ABSTRACT

Title of Dissertation:	COUPLING DNA LABELING AND NEXT-GENERATION SEQUENCING TECHNIQUES TO CHARACTERIZE METABOLICALLY-ACTIVE BACTERIA IN NONTRADITIONAL IRRIGATION WATER
	Leena Malayil, Doctor of Philosophy, 2019
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Bacteria are ubiquitous in irrigation water resources and can include pathogens that may compromise food safety and public health. However, only a small fraction of total bacterial community members in water can be identified through standard culture-based laboratory methods. 16S rRNA and shotgun sequencing techniques have improved the identification of non-culturable bacteria in water resources. Nevertheless, because sequencing approaches are nucleic-acid based, they are unable to differentiate between the proportion of bacterial communities identified that are live and metabolically-active versus those that are represented by free, relic DNA, not present in viable cells. To bridge this knowledge gap, my dissertation research coupled DNA-labeling (using 5-bromo-2'- deoxyuridine (BrdU) and propidium monazide (PMA)) with next-generation sequencing approaches to identify and comprehensively characterize metabolically-active bacteria in multiple

nontraditional irrigation water sources in the Mid-Atlantic region. My aims were as follows: 1) To characterize the metabolically-active fraction of bacterial communities, as well as antibiotic resistance genes and virulence gene profiles in nontraditional irrigation water sources; 2) To evaluate culture-dependent and -independent methods in the detection of metabolically-active pathogenic and non-pathogenic *Vibrio* species in four nontraditional irrigational water sources; and 3) To track metabolically-active bacterial communities from rooftop-harvested rainwater to irrigated produce in Maryland. Overall, we identified diverse metabolically-active bacterial communities in all nontraditional water sources. Notably, we observed the presence of viable bacteria of importance to both human and/or animal health (*Actinobacterium* spp., *Flavobacterium* spp., *Aeromonas* spp., *Pseudomonas* spp., and *Vibrio* spp.). Interestingly, diverse antimicrobial resistance and virulence genes were predominantly found in non-BrdU-treated samples, indicating that these genes can persist in relic DNA and could be transferred to other environmental bacteria through transformation events. We also source-tracked viable bacteria, including *Sphingomonas* spp., *Enterobacter* spp., *Enterococcus* spp., and *Citrobacter* spp. from rooftop-harvested irrigation water to produce. In summary, this work provides the first description of total, viable, and metabolically-active bacterial communities in different nontraditional irrigation water sources. These data can be used to improve risk characterization of these water sources, and ultimately inform the selection of appropriate cost-effective remediation methods to treat these waters prior to irrigation activities in order to prevent foodborne outbreaks.

COUPLING DNA LABELING AND NEXT-GENERATION SEQUENCING TECHNIQUES TO CHARACTERIZE METABOLICALLY-ACTIVE BACTERIA IN NONTRADITIONAL IRRIGATION WATER

by

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Dedication

To my dear boys Akhil and Nikhil Nair.

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"*Appreciation can make a day, even change a life. Your willingness to put it all into words is all that is necessary." —Margaret Cousins*

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Chapter 1: Introduction

Nontraditional water sources (e.g. reclaimed water, untreated ponds, creeks and rivers) are being sought out to irrigate food crops throughout the world in order to meet the demands of our growing population [1–3] . Existing freshwater sources, such as groundwater, are increasingly over-pumped and, in some cases, too polluted to be used for agricultural irrigation [4, 5]. At the same time, there is a growing body of literature showing that irrigation waters can be a source of pathogenic microorganisms on produce, resulting in food borne illnesses and outbreaks [6–8]. Therefore, the idea of using nontraditional water sources for food crop irrigation is appealing but requires caution since both microbiological and chemical constituents could be present in these waters, posing concerns for food safety and public health.

Currently, to detect bacteria in water, conventional culture-based assays and molecular-based methods are mainly used. Culture-dependent techniques are generally time consuming and are unable to detect non-culturable bacteria [9]. Molecular-based methods like polymerase chain reaction (PCR), quantitative PCR (qPCR) and multiplex PCR, though time-efficient and labor saving [10, 11], have limitations and cannot capture the overall bacterial diversity within tested water samples. Nowadays, next generation sequencing (NGS) techniques are increasingly used to characterize total bacterial communities in complex environmental media, including water [12]. These techniques have helped us gain insights into identifying and comparing additional non-culturable bacteria that are present [13]. These sequencing techniques also result in extensive data generated on the total bacterial diversity of environmental samples [14], but, because sequencing approaches are

nucleic-acid based, they are unable to differentiate between the proportion of bacterial communities identified that are live and metabolically-active versus those that are represented by free, relic DNA, not present in viable cells [15]. Knowledge of viable bacterial communities in our irrigation water systems is crucial, however, as these microorganisms can be transferred from water sources to food crops, potentially resulting in food-borne outbreaks [6]

To address this knowledge gap, the overarching goal of my dissertation was to couple next-generation sequencing technologies with novel DNA-labeling approaches utilizing 5-bromo-2'deoxyuridine (BrdU) or propidium monoazide (PMA) (discussed in detail in Chapter 2) to decipher the metabolically-active fraction of bacterial populations in diverse irrigation water sources. My specific goal was to utilize these coupled methods to address the following aims:

1. To comprehensively characterize the metabolically-active fraction of bacterial communities, as well as antibiotic resistance genes and virulence gene profiles in nontraditional irrigation water sources (pond and reclaimed water) in the Mid-Atlantic region.

2. To evaluate culture-dependent and -independent methods in the detection of metabolically-active pathogenic and non-pathogenic *Vibrio* species in four nontraditional irrigation water sources (reclaimed water, pond water, tidal brackish water and non-tidal fresh water) in the Mid-Atlantic region.

3. To track bacterial communities from rooftop harvested rainwater to irrigated produce in a raingarden in Maryland.

Each of the above-mentioned research aims is addressed in a separate manuscript included in this dissertation. Chapter 2 provides background information on the different water sources that are present in the United States that can be tapped as alternative irrigation water sources. This chapter also discusses agricultural water reuse activities and regulations in the United States; the presence of bacterial constituents and antimicrobial resistance and virulence genes in these water sources; and the characterization of bacterial communities in environmental samples. Chapter 2 closes by introducing in detail the innovative idea that is the basis of my primary research: coupling DNA labeling and next-generation sequencing techniques to increase understanding of the proportion of metabolically-active bacteria present in tested water samples.

Chapter 3 is a manuscript, entitled "Characterizing metabolically-active bacteria in reclaimed water and ponds using bromodeoxyuridine DNA labeling coupled with 16S rRNA and shotgun sequencing" that describes the presence of metabolically-active bacteria, antimicrobial resistance genes and virulence gene profiles in pond and reclaimed water sources in the Mid-Atlantic region. Chapter 4 is a manuscript, entitled "Coupled DNA-labeling and sequencing approach enables the detection of viable-but-non-culturable *Vibrio* spp. in irrigation water sources in the Chesapeake Bay watershed" that identifies the metabolically-active pathogenic and non-pathogenic *Vibrio* species in different water sources in the Mid-Atlantic region. Chapter 5 is a manuscript, entitled "Source tracking microbial communities from

rooftop harvested rainwater to irrigated soil and produce" that presents data from a field study that utilized DNA-labeling, coupled with sequencing, to track bacterial communities from rooftop harvested rainwater to irrigated crops. Lastly, Chapter 6 provides conclusions, a discussion of the public health significance of my research findings, and a summary of future research directions.

Chapter 2: Background

With the advent of the Food Safety Modernization Act (FSMA), implemented by the U.S. Food and Drug Administration (FDA), the nation's food safety system is shifting its focus from responding to foodborne outbreaks to preventing them [16, 17]. One of the foundational rules of FSMA is the Produce Safety Rule (PSR, 21 CFR 112) that establishes, for the first-time, science–based minimum standards for safe growing, harvesting, and handling of fresh produce that is grown for human consumption [18]. Within the PSR, new standards are being developed to ensure the microbiological quality of water that is in contact with produce (other than sprouts) [18]. In order to comply with these new standards, FDA requires farmers to do an initial survey using a minimum of 20 samples (for untreated surface water over the course of 2-4 years) and 4 samples (for ground water over the course of 1 year) collected as close to harvest time as possible [18]. These initial survey findings are then used to calculate two numerical criteria (geometric mean (GM), and statistical threshold value (STV)), both based on the presence of generic *E. coli* (an indicator of fecal contamination) in these water sources [18]. After obtaining these data, farmers are required to develop a Microbial Water Quality Profile (MWQP) that establishes: 1) whether water is of acceptable quality to contact produce directly (with a geometric mean (GM) and statistical threshold (STV) value of 126 colony forming units (CFU) or less and 410 CFU or less, respectively, of generic *E. coli* per 100 mL); 2) if mitigation strategies of some form are required prior to application (e.g. employing a die off rate of 0.5 log CFU/day to calculate the acceptable number of

days between the last irrigation event and harvest); or 3) if the water source should be used for other non-contact applications [18] (21 CFR 112). In addition to the water quality checks, with stricter rules and regulations put forth by FDA, farmers need to better understand and monitor the quality of the water sources that are being used to irrigate fresh produce in order to prevent future outbreaks.

Beyond the specific water definitions within the PSR, agricultural water, in general, is defined as water used to grow fresh produce and sustain livestock, and the typical sources include surface water (e.g. rivers, streams, ponds, lakes), groundwater and rainwater [19]. In 2018, the United States Geological Survey (USGS) released a report estimating U.S. water use [20]. According to the report, U.S. water use in 2015 was estimated to be 322 billion gallons per day (Bgal/d) of which 118 Bgal/d (surface water and groundwater combined) and 669 Mgal/d (reclaimed water) was used for irrigation purposes [20]. Of the total irrigation withdrawals, surface water and groundwater withdrawals accounted for 52 and 48 percent, respectively [20]. In addition to withdrawals from surface water and groundwater sources, reclaimed water has been used for various irrigation purposes [21].

To produce safe and wholesome fresh produce (fresh fruits and vegetables), accessibility to abundant sources of high-quality water plays a key role. However, access to safe, high-quality water is becoming increasingly difficult in many parts of the world due to climate change, population growth, over-pumping of ground water, and contamination of irrigation water sources from land use activities, to name a few [4, 5]. To meet the demands of our growing population, nontraditional irrigation water sources (e.g. reclaimed water; untreated ponds, creeks, and rivers; and rooftop

harvested rainwater) are being sought out to support food production throughout the world [1–3]. The idea of using alternative water sources for food production is appealing but requires caution since both microbiological and chemical constituents could be present in these waters, posing concerns for both food safety and public health. According to recent data from the Centers for Disease Control and Prevention, about 48 million people in the United States get sick, 128,000 are hospitalized, and 3,000 die each year from foodborne diseases [22]. Some of these foodborne illnesses/outbreaks have been attributed to produce grown with contaminated irrigation water, highlighting a significant public health burden that could be prevented.

Irrigation water sources

Oceans and seas account for the largest water body types on Earth and are usually unfit for agricultural irrigation purposes due to high salinity. Humankind depends on these water bodies for food (fish and other marine animals), for transport and for their influence on the atmosphere and global water and nutrient cycles. Inland water bodies are generally categorized into lentic or lotic habitats [23] and represent the major irrigation water sources. Most of these habitats are freshwater bodies, although, depending on their geological and climatic conditions, may include brackish estuaries [23]. The term *lentic* refers to standing motionless waters such as lakes and ponds (lacustrine) or swamps and marshes (paludal), while *lotic* refers to flowing water bodies (fluvial) like rivers, streams and creeks, and in coastal locations, brackish estuaries like the Chesapeake Bay [23]. Most of these inland water bodies

are good alternative surface waters that can play a key role as potential irrigation water sources and will be discussed in detail below in terms of their microbial loads and their use and regulation in the United States.

Microbial Quality of Surface waters: Pond water (Lentic), Creeks (Freshwater Lotic) and Brackish water (Saline Lotic)

Pathogenic viruses (norovirus, sapovirus, adenovirus, hepatitis E virus, enterovirus, hepatitis A virus and rotavirus), bacteria (*Campylobacter jejuni*, pathogenic and enterohemorrhagic *Escherichia coli*, *Salmonella enterica*, *Shigella* spp., *Legionella pneumophila,* and *Pseudomonas aeruginosa*), and protozoa (*Acanthamoeba* spp., *Cryptosporidium parvum,* and *Giardia intestinalis*) have been detected in freshwater sources (lentic and lotic) and have also caused waterborne outbreaks or sporadic infections in humans [24–26]. There is a growing body of literature demonstrating the presence of multiple bacterial phyla in freshwater systems [27, 28] but existing data are heavily derived from large lakes and rivers [29, 30] and hence, there is a need to better understand the microbial communities of smaller freshwater bodies such as creeks and ponds.

Agricultural freshwater ponds (lentic ecosystems) are potential alternative water sources that are increasingly utilized for irrigation purposes. While there is no universal definition of a pond, some groups have defined ponds as small bodies of freshwater (less than 5 hectare), shallow enough for vegetation to grow, usually stagnant and less stable when compared to lakes [31–33]. Despite their small size and shallow depth [31, 32], these ponds have been known to harbor a wide array of

aquatic plants and macroinvertebrates, even greater than that of larger water bodies such as rivers and lakes [31]. Freshwater ponds are also home to indigenous bacterial communities that are different from those of marine water systems [25]. Many of these microorganisms are key components in the biogeochemical cycling of elements such as carbon and nitrogen, and hence, play a major role in these water sources. Additionally, ponds are typically susceptible to multiple exposures (anthropogenic, agricultural, and environmental) that may influence their microbial communities. A recent study by our group also revealed diverse and dynamic bacterial and viral populations in an agricultural freshwater pond in the Mid-Atlantic region during the late season when nutrients and temperatures are at their lowest levels [34]. The studied pond [34] was dominated by *Actinobacteria*, *Proteobacteria*, *Bacteroidetes,* and temperate phages (*Siphovirus*), all of which have members known to cause foodborne illness and the potential to transfer virulence and antibiotic resistance genes [35].

Besides ponds, there are over 3 million miles of flowing water systems (lotic) in the U.S., including rivers, streams, creeks and brooks [36]. Like ponds, there is no universal definition for a creek; however, certain observations are used to differentiate a creek from rivers and streams, such as size (creeks are smaller than rivers) and flow direction (creeks flow into rivers) [36]. These flowing water systems can be impacted by associated tributaries and land use patterns in their catchment areas (e.g. agriculture, urban), which are greater than that of stagnant ponds and lakes [37]. Human and animal pathogens have been observed in the lotic ecosystem, especially rivers. For instance, the Danube river, located in central Europe has been

impacted by human activities with *Salmonella* spp., enteropathogenic *E. coli,* and *Vibrio* spp. [38]. In 2015, a comprehensive review of published findings, focused on microbial diversity in streams and rivers, found that lotic ecosystem studies examined bacterial communities (56%) predominantly, followed by fungal communities and archaeal or protozoan communities [39].

In addition to creeks, other lotic systems, brackish and saline waters, are currently being explored as potential sources for agricultural irrigation as groundwater levels continue to be variable in key food production areas [40, 41]. However, these water sources also can harbor important bacterial pathogens. *Vibrio* spp., for instance, are natural inhabitants of coastal, brackish waters of the Chesapeake Bay and its tributaries and include frank pathogens such as *V. cholera, V. parahaemolyticus* and *V. vulnificus* [42–44]. Human *Vibrio* infections can occur among people consuming raw or undercooked shellfish and among those working or recreating in contaminated waters [42, 43, 45, 46]. If *Vibrio*-contaminated water is also used to irrigate food crops that are eaten raw, this could represent an additional understudied exposure pathway for human *Vibrio* infections. Hence, there is a need to further our understanding of the prevalence of these microorganisms in potential alternative irrigation water sources. Nonetheless, previous studies have provided evidence that *Vibrio* spp. can enter a viable-but-non-culturable state [47–49], limiting the ability of traditional culture methods to assess the true prevalence of these microorganisms in water bodies.

Reclaimed water

Use of advanced treated municipal wastewater, also referred to as reclaimed water, for agricultural purposes has been practiced in many cities throughout the world for hundreds of years as an effective way to alleviate water pollution, improve ecological environments, and address agricultural water shortages [50–53]. The United States Environmental Protection Agency (US EPA) defines reclaimed water as treated municipal wastewater that meets specific water quality criteria which can then be used for a range of purposes [21]. Use of reclaimed water (also referred to as recycled water) for various purposes is practiced by most states in the US [51, 54]. The leading states with regard to agricultural use of reclaimed water are Florida, California, Colorado, Wyoming, Idaho, North Carolina, Nevada, Texas, Utah, Washington and Arizona [21, 55]. Currently in the U.S. there are no federal regulations directly governing reclaimed water use [21]. In the absence of federal standards and regulations concerning reclaimed water use, the U.S. EPA developed water reuse guidelines, the latest of which were published in 2012 [21]. States then interpreted the EPA guidelines and developed state-by-state approaches to regulate reclaimed water reuse, and this can be challenging. As mentioned earlier, reclaimed water is used for various purposes but here we discuss its use as an irrigation water source. The minimum suggested EPA regulatory guidelines for the use of reclaimed water for food crops intended for human consumption that are consumed raw requires no detectable fecal coliform CFUs/100 mL, while irrigation of food crops intended for human consumption that are commercially processed or non-food crops (not

consumed by humans) requires water quality standards to meet <200 CFU fecal coliforms/100 mL [21].

Though reclaimed water use can fulfill multiple needs, including but not limited to landscape irrigation (e.g. golf course and public parks) [56]; non-potable urban uses (cooling water for power plants and oil refineries, and toilet flushing) [57–60]; and environmental uses (e.g. stream augmentation and groundwater recharge), the use of this alternative water source could also result in both environmental and public health impacts [61] particularly when it is utilized for agricultural irrigation of raw produce [62]. A few outbreaks associated with food irrigated with raw sewage or primary treated wastewater have been reported in other parts of the world [63, 64]. However, to date, there have been no documented foodborne illnesses resulting from the use of reclaimed water (tertiary treated) in irrigation activities in the United States [65].

Previous studies have revealed the presence of bacterial pathogens [66–69], heavy metals [70], organics (e.g. industrial chemicals, pesticides, pharmaceuticals and personal care products) and antibiotics [71–74] in reclaimed water used for agricultural irrigation. Other studies have shown that it is possible for chemical and microbiological contaminants remaining in reclaimed water to be transferred to and persist on irrigated crops [62, 75]. Goldstein et al. (2012 and 2014) detected methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci in the influent and effluent of four U.S. (2 Mid-Atlantic and 2 Midwest) wastewater treatment plants [76, 77]. Another study reported bacterial contamination (fecal streptococci, *E. coli*, *Pseudomonas aeruginosa*, *Salmonella*, *Vibrio*, and

Listeria) of vegetable crops (radishes) and soil that were irrigated with reclaimed water in Saudi Arabia [78].

Rooftop harvested rainwater gardens

Beyond reclaimed water, in recent years, rooftop harvested rain water (RHRW) is becoming an alternative and eco-friendly water resource in countries such as Australia, Canada, Germany, New Zealand, Thailand, Japan, Denmark, India, and the United States [79–81]. Like reclaimed water use, rooftop harvested rainwater use is not regulated by the federal government. Rather, it is up to individual states to regulate its collection and use [82] and state-based rainwater harvesting regulations and policies vary widely [83]. Most states have no rainwater harvesting regulations, while some states have policies for both the collection and use of rainwater [84]. Texas and Ohio have enacted several laws regulating rainwater harvesting and these two states allow this water source to be used for potable purposes, a practice that is frequently excluded from other states' regulations and laws [83]. Regarding the purchase of rainwater harvesting equipment, certain states (Rhode Island, Texas and Virginia) offer tax credits or exemptions to homeowners [83]. In contrast, Colorado was the only U.S. state, until recently, where it was illegal to harvest rainwater [83, 84]. However, under a new Colorado law, House Bill 1005 (2016), residential owners can use two rain barrels (110 gallons total) to capture rainwater from their rooftops and use the water within their property [83].

RHRW is not only being used in toilet flushing, irrigation activities, and as a drinking water source when properly treated, but has also proved to help reduce storm

water runoff and can even be a part of the urban landscape [85]. Even though rooftop runoff rainwater is a very promising alternative irrigation water source, caution is needed as this water may contain more pollutants than rainwater. Feces of birds, insects, and mammals, as well as old roofing materials (shingles, copper, etc.), dirty drainage pipes and poorly-maintained storage tanks can all further contaminate the water as it runs down from the roof to the source of use [86, 87]. The presence of lead, zinc, chromium, manganese, molybdenum, silver, nickel, copper and cadmium in harvested rainwater has been demonstrated in multiple studies from Australia and Hebron (West Bank, Palestinian Territories) [88, 89]. The presence of lead, zinc, chromium, manganese, molybdenum, silver, nickel, copper and cadmium in harvested rainwater has been demonstrated in multiple studies from Australia and Hebron (West Bank, Palestinian Territories) [88, 89]. Besides the presence of heavy metals, several studies have shown the presence of enteric and opportunistic pathogens like *Enterococci* spp.*, Escherichia coli, Clostridium perfinges, Salmonella* spp.*, Campylobacter* spp.*, Legionella* spp.*, Aeromonas* spp.*, Pseudomonas* spp.*, Mycobacterium* spp.*, Shigella* spp.*, Vibrio* spp.*, Giardia* spp., and *Cryptosporidium* spp. in RHRW that could potentially be transmitted to vegetable crops if the water is used for irrigation purposes [86, 90, 91].

Characterization of bacterial communities using culture-dependent methods

Microorganisms predominate the biosphere—which contains about 1 trillion (10^{12}) microbial species [92]—and are present in a multitude of diverse environments, including water bodies [93]. It is estimated that there ~ 10^{30} cells of bacteria and

archaea inhabiting Earth [92, 94–96]. Despite their critical importance and enormous representation on Earth, very little is known about the diversity of bacterial communities in differing environments and this is largely due to our inability to culture the majority of bacterial species in laboratory settings [97]

Typically, biological diversity is measured via counting the number of validly described species that a given branch of the tree of life possesses [97], which is a persistent challenge in biology. However, estimating bacterial diversity is even more complicated since bacteria are not visible to the naked eye and cannot be easily differentiated morphologically [98]. Identification of bacteria in a given environment is accomplished using culture-dependent approaches, culture-independent methods or both.

For centuries, bacteria were identified by isolation in culture and characterized via enzymatic reactions and morphological analyses [99–101]. These culture-dependent methods have provided a good understanding of phenotypic characteristics, including expressed antibiotic resistance, of isolates recovered from various environments [102]. Yet, identifying environmental bacteria via culture-dependent methods provides limited information on the overall bacterial diversity of complex environmental niches [103, 104]. Moreover, culture-based detection methods are greatly hindered by the presence of viable-but-non-culturable (VBNC) microorganisms that cannot be cultured using known laboratory procedures [9].

The first evidence of VBNC bacteria was revealed via microscopy; the number of cells observed under a microscope far outweighed the number of colonies growing on a petri plate, which was termed as “The Great Plate Count Anomaly” by

Staley and Konopka (1985) [105]. On exposure to stressful conditions such as starvation and low temperatures many bacterial species enter the VBNC state as a survival strategy [106, 107]. This phenomenon is mainly due to a lack of sufficient biological information that allows for the development of specific culture methods to detect these environmental microorganisms in a laboratory setting [97].

Characterization of bacterial communities using culture-independent methods

In the past three decades, molecular detection methods, including polymerase chain reaction (PCR) assays followed by cloning, or direct sequencing of environmental samples has yielded additional insights into bacterial diversity [10, 11]. Molecular or culture-independent methods such as these depend on DNA-based analyses to assess microbial community structure, function and dynamics and do not require the cultivation of microorganisms in a laboratory. These DNA-based methods include analyses of either whole genomes or selected marker genes like 16S rRNA and 18S rRNA (ribosomal RNA). For bacterial identification, the 16S rRNA gene is the most commonly used molecular marker since these genes are ubiquitous across all bacterial species, structurally and functionally conserved, and contain variable and highly conserved regions [108]. Other conserved genes like the RNA polymerase beta subunit (*rpoB*), recombinase A (*recA*), gyrase beta subunit (*gyrB*) and heat shock protein (*hsp60*) have also been used in bacterial species differentiation and identification [109]. These unique markers have revealed a hidden treasure of bacterial diversity that had never been acknowledged by culture-dependent work. For

instance, more than 80 bacterial phyla have been identified to date, of which only half of these have members that can be cultured in the laboratory [9, 110–112].

Although the above-mentioned molecular markers have been commonly used in most microbial ecology surveys, species and strain level resolution is oftentimes not achieved [113]. Hence, to gain a more comprehensive view of bacterial genetic diversity, DNA sequencing technologies are commonly employed now. Some of the earliest sequencing techniques, Sanger and Max-Gilbert sequencing, have been completely superseded by next generation sequencing (NGS) techniques after the completion of human genome project (HMP) [114]. New developments in NGS biochemistries, bioinformatics and instrumentation have helped revolutionize the field of microbial ecology and genomics [13, 115]. NGS platforms such as Illumina/Solexa, are much faster and less expensive compared to traditional Sanger sequencing techniques. As a result, these sequencing approaches have allowed us to more deeply investigate microbial communities by generating billions of reads at a very low cost and high speed; and hence, have played a pivotal role in presenting a more comprehensive view of phylogenetic composition and functional diversities of environmental bacterial communities [116].

