alt, act, aerA*	Strong	No
9	*A. veronii*	Fall	NF	*ast, alt, flaA*	Strong	No
10	*A. veronii*	Fall	NF	*ast, alt, act, flaA*	Strong	No
11	*A. veronii*	Fall	RW	*ast, act, flaA*	Strong	No
12	*A. veronii*	Summer	NF	*ast, alt, flaA, aerA*	Strong	No
13	*A. veronii*	Summer	NF	*ast, alt, flaA*	Strong	No
Reference	*A. hydrophila*		ATCC 7966	*ast, alt, act, flaA*	Moderate	Yes
Reference	*A. veronii*		ATCC 35624	*ast, alt, act, aerA, flaA*	Strong	Yes

5.3.7 Attachment and persistence of probable pathogenic species to

biotic surface

To evaluate the possibility of transfer of presumptive pathogenic species of

Aeromonas from irrigation water to vegetable crops, we investigated the attachment

and persistence capability of select *Aeromonas* isolates to the lettuce leaf surface.

Seven representative isolates classified as *A. hydrophila, A. veronii* and *A, jandaei*,

determined to carry multiple virulence genes and having the ability to attach strongly

to a polystyrene surface, were selected (isolates 1-7 in Table 5.4).

Loosely and strongly attached cells were recovered from lettuce leaves for all test

isolates and the two reference strains, *A. veronii* ATCC 35624 and *A. hydrophila*

ATCC 7966. Out of 7 isolates, one (isolate 5, classified as *A. veronii* retrieved from

RW in summer) showed no significant reduction of microbial load between time of

inoculation and 120 h ($p>0.05$), while two other *A. veronii* isolates (isolate 6 was

collected from RW in fall and isolate 7 was collected from RW in summer) exhibited

an initial reduction in loosely adhering cells ($p<0.01$ and $p<0.001$, respectively) after

24 h, followed by an increase after 5 days ($p<0.01$ only for isolate 7). The rest of the

isolates (isolates 1, 3 and 4, classified as *A. hydrophila* and isolate 2, classified as *A.*

jandaei) showed a significant reduction in loosely attached bacterial cell counts over

time (from 0 h to 5 days) ($p<0.001$), similar to the reference strain *A. hydrophila*

ATCC 7966 (Figure 5.8).

Isolates 1 to 4 also exhibited a steady decline in bacterial cells strongly adhering to

lettuce leaves, with a significant reduction in bacterial count ($p<0.05$ for all) over

time (from 0 h to 5 days). No significant change in strongly attached cells was

detected for isolates 5 and 7, while isolate 6 exhibited an increase over time ($p<0.05$) (Figure 5.8).

We calculated the ratio of strongly to loosely attached cells (S:L) to assess attachment strength for each isolate (Table 5.5). Isolates 1-4 had a high level and isolates 5-7 a moderate level of strongly attached cells immediately after inoculation. This ratio remained proportional in isolates 1 and 2 after 24 h but decreased slightly in isolates 3 and 4, indicating a higher proportion of loosely to strongly attached cells. In all these isolates, strongly attached cells were an insignificant component of the population after 5 days. This was similar to the reference strain *A. hydrophila* ATCC 7966 (S:L from 0.66 to 0 by 120 h). Isolates 5, 6 and 7 exhibited an increase in the proportion of strongly attached cells after 24 h, as was detected for both reference strains. In isolates 6 and 7, the S:L ratio was proportional at 24 h. However, in contrast to *A. hydrophila* ATCC 7966, attachment strength in the *A. veronii* ATCC 35624 reference strain continued to augment over 120 h. This increase only occurred in one test isolate, isolate 5, which attained a proportional ratio of S:L cells by 120 h. Attachment strength in isolate 6 remained high to also retain a proportional ratio. On the other hand, isolate 7 exhibited a decline in the S:L ratio but unlike isolates 1-4, maintained a strongly attached population of cells.

Taken together, this indicated that for *A. veronii* isolates 5, 6 and 7 and the reference *A. veronii* ATCC 35624 strain, strongly attached cells became a dominant component of the population of leaf-associated cells (Figure 5.8). The assayed isolates classified as *A. hydrophila* and *A. jandaei* behaved similarly to the reference strain *A.*

hydrophila ATCC 7966, all exhibiting declines in loosely and strongly attached cells by the end of the 5-day incubation.
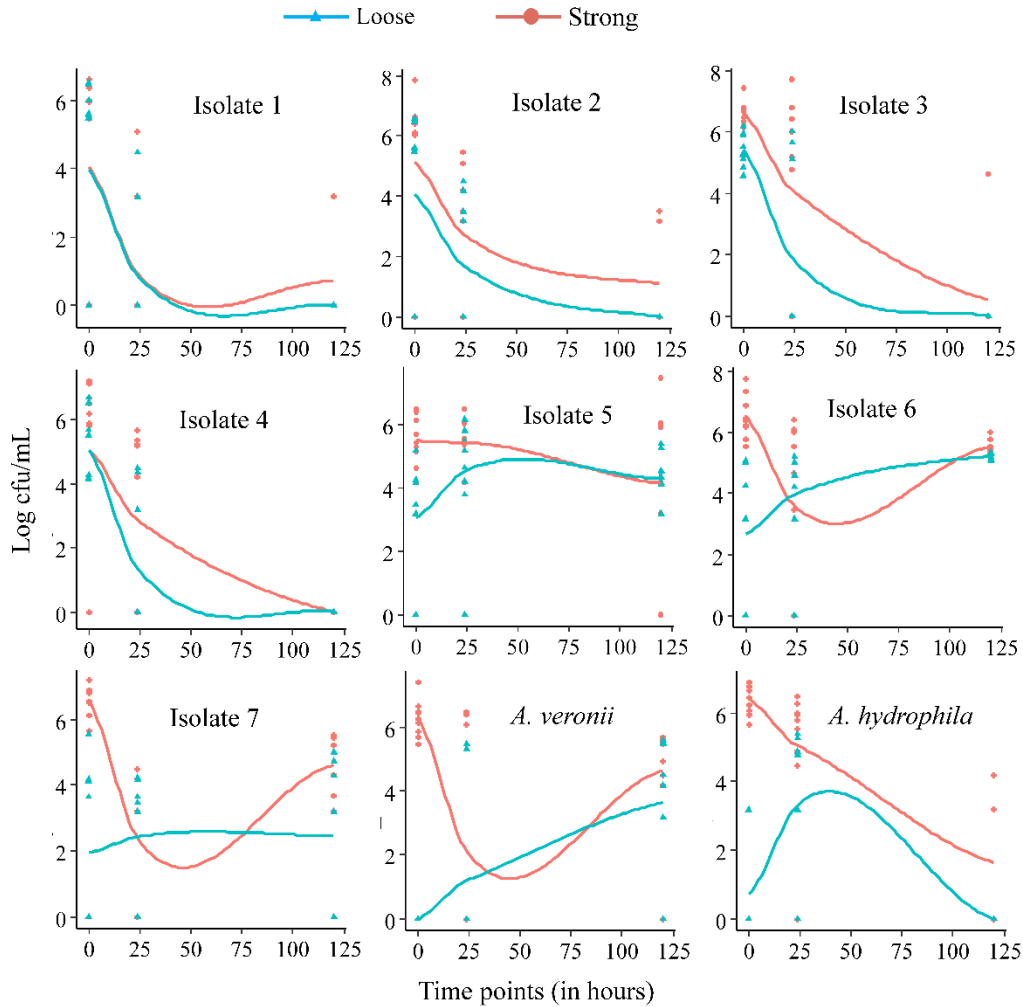
Figure 5.8: Change in number of strongly to loosely (S:L) attached *Aeromonas* spp. on a biotic surface (adaxial side of Romaine lettuce leaves cv. 'Sparx'). Seven isolates used were classified as *A. hydrophila* (isolates 1, 3 and 4), *A. jandaei* (isolate 2) and *A. veronii* (isolates 5-7), recovered from different water types and harboring different combinations of virulence genes as described in Table 3. *A. hydrophila* (Chester) Stanier ATCC 7966 and *A. veronii* Hickman-Brenner et al. ATCC 35624 were used as reference strains. Time points for cell

counting were 1 hour (0) post-inoculation, 24 hours (24) post-inoculation and 120 hours

(120) post-inoculation of leaf surfaces.

Table 5.7: Attachment strength of *Aeromonas* isolates in different time points

Isolate Nº·	Strong:Loose post-inoculation	Strong:Loose 24 h	Strong:Loose 120 h
Isolate 1	0.98	0.93	0.00
Isolate 2	0.79	0.62	0.00
Isolate 3	0.82	0.46	0.00
Isolate 4	1.00	0.47	0.00
Isolate 5	0.56	0.83	1.04
Isolate 6	0.41	1.11	0.94
Isolate 7	0.30	1.02	0.53
A. veronii ATCC 35624	0.00	0.57	0.78
A. hydrophila ATCC 7966	0.11	0.66	0.00

5.4. Discussion and Conclusion

Foodborne disease outbreaks of fruit and vegetable crops are frequently associated with irrigation water (Gelting and Baloch, 2013; Gelting et al., 2015; Steele et al., 2005). Understanding the ecology and pathogenicity of under-studied pathogens prevalent in agricultural water will help to ensure that strategies developed to mitigate foodborne illness are universally effective. Using phylogenetic analysis, spatial and temporal distribution analysis, virulence gene profiling and attachment assays, this study characterized *Aeromonas* isolates recovered from representative irrigation water sources and evaluated potential for establishment onto a leafy green crop. Water type and season of collection affected the spatial and temporal distribution of *Aeromonas* species detected in water environments. The highest diversity was observed in fresh and tidal rivers and the lowest in ponds. Seasonally, the highest diversity was obtained in winter and the lowest in summer. *Aeromonas* species known to cause human disease were isolated from all water types with the highest incidence in non-tidal fresh river water and reclaimed water. Bacterial isolates collected from non-tidal fresh river, reclaimed and on-farm pond water were identified predominantly as *A. hydrophila, A. veronii* and *A. jandaei* and almost ~75% of these isolates harbored multiple virulence gene in their genome. We also detected seasonal and water type effects on bacterial attachment to an abiotic surface. Importantly, *A. veronii* was able to attach and persist on Romaine lettuce leaves for up to 5 days. This study provides an ecological assessment of the under-studied bacterial taxon *Aeromonas* in surface and reclaimed waters and characterizes traits that may be relevant to food safety of fruit and vegetable crops that can contact this potential pathogen via irrigation water.

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Due to the complexity of this taxon, phenotypic identification methods are not sufficient to classify *Aeromonas* species. Multilocus phylogenetic analysis (MPLA) incorporating seven housekeeping gene fragments (*gyrB, rpoD, recA, dnaJ, gyrA, dnaX* and *atpD*) determined that *rpoD* exhibited the highest substitution rate between inter-species variation (Martinez-Murcia et al., 2011). This prompted us to use the *rpoD* gene to identify 228 isolates collected from four different water types. Approximately 65% of the isolates were classified as *A. hydrophila, A. veronii, A. jandaei, A. caviae* and *A. dhakensis*. Studies have shown that four of these, *A. hydrophila, A. veronii, A. caviae* and *A. dhakensis*, were more frequently associated with various human infections, while *A. jandaei* less so (Fernández-Bravo and Figueras, 2020; Figueras and Beaz-Hidalgo, 2015; Parker and Shaw, 2011).

In this study, *A. hydrophila, A. veronii, A. jandaei* and *A. dhakensis* were predominantly isolated at proportions of 25.9%, 21.4%, 15.6% and 2%, respectively, in freshwater samples (freshwater river and pond water). This differed from a study in China that reported 77% (n=40) of isolates as *A. veronii*, but only 5% as *A. hydrophila* and 5% as *A. jandaei* in pond water (Hu et al., 2012). In Malaysia 43% (n=122) of isolates recovered from a freshwater lake were identified as *A. veronii*, 37% as *A. jandaei*, but only 6% as *A. hydrophila* and 1% as *A. dhakensis* (Khor et al., 2015). In addition to surface water, we also recovered commonly known pathogenic species of *Aeromonas* (*A. hydrophila, A. caviae, A. veronii and A. jandaei*) from reclaimed water (from storage lagoons of tertiary treated wastewater treatment plants). This differed from the zero prevalence of *Aeromonas* from a study in Spain conducted with limited sample size (N=3) (Latif-Eugenín et al., 2017). Jjemba et al.

(2010) reported a higher recovery rate of *Aeromonas* spp. compared to indicator microorganisms including heterotrophic bacteria, coliforms, *E. coli* and enterococci, in storage reservoirs and distribution systems, but no detection in wastewater treatment plant effluents in Florida, California, Massachusetts and New York. This is in contrast to the detection of *Aeromonas* in chlorinated effluent as a result of its ability to multiply in low concentrations of chlorine residue (Al-Jassim et al., 2015). These reports attest to the adaptable capacity of *Aeromonas*; minimizing the risk of transfer of pathogenic *Aeromonas* species from reclaimed water to food crops requires strict compliance to recommendations for the adoption of reclaimed water before utilizing this water type for fruit and vegetable crop irrigation (https://www.epa.gov/waterreuse/water-reuse-action-plan).

Seasonal dynamics in species diversity were detected; although higher diversity was detected in the winter, the community had higher evenness and several species not associated with human disease were identified. By contrast, *A. hydrophila* and *A. veronii* predominated in summer and fall communities. This is concordant with other studies and might explain the frequent gastroenteritis cases/outbreaks in summer (Figueras and Beaz-Hidalgo, 2014; Figueras and Beaz-Hidalgo, 2015; Humphries and Linscott, 2015; USEPA, 2006). Interestingly, in this study, the summer and fall seasons were associated with the presence of two enterotoxin genes *ast* and *act* while the winter and spring were associated with the absence of these genes.

The ability of aeromonads to cause gastroenteritis in humans is multifactorial and depends on a wide range of virulence determinants (Fernández-Bravo and Figueras, 2020). Albert et al. (2000) reported that the presence of multiple enterotoxin gene

patterns (*alt+ast* or *alt+ast+act*) might be responsible for both pediatric and adult

diarrhea. In our study almost half of *A. hydrophila, A. veronii, A. jandaei, A.*

dhakensis and *A. caviae* carried different combinations of enterotoxin genes along

with attachment (*flaA*) and aerolysin (*aerA*) genes. This is similar to 40% reported in

A. hydrophila from environmental samples collected from surface water, sediment,

phytoplankton and zooplankton in Bangladesh (Albert et al., 2000) and 52% from

drinking water samples in the USA (Sen and Rodgers, 2004). Considering the high

prevalence of virulence genes in environmental isolates, it is plausible that clinical

and environmental strains are cycling through the host and the environment.

Biofilm formation in pathogenic microorganisms facilitates the disease transmission

from contaminated irrigation water via food crops (Choi et al., 2013). In the present

study, approximately 16% of *A. hydrophila*, 20% of *A. veronii*, 22% of *A. jandaei* and

50% of *A. caviae* from three water types (NF, PW, RW) showed strong biofilm

formation on a polystyrene surface. A previous analysis of 58 environmental isolates

from Turkey reported that 43% of *A. hydrophila*, 87% of *A. veronii* but no *A. caviae*

(Ormanci and Yucel, 2017) were capable of strong biofilm formation. Different

weather and geographical conditions might be responsible for dissimilar findings in

these two studies. However, in both cases, *A. veronii* showed a higher potential to

form biofilms on abiotic surfaces than *A. hydrophila*. In our study, non-human

pathogens were more likely to show strong attachment capabilities; *A. salmonicida*

(33%), *A. encheleia* (29%) and *A. piscicola* (21%) compared to *A. veronii* (19%) and

A. hydrophila (16%). Moderate and strong attachment associated with the presence of

the *flaA* gene which was also detected in high proportions in the former group of

species. Assessing the attachment and biofilm formation capability of *Aeromonas*

isolates gives us some insight on the potential for persistence in food processing

environments and possible risk of contamination of fresh crops.

In addition to evaluating adherence capability to an abiotic surface, representative

isolates were selected to assess their attachment to Romaine lettuce, with the aim of

evaluating the potential for *Aeromonas* to transfer from irrigation water to leafy

greens. These data are relevant as attachment to abiotic surfaces may not always be

directly equivalent to interactions with vegetable surfaces as shown for *A. hydrophila*

on cabbage and lettuce (Elhariry, 2011). There is evidence that *Aeromonas* can

transfer from irrigation water to fresh fruit and vegetables and act as a vehicle of

transmission of infections (Latif-Eugenín et al., 2016). Indirect evidence also comes

from sequence identity between two strains collected from irrigation water and fresh

produce from a farm in Catalonia North-East of Spain (Latif-Eugenín et al., 2017).

We turned our attention to the 5 *A. hydrophila*,7 *A. veronii*, and 1 *A. jandaei* isolates

from our study that exhibited strong attachment to the abiotic surface and

simultaneously carried multiple enterotoxin genes. Seven of these were assayed on

lettuce leaves. Both loosely and strongly associated cells were retrieved from lettuce

leaf surfaces, indicating the ability for *Aeromonas* to quickly adapt to this niche.

Although three *A. hydrophila* and one *A. jandaei* isolates displayed a gradual decrease

of both loosely and strongly associated cells after 5 days of leaf inoculation, the three

A. veronii isolates exhibited a strong attachment capability and persisted in the lettuce

phyllosphere for the duration of the 5-day incubation. These behaviors followed the

same patterns as the reference strains. All the *A. veronii* test isolates were recovered

from reclaimed water in summer and fall. This finding suggests that some *Aeromonas* species or strains can become established on lettuce. A high prevalence of *Aeromonas* spp. has been reported on packaged lettuce (55% positive, n=120), although most of those isolates were identified as presumptive *A. hydrophila* (Szabo et al., 2001). Our findings suggest *Aeromonas* persistence could span the period between an irrigation event and sale to a consumer and we identify *A. veronii* as a species particularly well adapted to persist on lettuce. *A. veronii* biotype *sobria* was a predominant cause of traveller's diarrhoea caused by *Aeromonas* spp. (Vila et al., 2003). For irrigation water that is compliant to microbial standards based on *Escherichia coli* levels, there is no recommendation regarding the time interval between the last application of spray or overhead irrigation and harvest. Moreover, some *Aeromonas* species can pose a significant public health risk due to their ability to multiply under refrigerated conditions and tolerate sanitizers. Foodborne illness outbreaks linked to fresh produce contaminated with *A. hydrophila* have been previously reported (Krovacek et al., 1995; Zhang et al., 2012). Therefore, contamination should be minimized as much as possible at pre-harvest levels, necessitating more research that investigates risks associated with the establishment of *Aeromonas* in fresh vegetable crop systems. Our study provides an integrated assessment of the diversity, distribution and potential for pathogenesis of *Aeromonas* prevalent in irrigation water. We investigated a variety of typical or potential water sources over a period of one year, revealing spatial and temporal patterns in species richness, evenness, virulence gene carriage and attachment behaviors. Taken together, these data can be used to infer risks of transfer and establishment potential of possible pathogenic strains from

irrigation water sources to fresh produce crops that are minimally processed and may be consumed raw. We found that surface river and pond water as well as reclaimed water are major reservoirs of pathogenic species of *Aeromonas*, raising the possibility that these species enter the food production chain and persist for extended periods. This bacterium, that poses a greater health problem in the elderly, children and immunocompromised populations, has so far been under-recognized as a food safety risk, yet its ubiquity and ability to withstand sanitizers, antimicrobials, high salt concentrations, low pH and low temperature conditions, elevate its threat. Data provided by epidemiological studies showed that infectious doses of this pathogen are relatively low for vulnerable populations (Fernández-Bravo and Figueras, 2020; Tomás, 2012). Moreover, *E. coli*, the microorganism used as a microbial standard to assess irrigation water quality, failed to indicate the presence of this bacterium (Solaiman et al., 2020), supporting the need to include this taxon in future enteropathogen-plant interaction studies.