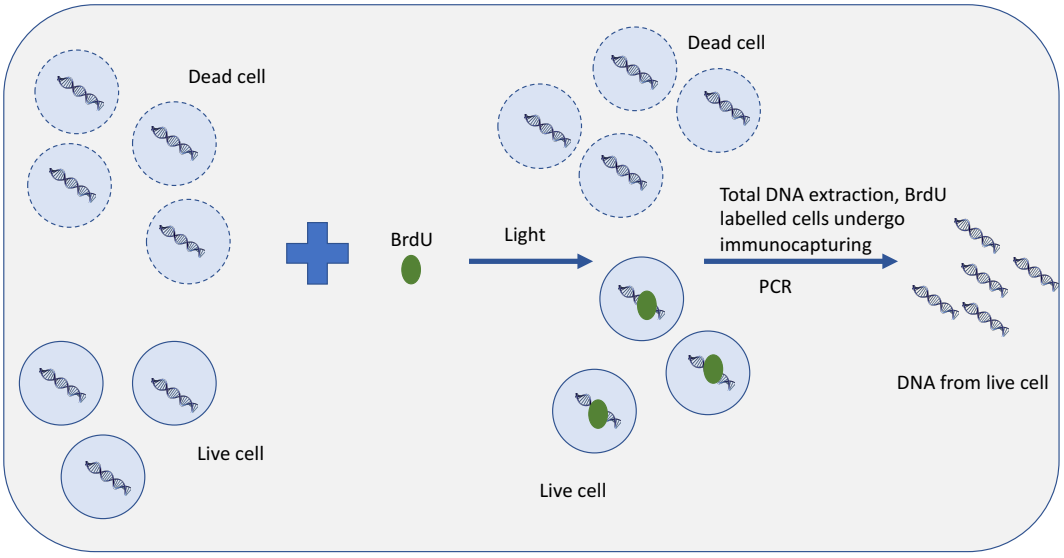
Nevertheless, similar to culture-dependent methods, DNA-based approaches have their own pitfalls and biases. The most common biases stem from DNA extraction and PCR steps performed prior to sequencing, and include issues such as the preferential lysis of certain bacterial cells, or interaction with inhibitory compounds, which can distort bacterial community composition, richness and structure [117]. This limitation can be greatly rectified by the incorporation of

internal amplification controls [117]. Another disadvantage of using DNA-based technologies is the lack of knowledge concerning whether the bacteria represented by the data are alive and viable or merely represented by persistent, relic DNA from dead microorganisms [118]. This challenge can be addressed by using RNA-based sequencing approaches instead of DNA-based methods, particularly those targeting mRNA (which is only produced by metabolically-active cells), thus indicating the presence of live cells [119]. One major issue, however, is that high quality RNA extraction is more challenging due to the rapid degradation of RNA which can occur because of inadequate sample processing and/or storage, or contamination with RNA-degrading enzymes like RNases [120]. Moreover, despite the labile nature of mRNA, false positive signals from residual transcripts have been shown in instances where high levels of dead bacteria ($> 10^4$ cells/mL) are present [121, 122].

Coupling DNA-labeling with sequencing methods for detection of metabolically-active bacteria

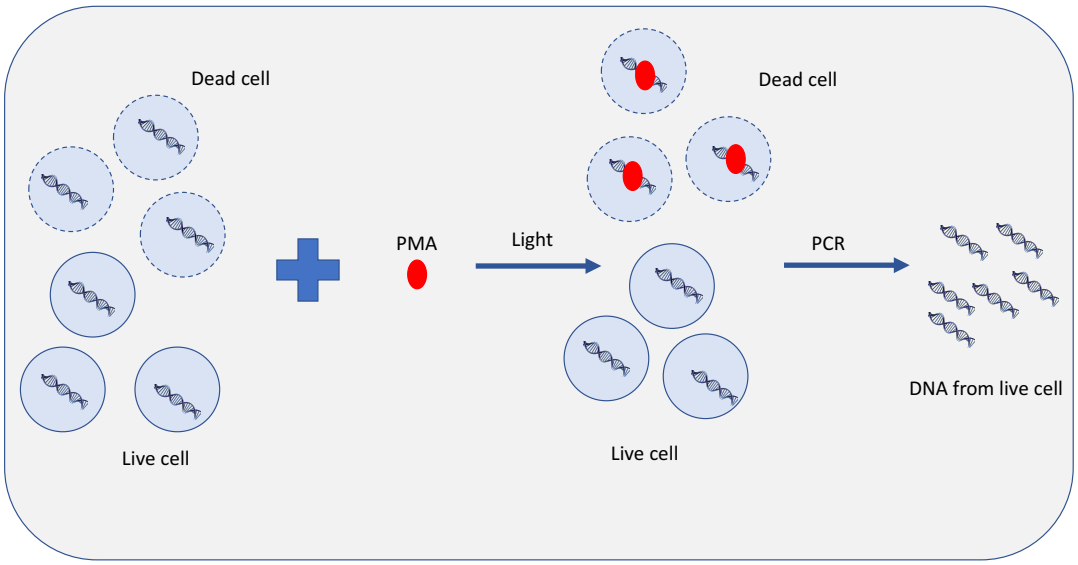
An alternative to RNA-based sequencing methods for the detection of live or metabolically-active bacteria includes the use of either bromodeoxyuridine (5-bromo-2'deoxyuridine, BrdU) or a photo-reactive DNA-binding dye such as ethidium monoazide (EMA) or propidium monoazide (PMA) [123–125]. BrdU is a synthetic thymidine analog that can incorporate into replicating or live DNA (Figure 1).

Figure 1: Description of 5-bromo-2'deoxyuridine (BrdU) labelling technique to identify metabolically active cells



The BrdU within this DNA can then be detected via antibody immunocapture techniques [125]. This BrdU labeling technique has been used to identify the metabolically-active fraction of bacteria present in aquatic and soil environments [126–128]. However, to our knowledge, no previous studies have coupled BrdU labeling and next generation sequencing to provide a comprehensive characterization of total, metabolically-active bacterial communities in water samples. PMA on the other hand can penetrate membrane-compromised (dead) cells and, following photo-activation, binds to free DNA (Figure 2).

Figure 2: Description of Propidium monoazide (PMA) labelling technique to identify metabolically active cells



Once inside a (dead) cell, PMA intercalates into the cell's DNA with high affinity, forming a covalent cross-linkage upon exposure to light. This bond between the dye and DNA results in strong inhibition of PCR amplification, preventing this DNA from being amplified and sequenced in 16S rRNA sequencing applications. PMA has been widely applied to characterize different environments [123, 129–131] and has been coupled with quantitative PCR (qPCR), and next-generation sequencing techniques [129, 132, 133].

Coupling BrdU- and/or PMA-labeling approaches with next-generation sequencing methods can further our knowledge of the overall diversity of the metabolically-active fraction of bacterial communities present in environmental samples. Knowledge of metabolically-active (live) bacteria in our agricultural

irrigation water systems is critical since there is increasing evidence that irrigation water can play a role in the microbial contamination of fresh produce, leading to foodborne outbreaks. The dissertation research described below demonstrates how coupling BrdU- and PMA-labeling with sequencing approaches can improve our understanding of multiple nontraditional irrigation water sources that are increasing in use as a result of our changing climate and rising water insecurity.

Chapter 3: Characterizing metabolically-active bacteria in reclaimed water and ponds using bromodeoxyuridine DNA labeling coupled with 16S rRNA and shotgun sequencing

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Abstract

Understanding the complex and varied microbiota of irrigation waters is vital to sectors of public health from plant pathology, sustainable agriculture and food safety, to surveillance of pathogens and antimicrobial resistance. Water is evaluated using a broad range of culture based and metagenomic methods, which provide valuable detection or profiling of microbiota associated with aquatic environments. Few approaches are capable of identifying the metabolically active constituents of microbial communities. Here we combine 5-bromo-2'-deoxyuridine (BrdU) labeling with 16S rRNA and shotgun sequencing to identify metabolically-active bacteria in reclaimed and pond water samples (n=56) from the Mid-Atlantic United States between March 2017 and January 2018. Metabolically-active genera in water samples included *Actinobacterium* spp*., Flavobacterium* spp*., Aeromonas* spp*., Propioniobacterium* spp*.,* and *Pseudomonas* spp. Lower alpha diversity was observed in BrdU-treated (metabolically-active) compared to non-BrdU-treated samples. Antimicrobial resistance genes and virulence genes were more abundant and in greater diversity in non-BrdU-treated reclaimed water samples, indicating that these genes may not be within active bacteria. Agricultural pond and reclaimed waters are important for the future of sustainable agriculture and thus the full understanding of the genetic potential of these waters is important to guide future treatment strategies to ensure appropriate water quality for intended purposes.

Introduction

Introduction

Alternative irrigation water sources, such as reclaimed water (treated wastewater) and agricultural ponds, are vitally important to the future of sustainable agriculture. These sources will likely be called upon more and more frequently to complement reserves from aquifers in multiple regions of the world [1–3]. These water sources have been exposed to significant anthropogenic and wildlife pressures, the full understanding of which will be important to a wide range of food safety and public health concerns.

Agricultural ponds are one of the most reliable and economical sources of irrigation water for farms, widely used for irrigation [134]. Previous studies of pond microbiota have described incidence of: *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Acidobacteria* and *Cyanobacteria* [135]. Fecal indicator bacteria (total coliforms, generic *Escherichia coli* and enterococci), *E. coli* O157:H7, *Salmonella* spp. and Shiga-toxin producing *E. coli* (STEC) genes have also been reported in agricultural ponds and in produce irrigated with pond water [136–138]. A *Salmonella* Newport outbreak that caused illness in more than 500 people in 26 states was attributed to consumption of tomatoes irrigated with pond water from the eastern shore of Virginia [139]. Another recent study describes the diverse and dynamic bacterial and viral populations in an agricultural freshwater pond in the Mid-Atlantic region [34]. The pond was dominated by bacterial taxa: *Actinobacteria*, *Proteobacteria*, *Bacteroidetes,* and temperate phages such as *Siphovirus*.

Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci have been detected in both influent and effluent water of U.S. wastewater treatment plants (Mid-Atlantic and Midwest)by Goldstein et al. (2012 and 2014) [76, 77]. Additionally Balkhair (2016) reported *E. coli*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Vibrio* spp., and *Listeria* spp. from soil and radishes irrigated with reclaimed water in Saudi Arabia [78]. These studies are some of the first to describe the bacterial pathogens that may persist in reclaimed water and potentially the produce managed with these waters.

In the mid-1970s, outbreaks associated with food irrigated with raw sewage or primary treated wastewater were described and [63] Katzenelson et al. (1976) reported incidence of shigellosis, salmonellosis, typhoid fever, and infectious hepatitis to be to four times higher in kibbutzes that used wastewater irrigation practices [64]. To date however, there have been no documented foodborne illnesses resulting from the use of reclaimed water (tertiary treated) in irrigation activities in the United States [65].

Currently, to ensure the microbiological quality of reclaimed water is suitable for irrigation applications, conventional culture-based methods have been used to evaluate water quality. Additionally, molecular assays are used to identify specific targets or to provide broad surveys of total environmental genomics. Next generation sequencing (NGS) techniques are commonly used to characterize microbial communities in complex environmental sources [12].

While all of these technologies have helped us gain insights into agricultural water ecosystems [13], most approaches do not differentiation between live and dead

bacteria [15]. As demand for reclaimed and alternative to ground water grows, it is vital to have a high resolution understanding of the pathogenic potential of water sources used to irrigate food crops [6]. To provide data to bridge this knowledge gap, we used a combination of bromodeoxyuridine (BrdU) DNA labeling and next-generation sequencing methods to characterize the live (metabolically-active) fraction of bacterial communities in multiple irrigation water sources (reclaimed water and ponds). BrdU is a synthetic thymidine analog that can incorporate into replicating DNA. The BrdU within this DNA can then be detected via antibody immunocapture and characterized using sequencing technologies. This enables one to distinguish between live (active) and dead (relic) bacterial communities [125, 140]. Additionally, we further described these bacterial communities by reporting antibiotic resistance (AMR) gene and virulence gene profiles by BrdU treatment and by water type. The data presented here demonstrate that the coupled use of BrdU labeling and sequencing provides an enhanced understanding of the metabolically-active fraction of bacterial communities in alternative irrigation water sources.

Materials and Methods

Sampling sites

Two water sources in the Mid-Atlantic, United States were included in the study: an agricultural pond and a tertiary wastewater treatment plant and reclamation facility. The agricultural pond is a temperate freshwater pond with a maximum depth of approximately 3.35 meters and a surface area of approximately 0.26ha. Permission to obtain pond water samples was granted by the farm manager. The tertiary wastewater treatment plant is located in a rural area and treats between 1,135.62 and

1,419.53 m^3 of domestic wastewater per day with a maximum daily capacity of 1,892.70 m^3. Raw influent consists of residential/municipal wastewater and light industry wastewater. The primary treatments involve the use of screens, grinders and grit chambers. Activated sludge reactors/aeration tanks and sedimentation tanks/secondary clarifiers are the secondary treatments used. The secondary clarified wastewater is then piped to an open-air lagoon and chlorinated before land application via spray irrigation to achieve further nutrient removal and ultimate groundwater recharge. Permission to collect reclaimed water for the purposes of this study was granted by the Town Administrator.

Sample collection

From March 2017 to January 2018, two liters of water from each source were collected during bi-weekly sampling trips. A total of 13 reclaimed water samples and 15 pond water samples (reclaimed water samples could not be obtained during the winter months of November, December and January) were used for further analyses. In addition, a ProDSS digital sampling system (YSI, Yellow Springs, OH, USA) was used to measure, in triplicate: water temperature (°C), conductivity (SPC uS/cm), pH, dissolved oxygen (%), oxidation/reduction potential (mV), turbidity (FNU), nitrate (mg/L), and chloride (mg/L).

Sample processing

200µl of 100mM BrdU was added to 1 L of water, while the other liter was not treated with BrdU. Both liters were then incubated for 2 days in the dark at room

temperature, allowing BrdU to incorporate into replicating DNA of the BrdU-treated samples and enabling us to detect the metabolically-active fractions of the bacterial communities within our water samples. Both the BrdU-treated and non-treated samples were then filtered through 0.2 μm, 47 mm filters (Pall Corporation, Port Washington, NY, USA) using sterile filter funnels (Thermo Fisher Scientific, Waltham, MA, USA). The filters were then dissected into four quadrants, placed in lysing matrix B tubes (MP Biomedicals, Solon, OH, USA) and stored at -80°C until DNA extraction.

DNA extraction

DNA extractions were performed using protocols previously published by our group [141, 142]. Briefly, 1 ml of PBS was added to the filters in the lysing matrix B tubes, before incubation in enzymatic cocktails containing lysozyme, mutanolysin, proteinase K and lysostaphin, after which the cells were mechanically lysed using an MP Biomedical FastPrep 24 (Santa Ana, CA). The DNA was then purified using the Qiagen QIAmp DNA mini kit (Germantown, MA) per the manufacturer's protocol.

Immunocapture of BrdU-treated samples

Immunocapture and isolation of BrdU-labelled DNA were performed using a previously published protocol [125]. Briefly, sheared and denatured herring sperm DNA (HS DNA) and monoclonal anti-BrdU (α-BrdU) antibody was mixed at a 1:9 ratio and incubated for 1 hour at room temperature to form the HS DNA/α-BrdU antibody complex. The extracted DNA from the water samples was then denatured by

heating for 5 min at 100^0C and transferred to ice. The mixture of HS DNA/α-BrdU antibody complex was then added to the denatured DNA from the water samples and incubated for 1 h in the dark at room temperature with agitation to form the DNA/HS DNA/α-BrdU antibody complex. Meanwhile, magnetic beads (Dynabeads, Dynal Inc., Invitrogen by Thermofisher Scientific) coated with goat anti-mouse immunoglobulin G were washed three times with 1mg/ml acetylated bovine serum albumin (BSA) in phosphate-buffered saline (PBS) buffer using a magnetic particle concentrator. The washed Dynabeads were then added to the DNA/HS DNA/α-BrdU antibody complex and incubated for an additional 1 h in the dark at room temperature. After incubation, the samples were washed in 0.5ml PBS-BSA, and the BrdU-containing DNA fraction was eluted by adding 1.7mM BrdU (in PBS-BSA) and incubating for 1 h in the dark at room temperature.

16S rRNA PCR amplification and sequencing

Extracted DNA was PCR amplified for the V3-V4 hypervariable region of the 16S rRNA gene using the universal primers 319F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) and sequenced on an Illumina HiSeq2500 (Illumina, San Diego, CA) using a method developed at the Institute for Genome Sciences [143] and described previously [141, 142]. Briefly, PCR reactions were carried out using Phusion High Fidelity DNA polymerase (Thermo Fisher, Waltham, MA, USA) and 2ng of template DNA in a total reaction volume of 25µl. An additional 0.375µl of bovine serum albumin (BSA; 20 mg/ml) was added to the PCR reactions to avoid potential PCR inhibition.

Negative controls without DNA template were included for both primer sets. The following PCR conditions were employed: 30s at 98°C, followed by 10s at 98°C, 15s at 66°C and 15s at 72°C, with a final step of 10 min at 72°C. Amplicon presence was confirmed using gel electrophoresis, after which the SequelPrep Normalization Kit (Invitrogen Inc., Carlsbad, CA, USA) was used for cleanup and normalization (25ng of 16S PCR amplicons from each sample were included), prior to pooling. 16S rRNA sequencing was performed on the Illumina HiSeq2500 (Illumina, San Diego, CA) per the manufacturer's protocol.

Shotgun library preparation and sequencing

DNA libraries for shotgun sequencing were prepared using the Truseq Nano prep kit (Illumina, SanDiego, CA, USA), per the manufacturer's specifications. Sequencing of the 56 water samples -26 reclaimed water (13 BrdU-treated and 13 non-BrdU treated) and 30 (15 BrdU treated and 15 non-BrdU treated) pond water was performed on the Illumina NextSeq 550 (Illumina, San Diego, CA).

16S rRNA sequence quality filtering and data analysis

Following sequencing, 16S rRNA paired-end read pairs were assembled using PANDAseq [144], de-multiplexed, trimmed of artificial barcodes and primers, and assessed for chimeras using UCHIME in *de novo* mode implemented in Quantitative Insights Into Microbial Ecology (QIIME; release v.1.9.1) [145]. Quality trimmed sequences were then clustered *de novo* into Operational Taxonomic Units (OTUs) and taxonomic assignments were performed using VSEARCH [146] with a minimum

confidence threshold of 0.97. The SILVA 16S database [147] in QIIME [145] was used for taxonomy assignments. Downstream data analysis and visualization was completed in RStudio (v.1.1.423) using R packages: biomformat [148] vegan [149], ggplot2 [150], phyloseq [151], Bioconductor [152] and metagenomeSeq [153]. All sequences taxonomically assigned to the Phylum *Cyanobacteria* were removed from further downstream analysis. When appropriate, data were normalized with metagenomeSeq's cumulative sum scaling (CSS) [153] to account for uneven sampling depth. Prior to normalization, alpha diversity was measured using both the Observed richness metric and the Shannon diversity index [154]. Bray-Curtis dissimilarity was used for calculating beta diversity and was compared using analysis of similarities (ANOSIM) on normalized data (999 permutations). Pearson correlation coefficients were calculated to identify associations between the water characteristics and the relative abundance of the bacterial phyla and visualized via heatmaps created in R via vegan heatplus [155].

Metagenomic data analysis

Unassembled metagenomic sequencing reads were directly analyzed using the Genius bioinformatics software package (CosmosID Inc., Rockville, MD), described elsewhere [43, 44] which aided in identification at the species, subspecies, and/or strain level and quantification of relative abundance. Briefly, the system utilizes curated genome databases (GenBook®) and a high-performance data-mining algorithm to disambiguate millions of metagenomic sequence reads into discrete microbial taxa. The GenBook databases are composed of over 150,000 microbial

genomes and gene sequences representing over 1,000 bacterial, 5,000 viral, 250 protists and 1,500 fungal species, as well as over 5,500 antibiotic resistance and virulence associated genes. The reference database, GenBook, constitutes both publicly available genomes or gene sequences through NCBI as well as a subset of genomes sequenced by CosmosID and its collaborators. The pipeline has two separable comparators. The first consists of a pre-computation phase for the reference database and a per-sample computation. The input to the pre-computation phase is a reference microbial genome or antibiotic resistance and virulence gene database, and its output is phylogeny trees, together with sets of variable length k-mer fingerprints (biomarkers) that are uniquely identified with distinct nodes, branches and leaves of the tree. The second per-sample, computational phase searches the millions of sequence reads against the fingerprint sets. The second comparator uses edit distance-scoring techniques to compare a target genome or gene with a reference set. The algorithm provides similar functionality to BLAST but sacrifices some recall precision for a one or two order of magnitude processing gain. The resulting statistics are analyzed to give fine-grain composition and relative abundance estimates at all nodes of the tree. Enhanced detection specificity is achieved by running the comparators in sequence. The first comparator finds reads in which there is an exact match with a k-mer uniquely identified with a reference genome or antibiotic resistance or virulence gene; the second comparator then statistically scores the entire read against the reference to verify that the read is indeed uniquely identified to that reference. For each sample, the reads from a species are assigned to the strain with the highest aggregation statistics.

To visualize the relative abundance of bacterial phyla, antimicrobial resistance genes (AMR), and virulence genes, stacked bar charts were generated using Tableau (version. 9.1). In addition, bacterial taxa were summarized and normalized using several R packages (vegan, dplyr, circlize [156], reshape2 [157] and stringr [158]) and those with a maximum relative abundance greater than 5%, 3% or 1% in at least one sample were used to build the microbial profile, shared and unique data based on the water type and treatments (BrdU or noBrdU).

Results

Water Characteristics

Water (pond and reclaimed) characteristics are described in Table 1. Overall, ambient temperature during sampling and water temperature in both pond and reclaimed water samples increased from March to September and then decreased from October to January. The pH of both water types was neutral to slightly basic across all sampling points. All other water characteristics showed slight variations between sampling months (Table 1).

16S rRNA Sequencing

A total of 56 samples (n=26 reclaimed water samples including 13 BrdU-treated and 13 non-BrdU-treated samples; and n=30 pond water samples including 15 BrdU-treated and 15 non-BrdU-treated samples) were successfully PCR amplified for the 16S rRNA gene and sequenced. Before sequence quality filtering, 7,570 OTUs were identified from a total of 2,839,267 sequences across all samples. Across all

samples, the minimum number of reads was 219 and the maximum was 147,717 with an average number of sequences per sample of 50,701.2 (±36,576.22 SD). Good's coverage of 0.90 was calculated for all samples and samples with Good's coverage <0.90 (4-reclaimed water) were removed to ensure appropriate read coverage in all samples analyzed downstream (Supplementary Figure S1). After removal of *Cyanobacteria* and pruning of low abundance taxa (OTUs with less than 10 sequences), the final dataset analyzed contained 2,650,795 sequences clustered into 3,786 OTUs from 52 samples.

Alpha and Beta Diversity

Alpha diversity metrics (Observed species and Shannon diversity) by site and by BrdU treatment were calculated on rarefied 16S rRNA sequence data (after down sampling each sample to 2,726 reads) (Figure 1A). Statistically significantly lower alpha diversity (both Observed species and Shannon diversity) was observed in BrdU-treated samples compared to non-BrdU-treated samples ($p < 0.0001$) from each site.

Beta diversity between all normalized samples was computed using PCoA plots of Bray-Curtis dissimilarity (Figure 1B) and showed the most significant clustering by site (ANOSIM R: 0.7667, $p = 0.001$), followed by BrdU treatment (ANOSIM R: 0.3078, $p = 0.001$). PCoA findings between water types (reclaimed and pond water) and treatments showed 26% variance between bacterial communities along the first principle component axis (Axis 1) and 14.5% along the second principle component axis (Axis 2).

16S rRNA Taxonomic Analysis by Site and by BrdU treatment

Analyzing our 16S rRNA data, predominant bacterial profiles observed in BrdU-treated pond water included hgcl-clade (*Actinobacteria*), *Flavobacterium*, Candidatus *Planktophila, Pseudarcicella,* and Uncl. *Planctomycetaceae,* while non-BrdU-treated samples were dominated by hgcl-clade (*Actinobacteria*), *Flavobacterium,* Candidatus *Planktophila,* CL500-29_marinie_group, and *Limnohabitans* (Figure 1C). Bacterial abundance when compared between different sampling months, we observed hgcl-clade (*Actinobacteria*) and *Pseudarcicella,* Candidatus *Planktophila were* present in all months irrespective of the treatments. Similarly, *Flavobacterium* also followed the same pattern, but higher abundance was observed from March-June and then from October-January. Uncl. Planctomycetaceae was observed in BrdU-treated pond water from June-September. Additionally, we also observed in October higher abundance of *Chryseobacterium, Rheinheimera, Pseudomonas* and *Aeromonas* (Supplementary Figure 2A).

The predominant bacterial profiles observed in the metabolically active fraction (BrdU) of the reclaimed water were *Flavobacterium* and *Aeromonas,* while in the non-BrdU-treated samples were Uncl. *PeM15* and *Flavobacterium* (Figure 1C). On comparing bacterial abundance between different sampling months, many of the profiles were observed in the non-BrdU treated samples. We observed *Flavobacterium* in all months irrespective of treatments but had higher abundance from March-June and then October-January. Higher abundance of *Aeromonas* was observed from March-August in BrdU-treated samples (Supplementary Figure 2B).

Correlation between Water Characteristics and 16S rRNA Bacterial Relative Abundance

Several bacterial phyla, irrespective of water sample type, were significantly correlated ($p < 0.05$) with some of the measured water characteristics (Figure 2). In pond water, the relative abundance of *Verrucomicrobia* was positively correlated with chloride and TM7 and *Planctomycetes* were positively correlated with turbidity. Additionally, *Planctomycetes* was positively correlated with ambient temperature and water temperature. Conversely, the relative abundance of *Proteobacteria* and *Chloroflexi* were negatively correlated with dissolved oxygen and *Bacteroidetes* was negatively correlated with ambient temperature, water temperature and turbidity. In reclaimed water, the relative abundance of *Bacteroidetes* and *OD1* were positively correlated with pH, while *Chloroflexi* was positively correlated with both conductivity and salinity. Negative correlations with nitrate and pH were observed for *Planctomycetes* and *Actinobacteria,* respectively, while *Verrucomicrobia* was negatively correlated with both dissolved oxygen and pH. Additionally, the relative abundance of *Bacteroidetes* in reclaimed water samples was negatively correlated with conductivity and salinity.

Shotgun Taxonomic Analysis by Site and by BrdU treatment

In analyzing our metagenomic data, the predominant bacterial profiles in BrdU-treated pond water samples included *Actinobacterium*, *Flavobacterium, Alpha proteobacteria, Propionibacterium, Polynucleobacter necessarius, Opitutaceae,*

Pseudomonas mandelii and *Sediminibacterium* spp., while non-BrdU treated samples were dominated by *Actinobacterium, Alpha proteobacteria, Sediminibacterium salmoneum, Opitutaceae, Polynucleobacter necessarius, Pseudomonas mandeii* and *Beta proteobacterium* (Figure 3A). The predominant bacterial profiles in reclaimed water samples treated with BrdU included *Flavobacterium, Sediminibacterium salmoneum, Aeromonas media, Propionibacterium, Pseudomonas, Aeromonas hydrophila, Flavobacterium sasangense, Pseudomonas fluorescens, Pseudomonas spp.* and *Arcobacter spp.,* while non-BrdU treated samples were dominated by *Actinobacterium, Sediminibacterium salmoneum, Opitutaceae, Polynucleobacter necessarius, Psuedomonas mandeii, Flavobacterium sasangense, Sediminibacterium spp.* and *Beta proteobacterium* (Figure 3A).

When bacterial relative abundances were compared between different sampling months, more bacterial profiles were abundant in the non-BrdU treated water compared to the BrdU-treated water, irrespective of water type (3B and 3C). In pond water (Figure 3B), a high relative abundance of *Actinobacterium* was observed in both treatments throughout the sampling period and was found to be highest in BrdU treated water samples during April, May and June. Though *Alpha proteobacteria* was observed throughout the sampling months, it was found to be abundant in the non-BrdU treated fraction. *Flavobacterium,* on the other hand, was characterized by patterns similar to those observed in reclaimed water samples. Additionally, we observed unclassified *Planctomycetaceae* abundant in the BrdU treated fraction from June to September.

Contrary to pond water, in reclaimed water (Figure 3C), *Actinobacterium* was typically observed at a higher relative abundance in the non-BrdU-treated fraction while *Flavobacterium* was present from August through November sampling months at a higher relative abundance in BrdU-treated water samples. *Aeromonas media* was predominant in the metabolically-active fraction (BrdU-treated) from April through June. Additionally, we observed the highest relative abundance of *Pseudomonas* (March) and *Propionibacterium* (July) in the metabolically-active fraction (BrdU-treated samples).

Shared and Unique Shotgun Bacterial Profiles by Site and by BrdU treatment

Bacterial profiles unique to pond water samples included *Sediminibacterium sp., Pseudomonas mandeii, Polynucleobacter necessaries, Opitutaceae* and *Alpha proteobacterium*. In contrast, bacterial profiles unique to reclaimed water samples included *Aeromonas media, Aeromonas hydrophila, Flavobacterium sasangense, Pseudomonas* and *Sediminibacterium salmonuem. Flavobacterium* spp. and *Actinobacterium* were shared between both water sample types (Figure 4A).

The BrdU-treated reclaimed water samples included *Rhizobium*, *Sphingopyxis sp.*, *Pseudomonas* spp., *Pseudomonas fluorescens*, *Propionibacterium*, *Arcobacter* spp., *Aeromonas media*, and *Aeromonas hydrophila* (Figure 4B). The unique bacterial profile found in the BrdU-treated pond water samples included *Actinobacterium*, *Pseudomonas fluorescens*, *Flavobacterium sp*. and *Raphidiopsis brookii* were (Figure 4C).

Antimicrobial Resistance Genes and Virulence Genes

The relative abundance of AMR genes was higher in reclaimed water samples compared to pond water samples, and in non-BrdU-treated samples compared to BrdU-treated samples for both water types (Figures 5A and 5B). Additionally, we observed that AMR genes could be detected throughout the year in reclaimed water samples (except in August and September), but were primarily detected only from August through October in pond water samples. The efflux pump gene msrE, beta-lactam resistance gene blaOXA, quinolone resistance gene qnrS2, macrolide resistance gene mphE, macrolide resistance gene emrF, aminoglycoside resistance gene aadA6, sulphonamide resistance gene sul2, and mphA gene that inactivates 14-membered-macrolides were the prominent AMR genes detected in BrdU-treated reclaimed water samples, while only the aminoglycoside resistance gene aph3' was detected in BrdU-treated pond water samples.

Similar to the AMR data, we observed a higher relative abundance of virulence genes detected at the species level throughout the sampling period in reclaimed water samples compared to pond water samples (Figure 6A and 6B). Virulence genes predicted to be present within *Klebsiella pneumoniae* dominated in BrdU treated reclaimed water while those predicted to be present within *E. coli* dominated in BrdU treated pond water. Other virulent species observed in BrdU treated reclaimed water included *Enterobacter aerogenes, Proteus mirabilis, Pseudomonas aerogenes, Vibrio cholerae, Salmonella typhimurium* and *Salmonella infantis*. In contrast, *Proteus mirabilis, Pseudomonas aerogenes* and *Pseudomonas*

putida were observed in BrdU treated pond water. *Bacteroides fragilis* was observed in both non-BrdU treated water types.

Discussion

While pond and reclaimed water sources may be attractive alternative irrigation water sources, our data show that additional water treatment may be needed to ensure that bacterial water quality is appropriate for the intended use. Our study showed that the bacterial communities of both reclaimed water samples and pond water samples are diverse and may include bacterial species of importance to human health. Moreover, through our novel approach of coupling BrdU-labeling with 16SrRNA and shotgun sequencing, we could tease out the metabolically-active fraction of the bacterial communities present in the tested water samples. Finally, our data showed that, while diverse antibiotic resistance and virulence genes were detected in both reclaimed water and pond water, these genes were more frequently identified in non-BrdU-treated samples compared to BrdU-treated samples, implying that these genes may be associated more with relic (inactive) DNA present in the water samples rather than viable, metabolically-active organisms.

The presence of *Actinobacterium* spp., *Flavobacterium* spp., *Aeromonas media*, *Aeromonas hydophila*, *Propioniobacterium* spp., *Pseudomonas fluorescens* and *Arcobacter* spp.—species or genera containing specific strains that have been associated with human and/or animal diseases—was observed in BrdU-treated water samples representing the metabolically-active fraction of the detected bacterial communities (Figure 3). *Actinobacterium* spp., in particular dominated in BrdU-treated pond water and via our 16S rRNA sequencing data we identified that hgcl-

clade was the *Actinobacterium* spp. that was predominant. This finding corroborates with other studies that have looked into freshwater microbiota [159, 160]. Our 16S rRNA sequencing data also revealed the presence of currently non-culturable members [161] of the phylum *Actinobacterium* (*Candidatus* Aquiluna*, Canditatus* Rhodoluna*, Candidatus* Planktoluna and *Candidatus* Planktophila*)* in both BrdU-treated and non-BrdU-treated samples. These bacterial species would have gone undetected in a culture-based study, and while they have been previously identified in water through sequencing studies [161, 162], to our knowledge, our data are the first to demonstrate that these organisms appear to be metabolically-active in both reclaimed water and pond water.

Aeromonas, *Arcobacter, Pseudomonas* and *Propionibacterium spp.* were observed at a high relative abundance across our BrdU-treated reclaimed water samples. *Aeromonas spp*. are ubiquitous in nature and found in terrestrial and aquatic milieus throughout the world [163]. They are detected globally in a broad range of foods, surface water, ground water, and bottled mineral water, as well as in chlorinated and non-chlorinated drinking water [164]. They are Gram-negative, rod-shaped, facultative anaerobes and some species are emerging as important enteric pathogens of concern to public health. *A. hydrophila*, a species detected at a high relative abundance in BrdU-treated reclaimed water is an important foodborne pathogen and is widely distributed in aquatic environments [164]. In 2012, a foodborne outbreak of *A. hydrophila* in a college in China was associated with salad ingredients washed in contaminated water [166]. Additionally, many strains of *A. hydrophila* are highly resistant to commonly prescribed antibiotics in clinical

medicine [163]. *Arcobacter spp*. [167–171] have been regarded as an underestimated enteropathogen present in environmental samples, especially in untreated water. These bacteria are very similar to *Campylobacter spp*. and hence have been misclassified and misdiagnosed in many instances [168, 170, 171].

Pseudomonas fluorescens, detected in BrdU treated pond and reclaimed water in this study*,* is another opportunistic pathogen that inhabits multiple environments including soil, water and plant surfaces [172]. They are typically resistant to a wide array of antibiotics [173] and have been associated with nosocomial infections like blood transfusion-related septicemia, catheter-related bacteremia, and peritonitis [174]. *Propionibacterium spp*. also detected at a high relative abundance in BrdU treated reclaimed samples, are nonsporulating Gram-positive bacilli and are common skin commensals [175]. These bacteria are usually non-pathogenic; however, some cases of endocarditis caused by *Propionibacterium spp*. have been reported [175]. The presence of these bacteria in BrdU-treated reclaimed water, indicating that they are likely metabolically-active, provides evidence that the type of reclaimed water tested in this study is likely to require additional treatment if the water is to be used for purposes such as the irrigation of fresh produce.

Some of the *Flavobacterium* spp*.* observed in our study across both water types (within the BrdU treated samples) were *F. aquaticum*, *F. psychrophilum*, *F. cauense*, *F. saliperosum* and F. *sasangense*. Most of these bacteria have been associated with infections in fish [176–182]. To date none of these species have been associated with human infections.

AMR is of growing concern due to use, overuse and misuse of antibiotics, as well as their improper disposal into the environment that has resulted in an increase in antimicrobial-resistant bacteria. In our study, we observed Beta lactam resistance genes (blaOXA) in our BrdU treated reclaimed water samples (Figure 5A). Additionally, we documented the presence of *Klebsiella pneumoniae*, a Gram negative opportunistic pathogen known for its high frequency and diversity of AMR genes (61) in BrdU-teated reclaimed water types (Figure 6A). These bacteria are known to cause a range of diseases (pneumonia, thrombophlebitis, urinary tract infection (UTI), bacteremia and septicemia) [184] and act as key traffickers of drug resistance genes from environmental to clinically-important Gram negative bacteria [183]. Previous studies have shown the presence of these blaOXA genes in *Klebsiella pneumoniae*, conferring resistance to ampicillin, ticarcillin, piperacillin, and cephalosporins [185].

Similarly, in BrdU treated pond water we observed aminoglycoside aph 3' genes and *E. coli* (Figure 5B and 6B). Aminoglycosides are an important class of antibiotics that includes clinically important drugs such as gentamicin, amikacin, tobramycin, and streptomycin that are extensively used to treat many bacterial diseases [186]. Recently, the emergence of aminoglycoside-resistant *E. coli* has been observed primarily due to the presence of modifying enzymes like aminoglycoside phosphotransferases (e.g. aph3') [187]. Infection with these AMR bacteria are difficult to treat in both humans and animals.

Conventional screening approaches for antibiotic-resistant bacteria involves plating different samples on non-selective or antibiotic selective agar plates, purifying

the bacterial colonies and using different methods to determine the minimum inhibitory concentration (MIC) for a wide array of antibiotics [188]. Since this is time-consuming, newer molecular based techniques like PCR, microarrays and next-generation sequencing techniques are being used. But, using all of the above-mentioned DNA based techniques, there is lack of knowledge whether the metabolically-active bacterial fraction of an environmental sample actually carries these genes or not. Here in our study, both AMR and virulence genes were observed at a higher relative abundance in reclaimed water samples compared to pond water samples. Additionally, we observed a lower abundance of these genes in the BrdU treated water samples, suggesting that AMR and virulence genes present in these water bodies are not predominantly within metabolically-active bacteria. Rather, these genetic determinants may be largely present in persisting relic DNA, and therefore, could be transferred to other environmental bacteria through transformation, rather than horizontal gene transfer, events.

In summary, we described diverse total and metabolically-active bacterial profiles in reclaimed water and an agricultural pond. Additionally, we observed both AMR and virulence gene profiles occurring predominantly in non-BrdU treated water samples. However, more work is required to understand whether the identified metabolically-active bacterial communities can be easily transferred to produce when these water sources are used for irrigation purposes. To our knowledge, this study is the first of its kind that couples BrdU labelling and DNA sequencing techniques to identify metabolically-active bacteria, AMR genes and virulence genes in alternative irrigation water sources in the United States.

Table 1: Water characteristics of reclaimed water and pond water during sampling

Samples	Sampling months	Ambient temp.(C)	Water temp.(C)	pH	DO (%)	Nitrate (mg/ml)	Chloride (mg/ml)	Turbidity (FNU)	Preci.[+] (in.)	Conductivity (SPC uS/cm)	ORP (mV)
Reclaimed water	Mar	-3	5.3	7.4	91.9	22.9	306.6	8.4	0.3	779.3	251.1
	Apr	16.1	18	7	27	2.1	185.2	9.6	0.6	918.7	188.4
	May	9	15.5	7.1	115.2	3.6	147.1	11	0.8	821.2	244.4
	Jun A	28	22.8	7.2	19.3	3.6	240.9	12.1	0.04	787.5	188.3
	Jun B	22	23.7	7	22.6	2	124.1	2.2	0.7	819	248.7
	Jul	27.8	25.4	7	17.2	0.6	206.3	11.1	0.6	791	222.9
	Aug A	21	23.2	7.3	78	3	39.8	12.9	1.1	688	156
	Aug B	27	25.9	8.1	78.7	9.7	84.7	15.5	0.9	732	123.9
	Sep A	18	19.6	8	127.3	5.4	75.9	7.2	1.1	741.3	92.3
	Sep B	28.9	24.2	7.6	59.6	26.3	259.1	7.2	0.02	724.3	290.3
	Oct A	13	18.4	7.5	47.8	20.8	110.2	3.6	0.6	748	235.5
	Oct B	8	15.5	6.8	22.5	11.9	174.9	1.7	1.5	779	191.6
	Nov	6	9	7.8	40.7	23.9	52.3	1.5	0.8	777	225.9
Pond water	Mar	-2.1	7	6.7	102.5	0.6	11.9	2.2	0.5	163.9	198.9
	Apr	21.6	20.5	6.7	103.5	0.2	18.6	2.3	0.6	157.9	219.7
	May	12.1	17.5	8.2	79.4	0.4	602.4	5.1	1.1	165.8	118.8
	Jun A	28.8	26.7	7.8	100.3	0.5	16.4	1.8	0.1	151.2	215.7
	Jun B	23.7	27.1	8.1	94.9	0.6	65.5	3.2	0.7	159.9	115.6
	Jul	28.9	29.5	8.3	113.8	0.3	23	6	0.6	168.5	156.7
	Aug A	30.1	27.9	8.6	104.6	0.6	14.5	0.9	0.6	145.7	199.9
	Aug B	21.7	25.3	7.9	71.4	0.3	19.9	4.9	2.8	147.1	165.8
	Sep A	18	21	8.2	81.4	0.2	11.9	4.3	1.2	151	196.7
	Sep B	26.1	24.6	7.7	60.2	0.4	18.1	3.6	0.01	154.9	170.5
	Oct A	15.5	20.3	9.2	62.2	1	6.6	3.2	0.7	160.1	40.9
	Oct B	9.1	14.7	9.1	86.5	0.4	36	1.1	1.8	162.2	115.3
	Nov	5.4	10	9.01	100.7	1.1	35.6	1.2	1.1	163.9	152.8
	Dec	5.7	5.5	9	99.6	1.2	76.5	0.8	0.2	168.2	238.9
	Jan	13.1	4.4	10.1	99.2	0.7	33.2	2.6	0.2	142.3	192.1

[+]24 hour prior to sampling
A and B- represents two sampling dates in the same month

Figures

Figure 1: (A) Violin plots of alpha diversity (Observed number of species and Shannon Index) across pond water and reclaimed water samples on rarefied data to minimum sampling depth. Alpha diversity of BrdU-treated samples represents the diversity observed in the metabolically-active fraction of bacterial communities present in each sample. Blue represents pond water and orange represents reclaimed water. (B) PCoA analysis of Bray Curtis computed distances between BrdU- and non-BrdU-treated pond and reclaimed water samples. Red depicts BrdU-treated samples and yellow depicts non-BrdU treated samples while the shape represents the sites: circle=pond water, and triangle=reclaimed water. Solid colored ellipses are drawn at 95% confidence intervals for sites, while dashed colored ellipses are drawn at 95% confidence intervals for treatments in each water source. (C) 16S rRNA taxonomy and relative abundance of top 25 bacterial profiles identified by site and by BrdU treatment.

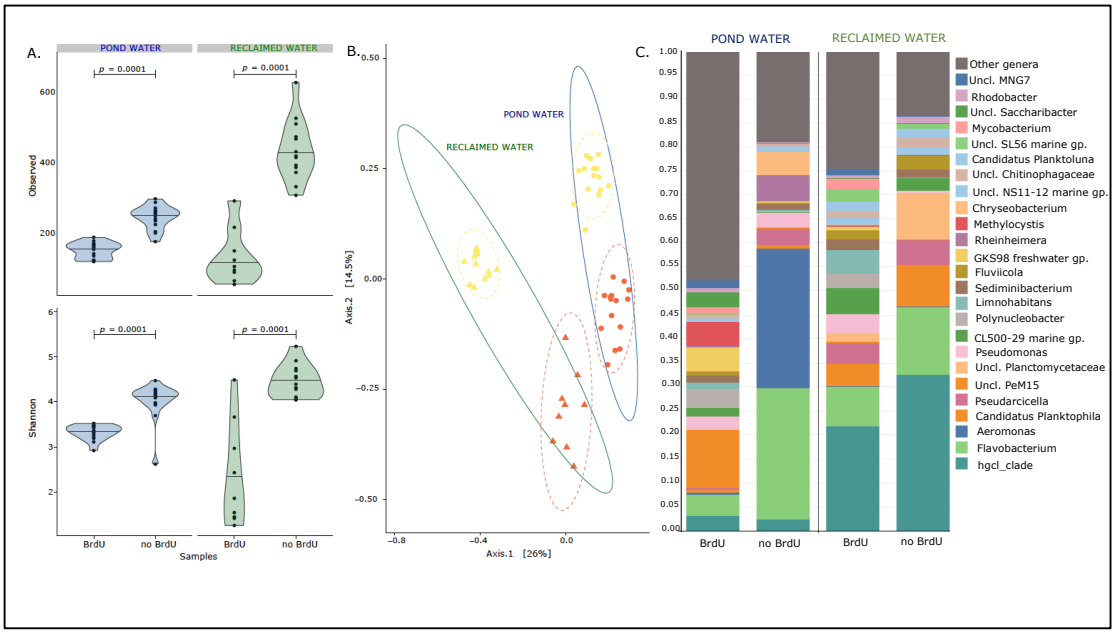


Figure 2: Heatmaps of Pearson's correlation coefficients between the water characteristics and relative abundance of bacterial phyla observed via 16S sequencing for the different water types. Color gradients reflect the different values of Pearson's correlation coefficients. ORP: Oxidation/reduction (mV), DO: Dissolved Oxygen (%).

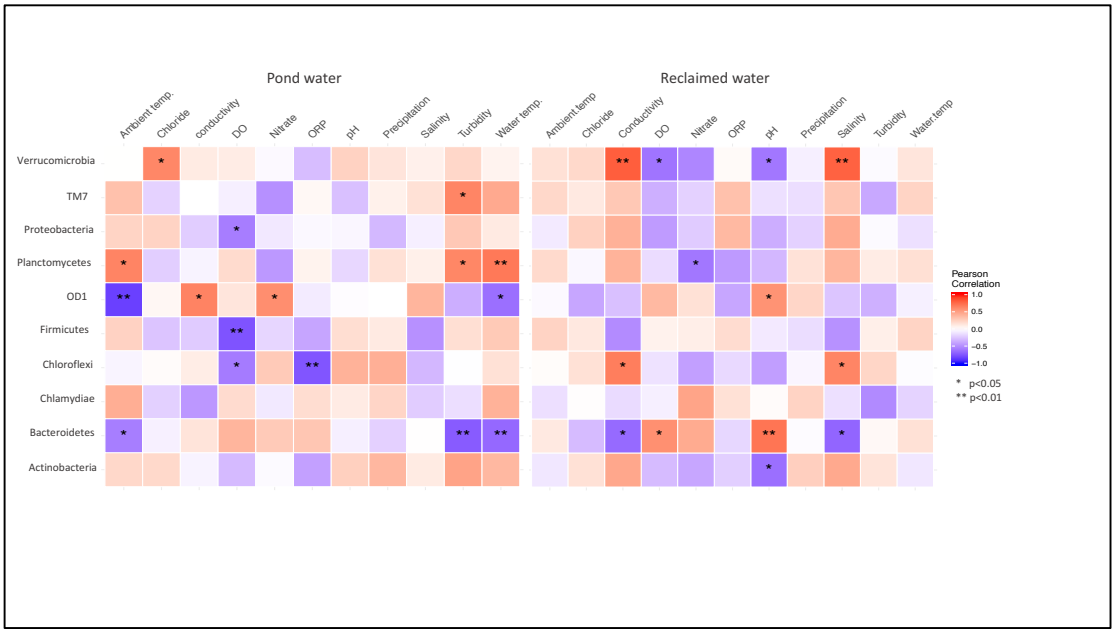


Figure 3: Shotgun taxonomic profiles of the bacterial microbiota of pond water and reclaimed water samples derived from shotgun metagenomic data. (A) Overall taxonomy and relative abundance of bacterial profiles identified by site and by BrdU treatment using a k-mer based approach developed by Cosmos ID. Relative abundance of bacterial profiles identified by treatment in (B) pond water and (C) reclaimed water over the entire sampling period.

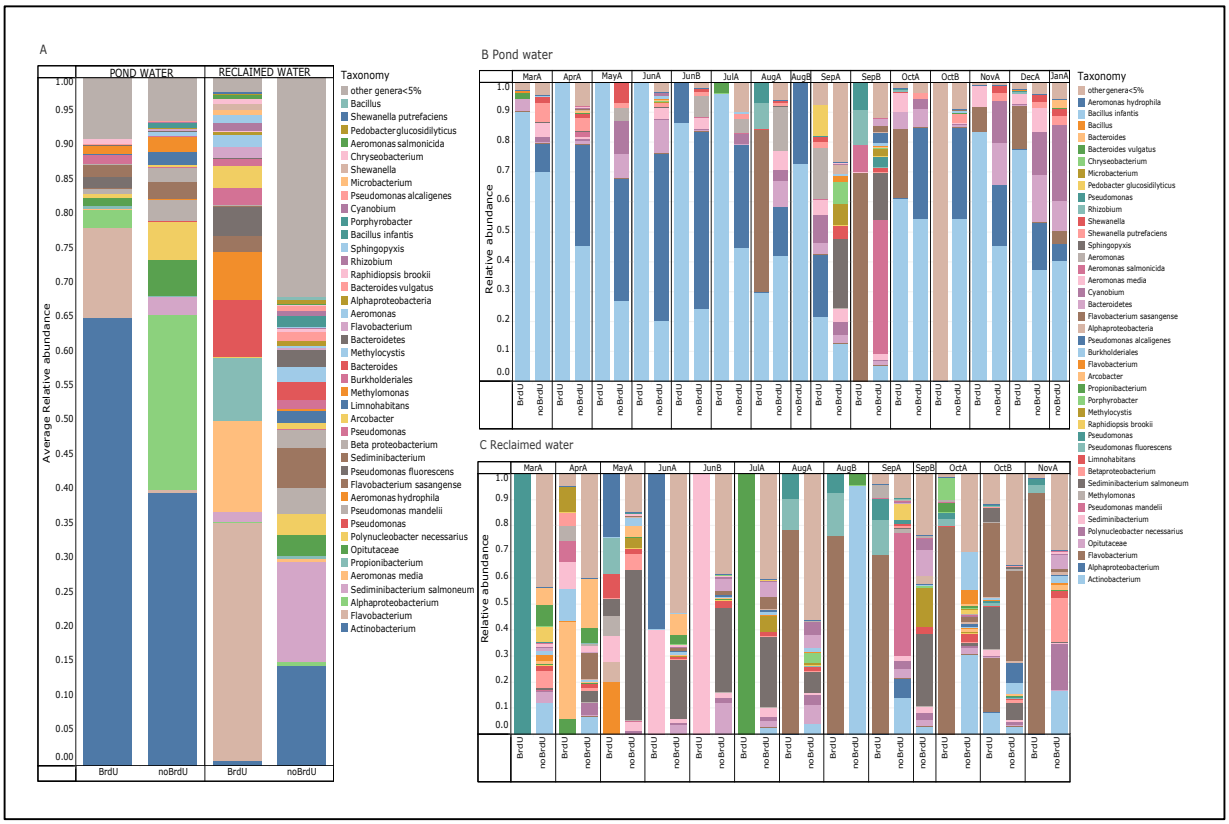


Figure 4: Shared and unique bacterial profiles visualized by chord plots between overall pond and reclaimed water samples (A); BrdU and non-BrdU-treated in reclaimed water samples (B); and BrdU and non-BrdU-treated in pond water samples (C).