Chapter 6: Investigating epiphytic lettuce crop microbiota in relation to irrigation with reclaimed and pond water

6.1 Introduction

Leaf surface supports the growth of different types of microorganisms including bacteria, fungi, archaea and others by providing nutrients and colonizable niches (Lindow and Brandl, 2003; Lindow and Leveau, 2002). Microbiota develop synergism, antagonism or commensalism with their host (Atlas and Bartha, 1998). Some members of the community that settle as commensals in the phyllosphere are pathogenic to humans (Atlas and Bartha, 1998). However, the shifts in the microbial community structure on plants is a natural phenomenon that can be influenced by many parameters including surrounding environment e.g. weather and neighboring plants (Rastogi et al., 2012; Vorholt, 2012), rainfall (Allard et al., 2020), irrigation type and plant age (Williams et al., 2013). Rhizosphere and soil microbiome are well studied topics and literature showed how rhizosphere and soil microbiome shift in response to change in meteorological conditions, geography, and seasons and disturbance of the community due to pathogen introduction (Huntington, 2006; Williams et al., 2013). These factors affect more the "transient" colonizer as opposed to "core" residents (Hirano and Upper, 1991; Rastogi et al., 2012). Studies have also focused on the effect of physiological condition of the plant on microbiome distribution as plant specifies the community structure on the leaf surface (Balint-Kurti et al., 2010; Hunter et al., 2010).

One of the major reasons to study the leaf microbiome in a leafy green crop that is eaten raw is to investigate what role leaf-microbiota associations play in initiating foodborne disease outbreaks. The microbial community structure on the lettuce surface may have a significant impact on foodborne illnesses associated with leafy greens. Pathogen introduction from contaminated irrigation water can interfere with the leaf microbiome and can persists for a long time even in a small concentration (Solaiman and Micallef, Submitted) that might be supported by the neighboring bacterial species. These hardy members of the community then might withstand the post-harvest treatment of the harvested leafy green crop. Several studies showed that soil and plant root microbiomes (rhizosphere) can be impacted by the irrigation water type and irrigation type (Gatica and Cytryn, 2013; Zolti et al., 2019). We are assuming that irrigation water might also have similar effect on leaf surface microbiome. Effects of irrigation water source and irrigation method on leafy green phyllosphere microbiomes is not as well studied. The phyllosphere is a good habitat for both plant and human pathogens. Therefore, for a comprehensive understanding of possibility of transfer of human pathogen to the surface of leafy green, we designed a longitudinal study to see the impact of irrigation on leaf surface microbiome and the temporal shift of the community on leafy green. In addition, we focused on abundance of antimicrobial resistance (ARG) and virulence (Vir) genes to determine the possibility of transfer of those gene to human.

6.2. Method

6.2.1 Field site and plot design

The field experiment was conducted at the Terpfarm, the research facility for the

University of Maryland in Upper Marlboro, Maryland from September 16th, 2019 to

October 18th, 2019. Lettuce plots were arranged in a Randomized complete block

design (RCBD) (Figure 1) with four replications, to apply four treatments based on

the type of irrigation water applied overhead. The whole plot was divided into four

different rows with 2′ buffer zone in each side of the field. Each row was divided into

four different sub-plots. Plots were 6′ apart from each other. There are 16 subplots

with 6.5′ in length and 3′ in width.

6.2.2 Lettuce transplant, overhead irrigation and sample collection

Romain Lettuce cultivar 'Sparx' seeds were planted and placed in the mist room in

the greenhouse, for germination. After germination, the seedlings were transferred to

the post germination room that maintained temperature 20°C during day and 18°C at

night. Three weeks old lettuce seedlings were transplanted in the field and planted

12″ apart from each other in 16 plots: 4 treatment with 4 replications. Overhead

irrigation with three different water types: reclaimed water (RW), pond water (PW)

and sterile water (SW) was applied on three groups of plants once in a week

according to Figure 1. The fourth group did not receive any overhead irrigation and

were denoted as "NO" in the figure. Drip irrigation were applied to all plants in the

field using special drip pipes regardless the treatment, with outlet located at plots.

Water spray using a handheld backpack sprayer was applied with 100 mL of

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reclaimed water from a wastewater treatment plant, 100 ml of pond water collected from Upper Marlboro CMREC and 100 mL of sterile water on each plant for first and second weeks. 300 mL and 400mL water was applied in third and fourth weeks respectively.
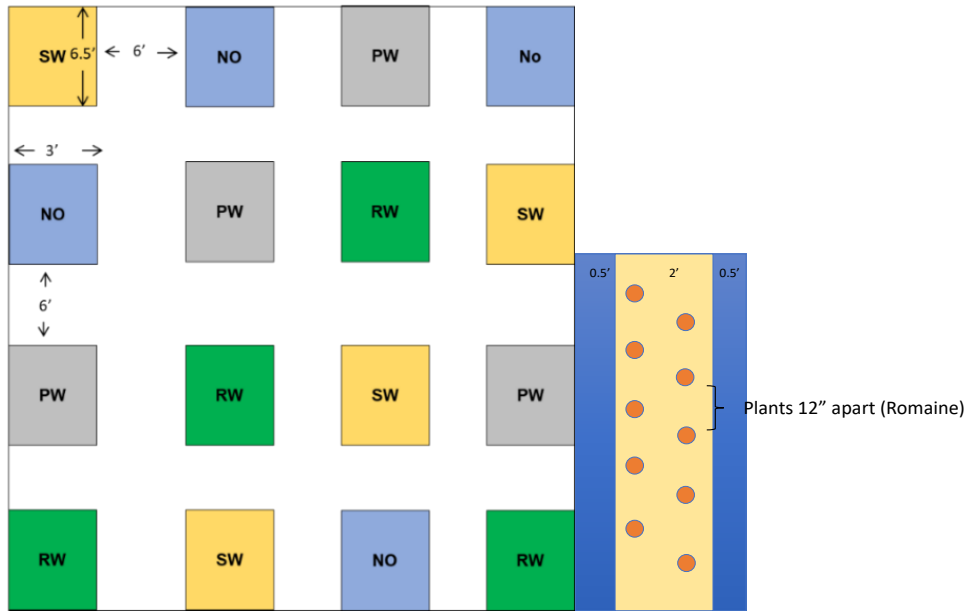
Figure 6.1: Randomized complete block design (RCBD) for lettuce field. Overhead irrigation with reclaimed water (RW) was applied on lettuce plant grown in RW blocks, pond water (PW) was applied on lettuce plant grown in PW blocks, sterile water (SW) was applied on lettuce plant grown in SW block. Blocks denoted as "NO" means lettuce grown in those blocks received no overhead irrigation. The second picture showing the organization of lettuce plant in each subplot.

Overhead irrigation continued for 5 weeks until crops were at a harvestable stage. Lettuce samples were collected weekly for retrieval of surface bacteria prior to overhead irrigation in sterile WhirlPak bags using sterile scissors. Altogether 52

lettuce samples at 4 timepoints before and after field transplantation and 4 irrigation water samples from reclaimed and pond water samples were collected. Samples were transported to the lab in cooler maintaining 4°C. Sampling frequency is shown in Table 6.1.

Table 6.1: Number and type of samples collected

Day	Type of sample	Type of water	Replications/each water type	Number of samples	Name of the replicates
Day 0	leaf	Greenhouse water (GW)	4	4	D0_GW_1 D0_GW_2 D0_GW_3 D0_GW_4
Day 4	Water	PW, RW	1	2	D4_P_1 D4_R_1
Day 11	leaf	PW, RW, SW, NO	4	16	D11_PW_1 D11_PW_2 D11_PW_3 D11_PW_4 D11_RW_1 D11_RW_2 D11_RW_3 D11_RW_4 D11_SW_1 D11_SW_2 D11_SW_3 D11_SW_4 D11_NO_1 D11_NO_2 D11_NO_3 D11_NO_4
Day 18	leaf	PW, RW, SW, NO	4	16	D18_PW_1 D18_PW_2 D18_PW_3 D18_PW_4 D18_RW_1 D18_RW_2 D18_RW_3 D18_RW_4 D18_SW_1

					D18_SW_2
					D18_SW_3
					D18_SW_4
					D18_NO_1
					D18_NO_2
					D18_NO_3
					D18_NO_4
Day 25	Water	PW, RW	1	2	D25_P_1 D25_R_1
Day 32	leaf	PW, RW, SW, NO	4	16	D32_PW_1
					D32_PW_2
					D32_PW_3
					D32_PW_4
					D32_RW_1
					D32_RW_2
					D32_RW_3
					D32_RW_4
					D32_SW_1
					D32_SW_2
					D32_SW_3
					D32_SW_4
					D32_NO_1
					D32_NO_2
					D32_NO_3
					D32_NO_4
Total				56	

6.2.3 Sample processing

Both lettuce and water samples were processed within 6 hours of collection. For each

lettuce sample, 25 g of leaves were placed in a Whirlpak bag (Nasco, Jackson,WI).

100 ml sterile deionized water were added in each bag. Hand massaging was applied

to submerged leaf samples for 30 s followed by shaking for 5 mins in shaker. One-

minute sonication at 200 rpm were applied to collect strongly attached cells. Then the

lettuce washes were filtered through sterile 0.45 μm nitrate-ester filters (Pall,

Rochester, NY), and filters were stored at -80°C for downstream analysis. Similarly, one liter of each water sample was also filtered, and filters were stored at -80°C.