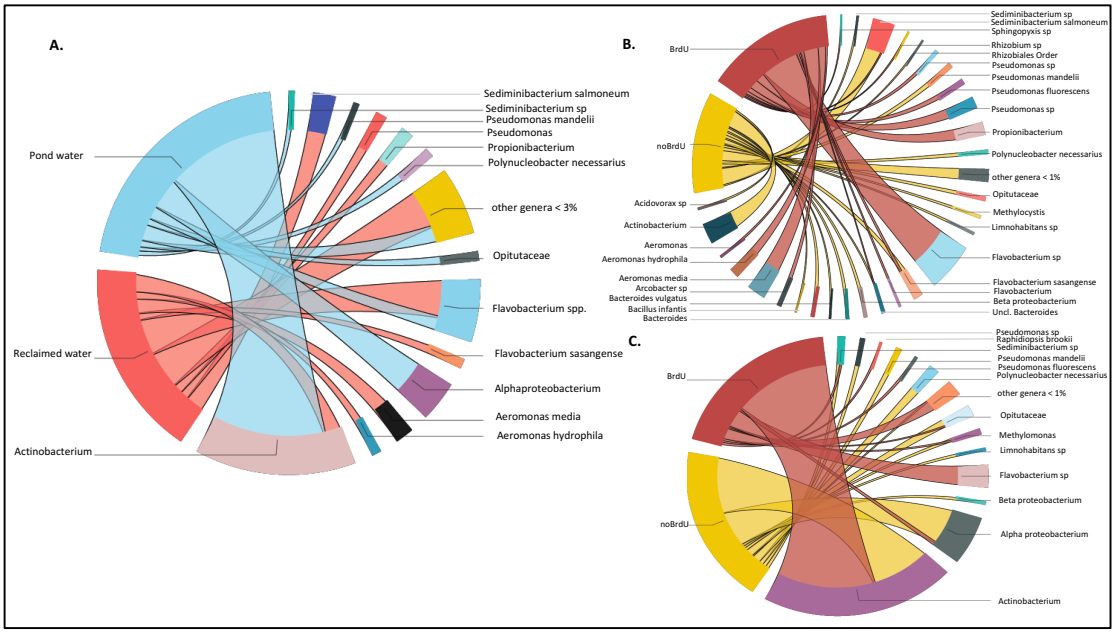


Figure 5: Relative abundance of antimicrobial resistance genes in reclaimed water (A) and pond water (B) samples by BrdU-treatment and by sampling month.

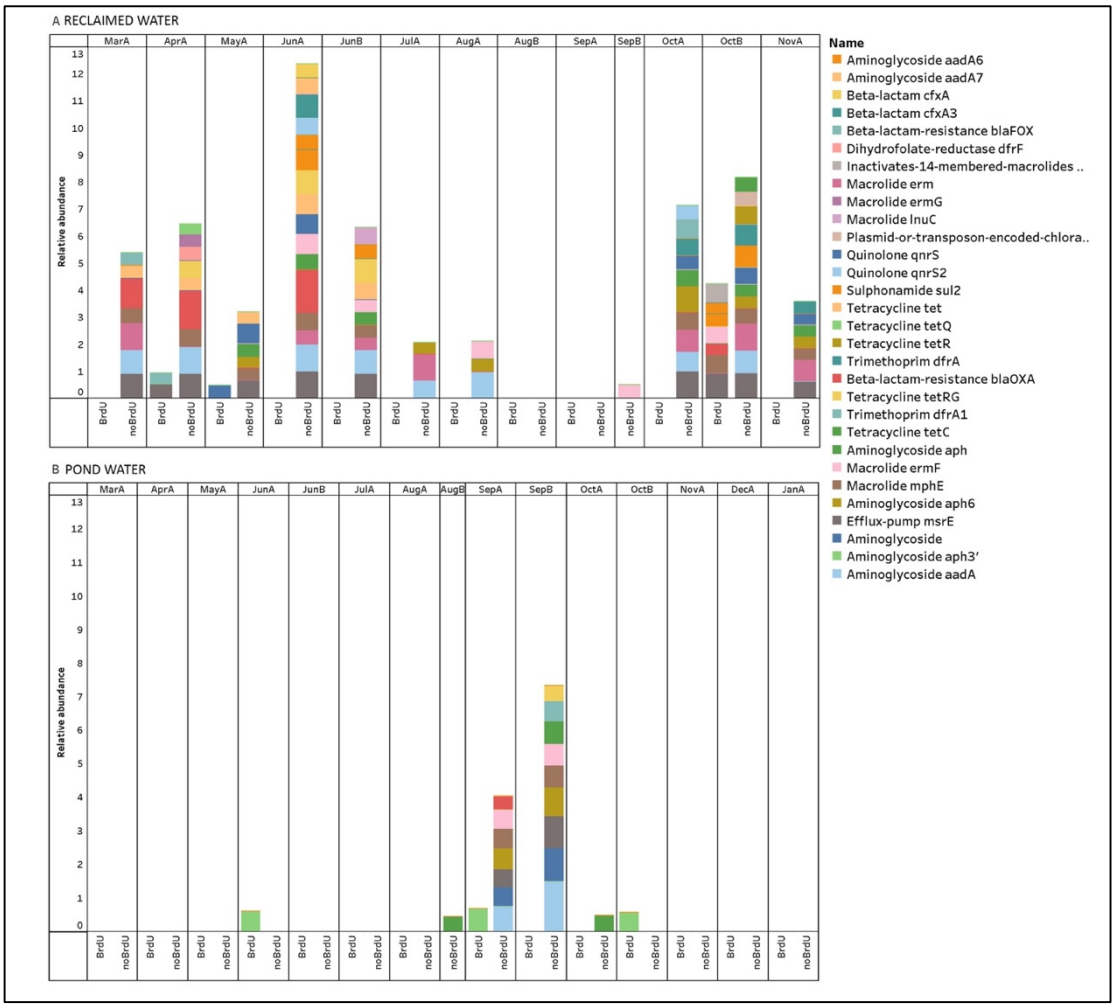
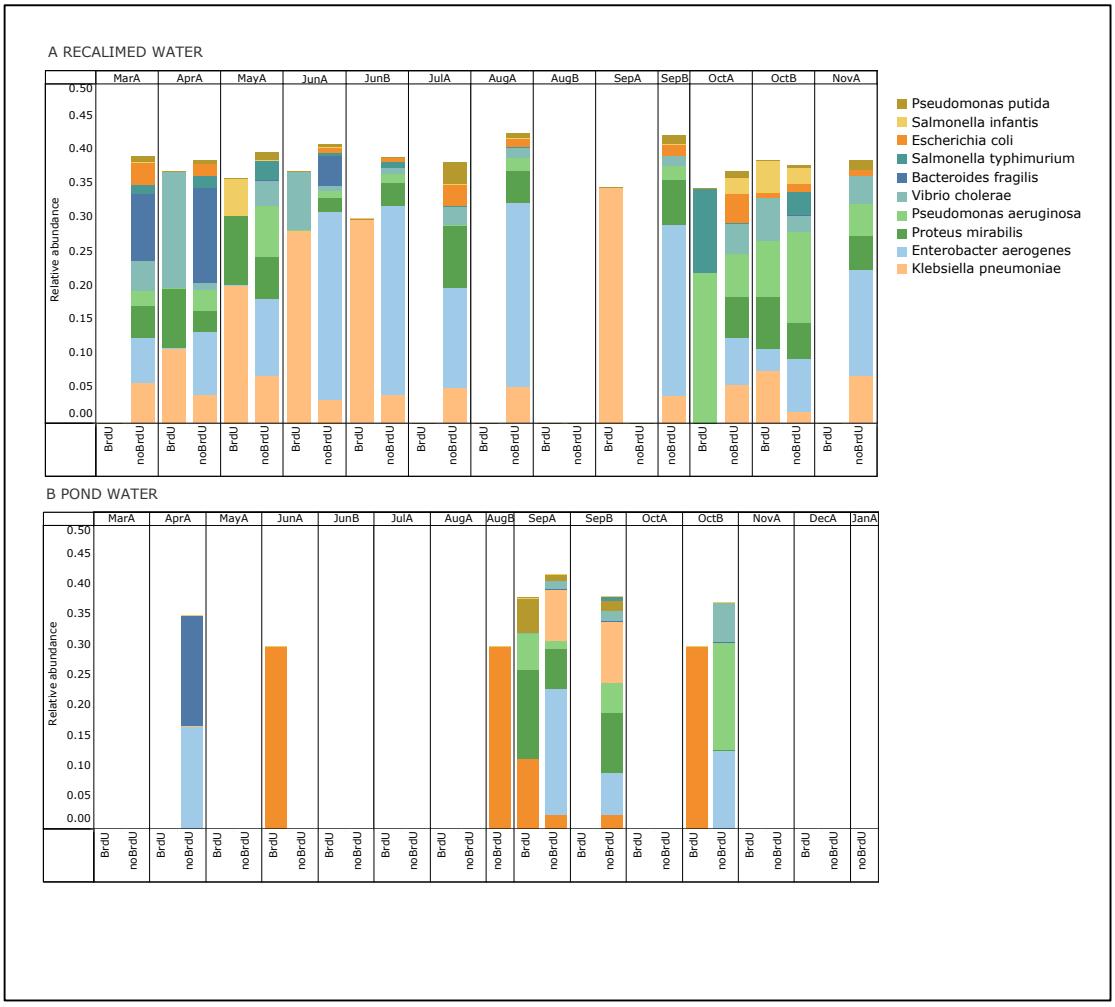
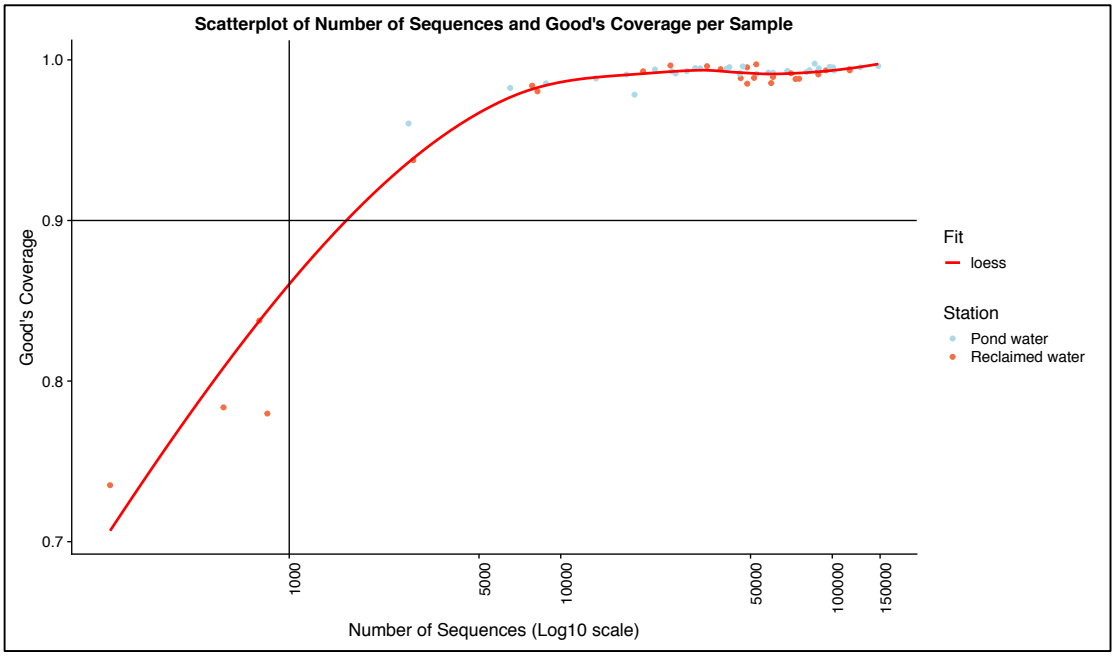


Figure 6: Virulence genes detected at the species level in (A) reclaimed water and (B) pond water by sampling month.

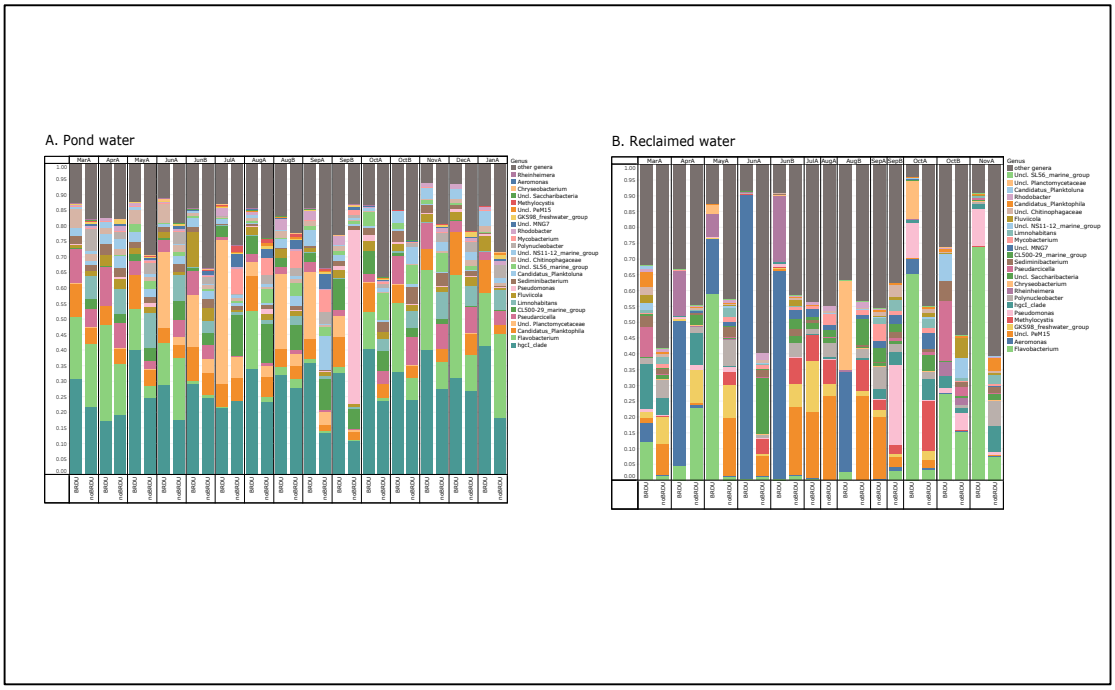


Supplementary Figures

S1: Good's coverage among reclaimed and pond water samples (n=56)



Supplementary Figure 2: Relative abundance of top 25 bacterial profiles identified by treatment in (B) pond water and (C) reclaimed water over the entire sampling period



Chapter 4: Coupled DNA-labeling and sequencing approach enables the detection of viable-but-non-culturable *Vibrio* spp. in irrigation water sources in the Chesapeake Bay watershed

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Abstract

Brackish waters are being explored as potential irrigation water sources to ensure future food security due to immense pressure on existing freshwater resources. However, brackish waters may harbor human pathogens like *Vibrio* species. Thus, there is a need to improve understanding of the prevalence of *Vibrios* in tidal brackish water intended for use as an irrigation water source. Nevertheless, the presence of viable-but-nonculturable (VBNC) *Vibrio* spp. in brackish water stymies our existing detection methods. To overcome this knowledge gap, we used a combination of 5-bromo-2'-deoxyuridine (BrdU) labeling, enrichment techniques, along with 16S rRNA sequencing to identify the metabolically-active fraction of *Vibrio* spp. in irrigation water from four sites (reclamation plant, pond, non-tidal freshwater creek and tidal brackish water creek) from May to September 2018 (n=180 samples). Additionally, standard culture methods were used to enumerate *Vibrios* in all enriched non-BrdU treated water samples. Our coupled DNA-labeling and sequencing method revealed the presence of metabolically-active *Vibrio* spp. in all sampling sites, while the culture method only showed the presence of *Vibrios* in three of the four sampling sites. We observed the presence of *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus* using both methods. Interestingly, we were also able to detect the presence of metabolically-active *Vibrios* in non-enriched BrdU treated samples. This approach not only refines our understanding of the prevalence of live VBNC *Vibrios* but can be applied to develop appropriate on-farm water treatment technologies that may be necessary to improve the quality of brackish water sources as climate change continues to impact our freshwater resources.

Importance *Vibrio* spp. tend to harbor in brackish waters, which are being explored as alternative irrigation water sources due to increasing pressure on our freshwater irrigation resources. Previous studies have attributed foodborne illness to the presence of *Vibrio* spp. in irrigation waters. Current detection methods lack the ability to identify the presence of VBNC *Vibrio* spp. in brackish waters. Hence, in our study we used a culture independent method using 5-bromo-2'-deoxyuridine (BrdU) labelling in tandem with DNA sequencing and traditional culture methods to identify the metabolically-active VBNC *Vibrio* spp. in four sites in the Mid-Atlantic region from May to September 2018. The significance of our research is that a refined understanding of the prevalence of *Vibrio* spp. in these irrigation waters was achievable via our novel approach, which in turn could help farmers to plan for on farm mitigation strategies for intended irrigation use.

Introduction

As global freshwater resources are rapidly being depleted—due to population growth, climate change, overpumping of aquifers and other factors—states and nations are relying more heavily on nontraditional irrigation water sources (e.g. recycled water, brackish water) to ensure agricultural water security and prevent food insecurity [189, 190]. In some semi-arid and arid regions of the world, brackish water is the only remaining irrigation water source available to farmers [191, 192]. The United States Geological Survey (USGS) defines brackish waters as having a dissolved-solids concentration between 1,000 and 10,000 mg/L, which is greater than that of freshwater (> 1,000 mg/L), but lesser than that of seawater (35,000mg/L) [193]. In the semi-arid and arid regions of United States and other regions, brackish water use has been largely restricted to relatively salt tolerant crops including cotton, sugarbeets, barley, wheat, safflower, sorghum, soybeans and tomatoes [192, 194–197]. The effects of salt stress on plants when irrigated with brackish water are well described in the literature [198–200], and multiple mitigation strategies are being explored to enable these water sources to be suitable for irrigation purposes [194–197].

Besides salinity, these brackish water sources are known to harbor important human pathogens. *Vibrio* spp., for instance, are natural inhabitants of coastal, brackish waters of the Chesapeake Bay, the Pacific ocean and the Gulf of Mexico and include frank pathogens such as *V. cholera*, *V. parahaemolyticus* and *V. vulnificus* [42–44, 201]. Additionally, *Vibrio* spp. have been recovered from surface waters, such as rivers, creeks, and irrigation canals [202–204], as well as reclaimed water

[203, 205, 206]. Human *Vibrio* infections can occur among people consuming raw or undercooked shellfish and among those working or recreating in contaminated waters [42, 43, 45, 46]. If *Vibrio*-contaminated water is also used to irrigate food crops that are eaten raw, this practice could represent an additional exposure pathway for human *Vibrio* infections [207, 208].

Hence, there is a need to further our understanding of the prevalence of *Vibrio* spp. in potential irrigation water sources. Nevertheless, previous studies have provided evidence that *Vibrio* spp. can enter a viable-but-non-culturable (VBNC) state [47–49, 209, 210], limiting the ability of traditional culture methods to assess the true prevalence of these microorganisms in water bodies. On the other hand, use of culture-independent, DNA-based techniques such as PCR and sequencing alone do not provide information on the viability of detected *Vibrios* in these water sources, since DNA detected through these methods can be derived from either dead or live organisms [10, 11, 15]. Thus, the goal of this study was to evaluate whether coupling 5-bromo-2'-deoxyuridine- (BrdU) labeling with next generation sequencing methods could enable the detection of VBNC *Vibrios,* as well as the differentiation between metabolically-active and dead organisms in nontraditional irrigation water sources. BrdU binds to replicating DNA; therefore, bacteria detected in BrdU-treated samples are interpreted to be metabolically-active, viable members of the tested bacterial community [125].

Methods

Sampling sites and sample collection

Existing sampling sites characterized through CONSERVE: A Center of Excellence at the Nexus of Sustainable Water Reuse, Food and Health (www.conservewaterforfood.org) were leveraged for this study: one tidal brackish water river, one non-tidal freshwater creek, one agricultural pond and one water reclamation facility. Preliminary biweekly bacterial monitoring data from these sites for the period of September 2016 to September 2017 (data not shown) revealed the presence of *Vibrio* spp. Hence, 4 L grab samples from each site were then collected over the course of five months (May 2018 to September 2018) to further characterize the presence of *Vibrio spp*. via culture-dependent and -independent methods.

Additionally, throughout our sampling period, the following water quality parameters were measured in triplicate using a ProDSS digital sampling system (YSI, Yellow Springs, OH, USA): water temperature (°C), conductivity (SPC uS/cm), pH, dissolved oxygen (%), oxidation/reduction potential (mV), turbidity (FNU), nitrate (mg/L), and chloride (mg/L) and and precipitation (inches) data within the last 14 days were also obtained from Weather Underground (https://www.wunderground.com/)

Sample Processing

All samples were subjected to both BrdU labeling (1,500 mL) and non-labeling (control subsamples, 1,500 mL).

Non-BrdU labeled water samples: Three 500 mL aliquots of each water sample were filtered through a 0.2μM filter and then subjected to one of the following: 1) enrichment with alkaline peptone water (APW) (30 mL); 2) enrichment with estuarine peptone water (EPW) (30 mL); or 3) no enrichment (control sample).

BrdU labelled water samples: A separate 500mL aliquot of each water sample was also subjected to BrdU treatment (100mL of 100mM BrdU per 500mL) and incubated for 2 days in the dark at room temperature. After incubation, the BrdU-labeled water sample was subjected to one of the following: 1) enrichment in APW; 2) enrichment in EPW; or 3) no enrichment (control sample).

Sample incubation and cultivation: All enrichments (BrdU labeled or not) and non-enriched control samples were incubated at 30°C for 18-20 h. Additionally, a loopful of growth from the enriched water samples (non-BrdU treated) were transferred to thiocitrate bile salt (TCBS) agar and incubated for 16-24 hours at 35°C. All colonies presenting as yellow (sucrose positive) or green (sucrose negative) on TCBS were selected and subjected to three rounds of streaking for purification and isolation. DNA of resulting purified isolates was then extracted using a heat shock method, which involves isolates being exposed to 100^0C heat and then suddenly being shocked by placing in ice.

Multiplex PCR detection of Vibrio genus

To detect five pathogenic *Vibrio* species, a multiplex PCR amplification of the heat shocked isolates was performed following a published protocol [211]. The amplified products were then viewed via gel electrophoresis.

DNA extraction and 16SrRNA Gene Amplification and Sequencing

DNA extractions on all enriched and non-enriched BrdU-labeled and non-labeled water samples were performed using protocols previously published by our group [141, 142]. Briefly, 1 mL of PBS was added to 1) filters (non-enriched samples) and 2) cells that were pelleted , by centrifuging at 2,450 x *g* for 20 min (enriched samples) in the lysing matrix B tubes, before incubation in enzymatic cocktails containing lysozyme, mutanolysin, proteinase K and lysostaphin, after which the cells were mechanically lysed using an MP Biomedical FastPrep 24 (Santa Ana, CA). The DNA was then purified using the Qiagen QIAmp DNA mini kit (Germantown, MA) per the manufacturer's protocol.

Extracted DNA was then PCR amplified for the V3-V4 hypervariable region of the 16SrRNA gene using the universal primers 319F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) and sequenced on an Illumina HiSeq2500 (Illumina, San Diego, CA) using a method developed at the Institute for Genome Sciences [212] and described previously [141, 142].

16S rRNA sequencing analysis

Following sequencing, 16S rRNA paired-end read pairs were assembled using PANDAseq [144], de-multiplexed, trimmed of artificial barcodes and primers, and assessed for chimeras using UCHIME in *de novo* mode implemented in Quantitative Insights Into Microbial Ecology (QIIME; release v.1.9.1) [145]. Quality trimmed

sequences were then clustered *de novo* into Operational Taxonomic Units (OTUs) and taxonomic assignments were performed using VSEARCH [146] with a minimum confidence threshold of 0.97. The SILVA 16S database [147] in QIIME [145] was used for taxonomic assignments. Downstream data analysis and visualization was completed in RStudio (v.1.1.423) using R packages: biomformat (v.1.2.0) [148] vegan (v.2.4-5) [149], ggplot2 (v.3.1.0) [150], phyloseq (v.1.19.1) [151], and metagenomeSeq (v.1.16.0) [153]. All sequences taxonomically assigned to the Phylum *Cyanobacteria* were removed from further downstream analysis. When appropriate, data were normalized with metagenomeSeq's cumulative sum scaling (CSS) [153] to account for uneven sampling depth. Prior to normalization, alpha diversity was measured using both the Observed richness metric and the Shannon diversity index [154]. Bray-Curtis dissimilarity was used for calculating beta diversity and was compared using analysis of similarities (ANOSIM) on normalized data (999 permutations).

Results

Water quality characteristics

Water quality characteristics of the four sampling sites (non-tidal freshwater creek, reclaimed water, tidal brackish river and freshwater pond) are shown in Table 1. Overall, ambient temperatures, irrespective of sampling site, increased from May to September 2018. Conductivity, nitrate and chloride levels were higher in the tidal brackish creek compared to the other sampling sites. pH ranged from slightly basic to

neutral. Dissolved oxygen was higher in the freshwater pond compared to all other sampling sites.

Culture data

After 3 rounds of isolation and purification, 87 sucrose-positive (yellow) and 28 sucrose-negative (green) pure colonies were obtained from TCBS agar resulting in a total of 115 presumptive *Vibrio* isolates from the four sites during the entire sampling period. Of the 115 presumptive *Vibrio* isolates, 28 (24%) isolates were confirmed via multiplex PCR as *Vibrio*s: 16 (13.9%) were positive for *V. cholerae*, 11 (9.6%) were positive for *V. parahaemolyticus* and 1 (0.87%) was positive for *V. vulnificus*. The *V. cholerae* isolates were predominantly from the tidal brackish water (13 isolates), followed by the non-tidal freshwater creek (2 isolates) and the reclaimed water (1 isolate). *V. parahaemolyticus* isolates were also predominantly from the tidal brackish water (9 isolates), and two isolates were obtained from the reclaimed water sample. The one *V. vulnificus* isolate was recovered from the reclaimed water source.