6.2.4 DNA extraction and library preparation

After filtration, DNA was extracted using QIAGEN DNeasy PowerWater Kit (QIAGEN, Valencia, CA, USA) from the filters according to the manufacturer's instructions. Extracted DNA was further purified using Monarch® PCR & DNA Cleanup Kit (NEB). Quality control was done in Qubit HS DNA & RNA quantitation kits. Nextera DNA libraries were prepared for each of the 56 samples separately using Nextera DNA XT Library Preparation Kit and Nextera Index Kit (Illumina, San Diego, CA, USA) followed by pooling to equimolar DNA concentration from each sample according to the manufacturer's instructions. Paired-end sequencing (2×300 bp) was conducted on an Illumina MiSeq sequencing platform with a MiSeq® Reagent Kit V3 high output flow cell.

6.2.5 Metagenomic datasets analysis

Demultiplexed sequence data were obtained from Illumina Basespace (Illumina Way, San Diego, CA, USA). Metagenomic reads were cleaned and curated using Trimmomatic v.0.36 (options: leading 20, trailing 20, sliding 4:20, min len 100) (Allard et al., 2020; Bolger et al., 2014). To investigate the taxonomic profiles of the leafy green surfaces, reads passing quality filters were classified using Kraken 2 v.2.1.1 with the "--paired" option (Wood et al., 2019). The standard Kraken 2 database was prepared using NCBI taxonomic information available on 12/09/2020.

Taxonomic classification of the reads was generated using "--report" option in Kraken 2. Taxonomic abundances were normalized by dividing the number of reads assigned to a specific taxon in a sample by the total number of reads in that sample. To investigate the sequencing depth, rarefaction curves and the alpha diversity indices (e.g., Shannon, Simpson, ACE, Fisher) were generated using the vegan and phyloseq packages in R v.4.0.3 (McMurdie and Holmes, 2013). Non-metric multi-dimensional scaling (NMDS) on Bray-Curtis distances between the normalized abundances of bacterial genera were performed with "vegan" (Oksanen et al., 2019). Analysis of similarities (ANOSIM) between the metagenomes was carried out with the "anosim" function in vegan. Interrogation of quality filtered reads for identity to antibiotic resistance genes (ARGs) and virulence factors (VFs) were performed using BLASTn v.2.7.1 (sequence identity > 95%, matched sequence length > 50 bp). Reads assigned to ARGs and VFs were identified using the Comprehensive Antibiotic Resistance Database (McArthur et al., 2013) and the Virulence Factors of Pathogenic Bacteria Database (VFDB full dataset; http://www. mgc.ac.cn/VFs/), respectively. The best-hits on the BLASTn analysis were determined using percent sequence identity and ARGs/VFs length coverage. Relative abundances of ARGs/VFs were calculated by dividing the number of reads assigned to a specific ARG/VF in a sample by the total number of reads in that sample. Metagenomic reads were further assembled with metaSPAdes v.3.13.0. Identity of the metagenomic contigs harboring ARGs and VFs were determined using the MEGABLAST algorithm and the nucleotide database collection (nr/nt) via the National Center for Biotechnology Information (NCBI) web server(http://www.ncbi.nlm.nih.gov/).

3. Results

3.3.1 Comparisons of phyllosphere microbial communities based on irrigation

   water

To assess the effect of types of irrigation water on lettuce surface microbiome using

the next-generation shotgun sequencing, 56 samples were analyzed (Table 6.1. Four

lettuce samples were taken from the greenhouse seedlings right before transplantation

in the field. From the field, 36 leaf samples irrigated with 3 different water types and

12 with no overhead irrigation were analyzed from three time points (day 11, day 18

and day 32).

After cleaning, curation, and quality filtering, the total number of reads in each

sample ranged from 2×10^5 to 5×10^6. A rarefaction curve using rarefied number of

taxa using ~8900 that tend to form plateau confirmed adequacy of sequence coverage

for the biodiversity in each sample (Figure 6.2).

Higher species richness was observed in all lettuce samples (irrigated with pond water

"PW", sterile water "SW", reclaimed water "RW" and only drip irrigation "NO")

collected in day 32 after transplantation compared with the samples collected before

transplantation, at day11 and at day 18. Although the statistical analysis showed no

significant differences in alpha-diversity index values (ACE, Shannon, Simpson, and
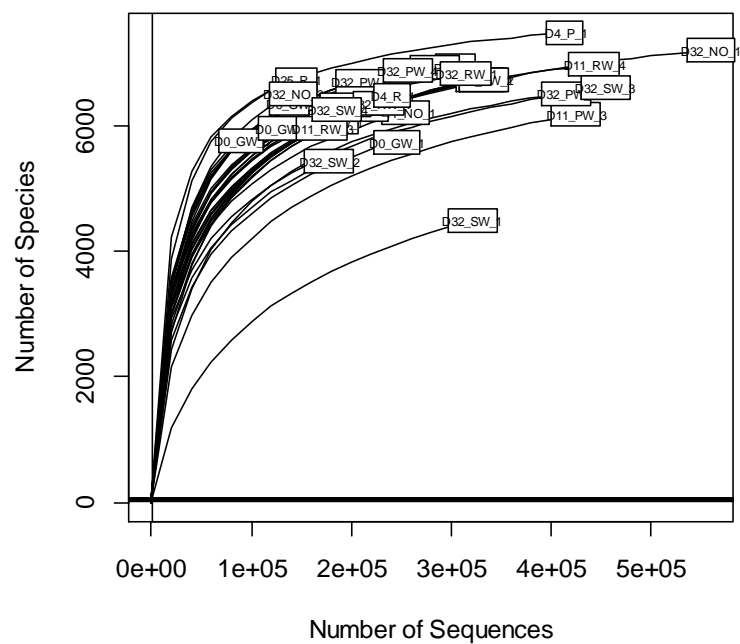
Fisher) (Figure 6.3).

Figure 6.2: The rarefaction curves of all samples including lettuce and water samples indicating rarefied number of taxa at increasing sequencing depth of samples.

Figure 6.3: Different alpha-diversity indices (ACE, Shannon, Simpson and Fisher) measures variation of species richness in samples irrigated with greenhouse water (GW) (after germination and before transplantation), pond water (PW), reclaimed water (RW), sterile water (SW) and samples irrigated with only drip irrigation system (NO). Colored dot indicates samples taken in different time points: day 0 (D0), day 11 (D11), day 18 (D18) and day 32 (D32).

The Non-metric Multi-dimensional Scaling (NMDS) plot and Analysis of similarity (ANOSIM) on the Bray-Curtis distances revealed the differences between the diversity of microbial community structure on initial (day 0) and day 11/day 18/day 32 samples. The community structure were found different on day 0 and day 11 at genus level (ANOSIM R = 0.155, p < 0.05, stress=0.06), on day 0 and day 18 (ANOSIM R = 0.24, p < 0.05, stress=0.06). The differences were also noticed between day 0 and day 32 samples (ANOSIM R = 0.53, p < 0.05, stress=0.52). NMDS plots showed that all day 11 samples were clustered together, and all were different from day 0 samples (one

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water type Vs another water type). Similar trend was noticed in day 0 vs day 18 samples.

Samples from day 32 showed different trend. All samples together were different from day 0 and also different from each other. Both sample types, irrigated with PW and RW showed significant difference from day 0 (GW) samples (for both type samples $p<0.05$) (Fig. 6.4). NMDS plot including all samples taken from all four days showed that day 11 and day 18 samples had similar community structure while day 0 and day 32 both had different community structure. The cluster dendrograms in Fig. 6.5 confirmed the data obtained from NMDS plots (Figure 6.5).
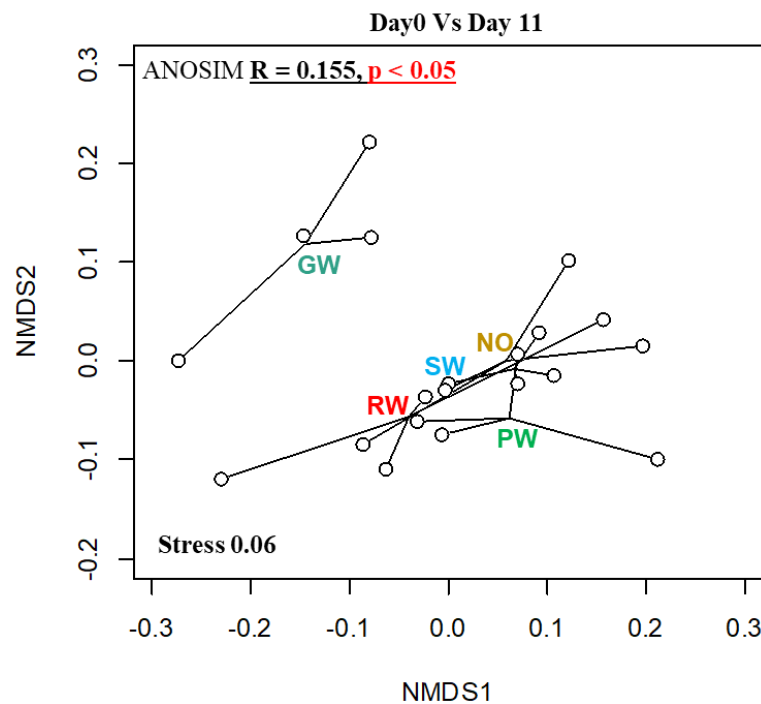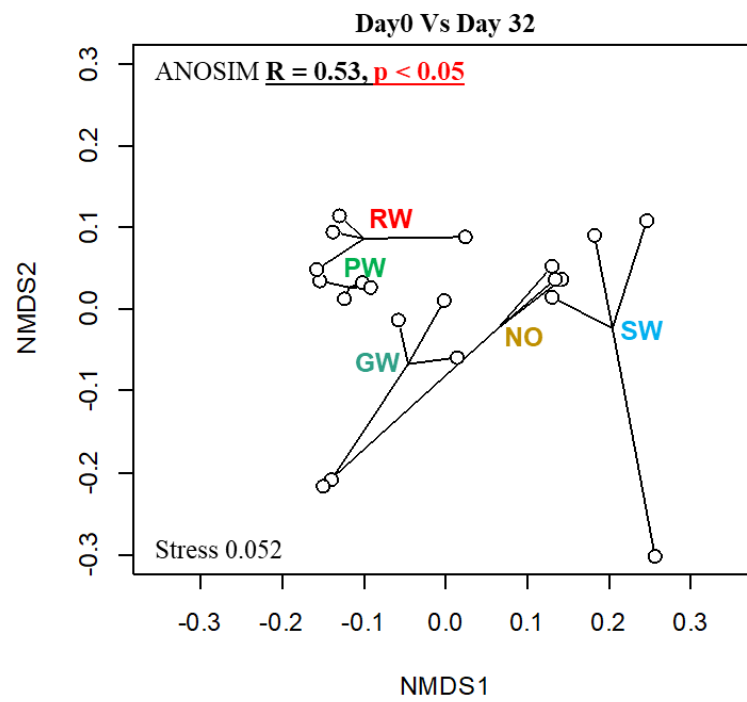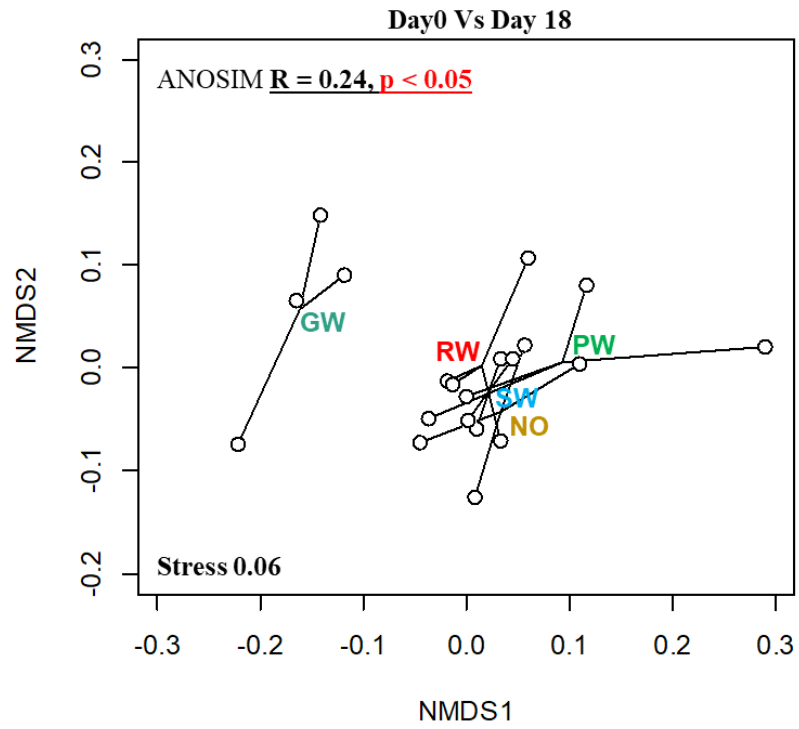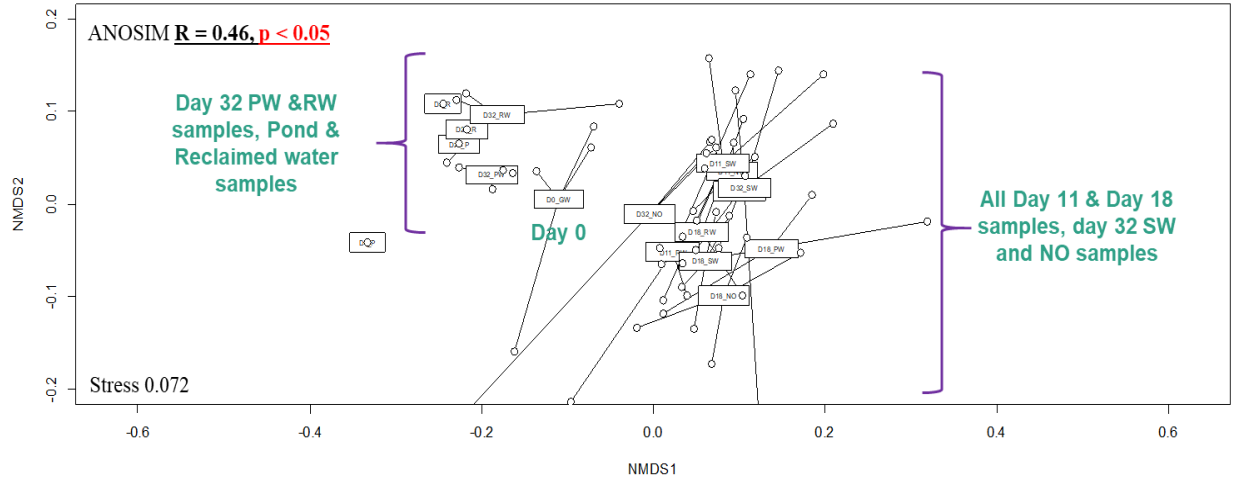
Day0 Vs Day 18

ANOSIM **R = 0.24,** **p < 0.05**

Stress 0.06

Day0 Vs Day 32

ANOSIM **R = 0.53,** **p < 0.05**

Stress 0.052

Figure 6.4: Non-metric multidimensional scaling (NMDS) plots based on Bray-Curtis distance matrix showing similarity and dissimilarity of bacterial community structure at genus level among lettuce samples irrigated with greenhouse water (GW) (on day 0) before transplantation, pond water (PW), reclaimed water (RW), sterile water (SW) and no overhead irrigation (NO). The first figure showing the differences between community structure on day 0 and day 11, second figure showing differences between day 0 and day 18, third figure showing differences between day 0 and day 32. The fourth figure showing differences among day 0, day 11, day 18 and day 32. Stress in all figures (0.06, 0.06 0.052 and 0.072 for first, second, third and fourth figure respectively) indicating the data is a good fit for the multidimensional ordination. Analysis of similarity (ANOSIM) was applied to assess the significance among samples.

Figure 6.5: Cluster dendrogram showing the similarities of community structure between samples collected on day 0, day11, day 18 and day32. First panel does not show any pattern other than clustering all day 0 samples together. Second panel showing similar result, though some replicates from each day and water types tend to cluster together, day 32 lettuce samples irrigated with sterile water (SW) and samples that received only drip irrigation, appeared to be closely related than lettuce samples irrigated with PW.

Bacterial composition at the phylum level was similar in all samples with some outliers. Proteobacteria and Actinobacteria were the two most dominant phyla

identified in all lettuce samples regardless of the type of irrigation water, except one

day11_PW lettuce sample, all day 32_PW lettuce samples and day 25 pond water

(D25_P_1) sample. Approximately 90% of the community was comprised of

Proteobacteria and Actinobacteria. In PW lettuce samples, Cyanobacteria was the

most frequent phylum along with Proteobacteria, ~90% of total community (Figure

6.6).
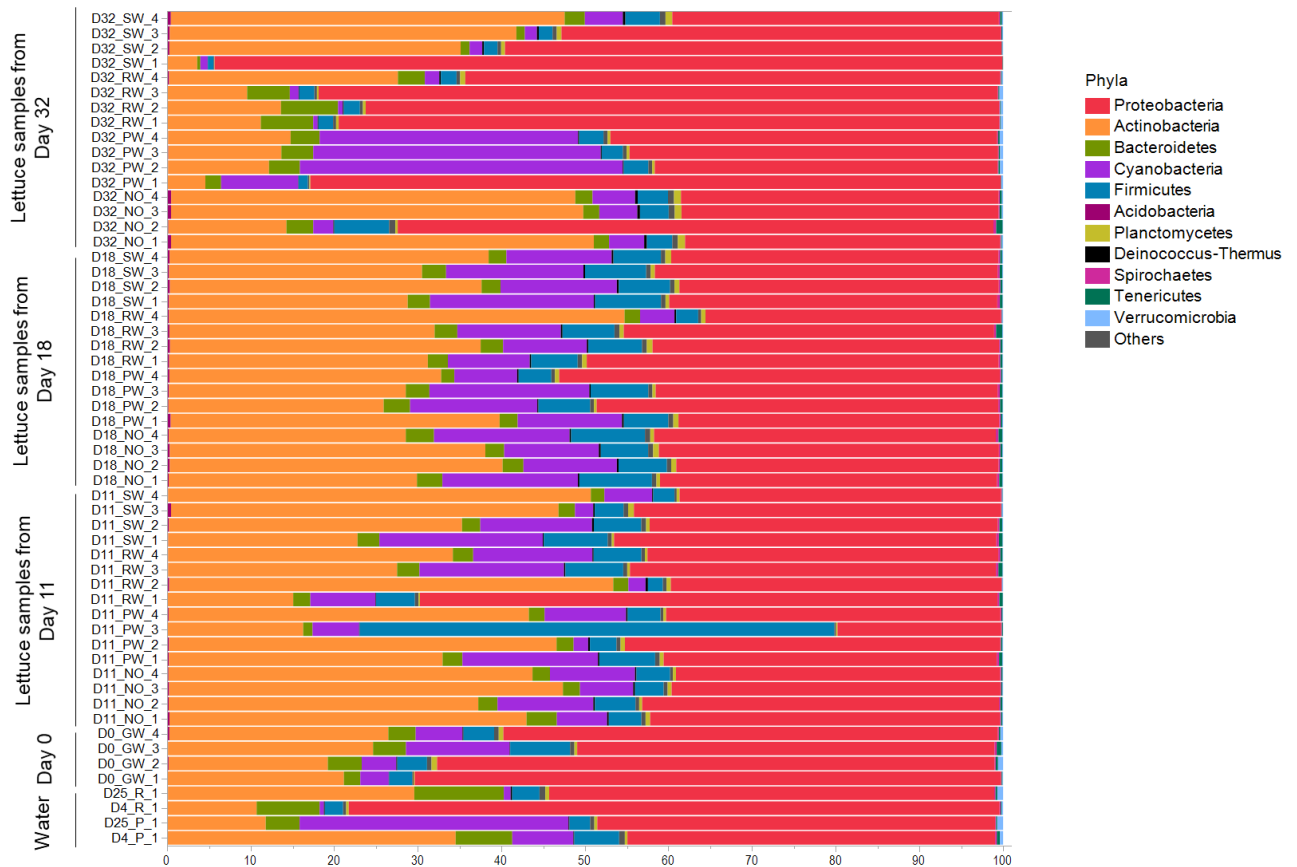
Figure 6.6: 100% stacked bar plot showing relative abundance (%) of bacterial phyla on

lettuce surface collected in 3 different days and irrigated with three different irrigation water

types and water samples collected from pond and wastewater treatment plant.