16S rRNA sequencing dataset

Extracted DNA from a total of 180 samples as described in Table 2 (n=48 pond water, n=48 reclaimed water, n=36 non-tidal fresh water creek and n=48 tidal brackish water) was PCR amplified for the 16S rRNA gene and sequenced using the Illumina HiSeq platform. 6,302,683 sequences were generated in total across all samples, and clustered into 17,237 operational taxonomic units (OTUs). Across all samples, the minimum number of reads was 357 and the maximum was 99,944, with

an average number of sequences per sample of 35,014.91 (+/-14,897.3 SD). A Goods estimate coverage of 0.90 was calculated for all samples and 3 control samples that were not enriched (1 reclaimed water, 1 pond water and 1 non-tidal fresh water creek sample) and had a Good's estimate coverage <0.90 were removed to ensure appropriate read coverage across all samples analyzed downstream (Supplementary Figure S1). After data cleanup (removing reads assigned to taxa '*Cyanobacteria*' and OTUs with less than 10 reads), the total number of sequences used in downstream analyses was 6,020,192 from 177 samples (n=47 pond water, n=47 reclaimed water, n=35 non-tidal fresh water creek and n=48 tidal brackish water), clustered into 7,298 OTUs.

Alpha and Beta Diversity

Alpha diversity metrics (Shannon diversity) were calculated on both rarefied (after down-sampling each sample to 2,901) and non-rarefied data (data not shown) to avoid sequence coverage issues. Since no differences were observed between the rarefied and non-rarefied analysis, we only presented alpha-diversity analysis performed on the rarefied dataset in Figure 1A. Irrespective of sampling site/water type, the alpha diversity of BrdU-treated samples (Shannon: 156.74 +/- 89.69) was significantly lower ($p < 0.001$) when compared to non-BrdU treated samples (Figure 1A).

Principal coordinate analysis using Bray Curtis distances was implemented to quantify the inter-sample diversity (beta diversity). The analysis revealed that bacterial profiles associated with BrdU-treated samples were similar to the non-BrdU

treated samples and showed slight variation by treatment (ANOSIM R value =0.2241, p=0.001) (Figure 1B.).

Taxonomical analysis

The top five bacterial phyla identified across all sampling sites irrespective of treatments and enrichments were *Proteobacteria*, *Firmicutes*, *Bacteroidetes, Actinobacteria*, and *Fusobacteria*. The most predominant phyla with an average relative abundance of 44.55% (+/-0.21) was *Proteobacteria*, followed by *Firmicutes* that had an average relative abundance of 24.40% (+/-0.26). *Bacteroidetes, Actinobacteria* and *Fusobacteria* had an average relative abundance of 15.58% (+/- 0.18), 11.45% (+/- 0.13) and 0.96% (+/- 0.04) respectively.

In total, 2,205 (30%) OTUs were assigned to the genus level of which only 351 (5%) could be identified to the species level. The top 25 bacteria across all sampling sites, enrichments and treatments were *Clostridium bifermentans,* Unclassified *Aeromonadaceae,* Unclassified *Pseudomonas, Bacillus cereus, Flavobacterum succinicans,* Unclassified *Citrobacter* Unclassified ACK-M1*,* Unclassified *Flavobacterium,* Unclassified *Actinomycetales, Lysinibacillus boronitolerans,* Unclassified *Enterobacteriaceae,* Unclassified *Serratia,* Unclassified *Cytophagaceae,* Unclassified *Rummeliibacillus, Clostridium metallolevans,* Unclassified *Rhodobacter, Unclassified C111,* Unclassified *Exiguobacterium,* Unclassified *Fluviicola, Unclassified Novosphingobium, Plesiomonas shigelloides,* Unclassified *Chitinophagaceae*, Unclassified *Microbacteriaceae*, Unclassified C39 and Unclassified *Vibrio* (Figure 2).

Differential abundance analysis was performed to identify bacterial genera that were significantly different ($p < 0.05$) between enrichments (APW versus no enrichment and EPW versus no enrichment) in all BrdU treated samples (Figure 3). *Vibrio cholerae, Vibrio vulnificus*, *Clostridium metallolevans, Lysinibacillus boronitolerans*, *Flavobacterum succinicans, Enterobacter cloacae*, *Cetobacterium somerae*, *Bacillus cereus, Plesiomonas shigelloides* and *Clostridium bifermentans* were found at a significantly higher abundance in BrdU-treated, non-enriched samples. Additionally, all BrdU-treated enriched (APW and EPW) samples were characterized by a higher relative abundance of Candidatus *Aquiluna rubra*.

Vibrio taxonomy

Irrespective of sampling site, treatments and enrichments, we were able to observe *Vibrios* in all samples at a low relative abundance (Figure 4). Among the sampling sites, tidal brackish creek samples were characterized by the highest relative abundance of *Vibrios,* followed by reclaimed water samples. Additionally, in the non-enriched BrdU-treated tidal brackish creek samples, we observed *Vibrio* spp., indicating the detection of metabolically-active, viable *Vibrios,* including *V. vulnificus,* without the aid of enrichment techniques. In non-tidal freshwater creek, reclaimed water and pond water samples a higher relative abundance of metabolically-active *Vibrios* coincided with the use of enrichment techniques. Some of the species observed were *V. cholerae*, *V. vulnificus*, *V. parahaemolyticus* and *V. aestaurinus* and *V. shilonii*.

Discussion

The irrigation water sources tested in our study harbored diverse bacterial communities (some of which are of concern to public health) and hence, would require mitigation strategies prior to their use for food crop irrigation. Through this study we were able to detect the presence of *Vibrios* across all sampling sites. Our culture-based methods were only able to detect *Vibrios* in three of the sampling sites while our 16S rRNA sequencing data revealed the presence of *Vibrios* in all four sampling sites. Additionally, our novel approach of coupling BrdU labeling with 16S rRNA sequencing teased out the likely metabolically-active *Vibrios* in the tested water samples. We also could detect live *Vibrios* in the non-enriched samples from all the sampling sites, indicating that the novel coupled method could be helpful in identifying VBNC Vibrios, which would have been undetected using only culture methods.

The bacterial genus *Vibrio* is ubiquitous and widely distributed in aquatic environments from brackish to deep seawater, worldwide [213]. From time to time these bacteria have been found in different surface waters [202–204] and reclaimed water [203, 205, 206]. Most *Vibrio*-associated illnesses have been associated with either foodborne infections caused by consumption of raw or undercooked seafood or wound infections acquired while involved in aquatic activities in coastal or estuarine waters [213]. Very rarely, instances of *Vibrio* outbreaks associated with the consumption of raw vegetables have been reported [207, 208, 214, 215]. For instance *Vibrio* contamination of vegetables irrigated with partially-treated municipal wastewater in Varanasi, India was reported [215]. Additionally, *V. cholerae* O1 was

detected in vegetables that were irrigated using waste water and stabilization ponds in Tanzania [208]. The prevalence of *V. parahaemolyticus* in raw salad vegetables at the retail level was also observed in Malaysia [207]. Nevertheless, to date no *Vibrio* outbreaks in the U.S. have been associated with the consumption of fresh produce.

Besides *Vibrios*, our study also revealed the presence of other human bacterial pathogens in BrdU-treated enriched samples including: *Clostridium bifermentans*, *Enterobacter cloacae*, *Plesiomonas shigelloides*, and Bacillus *cereus* (Figure 2). *B. cereus* is a Gram-positive, aerobic-to-facultative, spore-forming rod that is widespread in nature and has been frequently isolated from soil and growing plants[216] and has been associated with food-associated illness [217, 218]. An outbreak of *B. cereus* was reported as a result of consumption of contaminated vegetable sprouts [219] and refried beans served by a fast food restaurant chain in upstate New York [220]. In addition, Valero et al. 2002 [221] characterized *B. cereus* isolates from nearly 56 samples of fresh vegetables (peppers, cucumbers, tomatoes, carrots, zucchini, garlic and onions) and in refrigerated, minimally processed foods that had these vegetables as the ingredients. The presence of these species in refrigerated, minimally processed foods demonstrates their persistence through food processing methods.

A total of 11 outbreaks associated with *Plesiomonas shigelloides* have been reported worldwide from 1961 to 2003, of which four outbreaks occurred in the US [222]. Sources for these outbreaks were mainly contaminated shellfish, fish, meat products, and contaminated water sources (tap, well and freshwater) [222]. The common environmental reservoirs for these bacteria include freshwater ecosystems

and estuaries [222].Indirect involvement of *Plesiomonas shigelloides* after major natural aquatic disasters have also been reported [223]. For instance, after the 2004 tsunami episode in India, along with pathogenic *Vibrios*, *Aeromonas* and *Plesiomonas* were also isolated from hand pumps and wells in several communities [224].

Enterobacter cloacae, another pathogen detected using our coupled BrdU/sequencing method, has been reported as opportunistic and multi-drug resistant bacterial pathogens involved in significant hospital associated outbreaks between 1993 and 2003 in Europe [225]. *E. cloacae* is ubiquitous in terrestrial and aquatic environments and occurs as commensals in the intestines of humans and animals, making it a perfect candidate for transfer from irrigated produce to humans. *E. cloacae* have been isolated from ready-to-eat salads served in a primary school in Valencia city [226] and from vegetables irrigated with untreated wastewater in Morocco [227].

Other nucleic acid intercalating dyes like ethidium monoazide (EMA) and propidium monoazide (PMA) have been used to detect foodborne pathogens to limit the underestimation of total viable cells in environmental samples [228–230]. Recently Cao et al. (2019) was able to detect VBNC *Vibrio parahaemolyticus* in shrimp samples with the help of PMA dyes [230]. Though this intercalating dye looks promising and is being widely now used to detect metabolically active bacteria a recent study by Li et al. (2017) found that on comparing DNA-, PMA and RNA-based 16S rRNA sequencing the PMA- based approaches tend to overestimate the live bacterial population when compared to RNA-based methods. Till date there has been no study using BrdU in tandem with sequencing but several studies have used

this dye to detect metabolically active bacteria in different environmental samples [231, 232] and cell proliferation studies especially in studying adult neurogenesis [233, 234]. Additionally, BrdU method coupled with qPCR has been extensively used to detect persistent fecal bacteria in sewage effluent [235], psychrotolerant in polluted sea sediments [236] and the impact of mycorrhizal fungi on bacterial communities in soil [237], and hence seems promising for our study to identify the live *Vibrio* in these nontraditional water sources.

In summary, coupling BrdU-labeling with 16S rRNA sequencing can help refine our understanding of the prevalence of metabolically-active VBNC *Vibrios,* and other important bacterial pathogens, in Chesapeake Bay tributaries and other associated water sources that are used for agricultural purposes. In addition to the knowledge of viability of *Vibrios*, sample size, the ability to reproduce these results and a thorough statistical analysis of the sequencing data are the strengths of our study. Some of the limitations of the study as with all 16S rRNA-based sequencing techniques are PCR amplification biases, limited ability to assign species-level classifications and with the culture dependent studies we run into false positive or negative results causing overestimation or underestimation of *Vibrios*. Our findings can easily be applied to develop appropriate on-farm water treatment technologies that may be necessary to improve the quality of nontraditional irrigation water sources as climate change continues to play a role in the depletion of our freshwater resources.

Table 1: Water quality characteristics of the sampling sites throughout the sampling period.

Sampling sites	Sampling months	Precipitation (14d)	Water temp.(C)	DO%	Conductivity (SPC uS/cm)	pH	ORP mV	Turbidity (FNU)	Nitrate (mg/L)	Chloride (mg/L)
Non-tidal freshwater creek	May A	1.79	15.7	97.5	211.4	7.28	150.8	2.8	0.53	11.74
	May B	4.65	17.5	93.5	141.8	6.93	144.5	13.5	1.1	1.36
	Jun A	4.03	16.45	92.8	202.65	7.07	114.25	11.1	0.93	11.24
	Jun B	1.43	20.15	94.9	164	7.05	105.1	5.3	0.885	1.34
	Jul	0	22.65	95.5	204.7	7.29	87.97	0.2	0.23	0.09
	Aug	4.51	22.15	93	162.8	7.22	80.2	5.47	0.38	0.64
	Aug B	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Sep	NA	NA	NA	NA	NA	NA	NA	NA	NA
Reclaimed water	May A	1.11	18.2	120.5	808	7.95	275.5	16.2	10.99	116.05
	May B	3.84	28.4	83.2	1.3	7.76	68.8	36.3	41.4	807.39
	Jun A	2.53	19.8	14.8	874	7.17	199.8	3.4	0.7	67.04
	Jun B	1.01	23.75	22.57	951	6.81	-158.3	20.7	2.59	78.46
	Jul	0.03	25.5	12.83	1083	6.9	-161.5	-1.8	0.33	47.08
	Aug	8.12	23.85	100.6	821.5	6.85	246.95	3.55	1	16.63
	Aug B	0.98	22.45	29.9	859	NA	NA	10.8	2.13	36.54
	Sep	0	17.85	31.1	696.5	7.52	222.1	7.5	5.35	1703.39
Tidal brackish creek	May A	0.89	20.338	58.9	19815.4	6.74	275.5	2.86	12.89	8795.25
	May B	4.01	24.139	31.5	2494.3	6.22	184.7	3.67	6.5	852.57
	Jun A	7	20.433	32.9	2438.9	6.48	189.7	8.19	6.5	1016.76
	Jun B	2.36	27.37	27.1	12140.4	6.76	156.2	4.32	22.34	4656.02
	Jul	0.09	27.09	32.6	20524	6.77	257	-0.315	34.03	9823.28
	Aug	4.72	29.17	29.4	11771	7	167.3	5.12	30.58	6507.61
	Aug B	0.64	28.17	26.1	21921	7.09	129.3	1.93	41.53	13797.6
	Sep	4.08	23.26	23.9	20933.2	7.27	150.5	2.26	25.39	14127.69
Freshwater pond	May A	1.79	19.9	111.9	145.4	7.99	234	1.6	0.25	7.22
	May B	4.65	22.9	41.47	125.2	6.89	114.95	15	0.67	2.61
	Jun A	4.03	18.6	41.05	99.03	6.7	177.85	11.6	1.03	3.99
	Jun B	1.43	27.3	111.8	104	7.13	151.8	11.17	0.43	0.73
	Jul	0	27.95	103.5	133.9	7.24	100.2	0.97	0.09	0.12
	Aug	4.51	27.8	164	85.625	7.92	119.9	2.8	0.23	2.76
	Aug B	0.48	27.15	96.7	97.85	7.47	136.1	4.6	0.22	0
	Sep	3.71	23.1	103.8	114.7	7.06	110.6	-3.1	0.23	0.4

Table 2: Sample summary of the four nontraditional irrigational water sources from sampling months (May ’18 – Sept ’18)

Sampling site	BrdU treatment (APW and EPW enrichment)	BrdU treatment (No enrichment)	No BrdU treatment (APW and EPW enrichment)	No BrdU treatment (No enrichment)	Total (N)
Pond water	16	8	16	8	**48**
Reclaimed water	16	8	16	8	**48**
Non-tidal Fresh water creek	12	6	12	6	**36**
Tidal brackish water	16	8	16	8	**48**
Total (N)					**180**

Figure 1: (A) Box plot of alpha diversity (Shannon Index) across all samples on rarefied data to minimum sampling depth. Alpha diversity of BrdU-treated samples represents the diversity observed in the metabolically-active fraction of bacterial communities present in each sample. Red represents BrdU-treated samples and green represents non-BrdU treated samples. (B) PCoA analysis of Bray Curtis computed distances between BrdU- and non-BrdU-treated water samples. Solid colored ellipses are drawn at 95% confidence intervals by treatment type of the water samples.

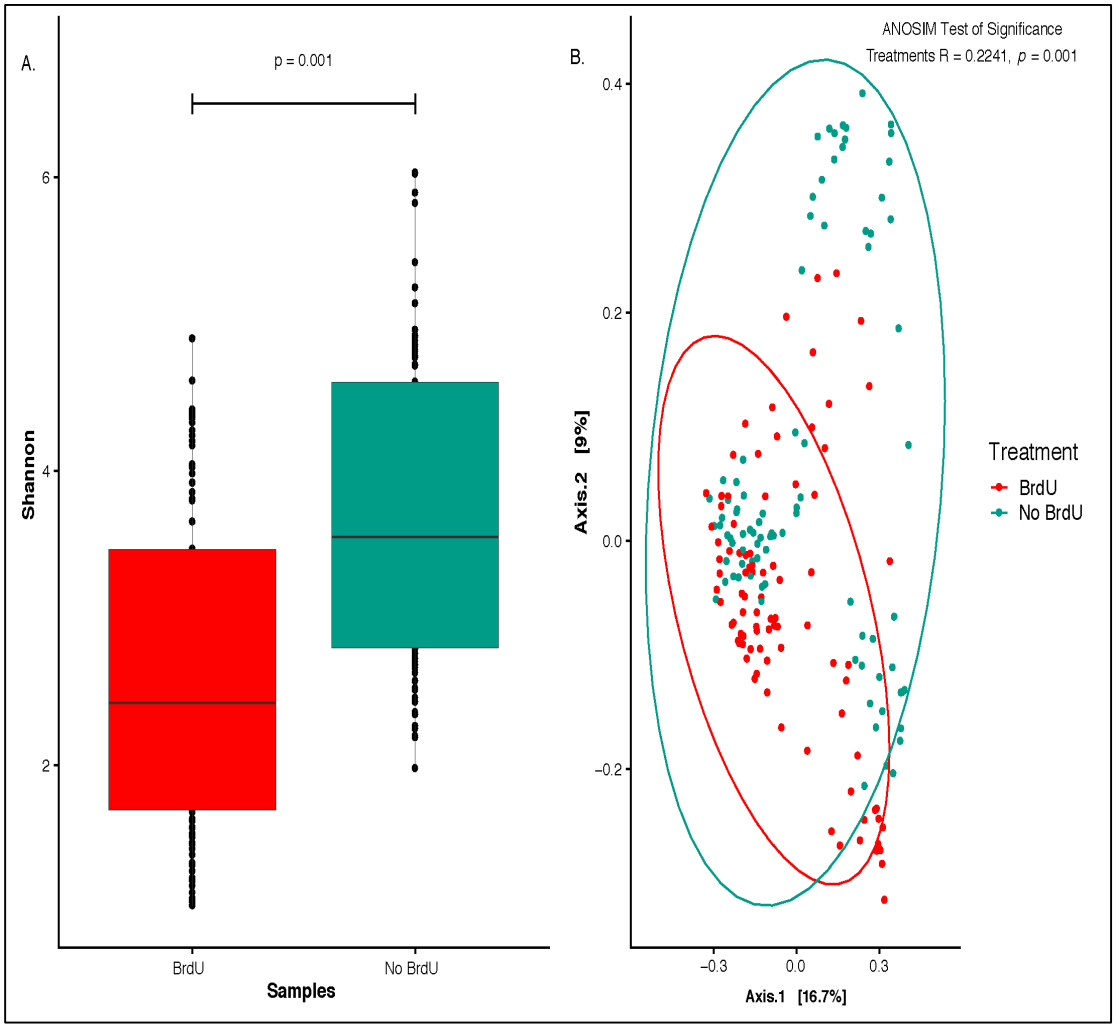


Figure 2: Taxonomic profiles of the top 25 bacteria detected in pond water, tidal brackish creek, reclaimed water and non-tidal freshwater creek water derived from 16S rRNA sequencing data are represented by site and by BrdU-treatment.

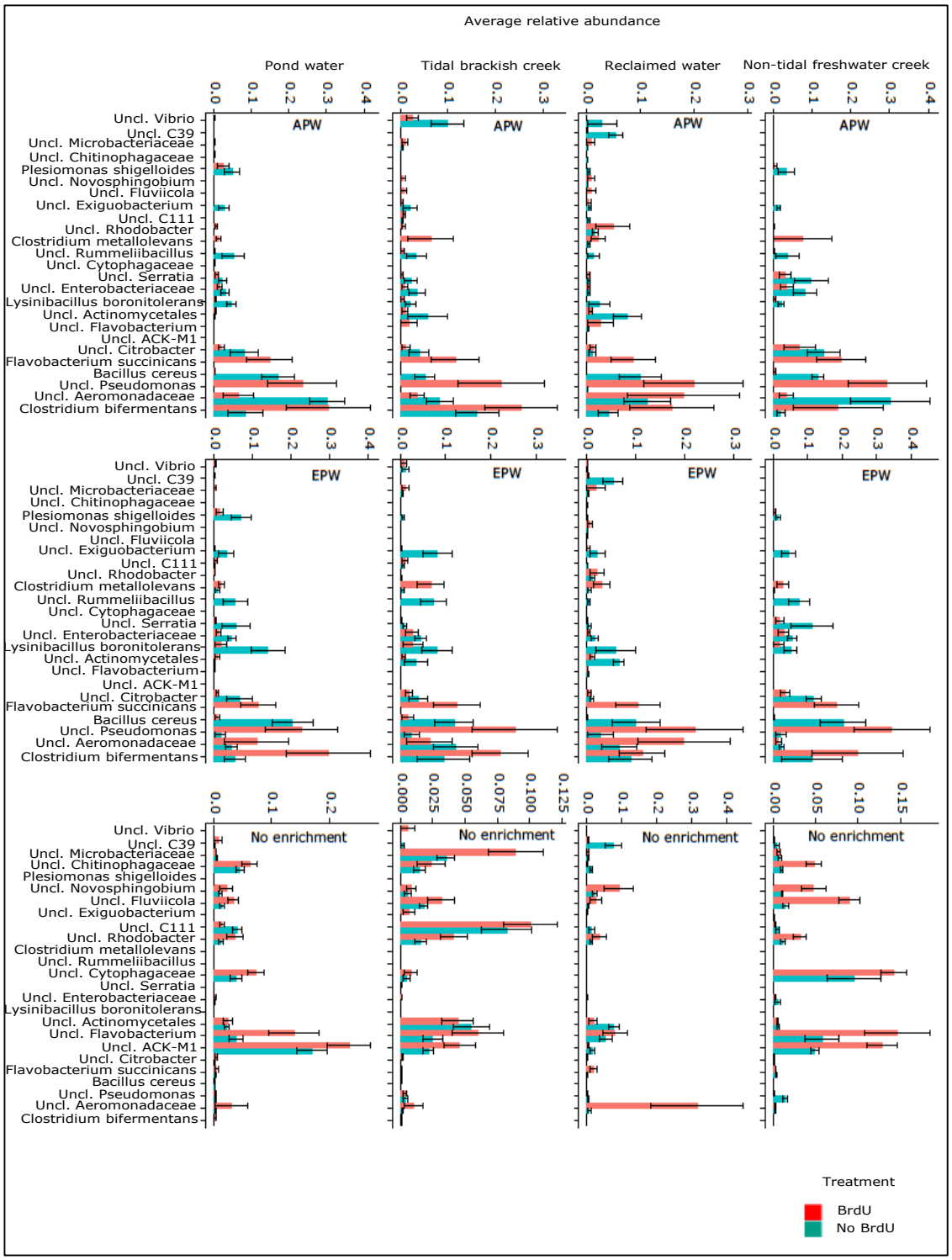


Figure 3: Differential abundances of bacterial genera that were statistically significant ($p<0.05$) in BrdU treated samples between enrichments: No enrichment versus APW and No enrichment versus EPW. A positive log2-fold change denotes a bacterial taxonomy that is significantly higher in either enrichments (APW or EPW), while a negative log2-fold change indicates a bacterial taxonomy that is significantly higher in no enrichment BrdU-treated samples. The grey line and arrows highlight the conversion in log2-fold change from negative to positive values.