There were some common genera that were found in all samples. *Pseudomonas,*
Streptomyces and *Pasteurella* were dominant in all samples regardless of the
irrigation water type. *Mycolicibacterium, Microbacterium* and *Sphingomonas* were
found in most samples whereas, *Bradyrhizobium* was found dominant in only GW,
NO and SW samples. *Methylobacterium* and *Curtobacterium* were found only in all
day 11 samples (Figure 6.7). Lettuce samples collected on day 32 and irrigated with
RW (D32_RW) showed different patterns from all other samples.
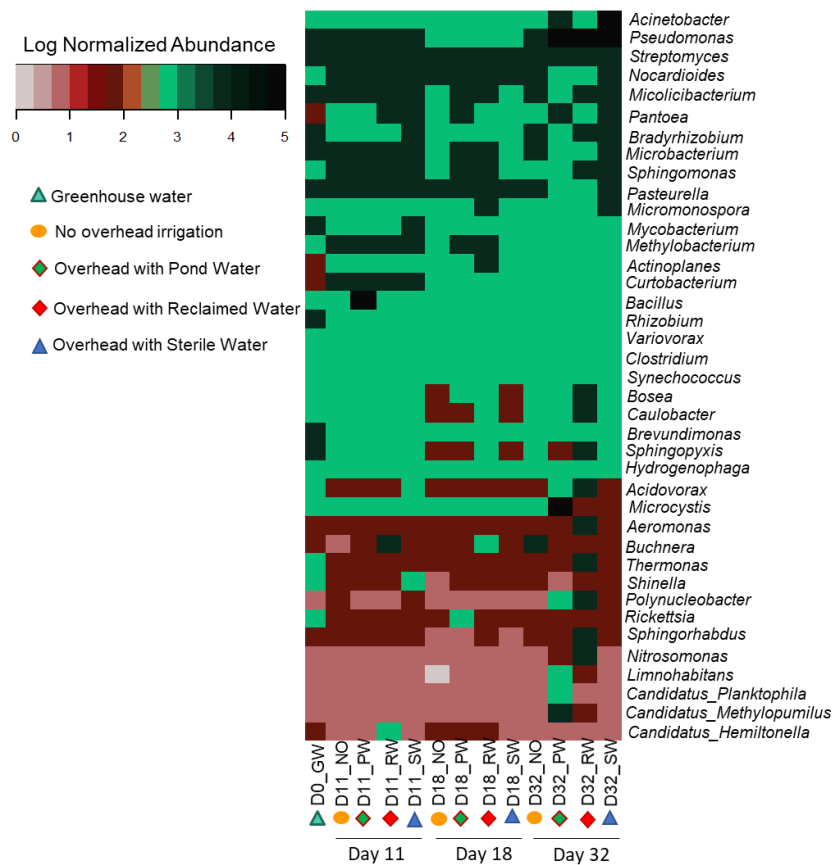
Figure 6.7: Forty most dominant bacterial genera found on lettuce surface. Heatmap was
plotted based on the average normalized abundance of the most dominant genera in all lettuce
samples.

6.3.2 Antimicrobial resistance genes in lettuce samples and water

To investigate the abundance of antimicrobial genes on lettuce and in water samples, metagenomic sequences were aligned with CARD database. Only 9 antimicrobial resistant genes were identified from day 32 lettuce samples irrigated with reclaimed water, and both reclaimed water samples. In total five ARG classes (tetracycline, macrolide, β-lactam, aminoglycoside and multidrug resistant) were detected. Tetracycline, macrolide and multidrug resistant class were most abundant class found in lettuce (day 32) irrigated with RW and water (RW) samples collected in day4. To get the possible assigned phyla, assembled contigs were aligned with NCBI database. Almost all (7/9) genes appeared to be derived from Gammaproteobacteria. The genome sequences from two genes, *mphE* and *aadA6* (resistant against macrolides and aminoglycosides respectively) were mapped with both beta and gamma proteobacteria, therefore, a putative host could not be assigned (Figure 6.8).
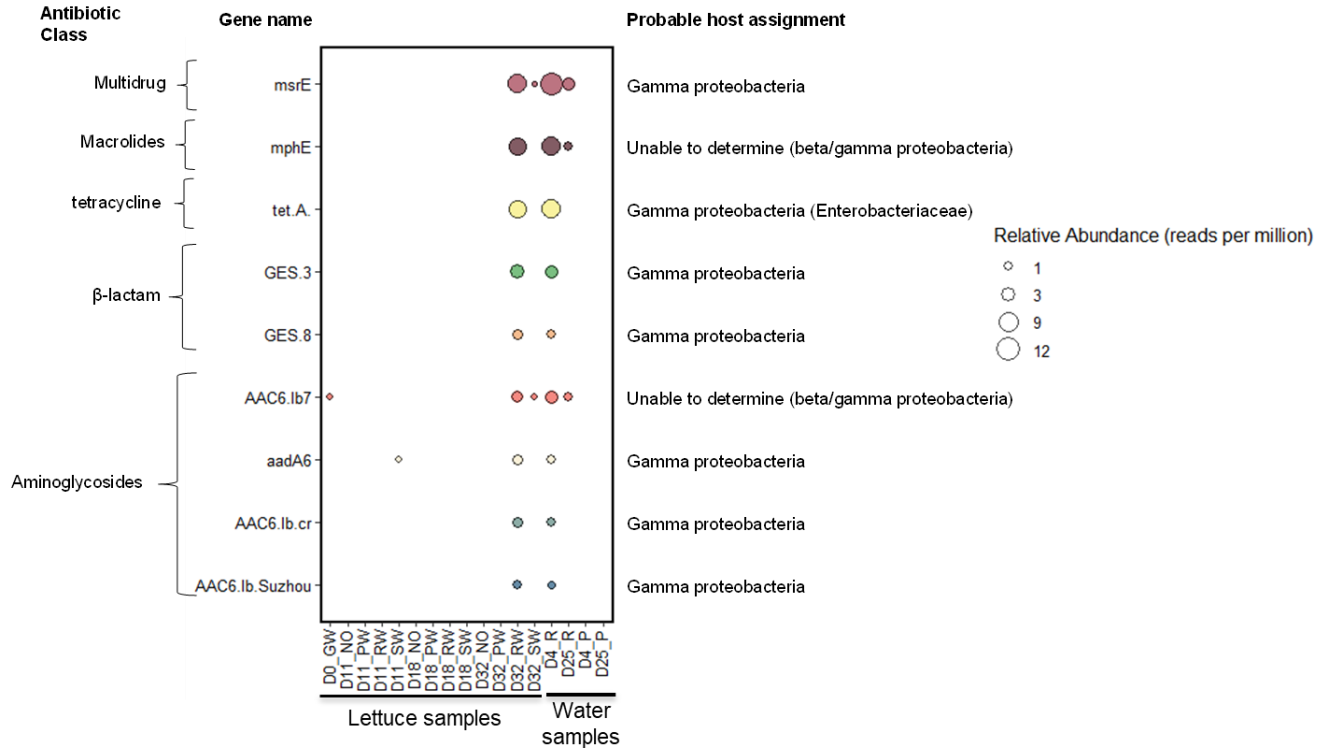
Figure 6.8: Possible antimicrobial resistance gene (ARGs) in lettuce samples irrigated with different water types and in different irrigation water. The size of each bubble is equivalent to the normalized ARGs abundance. X axis showing the sample type.

6.3.3 Virulence factors in lettuce and water samples

To investigate the abundance of possible virulence factors on lettuce and in water samples, metagenomic sequences were aligned with Vir-DB database. In total 35 virulence factors were identified from lettuce samples irrigated with different water types, and in reclaimed water samples. Possible proteins involved in ATP degradation, type VI secretion system, transposon, toxin production, toxin transporter, RNA polymerase, outer membrane protein, hemolysin, different types flagella biosynthesis protein and cell surface antigens were identified. Most of the virulence factors were identified in lettuce samples irrigated with reclaimed water that collected

in day 32 and reclaimed irrigation water samples collected initially (on day 4) except

for mbtF, sca4 and rvhB6b that were only identified in lettuce samples from green

house (before transplantation to the field). sca4 and rvhB6b were also identified in

lettuce samples from day 18, irrigated with PW. (Figure 6.9).
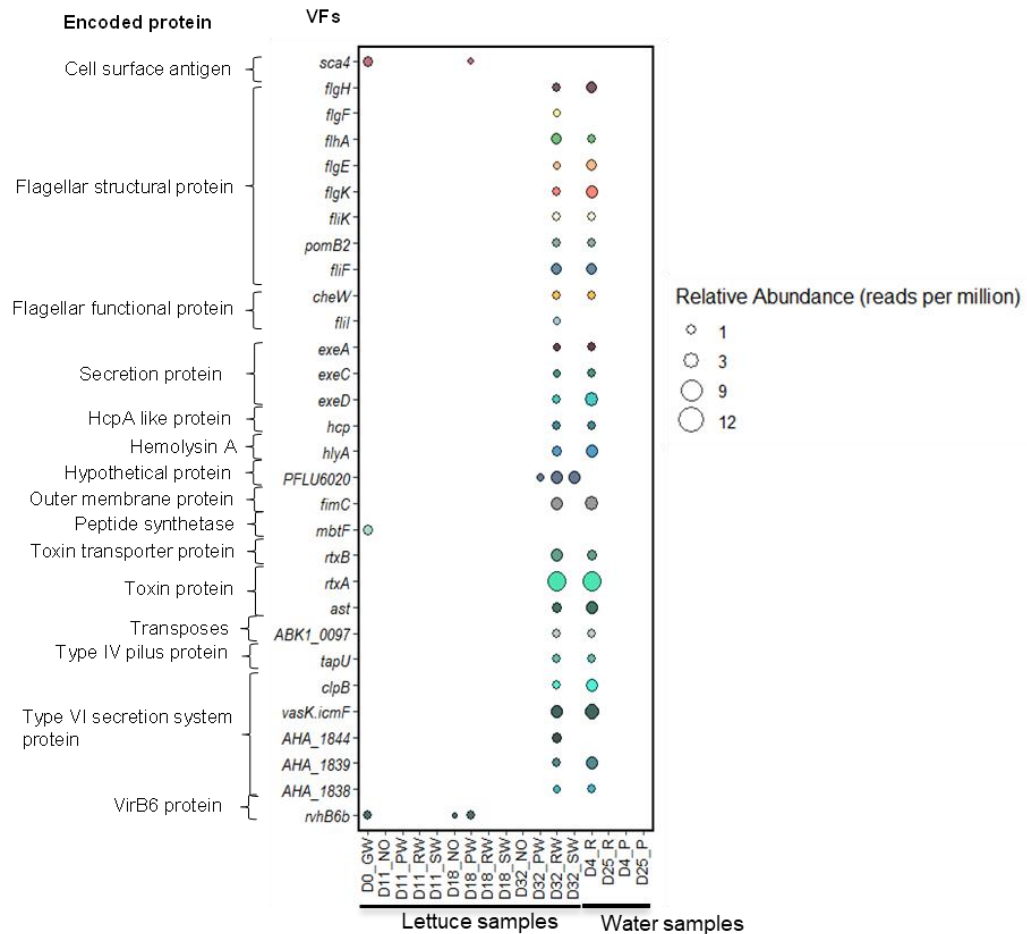
Figure 6.9: Possible Virulence factors (VFs) in lettuce samples irrigated with different water

types and different irrigation water. The size of each bubble is equivalent to the normalized

VFs abundance. X axis showing the sample type. D0, D11, D32, D4 and D25 denotes ARGs

found in lettuce and water samples collected on day 0, 11, 32, 4 and 25 days irrigated with

greenhouse water (GW), no overhead (NO), pond water (PW), reclaimed water (RW) and

sterile water (SW).

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6.4. Discussion and Conclusion

Microbial communities on fresh produce surfaces play an important role not only in facilitating the growth and health of plants in the agricultural environment, but also in maintaining food safety. Despite the importance of studying surface microbiota on fresh produce crops, very few studies have been conducted and mostly in the greenhouse (Erlacher et al., 2015; Williams et al., 2013). The impact that irrigation water has on temporal shift of surface microbiome are far less studied compared with soil or rhizosphere microbiome (Chaparro et al., 2014; Micallef et al., 2009; Pérez-Jaramillo et al., 2016). In this study, we present the effect of irrigation water type and overhead irrigation on the lettuce surface microbiome grown in the field, through a temporal survey spanning the entire crop cycle until time to harvest. We also presented antimicrobial and virulence gene abundance in the irrigation water and the possibility to transfer to the lettuce surface.

Proteobacteria and Actinobacteria were found the two most dominant phyla on lettuce surface collected on day 0, day 11 and day 32 samples, irrigated with sterile water or no water, are partially similar to a field study in the Salinas Valley, CA, USA. The study found Proteobacteria, Firmicutes, and Actinobacteria dominantly colonized on romaine lettuce. However, in this study lettuce samples were inoculated with *Escherichia coli* O157:H7 which might be a reason of Firmicutes inclusion in the community (Williams et al., 2013). Although one other study found higher proportion Firmicutes on lettuce surface (Zwielehner et al., 2008), this phylum is not commonly found in lettuce (Hunter et al., 2010; Rastogi et al., 2012). Actinobacteria was most abundant phylum in samples irrigated with RW while Proteobacteria and

Cyanobacteria were the dominant phyla in samples irrigated with PW. The community composition in pond water samples also showed similar data that indicated the shift of community due to irrigation water types. Although due to having one replicate for irrigation water in our study, this implication might not have strong backup.

Pseudomonas, Streptomyces and *Pasteurella* were three most dominant bacterial genera that were commonly found in all samples. *Pseudomonas* and *Streptomyces* are commonly found in water, soil and plant surface (Kieser et al., 2000; Palleroni, 2008) while *Pasteurella* is commonly found in healthy livestock and domestic animal (Boyce et al., 2010). The most abundant genera found in lettuce samples irrigated with sterile water and no water in both sampling time (day11 and day32), with pond water in day 11 and with reclaimed water in day 32 appeared to be similar; *Mycolicibacterium, Microbacterium, Bradyrhizobium,* and *Sphingomonas*. These four genera were commensal members of the bacterial community in soil, water and plant surface (Bach-Griera et al., 2020; Gneiding et al., 2008; Martinez-Romero and Ormeño-Orrillo, 2019). *Actinobacteria*, another mostly commensal but occasionally human pathogen were abundant in only the samples collected on day 32. These genera might introduce in lettuce samples from soil or from the irrigation water. Another genus *Aeromonas* were found as one of the most abundant genera in lettuce samples irrigated with reclaimed water (RW) that supports our previous study. We found *Aeromonas* in both reclaimed water and pond water (Solaiman and Micallef, Submitted). Therefore, it can be inferred that this genus surely introduced on the lettuce surface community from the irrigation water.

In addition to investigating the community structure, we also focused on the abundance and diversity of antimicrobial resistance genes (ARGs) and virulence factors (VFs). ARGs were detected in lettuce samples collected in day 32 that was irrigated with RW, similar data were also obtained from the both reclaimed water samples (D4_R_1 and D25_R_1) that was used for the overhead irrigation. We can deduce that the resistant genes were transmitted in the lettuce as a result of using reclaimed water. No ARGs were identified in day 11 lettuce samples. However, our study showed that ARGs can transfer to food crops as the irrigation period extends over a crop cycle. A single irrigation event might not affect the ARGs abundance on crop surface. Similar data was obtained in case of VFs. VFs were mostly detected in lettuce samples collected in day 32 that was irrigated with RW, and the reclaimed water samples that was used for the overhead irrigation in day 4. However, no VFs were detected in water samples in day 25. Similar to ARGs, VFs might also transmit to the lettuce from reclaimed water. However, having only one replication of water samples obstruct the conclusion that ARGs and VFs can spread to human via food crops as a result of using contaminated irrigation water.

Our study provides a baseline data for temporal shift of microbial community structure on fresh produce surface grown in the field. Data showed that lettuce leaf microbial community structure changes as the irrigation period extends. The antimicrobial resistance genes and virulence factors also can be transferred to the fresh produce crops with the extension of irrigation period.

Chapter 7: Conclusion and Future direction

Climate change, population growth and overuse of groundwater have necessitated the exploration of safe alternative water (river water, produce wash water or treated wastewater) for food crop irrigation (Dunne, 2020). Due to microbial contamination in agricultural water and enhanced microbial contamination trace-back tools, foodborne outbreaks/illnesses associated with fresh produce are getting higher day by day (Greene et al., 2008; Iwu and Okoh, 2019). Understanding the dynamics of bacterial ecology in agricultural water will allow us to continue investigation of distribution of pathogenic and antimicrobial resistant bacteria, their persistence in the water environment, transfer potential to food crops and adaptability and persistence on the food crops for long periods of time. These will enable us to find a transformative solution to mitigate microbial contamination issues in alternative or nontraditional irrigation water sources.

In this study, the prospective irrigation water sources were found highly contaminated with potential human pathogens and indicator bacteria (TC, *E. coli* and enterococci). **Chapter 3** highlights the prevalence of a possible bacterial pathogen (*Aeromonas* spp.), indicators (TC and enterococci) and fecal indicator (*E. coli*) in different types of alternative irrigation water, their correlation with each other and with different physical chemical parameters of the water. Findings from this part of the research can help identify safe irrigation water sources for the most sensitive of food crops i.e. fresh produce crops. This objective found that on-farm pond water sources were safer sources compared with treated wastewater, and nontidal fresh and tidal brackish river water for fresh produce irrigation due to low microbial count. Only pond water

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complied with Food Safety Modernization Act (FSMA) microbial standards for irrigation of fruit and vegetables. Bacterial counts in reclaimed water, a resource that is not universally allowed on fresh produce in the USA, generally met microbial standards or needed minimal mitigation. Highest microbial count was found in river water that might require substantial treatment to comply with FSMA standards (Solaiman et al., 2020).

Though *E. coli* is a universal water quality indicator and denoted as a mostly commensal taxon, it shows the potential to be pathogenic, causing gastroenteritis and other extra-intestinal diseases. A wide distribution of pathogenic and antimicrobial resistant Escherichia coli and the prevalence of resistance genes is another concern related to alternative irrigation water usage. The study described in **Chapter 4** provided an integrated assessment of the phylogenetic analysis of potentially pathogenic *E coli*, revealed their ESBL antibiotic resistance phenotypes and resistance gene carriage prevalent in alternative irrigation water. The findings found here were consistent with previous parts of the research. River water harbored high levels of phylogroups that are responsible for gastroenteritis, compared to other water types. Similarly, higher proportions of antibiotic resistant isolates were recovered from river water. This finding led us to infer the risk of transfer of potentially pathogenic and antimicrobial resistant *E. coli* from irrigation water via food crops and the transfer of mobile resistant genes from one microbial member to another. Data showed, along with generic *E. coli*, that many pathogenic strains we detected in irrigation water are known to cause adult and neonatal gastroenteritis and extraintestinal diseases.