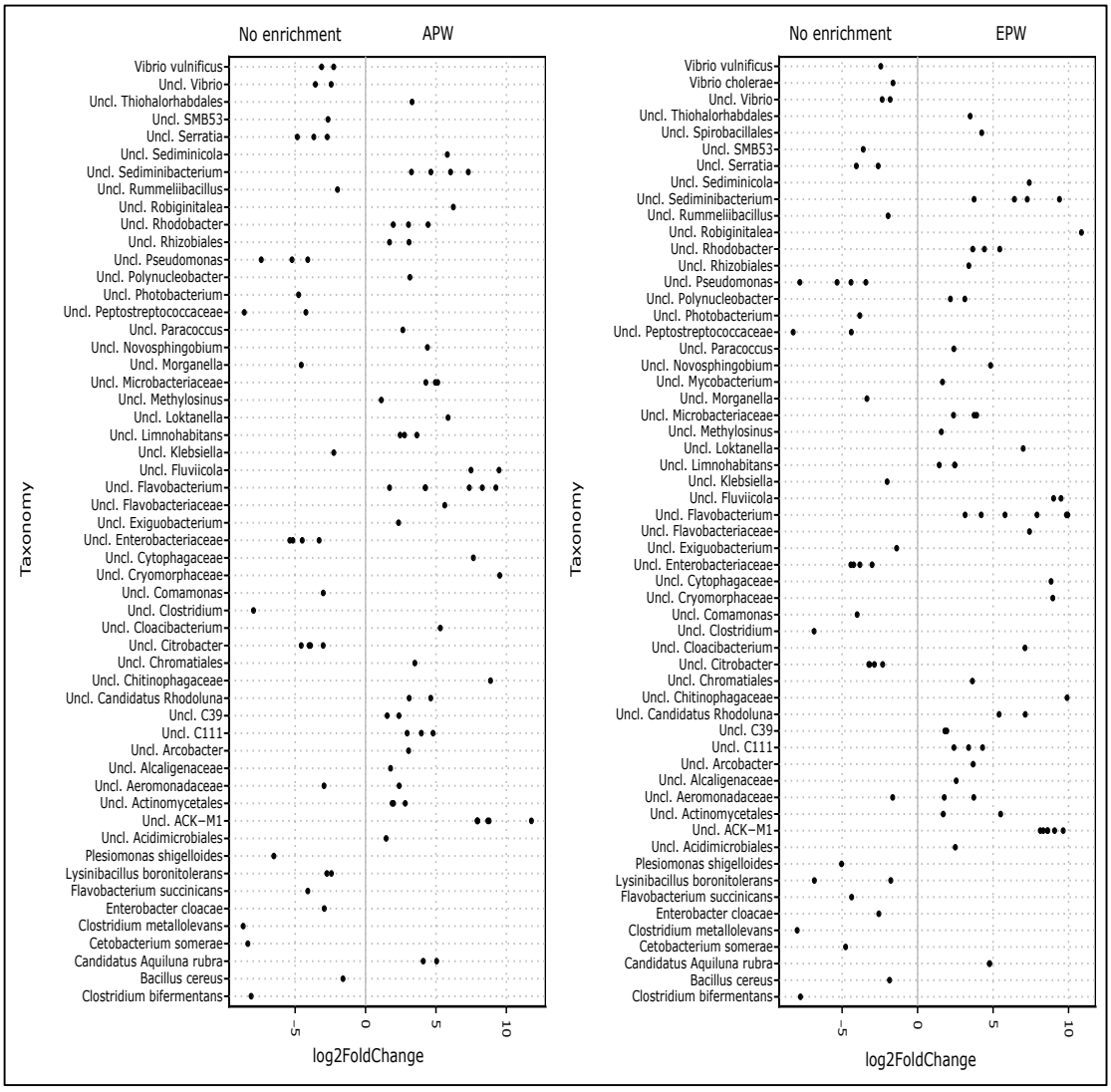
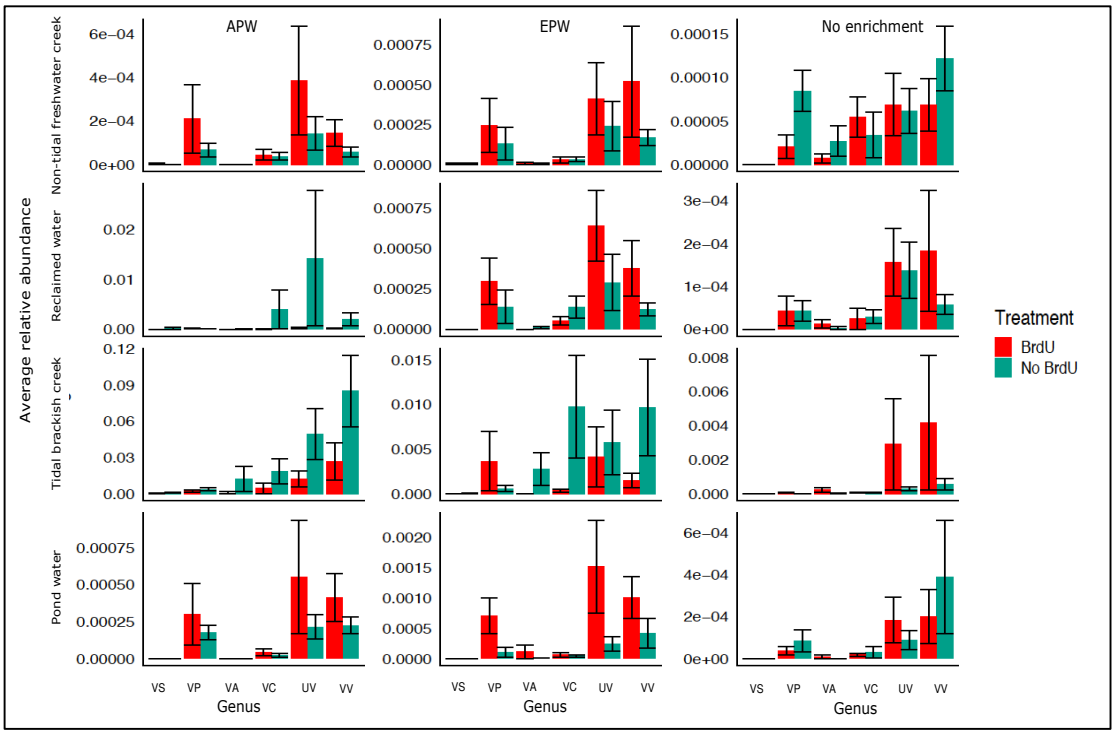


Figure 4: Average relative abundance of *Vibrio* species in pond water, tidal brackish creek, reclaimed water and non-tidal freshwater creek samples in different enrichments (APW, EPW and no enrichment). *Vibrio* species abbreviation: VS= *V. shilonii*, VP= *V. parahaemolyticus*, VA= *V. aestaurinus*, VC= *V. cholerae*, UV= Unclassified *Vibrio* and VV- *V. vulnificus.*



Chapter 5: Source tracking microbial communities from rooftop harvested rainwater to irrigated soil and produce

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Abstract

Rooftop harvested rainwater (RHRW) is gaining interest as a potential irrigation water source. Previous studies have indicated that both biotic and abiotic factors can affect RHRW quality. However, very few studies have investigated the potential transfer of microorganisms from RHRW to irrigated produce. To bridge this knowledge gap, we characterized and tracked metabolically-active bacteria from a RHRW system (ambient rain, first flush tanks, secondary tanks and municipal water) to irrigated produce (chard) by using a combination of two different DNA-labeling techniques (5-bromo-2'-deoxyuridine (BrdU) and propidium monoazide (PMA)) along with next generation sequencing techniques. A total of 186 samples (n=36 irrigation water, n=90 soil and n=60 chard samples) were collected from a RHRW system in Maryland, U.S.A from June to August 2018. Subsamples were treated with BrdU and PMA. DNA from all treated and non-treated samples was extracted and PCR-amplified for the V3-V4 hypervariable region of the 16S rRNA gene and sequenced using the Illumina HiSeq 2500. Data were analyzed using QIIME, R and SourceTracker. Additionally, both water and soil characteristics were recorded for the sampling period. Irrespective of sample type, statistically significantly lower alpha diversity was observed among BrdU-treated samples. The top ten bacterial phyla identified across all samples irrespective of treatment were *Proteobacteria*, *Actinobacteria*, *Bacteroidetes, Firmicutes*, *Chloroflexi*, *Acidobacteria*, *TM7*, *Gemmatimonadetes*, *Planctomycetes* and *Thermi*. Bacterial profiles present in all sample types were *Pseudomonas veronii*, *Pseudomonas lurida*, *Sphingomonas* spp.,

Aeromonas spp*., Pseudomonas* spp*., Arthrobacter* spp*., Sediminibacterium* spp*., Bacillus* spp*., Janthinobacterium lividum, Curvibacter lanceolatus* and *Geobacillus thermodentrificans.* Additionally, metabolically-active *Escherichia, Enterobacter, Citrobacter and Enterococcus* were observed in all sample types at a lower relative abundance compared to the above-mentioned bacterial profiles. Using the SourceTracker tool we were able to track and quantify the relative contributions of each RHRW irrigation system component to the bacterial communities of the produce. Our findings can be applied to develop appropriate RHRW treatment technologies that may be necessary prior to the use of these water sources to irrigate food crops.

1. Introduction

In recent years, rooftop harvested rain water (RHRW) has garnered increasing interest as an alternative, eco-friendly water resource in countries such as Australia, Canada, Germany, New Zealand, Thailand, Japan, Denmark, India, and the United States [79–81]. RHRW is not only being used for toilet flushing, irrigation, and as a drinking water source when properly treated, but also has helped reduce storm water runoff and can even be a part of the urban landscape [85].

In the United States, RHRW collection and use is solely regulated by individual states and not by the federal government [82], and hence, regulations and policies vary widely [83]. Rainwater and harvested rainwater are generally considered to be of relatively good microbial quality that can be relied upon as an irrigation water source. Depending on atmospheric pollution, environmental conditions, wind speeds/direction, and the harvesting and storage method of rainwater, the quality of harvested rainwater may fluctuate and could be compromised by avian feces, insects, and mammals, as well as old roofing material (shingles, copper, etc.), dirty drainage pipes and poorly-maintained storage tanks [86, 87, 238]. The presence of lead, zinc, chromium, manganese, molybdenum, silver, nickel, copper and cadmium content in harvested rainwater has been demonstrated in multiple studies from Australia and Hebron (West Bank, Palestinian Territories) [88, 89]. Besides the presence of heavy metals, several studies have identified enteric and opportunistic pathogens like *Enterococci, E. coli, Clostridium perfinges, Salmonella, Campylobacter, Legionella, Aeromonas, Pseudomonas, Mycobacterium, Shigella, Vibrio, Giardia* and *Cryptosporidium* in RHRW that could potentially be transmitted to vegetable crops if

RHRW is used for irrigation purposes [86, 90, 91]. Hence, although harvested rainwater is an eco-friendly alternative irrigation water source, there is a need to better understand both the chemical and microbial constituents of these waters before their intended use.

To address this need, we 1) characterized the total and metabolically-active (live) bacterial communities from RHRW, as well as irrigated soil and produce (chard); and 2) assessed the relative contributions of specific components of the RHRW system (ambient rain, municipal water, rooftop harvested rainwater and soil) to the bacterial communities of irrigated produce. First, to characterize the metabolically-active bacterial populations in these samples, we used a combination of two different labeling techniques (5-bromo-2'-deoxyuridine (BrdU) and propidium monoazide (PMA)) along with next-generation sequencing techniques. Use of these labeling techniques enables the differentiation between dead and metabolically-active (live) bacteria in tested samples. BrdU is a synthetic thymidine analog that can incorporate into replicating DNA [125], while PMA is a photoreactive DNA-binding dye that can penetrate membrane-compromised cells and, following photo-activation, binds to free DNA [239]. The BrdU and PMA labeling techniques have been used to identify metabolically-active bacteria in various environments [125, 129, 140, 228, 240]. Second, to track bacterial communities from different elements of the RHRW system to irrigated produce, we performed SourceTracker analyses on our sequencing data. The SourceTracker uses a Bayesian approach to estimate the proportion of each source contributing to a designated sink sample (chard, in this case) [241]. This approach has been used to track microbial communities in multiple sources including

sewage, coastal waters, lakes, estuaries, public restrooms, neonatal care units, indoor air, and urban storm water [241–243]. Additionally, we evaluated whether the characterized bacterial communities in irrigated chard were correlated with soil and water characteristics.

2. Materials and Methods

2.1 Sampling site

The study site is located in Maryland, U.S.A., and includes a RHRW system based off of a design developed in Melbourne, Australia [244]. The system comprises four separate raised vegetable beds that utilize rainwater harvested from a rooftop with a roof area of 135m^2. The vegetable beds are 1.22m x 2.44m and were built with pressure-treated dimensional lumber and lined with waterproof lining to act as a vertical waterproof barrier. No ground level barrier was installed in order to allow for ground drainage. Each of these raised beds have a perforated supply pipe at the bottom to allow for the water to be dispersed throughout the bed. The supply pipe is connected to secondary and first flush systems and then to the roof's downspout. Additionally, a separate raised bed that uses municipal drinking water and natural rain for irrigation was also tested as our control bed. Our sampling site is summarized in Figure 1.

2.2 Sample Collection and processing

2.2.1 Irrigation water samples

From June to August 2018, 600 mL grab samples of water (n=36) associated with each raised bed were collected over three sampling dates and included samples from the first flush tanks, secondary tanks, municipal water (control) and ambient rain. Samples from secondary tanks and ambient rain were collected only in the month of July, due to lack of rainfall events during the other two sampling dates. A summary of samples is described in detail in Table 1. We also used a ProDSS digital sampling system (YSI, Yellow Springs, OH, USA) to measure, in triplicate: water temperature (°C), conductivity (SPC uS/cm), pH, dissolved oxygen (%), oxidation/reduction potential (mV), turbidity (FNU), nitrate (mg/L), and chloride (mg/L). Table 2 describes the water characteristics that were measured during our sampling period. Additionally, we tested for the presence of four metals (lead (mg/L), copper (mg/L), aluminum (mg/L) and zinc (mg/L)) in the first flush tanks, secondary tanks and control water. Precipitation (inches) was also measured using rain gauges

40μL of 100 mM BrdU was added to 200 mL of water, while the other 200 mL was not subjected to any treatments. Both samples were then incubated for 2 days in the dark at room temperature, allowing the BrdU to incorporate into replicating DNA of the BrdU-treated samples, thereby enabling us to detect metabolically-active fractions of the bacterial communities in these water samples. The BrdU-treated, non-treated samples and a separate 200 mL (subjected to PMA treatment, described below) were filtered through 0.2 μm, 47 mm filters (Pall Corporation, Port Washington, NY, USA) and the filters were then dissected into four quadrants and placed in lysing matrix B tubes (MP Biomedicals, Solon, OH, USA). To the filters for PMA treatment, 3 μL of 50μM PMA was added, as described previously [245]. The

PMA-treated samples were then subjected to a 5-minute dark cycle and then exposed to a 650 W halogen lamp placed 20cm from the sample tubes for 5 minutes. All of the filters (treated and not treated) were then stored at -80^{0}C until DNA extraction.

2.2.2 Soil samples

20 g soil samples from two sides of the raised beds (n=5) were collected, from which 0.2g was weighed to perform the treatments (BrdU and PMA) or no treatments (controls), resulting in a total of 90 soil samples. Additionally, soil analysis was performed by Waypoint analytical, Richmond, Virginia, U.S.A to measure organic matter (%), estimated nitrogen release (lbs/A), cation exchange capacity (milli-equivalent/100g) phosphorous (ppm), calcium (ppm), potassium (ppm), magnesium (ppm) and pH. We also tested surface soil moisture (SM1) and soil moisture 12 inches below the surface (SM2) (Table 3).

For PMA treatment, 0.2 g of soil was treated with 3 µl of 50µM PMA, and then subjected to a 5-minute dark cycle and then exposed to a 650 W halogen lamp placed 20cm from the sample tubes for 5 minutes. For the BrdU treatment, 0.2g of the soil was incubated with 26 µl of 7.69mM BrdU and kept in the dark at room temperature for 48 hours. All 90 samples (described in Table 1), treated (PMA or BrdU) or not treated, were stored at -80^{0}C until DNA extraction.

2.2.3 Produce samples

Chard leaves were collected from both sides of each raised beds resulting in a total of 60 samples (leaves had not grown during our first sampling trip). Similar to water and soil samples, chard samples were also subjected to treatments (BrdU and PMA) and no treatment.

200 mL of sterile water was added to Chard samples in Whirl-Pak® bags, hand-massaged for 30 s, and then the resulting wash water was transferred by pipette to the filtration setup. For each sample, the total volume of surface wash water was filtered through one 0.2 μm, 47 mm filter and filters were cut and transferred to lysing matrix B tubes. PMA and BrdU treatments of these filters were performed similarly to that of the water samples, described above. All 60 samples (described in Table 1) were stored at -80°C until DNA extractions could be completed.

2.3. Immunocapturing of BrdU treated samples

Immunocapture and isolation of BrdU-labeled DNA were performed using a previously published protocol [125]. Briefly, to sheared and denatured herring sperm DNA (HS DNA), monoclonal anti-BrdU (α-BrdU) antibody was mixed at a 1:9 ratio and incubated for 1 hour at room temperature to form the HS DNA/α-BrdU antibody complex. The extracted DNA from all samples (water, soil and produce) was then denatured by heating for 5 min at 100^0C and transferred to ice. The mixture of HS DNA/α-BrdU antibody complex was then added to the denatured DNA from all samples and incubated for 1 h in the dark at room temperature with agitation to form the DNA/HS DNA/α-BrdU antibody complex. Meanwhile, magnetic beads (Dynabeads, Dynal Inc., Invitrogen by Thermofisher Scientific) coated with goat anti-

mouse immunoglobulin G were washed three times with 1mg/mL acetylated bovine serum albumin (BSA) in phosphate-buffered saline (PBS) buffer using a magnetic particle concentrator. The washed Dynabeads were then added to the DNA/HS DNA/α-BrdU antibody complex and incubated for an additional 1 h in the dark at room temperature. After incubation, the samples were washed in 0.5mL PBS-BSA, and the BrdU-containing DNA fraction was eluted by adding 1.7mM BrdU (in PBS-BSA) and incubating for 1 h in the dark at room temperature.

2.4 DNA extraction and 16S rRNA Gene Amplification and Sequencing

DNA extractions were performed using protocols previously published by our group [141, 142]. Briefly, 1 mL of PBS was added to the filters and soil samples in the lysing matrix B tubes, before incubation in enzymatic cocktails containing lysozyme, mutanolysin, proteinase K and lysostaphin, after which the cells were mechanically lysed using an MP Biomedical FastPrep 24 (Santa Ana, CA). The DNA was then purified using the Qiagen QIAmp DNA mini kit (Germantown, MA) per the manufacturer's protocol.

Extracted DNA was PCR amplified for the V3-V4 hypervariable region of the 16S rRNA gene using the universal primers 319F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) and sequenced on an Illumina HiSeq2500 (Illumina, San Diego, CA) using a method developed at the Institute for Genome Sciences [212] and described previously [141, 142].

2.5 16S rRNA sequencing analysis

Following sequencing, 16S rRNA paired-end read pairs were assembled using PANDAseq [144], de-multiplexed, trimmed of artificial barcodes and primers, and assessed for chimeras using UCHIME in *de novo* mode implemented in Quantitative Insights Into Microbial Ecology (QIIME; release v.1.9.1) [145]. Quality trimmed sequences were then clustered *de novo* into Operational Taxonomic Units (OTUs) and taxonomic assignments were performed using VSEARCH [146] with a minimum confidence threshold of 0.97. A combination of Greengenes [246] and SILVA 16S database [147] in QIIME [145] was used for taxonomy assignments. Downstream data analysis and visualization was completed in RStudio (v.1.1.423) using R packages: biomformat (v.1.2.0) [148] vegan (v.2.4-5) [149], ggplot2 (v.3.1.0) [150], phyloseq (v.1.19.1) [151], and metagenomeSeq (v.1.16.0) [153]. All sequences taxonomically assigned to the Phylum *Cyanobacteria* were removed from further downstream analysis. When appropriate, data were normalized with metagenomeSeq's cumulative sum scaling (CSS) [153] to account for uneven sampling depth. Prior to normalization, alpha diversity was measured using both the Observed richness metric and the Shannon diversity index [154]. Bray-Curtis dissimilarity was used for calculating beta diversity and was compared using analysis of similarities (ANOSIM) on normalized data (999 permutations). Pearson correlation coefficients were calculated to identify associations between the water and soil characteristics and the relative abundance of the bacterial phyla and visualized via heatmap created in R via vegan heatplus [155]. A core bacterial microbiome was determined comprising OTUs present in 100% of samples and a Venn diagram (v.1.6.20) [247] was generated with limma (v.3.30.13) [248] package in R to visualize

these data. To track bacterial communities from the water source to produce, we used the SourceTracker [241] 0.9.5 software with QIIME [243]. For our SourceTracker analyses, ambient rain, first flush tanks, secondary tanks, municipal water (control tank) and soils from the five beds were considered as the sources and chard samples (produce) were considered the sink. This analysis has been used to track microbial communities from multiple water sources [242, 249, 250], public restrooms [251], neonatal intensive care units [252], etc.

3 Results

3.1 Water Characteristics

Water characteristics from the first flush tanks (A and B) and control tanks during our sampling period (June- August 2018) are described in Table 2. Both flush tanks showed similar water characteristics during the entire sampling period except for conductivity, where flush tank A showed an increase while, tank B showed a decrease. Control tanks were characterized by a decrease in water temperature and conductivity, and an increase in precipitation and oxidation and reduction potential levels. Additionally, water characteristics for the secondary tanks (A and B) and ambient rain are described for the July sampling date in Table 2. All tanks and ambient rain showed pH ranging from neutral to basic during the entire sampling period. The total rainfall measured using a rain gauge was 19.75 inches during our sampling period. None of the metals tested (Supplementary Figure S1) exceeded the maximum concentration level suggested by US EPA guidelines for irrigation purposes.

3.2 Soil Characteristics

Soil characteristics for the 4 raised beds (Beds 1-4) and 1 control bed (Bed 5) are described in Table 3. Overall, all beds had high phosphorus and calcium content while potassium and magnesium content were at medium levels. Additionally, beds 2,3,4 and 5 showed very high organic matter while bed 1 had medium levels. Soil pH was observed to be slightly basic in nature.

3.3 Sequencing dataset

A total of 186 samples (n=36 water samples including 9 flush tank A, 9 flush tank B, 9 control, 3 secondary tank A, 3 secondary tank B and 3 ambient rain; n=90 soil samples and n=60 chard surface samples) were PCR amplified for 16S rRNA gene and sequenced. 5,975,496 sequences were generated in total across all samples, and clustered into 15,071 operational taxonomic units (OTUs). Across all samples, the minimum number of reads was 445 and the maximum was 62,676, with an average number of sequences per sample of 32,126.32 (+/- 11,403.18 SD). Goods estimate coverage of 0.90 was calculated for all samples and samples with Good's <0.90 (1- no treatment soil and 1- PMA treated produce) were removed to ensure appropriate read coverage in all samples analyzed downstream (Supplementary figure S2). After data cleanup (removing reads assigned to taxa '*Cyanobacteria*' and OTUs with less than 10 reads), the total number of sequences used in downstream analyses

was 5,582,816 from 184 samples (n=36 water samples, n=89 soil sample and n=59 chard surface samples), clustered into 8,791 OTUs.

3.4 Microbiota differences between Samples and treatments

3.4.1 Alpha diversity

To avoid sequence coverage issues, alpha diversity metrics (Observed species and Shannon diversity) by different sample types (produce, soil and water) and by treatments were calculated on both rarefied (after downsampling each sample to 6474 reads) (Figure 2A) and non-rarefied data (data not shown). Since no differences were observed between the rarefied and non-rarefied analysis, we only presented alpha-diversity analysis performed on the rarefied dataset. Statistically significantly lower alpha diversity (both Observed species and Shannon diversity) was observed in BrdU-treated samples compared to non-BrdU-treated and PMA treated samples ($p< 0.0001$) for each sample type. Additionally, irrespective of the treatments, alpha diversity (Observed and Shannon) was significantly higher in soil samples compared to both produce and water samples ($p< 0.0001$).

3.4.2 Beta diversity

Beta diversity between all normalized samples was computed using PCoA plots of Bray-Curtis dissimilarity (Figure 2B) and showed the most significant clustering by sample type (ANOSIM R: 0.6424, p=0.001), followed by treatment (ANOSIM R: 0.2037, p=0.001). PCoA findings between treatments within sample types showed 28.9% variance between bacterial communities along the first principle

component axis (Axis 1) and 6.2% along the second principle component axis (Axis 2).

3.5 Correlation studies

3.5.1 Soil characteristics and bacterial abundance correlation

Several bacterial phyla irrespective of the soil beds showed significant correlations ($p < 0.05$) with the measured soil characteristics (Figure 3A). *Proteobacteria* and *Bacteroidetes* were positively correlated while *Planctomycetes* and *Chloroflexi* were negatively correlated with calcium. *Planctomycetes* and *Gemmatimonadetes* showed negative correlation ($p < 0.001$, $p < 0.05$ respectively) with phosphorus. *Bacteroidetes* were negatively correlated ($p < 0.001$) to soil moisture measured 12 inches (SM2) below surface during the sampling period.

3.5.2 Water characteristics and bacterial abundance correlation

Despite the short sampling period, three bacterial phyla irrespective of the different water types showed significant correlations ($p < 0.05$) with the measured water characteristics (Figure 3B). *Thermi* and *Firmicutes* were positively correlated ($p < 0.05$) to pH and turbidity respectively, while *Chloroflexi* was negatively correlated ($p < 0.05$) with dissolved oxygen and precipitation. The metals tested showed no significant correlations with any of the bacterial phyla.

3.6 Taxonomical analysis by sample and treatments

The top ten bacterial phyla identified across all samples irrespective of treatment were *Proteobacteria*, *Actinobacteria*, *Bacteroidetes Firmicutes*,

Chloroflexi, *Acidobacteria*, *TM7*, *Gemmatimonadetes*, *Planctomycetes* and *Thermi*. The most predominant bacterial phyla observed in all samples irrespective of treatments was *Proteobacteria* with an average relative abundance of 69.15 % (+/- 13.45) in produce, 44.5 % (+/- 7.25) in soil, and 67.01 % (+/- 21.60) in water.

Only 45% of the total 8,791 OTUs were assigned to the genus level (3940 OTUs) of which only 525 could be identified to the species level (6%). The top 25 bacterial taxonomy among all samples with respect to treatments (Figure 4) included *Pseudomonas veronii*, *Pseudomonas lurida*, *Pseudomonas fluorescens, Sphingomonas* spp*., Aeromonas* spp*., Pseudomonas* spp*., Arthrobacter* spp*., Sediminibacterium* spp*., Bacillus* spp*., Janthinobacterium lividum, Curvibacter lanceolatus*, *Geobacillus thermodentrificans*, *Chryseobacterium* spp*., Microbacterium* spp*., Flavobacterium, Enterobacter* spp*., Undibacterium* spp*., Rhizobium* spp*., Chryseobacterium taiwanense, Methylobacterium* spp., *Marmoricola* spp., Uncl. JG30-KF-CM45, Uncl. *Acidobacteria* Subgroup _6, and Uncl. KD4-96.

A low average relative abundance of coliform bacteria (e.g. *Escherichia*, *Enterobacter* and *Citrobacter*) and *Enterococcus* was observed in all sample types (Figure 5 A, B, C and D). In BrdU-treated produce samples, *Enterobacter* and *Citrobacter* showed higher average relative abundance of 4% and 1%, respectively, when compared to *Escherichia* which had an average relative abundance of 0.12%, Similarly, average relative abundance of *Enterococcus* was low in all sample types tested during our sample period.

3.7 Core microbiome analysis

Core microbiome analysis showed 68 OTUs and 157 OTUs were present in all chard samples and soil beds respectively and no unique OTUs were observed between the chard samples and soil beds (supplementary figure S3 and S4). The species observed as members of the chard core microbiome were *Clostridium bifermentans*, *Bacillus cereus*, *Enterobacter cloacae*, *Escherichia coli*, *Veillonella dispar*, *Streptococcus infantis*, *Janthinobacterium lividum*, *Haemophilus parainfluenza*, *Geobacillus thermodenitrificans*, *Pseudomonas veronii*, and *Sphingomonas yabuuchiae* (Supplementary Table S1). The species observed as members of the core microbiome in soil were *Pseudomonas veronii*, *Curvibacter lanceolatus*, *Geobacillus thermodenitrificans*, *Janthinobacterium lividum*, *Clostridium bifermentans*, *Escherichia coli*, *Thermobispora bispora*, *Bacillus flexus*, *Streptococcus infantis*, *Rothia mucilaginosa*, *Veillonella dispar*, *Nocardioides dilutus*, *Geobacillus thermodenitrificans*, *Streptomyces mirabilis* and *Enterobacter cloacae* (Supplementary Table S2). Between the different water types (ambient rain, first flush tanks, secondary tanks and control tank), 227 OTUs were shared of which 171 OTUs were shared by all water types, 23 OTUs were shared by first flush tanks, secondary tanks and control tank, 16 OTUs were shared between first flush tank and secondary tanks, 7 OTUs were shared between first flush tanks, secondary tanks and ambient rain, 3 OTUs between secondary tanks, control tank and ambient rain, 2 OTUs were shared between ambient rain and secondary tank and 1 OTU was shared between first flush tank and ambient rain (supplementary figure S5).

On observing the core microbiota shared between produce, soil and water we found 15 OTUs namely *Curvibacter lanceolatus* (5)*, Pseudomonas veronii* (1),

Pseudomonas fluorescens (46), *Janthinobacterium lividum* (8), Uncl. *Microbacterium* (17), Uncl. *Undibacterium* (12), Uncl. *Enterobacter* (26), *Pseudomonas lurida* (832), Uncl. *Sphingomonas* (10), Uncl. *Arthrobacter* (6), Uncl. *Aeromonas* (338), Uncl. *Aeromonas* (14), Uncl. *Pseudomonas* (1136), Uncl. *Pseudomonas* (4548), and Uncl. *Pseudomonas* (12144). There were 4 unique OTUs observed in produce: *Enterobacter cloacae* (10566), *Citrobacter freundii* (8563), *Streptococcus infantis* (160), and Uncl. *Janthinobacterium* (2449). In soil, 8 unique OTUs observed were *Aeromicrobium ginsengisoli* (678), Uncl. *Cryobacterium* (269), Uncl. *Janibacter* (64), Uncl. *Chloroflex*i (42), Uncl. *Thermopolyspora* (21) Uncl. *Kaistobacter* (3880), *Microvirga lupini* (45), and *Microvirga zambiensis* (5372). Additionally, 3 OTUs, Uncl. *Marmoricola* (51), Uncl. *Bacillus* (3) and *Bacillus cereus* (28), were shared between soil and produce, 1 OTU was shared between water and soil, *Geobacillus thermodenitrificans* (4), and 2 OTUs were shared between water and produce, Uncl. *Pseudomonas* (144) and Uncl. *Pseudomonas* (13062) (Figure 6). The Sankey plot (Figure 7) shows how the core microbiota shared between produce, soil and water is distributed among treatments.

3.8 Source tracking bacterial communities from water to produce

Bacterial communities present on the surface of chard collected after a rain event (July sampling month) and sampled from soil beds 1-3 suggested that the microbial sources were predominantly the first flush tank B and secondary tanks, while the microbial sources of tested chard growing in soil bed 4 were mainly municipal water (control tank), ambient rain and soil from the control bed (soil-

control). Bacterial communities on chard grown in the control beds seemed to be primarily tracked to the first flush tank (A and B), municipal water (control tank), ambient rain, soil from the control bed and unknown sources (Figure 7).

Discussion

In this study, we investigated the potential for bacterial transfer from irrigation water to fresh produce by tracking bacterial communities in raised beds irrigated with rooftop harvested rainwater. Our study showed that bacterial communities of irrigation water (ambient rain, first flush tanks, secondary tanks and municipal water), soil and produce are diverse. Additionally, the study also provides insights into bacterial communities that are being transferred from roof top harvested rain water to produce that may include bacterial species of importance to human health. Moreover, we detected heavy metals in the first flush tanks, secondary tanks and municipal water, and surprisingly, we observed that municipal water had higher levels of metals compared to the other water samples. However, all detected heavy metal levels were still below the maximum concentration levels suggested by US EPA for irrigation standards.

PMA, a photoreactive DNA binding dye has been widely applied to characterize live bacteria in different environments [123, 129–131] and has been coupled with quantitative PCR (qPCR), and next-generation sequencing techniques [129, 132, 133] and is a promising method to detect viable cells. But in our study, we observed that PMA treated samples showed higher alpha diversity compared to BrdU treated samples, indicating an overestimation of metabolically-active samples with

the PMA method. Li et al. (2017) also showed that, in environmental samples, PMA-based 16S rRNA sequencing to detect live bacteria usually overestimates the bacterial community richness [132]. Additionally, many factors have been shown to influence the effectiveness of PMA assays like light spectrum and intensity used, source and concentration of dye, bacterial community composition, incubation time and temperature, and the properties of the sample being tested [253]. Hence, use of PMA may require more standardization depending on the environmental source being tested. Use of BrdU, on the other hand, has shown stability in results across various environmental samples (cigarette tobacco, hookah, little cigars, agricultural pond water, reclaimed water, tidal brackish water and non-tidal freshwater creek) tested in our lab (data not published), and hence, may be a more reliable dye to couple with sequencing methods in order to determine the proportion of viable cells in different environments.

Soils provides a wide range of niches to sustain microbial diversity and soil chemistry has shown to play a key role in the presence and absence of certain bacterial taxonomy [254]. In our study, we observed *Bacteroidetes* and *Proteobacteria* to be positively correlated, while *Planctomycetes* and *Chloroflexi* to be negatively correlated with soil calcium (Figure 3A). Similar results have been observed in a study conducted at the Hubbard Brook Experimental Forest in New Hampshire, USA corroborating our findings [255]. In the case of *Bacteroidetes,* we observed a negative correlation with soil moisture which could be explained since an increase in soil moisture, decreases oxygen diffusion, resulting in microbial activity reduction [256]. Similar to soil chemistry, water parameters have also been shown to

influence presence and absence of bacterial communities in previous studies [257, 258]. A recent study that characterized bacterial community in cloud water reported that *Firmicutes* had a positive correlation to pH which corroborates our findings [258].

Genera that include human bacterial pathogens such as *Pseudomonas* spp., *Aeromonas* spp., *Escherichia* spp, *Citrobacter* spp., *Sphingomonas* spp., and *Curvibacter* spp., detected in previous rainwater-associated studies via culture dependent or independent methods, were also observed in our BrdU-treated water, soil and produce samples (Figure 4 and 5) indicating their viability. Most of these genera include species that have been associated with foodborne illness [259–261]. Additionally, we identified uncultured soil bacteria (Uncl. JG30-KF-CM45, Uncl. KD4-96 and *Acidobacteria* Subgroup_6), that have been previously identified only via next-generation sequencing [262], predominantly abundant in PMA-treated and non-treated samples, indicating that they are relic DNA and being overestimated via PMA-based studies.

Our SourceTracker analysis helped us to identify potential sources of the bacterial communities that were characterized on chard samples. Predominantly, chard from the tested raised beds (1-3) had bacterial communities from the first flush tanks, while bed 4 had a higher proportion of bacterial communities from the control tank (municipal water) and ambient rain. Additionally, the control bed showed bacterial influence predominantly from first flush tanks, the control tank and ambient rain. A logical reason for this finding is that bed 4 and the control beds were close to each other compared to other beds, and hence could have experienced some cross

contamination due to leak in the pipes connecting beds to the flush tanks, leaching from the soil, etc.

In summary, our findings suggest the presence of metabolically-active and diverse bacterial communities in all tested samples. The bacterial communities identified in the tested samples also included genera of human health importance. Additionally, with the help of SourceTracker we able to identify and quantify the relative proportion contribution of each of the sources (RHRW irrigation system components and soil) to the bacterial communities of the produce. To our knowledge it is the first comprehensive study that characterizes the total, live and metabolically-active bacterial communities in RHRW irrigation system, soil and produce using this DNA labeling techniques (BrdU and PMA) in tandem with 16S rRNA sequencing technique. The study emphasizes that irrigation water quality greatly influences the bacterial dynamics of the irrigated crop and would require cost-effective on farm mitigation strategies prior to use of these water sources for intended irrigation purposes.

Tables

Table 1: Summary of rooftop harvested rainwater system samples collected and treated throughout the sampling period.

Samples	BrdU treatment	PMA treatment	No treatment	Sampling months	Total (N)
Flush tank A	3	3	3	Jun ’18 – Aug ‘18	**9**
Flush tank B	3	3	3	Jun ’18 – Aug ‘18	**9**
Flush tank C	3	3	3	Jun ’18 – Aug ‘18	**9**
Secondary tank A	1	1	1	Jul’18	**3**
Secondary tank B	1	1	1	Jul’18	**3**
Ambient rain	1	1	1	Jul’18	**3**
Soil beds (1- 5)	30	30	30	Jun ’18 – Aug ‘18	**90**
Chard (1- 5)	20	20	20	Jun ’18 – Aug ‘18	**60**
Total (N)					**186**

Table 2: Water characteristics within a rooftop harvested rainwater system during the sampling period.

Samples	Sampling Months	Water temp. (^{0}C)	pH	DO (%)	Nitrate (mg/mL)	Chloride (mg/mL)	Turbidity (FNU)	Precipitation (inches)	Conductivity (SPC uS/cm)	ORP (mV)
	June	26.17	7.54	92.37	0.95	0.78	0.44	0.29	53.47	149.87
First flush tank A	July	24.67	7.55	106.07	0.05	0.06	2.5	2.46	70.4	137.04
	August	24.1	8.28	99.94	0.65	0.02	0.04	2.72	79.57	132.27
	June	26.23	7.32	93.83	0.55	0.07	0.3	0.29	87.67	144.07
First flush tank B	July	25.3	7.68	106.27	0.04	0.04	2.53	2.46	72.17	94.3
	August	24	7.78	100.2	0.48	0.01	-2.03	2.72	61.6	119.03
	June	29.1	7.57	95.27	1.07	1.07	0.1	0.29	1575.83	181.03
Control tank	July	27.8	7.31	106.6	0.16	0.02	4.43	2.46	485.13	230.33
	August	27.03	7.48	95.8	1.3	1.51	-2.47	2.72	347.03	372.03
Secondary tank A	July	24.6	8.3	106.03	0.03	1.11	1.17	0.29	79	100.97
Secondary tank B	July	24.67	7.88	104.2	0.01	0.23	2.43	2.46	75.97	122.17
Ambient Rain	July	25.1	7.82	110.37	0	0.06	0.1	2.72	15.9	91.97

Table 3: Soil characteristics within a rooftop harvested rainwater system during the sampling period.

Soil characteristics	Soil Bed 1	Soil Bed 2	Soil Bed 3	Soil Bed 4	Soil Bed 5
OM (%)	3.8	7.5	8	8.2	10.4
ENR (lbs/acre)	106	150	150	150	150
pH	7.4	7.6	7.6	7.5	7.5
CEC (meq/100g)	13.8	4.3	13.9	14	14.5
Phosphorus (ppm)	75	69	69	75	91
Potassium (ppm)	114	156	126	105	143
Magnesium (ppm)	188	218	212	204	213
Calcium (ppm)	2393	2416	2371	2414	2479
SM1 (%)	28.48	28.58	30.89	30.02	32.14
SM2 (%)	57.58	50.74	57.24	54.53	48.08

Figures

Figure 1: Rooftop rainwater harvesting study design.

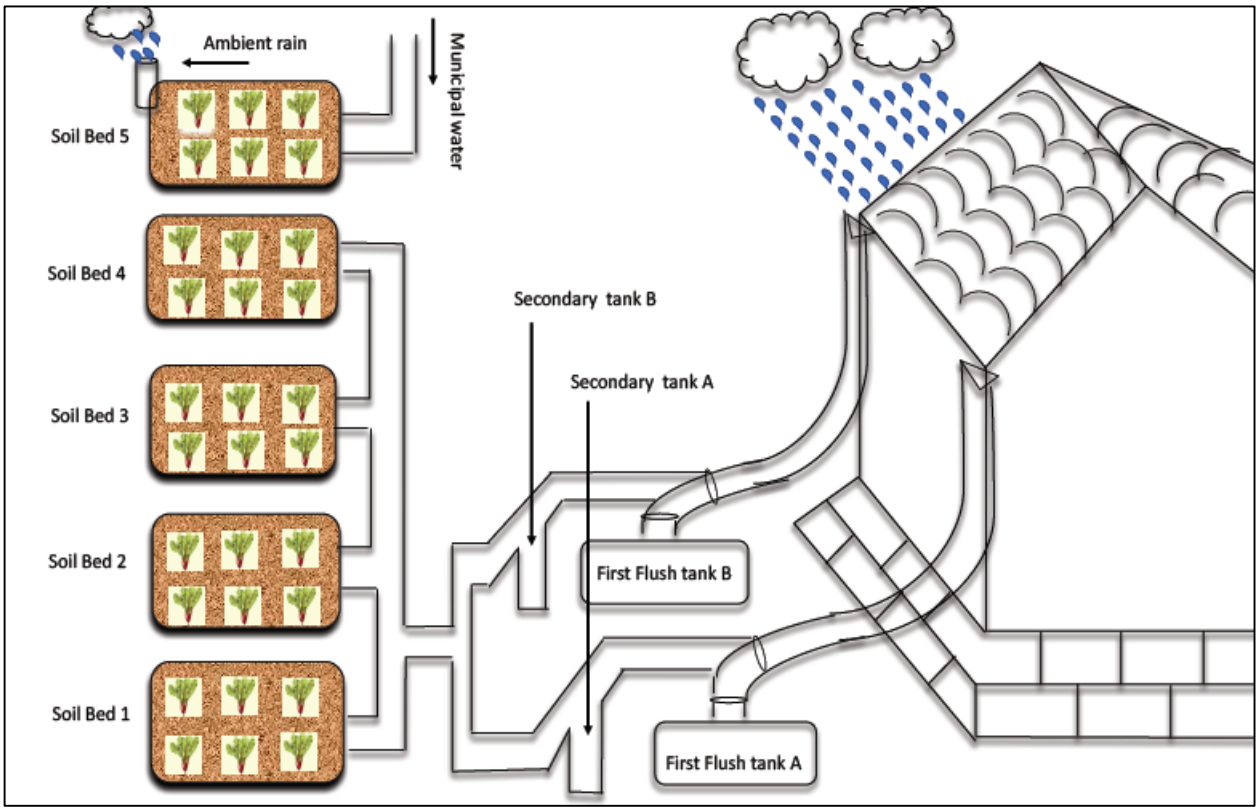


Figure 2: Bacterial diversity plots. (A) Box plots of alpha diversity (Observed number of species and Shannon Index) across produce, soil and water with treatments (BRDU and PMA) and no treatments (noTRT) on rarefied data to minimum sampling depth. Alpha diversity of BrdU-treated (red) and PMA-treated (orange) samples represents the diversity observed in the metabolically-active fraction of bacterial communities presents in each sample. (B) PCoA analysis of Bray Curtis computed distances between treated (BrdU and PMA) and non-treated (noTRT) produce, soil and water samples. Red depicts BrdU-treated samples orange depicts PMA-treated samples and green depicts non-treated samples. Solid colored ellipses are drawn at 95% confidence intervals for treatments in each sample type.

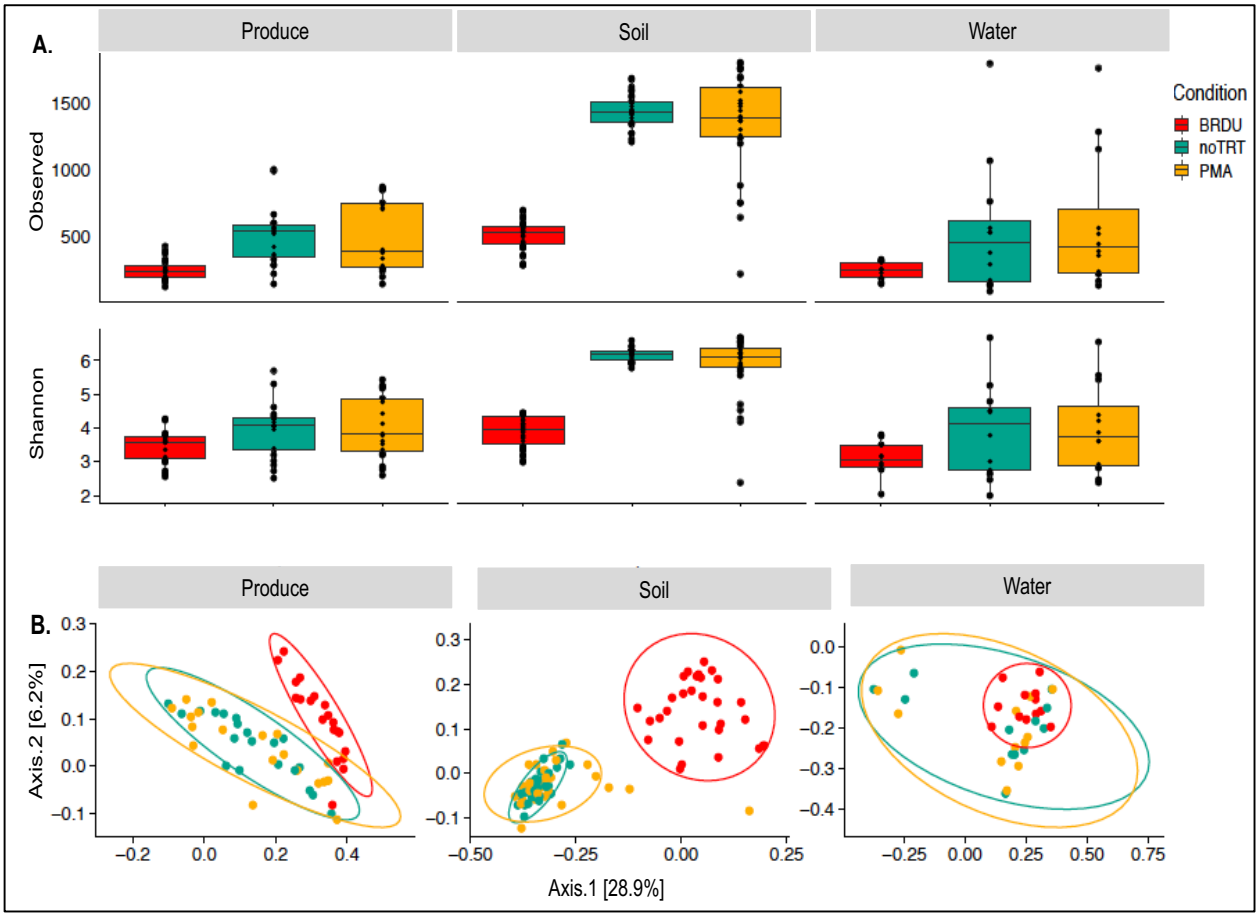


Figure 3: Heatmaps of the Pearson's correlation coefficients between the soil characteristics (A), water characteristics (B) and relative abundance of bacterial phyla. Color gradients reflect the different values of Pearson's correlation coefficients. CEC: Cation Exchange Capacity (meq/100g), ENR: Estimated Nitrogen Release (lbs/A), OM: Organic Matter (%), SM1:surface Soil Moisture (%) and SM2: soil moisture measured 12 inches below surface (%). Precipitation (inches), ORP: Oxidation/reduction (mV), DO: Dissolved Oxygen (%), Aluminium (mg/L).

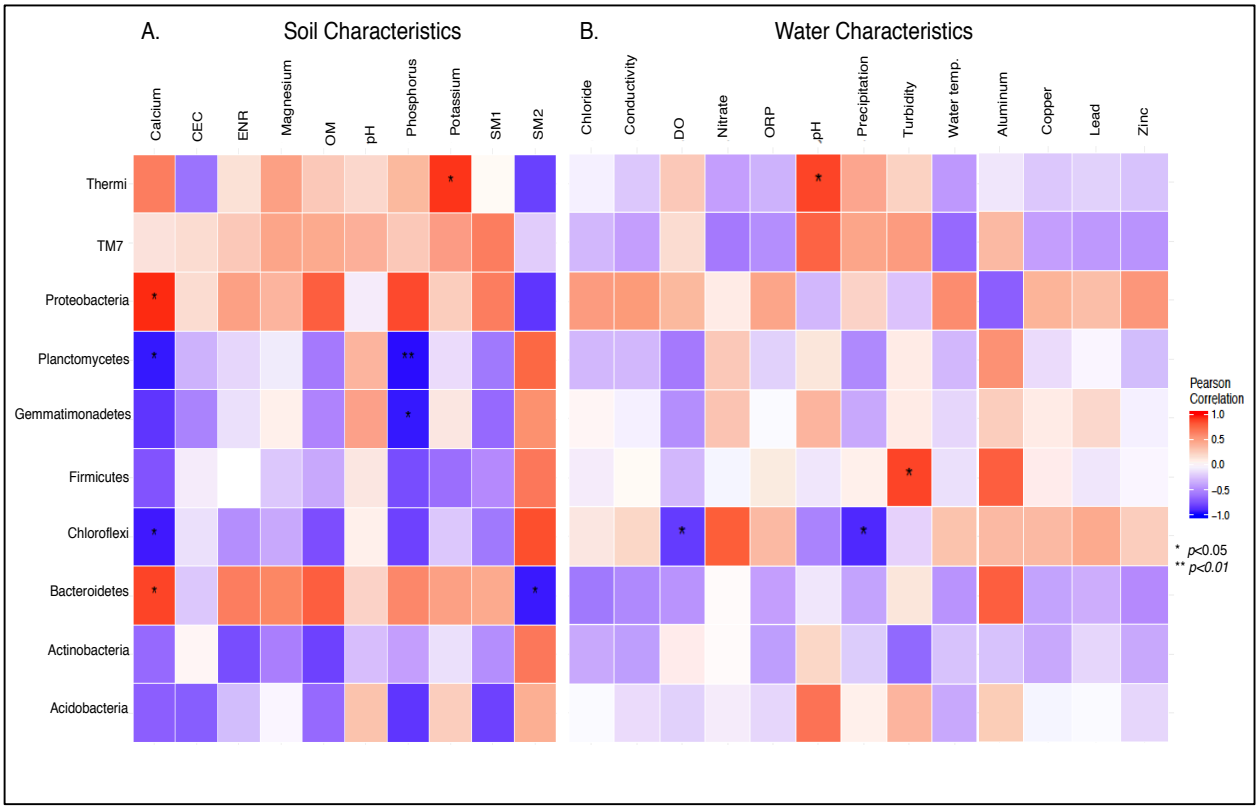


Figure 4: Average relative abundance of the top 25 bacterial profiles in all samples across all treatments. The colored bars represent the sample types, green - produce samples, blue - water samples and yellow - soil samples.

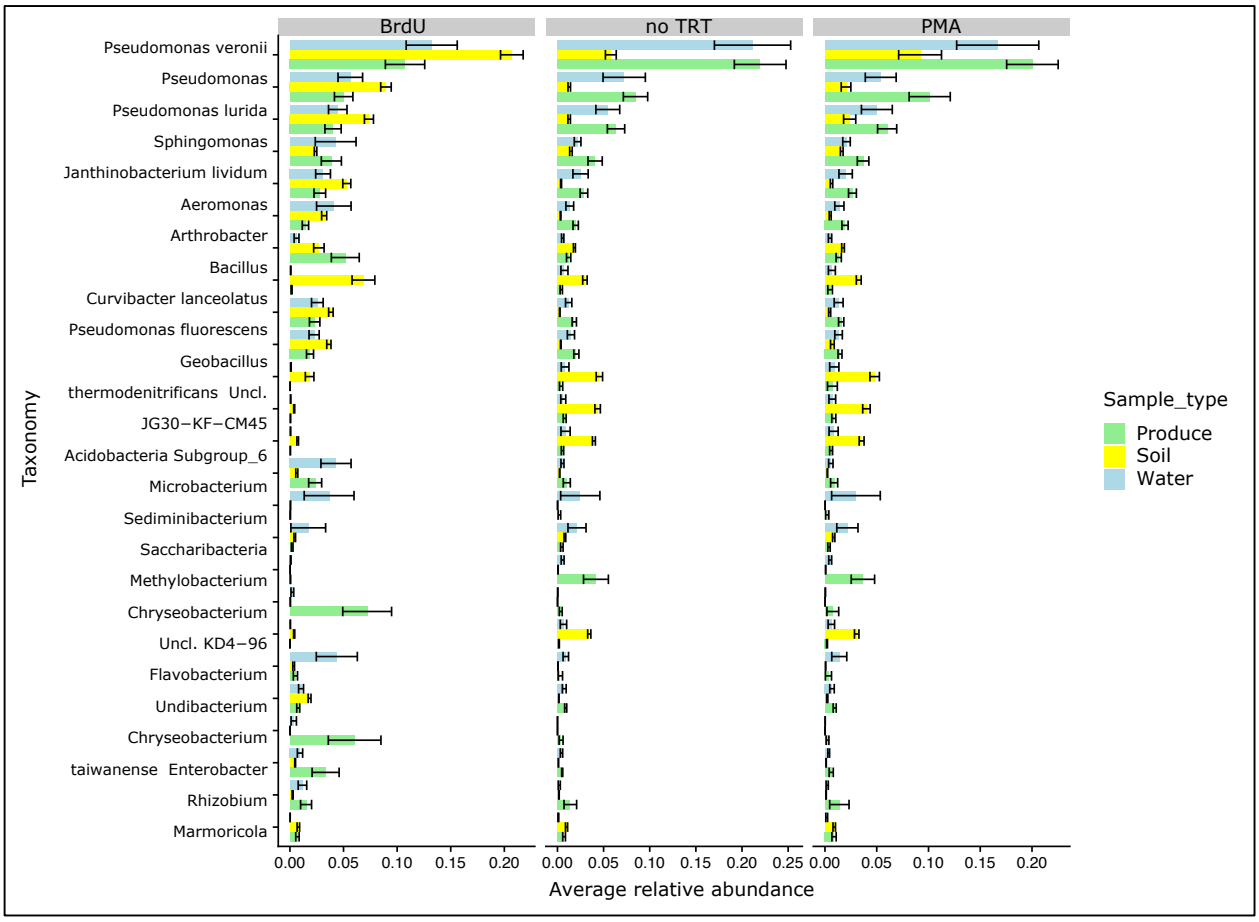


Figure 5: Average relative abundance of the coliform bacteria (*Escherichia*, *Citrobacter* and *Enterobacter*) and *Enterococcus* in the different sample types. The colored bars represent the treatments, red- BrdU treated, green- no treatment and orange – PMA treated in all sample types for the sampling period.