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An under-studied aquatic bacterial pathogen, *Aeromonas* spp. that causes

gastroenteritis was also included in this study. Although it is reported as widespread,

data was scarce on the transfer potential of the virulent strains from irrigation water to

human via fresh produce crops. In **Chapter 5**, an extensive assessment of the

diversity, distribution and potential for pathogenesis of *Aeromonas* prevalent in

irrigation water was provided. Our study revealed that species diversity, virulence

gene carriage and attachment potential on both biotic and abiotic surface differed with

seasonal and water type variation. Taken together, these data can be used to infer

risks of transfer and establishment potential of possible pathogenic strains from

irrigation water sources to minimally processed fresh produce crops. We found that

river, pond and reclaimed water sources are all major reservoirs of pathogenic species

of Aeromonas, raising the possibility that this taxon enter the food production chain

and persist for extended periods when these water sources are used for irrigation. This

genus, that poses a greater health problem in the elderly, children and

immunocompromised populations, has so far been under-recognized as a food safety

risk, yet its ubiquity and ability to withstand sanitizers, antimicrobials, high salt

concentrations, low pH and temperature conditions, elevate its threat. Due to lack of

correlation with indicator bacteria, *Aeromonas* should be included in microbial

quality assessment of the irrigation water.

These microbially contaminated water sources might be strong driver of shifting

overall bacterial community structure on plants by perturbing the existing microbiota

(Zolti et al., 2019). Both plant and human pathogens introduction from the treated

wastewater and other contaminated surface water can cause community imbalances

(Hidri et al., 2010). Competitive exclusion might reduce the beneficial ones and favour the pathogens. In **Chapter 6**, we used high-throughput DNA sequencing to compare the lettuce surface microbiome irrigated with three different water types: treated wastewater, pond water and sterile water throughout the crop life cycle. The effect of irrigation water application method was also investigated, and we found reclaimed water was less fit to use as irrigation water compared with pond and sterile (control) water. We found that samples that received only drip irrigation and samples that received both drip irrigation and overhead irrigation with sterile water have the similar effect on lettuce surface microbiome. However, further study on transcriptome analysis could help to evaluate the active members of the microbial community.

In the future, an extensive study along the farm to fork continuum including a field study showing the transferability and persistence of specific foodborne pathogens may be able to confirm the risks of using reclaimed water. In addition, mRNA sequencing from surface microbiome can be added to investigate the live community and the live members of the community who carry ARGs and VFs. The data from all these experiments will help to investigate the suitability of recycled and surface water for irrigation purposes based on irrigation methods. The findings will help policy makers to establish guidelines, whether those water sources should be treated to make them free of pathogens before being used to irrigate crops. Thus, the project will reduce the pressure on existing groundwater supplies and reduce water and food associated health risk. Alternative water sources will help farmers to mitigate risks, increase resilience when facing variability in climate and increase crop production. Overall, the project will help researchers, farmers and policy makers to understand

the benefit and risk of using surface and recycled water to irrigate fresh produce

crops.

Appendix 1: Supplementary tables

Table S1. Positive (red) and negative (blue) correlation coefficients between counts of bacterial taxa and physicochemical parameters (dissolved oxygen (%DO), water temperature, pH, conductivity, chloride, salinity, turbidity and nitrate) of non-tidal river (NF), tidal river (TB), reclaimed (RW) and pond (PW) water samples. Only relationships with statistically significant correlation coefficients are given.

Water Type	Taxon	Physico-chemical parameters	Correlation Coefficient r	Lower 95%	Upper 95%	p-value
NF	*E. coli*	DO%	-0.292	-0.43	-0.14	<0.001
		Nitrate	-0.184	-0.35	-0.01	<0.05
		Turbidity	0.165	-0.01	0.33	=0.06
		ORP	0.234	0.07	0.39	<0.01
	Total coliforms	DO%	-0.278	-0.42	-0.13	<0.001
	Enterococcus	DO%	-0.329	-0.46	-0.18	<0.0001
		Turbidity	0.214	0.04	0.37	<0.05
		Nitrate	-0.248	-0.40	-0.08	<0.05
		ORP	0.196	0.03	0.35	<0.05
TB	*Enterococcus*	Turbidity	0.337	-0.01	0.61	=0.06
		Conductivity	-0.352	-0.62	-0.004	<0.05
		Chloride	-0.570	-0.77	-0.28	<0.001
		Salinity	-0.355	-0.63	-0.01	<0.05
PW	*E. coli*	Temperature	0.233	0.03	0.47	=0.05
		pH	-0.335	-0.53	-0.10	<0.01
		Chloride	-0.344	-0.62	0.01	=0.05
	Enterococcus	Chloride	-0.551	-0.76	-0.25	<0.01
	Aeromonas	Turbidity	0.469	0.05	0.75	<0.05
		Nitrate	0.764	0.20	0.95	<0.05
		Salinity	-0.521	-0.75	-0.18	<0.01
RW	*E. coli*	Temperature	0.237	-0.01	0.46	=0.06
		Conductivity	0.332	0.09	0.54	<0.01
		Salinity	0.316	0.07	0.53	=0.01
		ORP	-0.276	-0.52	0.002	=0.05
	Total coliforms	Temperature	0.304	0.07	0.52	<0.05
		ORP	-0.309	-0.54	-0.03	<0.05
	Enterococcus	Chloride	0.394	0.14	0.60	<0.01

Table S2: Zone of inhibition criterion for Enterobacteriaceae (CSLI, 2020)

	Ceftazidime (CAZ30)	Cefotaxime (CTX30)	Ceftriaxone (CRO30)	Cefuroxime (CXM30)	Cefoxitine (FOX30)
Sensitive	≥ 21	≥ 26	≥ 23	≥ 18	≥ 18
Intermediate	18-20	23-25	20-22	15-17	15-17
Resistant	≤ 17	≤ 22	≤ 19	≤ 14	≤ 14

Table S3: Interpretation of biofilm production. The cut-off Optical Density (ODc) was calculated as three standard deviations above the mean OD of the negative control.

Average OD value	Biofilm production
$ODc < OD \leq 2ODc$	Weak/No biofilm producer
$2ODc < OD \leq 4ODc$	Moderate
$4ODc < OD.$	Strong

Appendix 2: Supplementary Figures
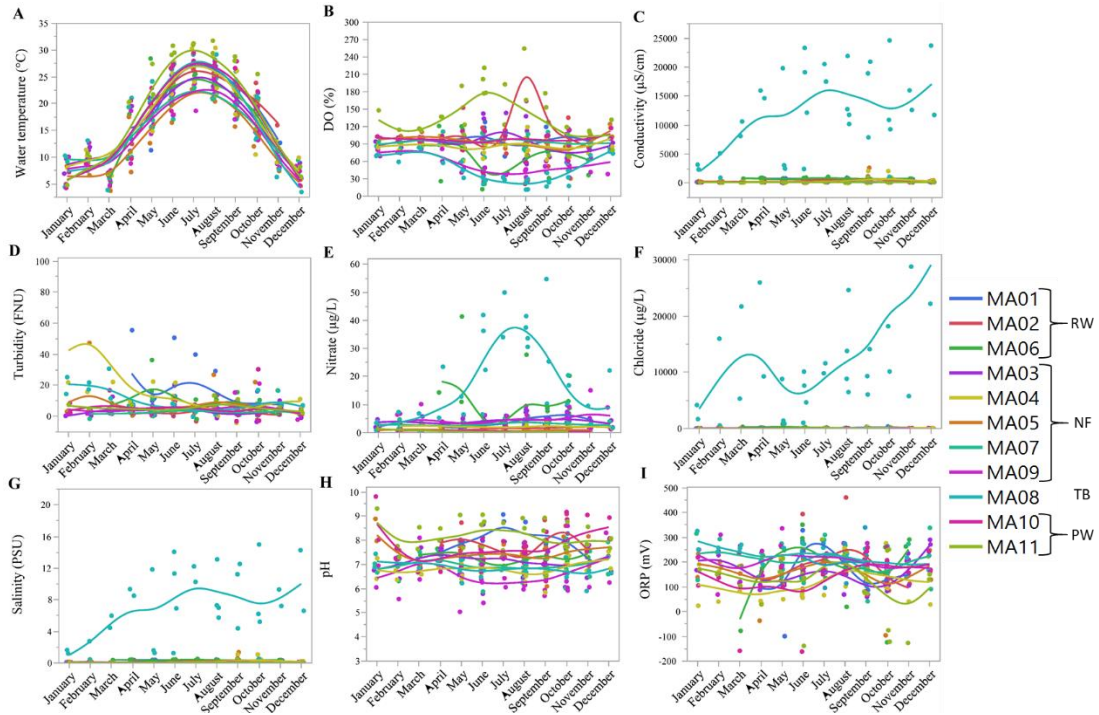
Figure S1: Physicochemical parameters of water by site (MA01, 02, 06=reclaimed water (RW); MA03, 04, 05, 07, 09=non-tidal freshwater rivers/creeks (NF); MA08=tidal brackish river (TB); MA10, 11=pond water (PW)), averaged by month for period September 2016 – October 2018. Panels are as follows: A) water temperature (°C), B) % dissolve oxygen (DO), C) conductivity/specific conductance (SPC; µS/cm), D) turbidity (FNU), E) nitrate (mg/L), F) chloride (mg/L), G) Salinity (PSU), H) pH, I) oxidation reduction potential (ORP; mV).

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