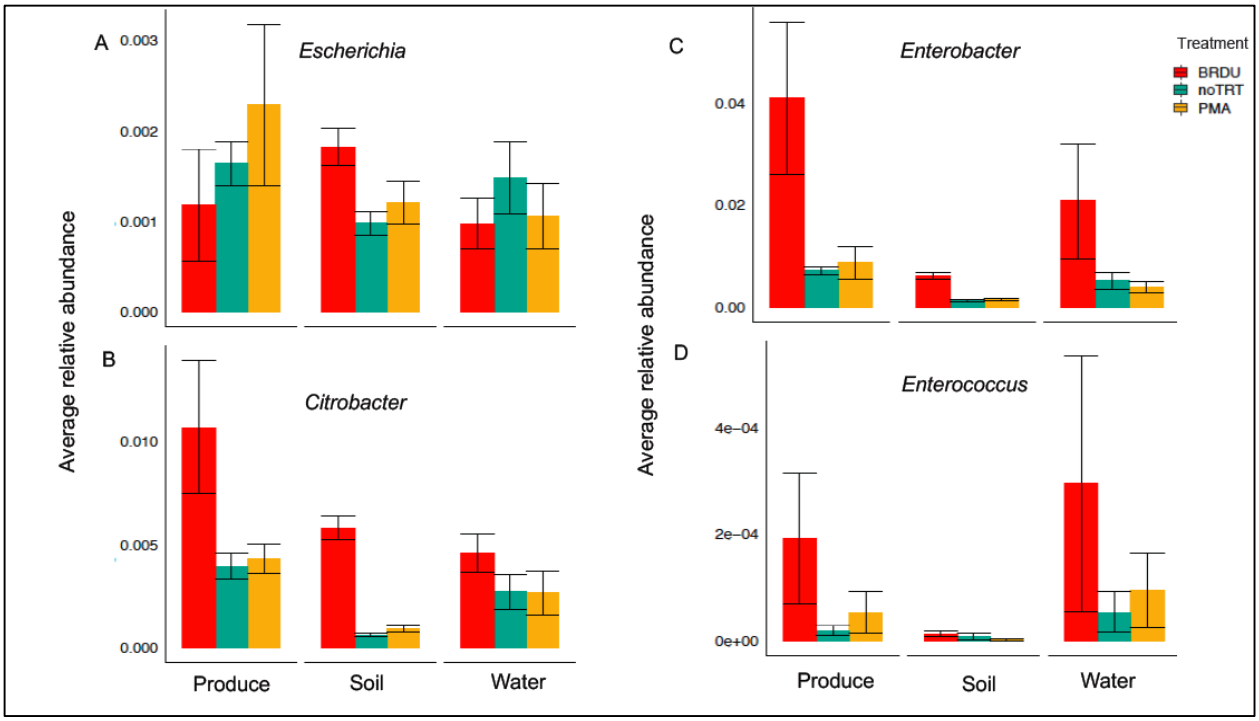


Figure 6: Core microbiome analysis indicating the number of observed taxonomic units (OTUs) shared between soil (yellow), produce (green) and water (blue).

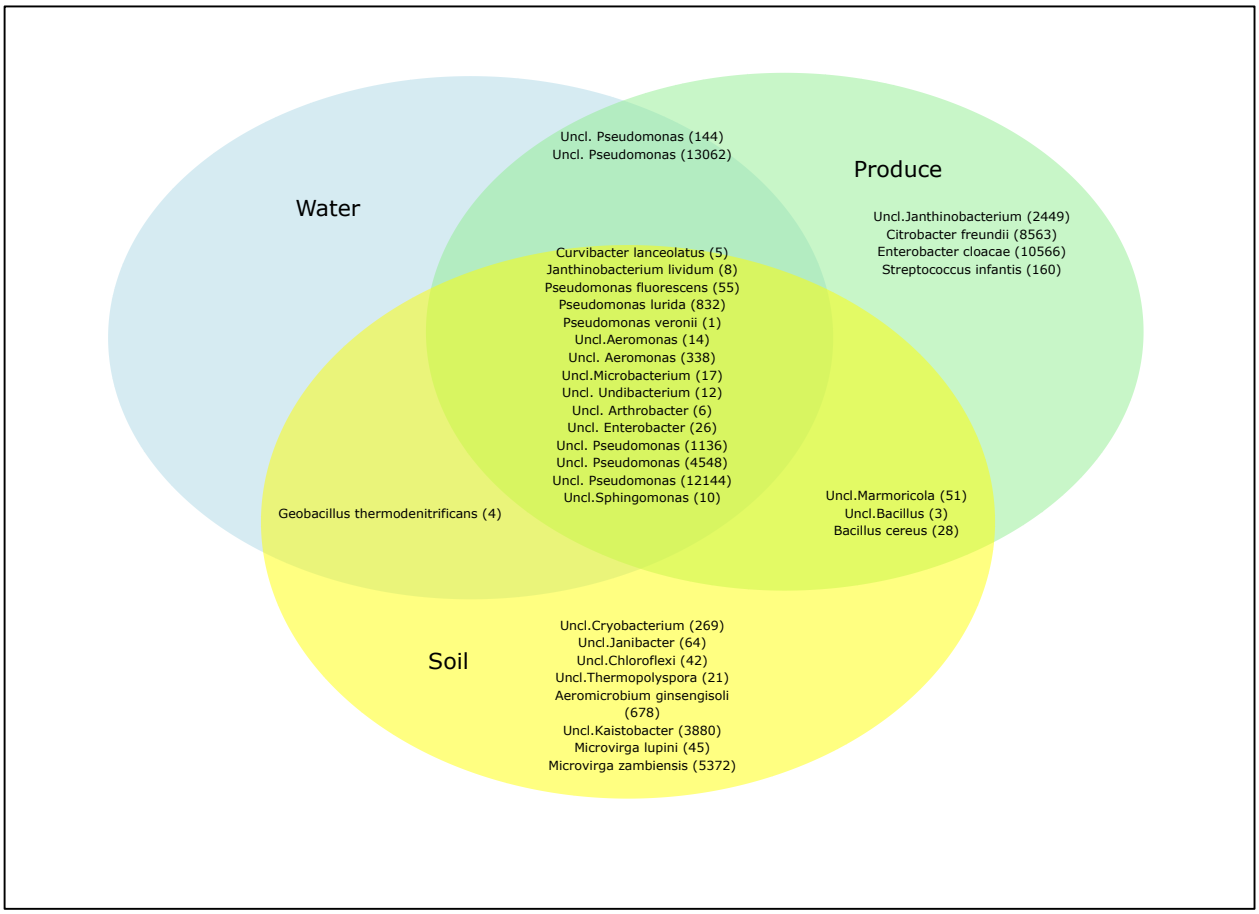


Figure 7: Distribution of core OTUs shared between soil, produce and water among different treatments.

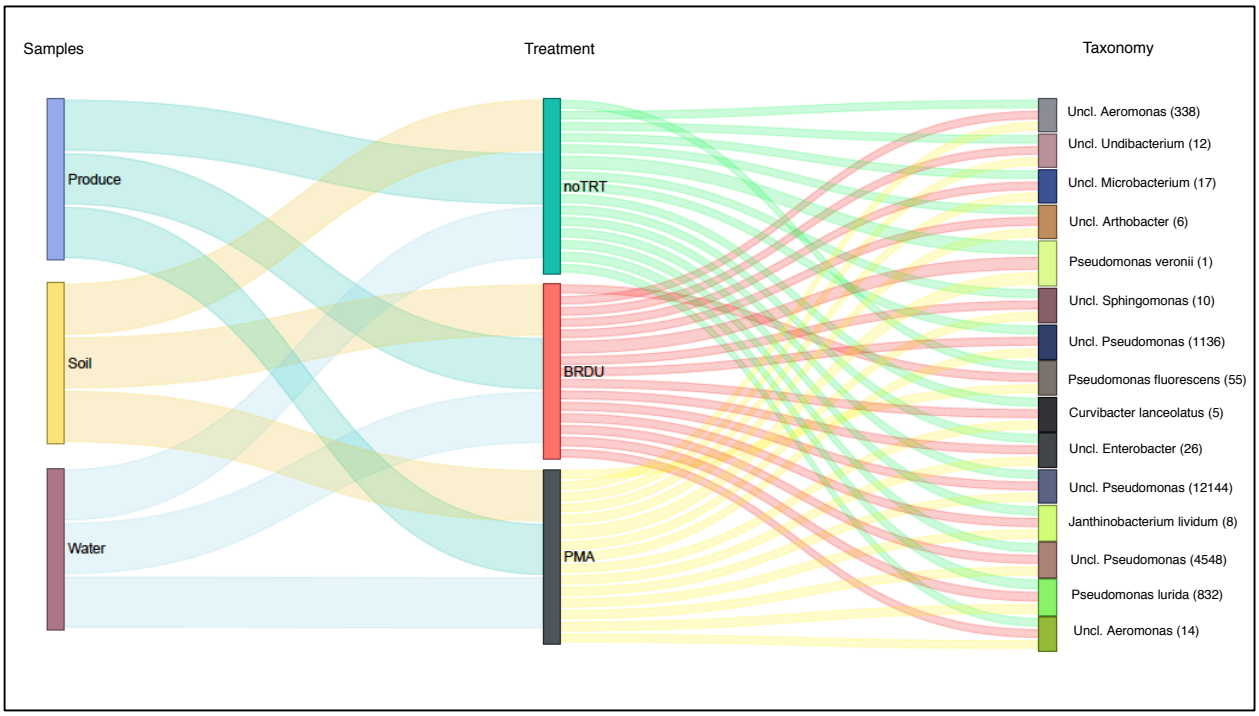
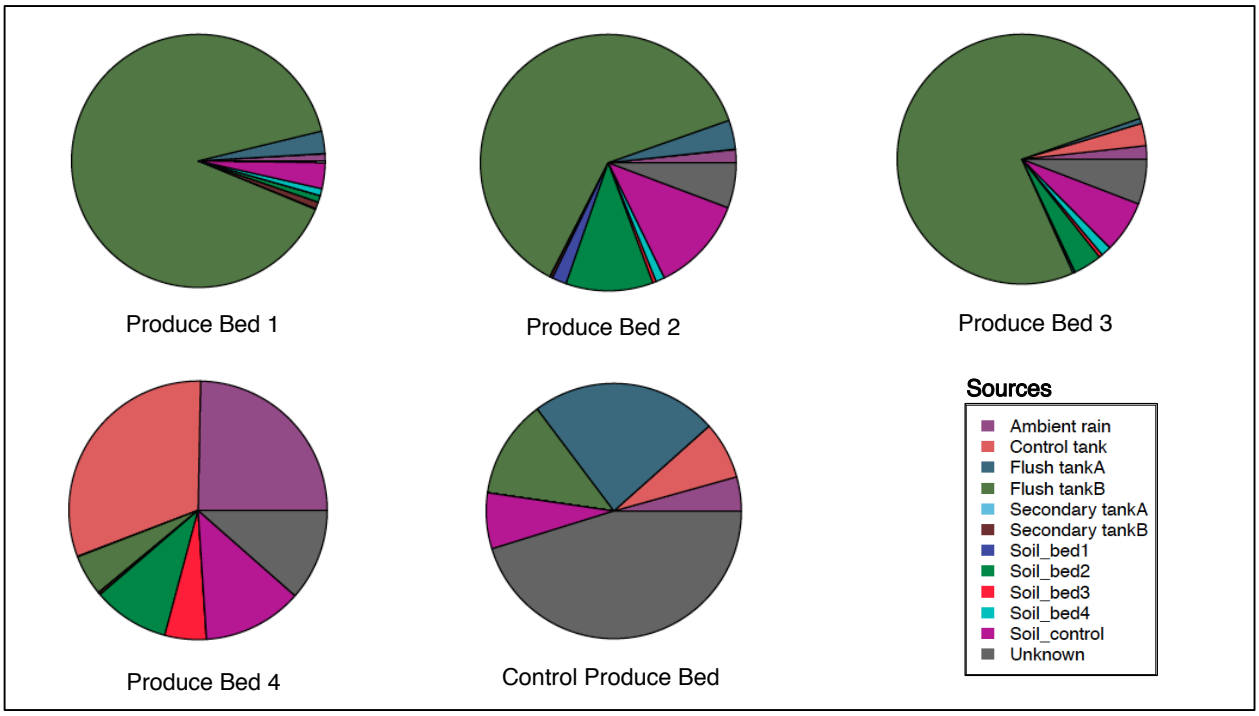
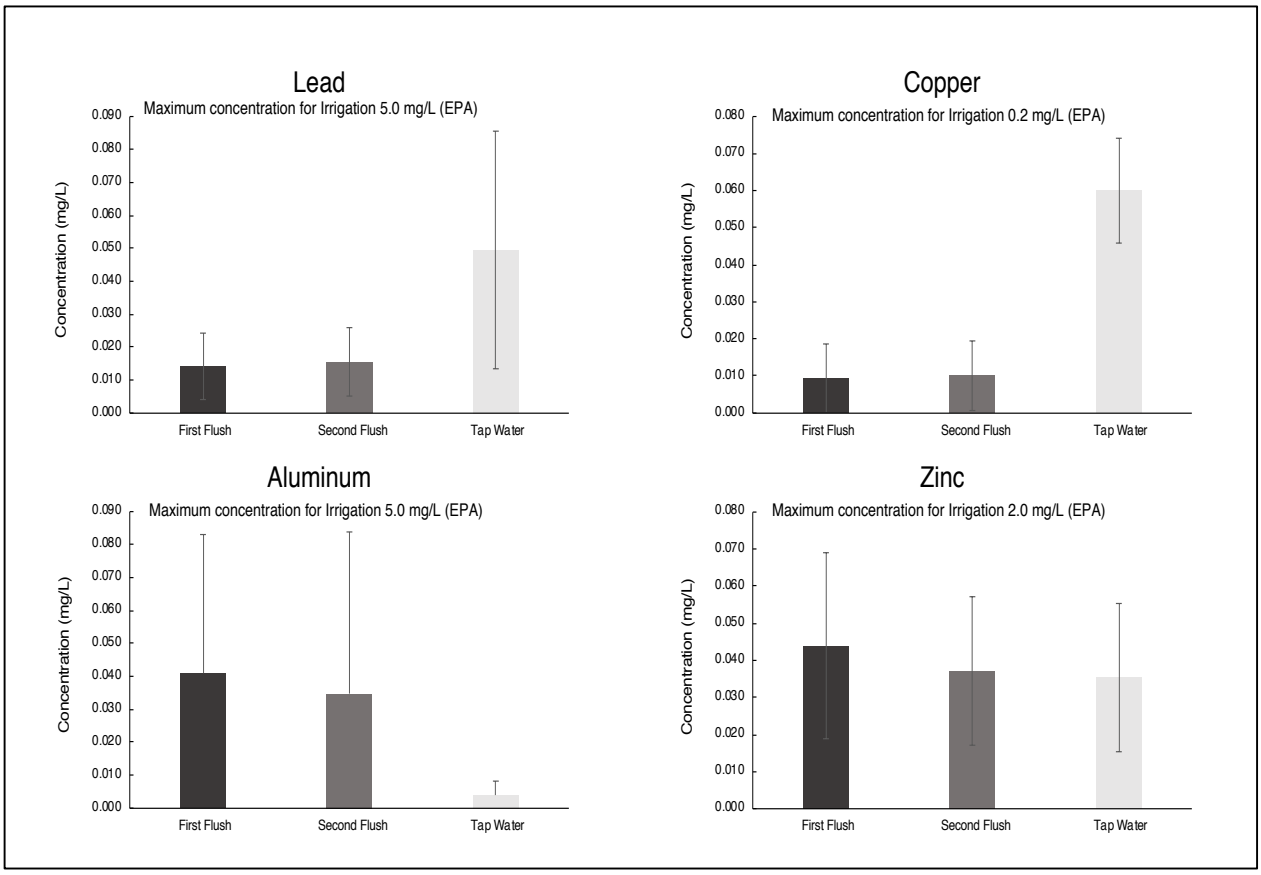


Figure 8: Pie charts representing the likely source of microbial communities on the surface of chard leaves taken from each soil bed during a rain event. The colors in the pie chart represent one of the sources- ambient rain (purple), first flush tank A (teal), First flush tank B (dark green), control- municipal water (peach), secondary tank A (light blue), secondary tank B (brown), soil from bed1 (dark blue), bed 2 (light green), bed 3 (red), control bed (pink) and unknown (gray) for a subset of produce (chard) samples from 4 rainwater fed gardens and a control bed that used municipal water source.

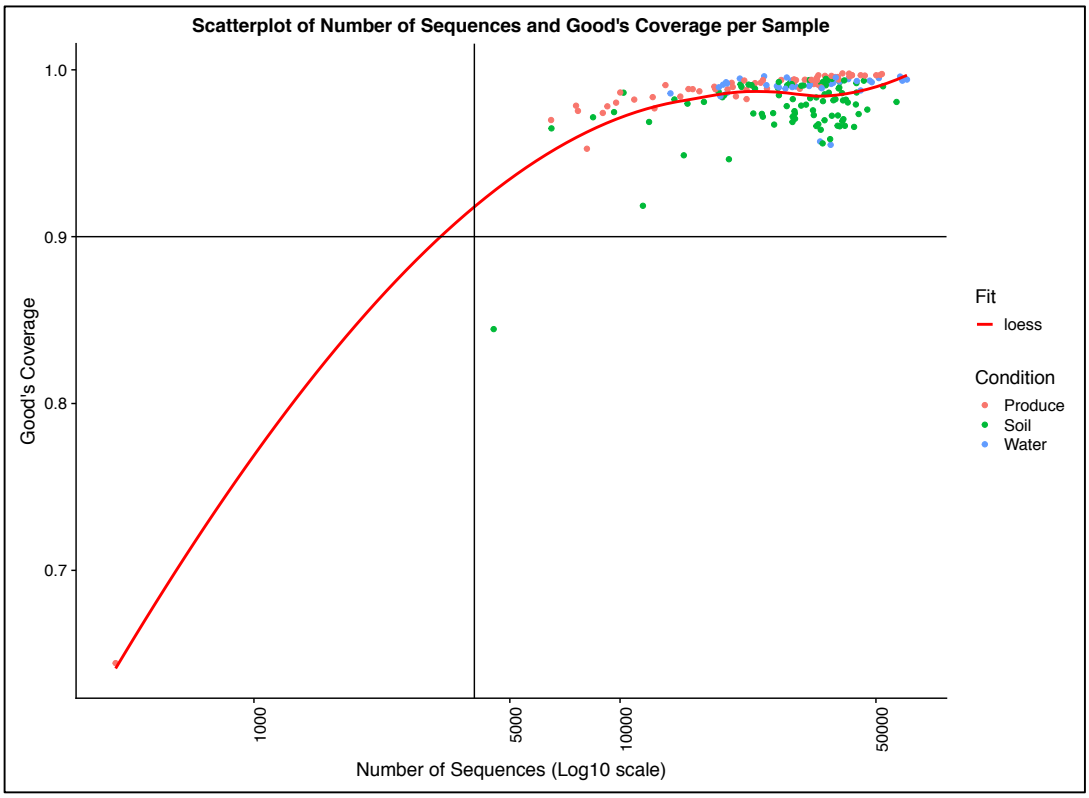


Supplementary figures

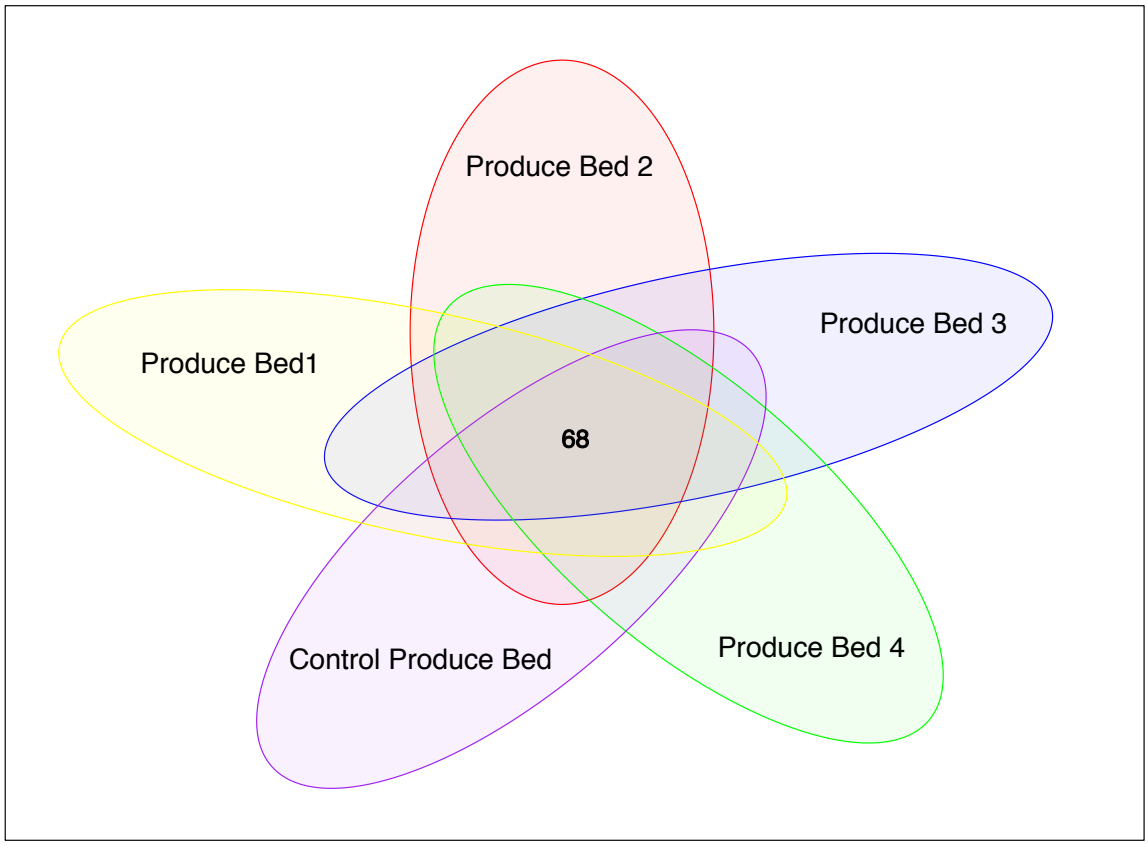
S1. Water metal data



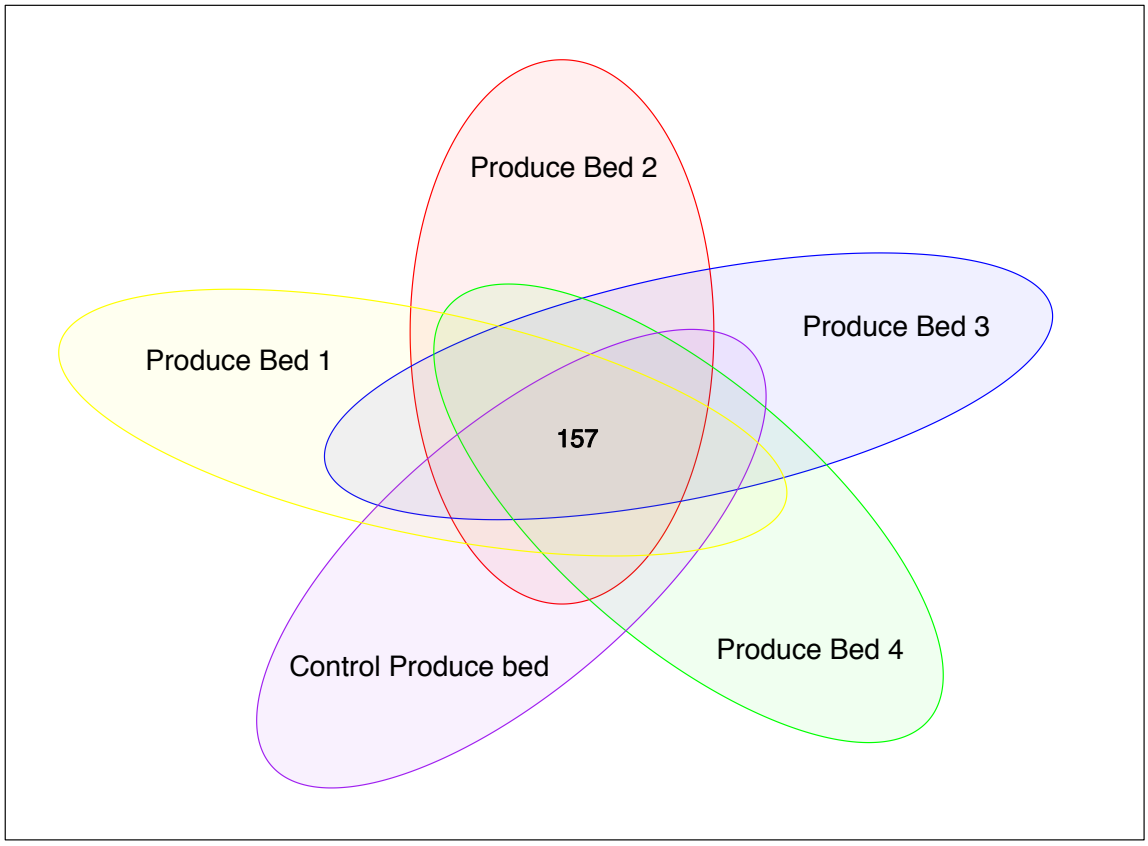
S2. Goods estimate



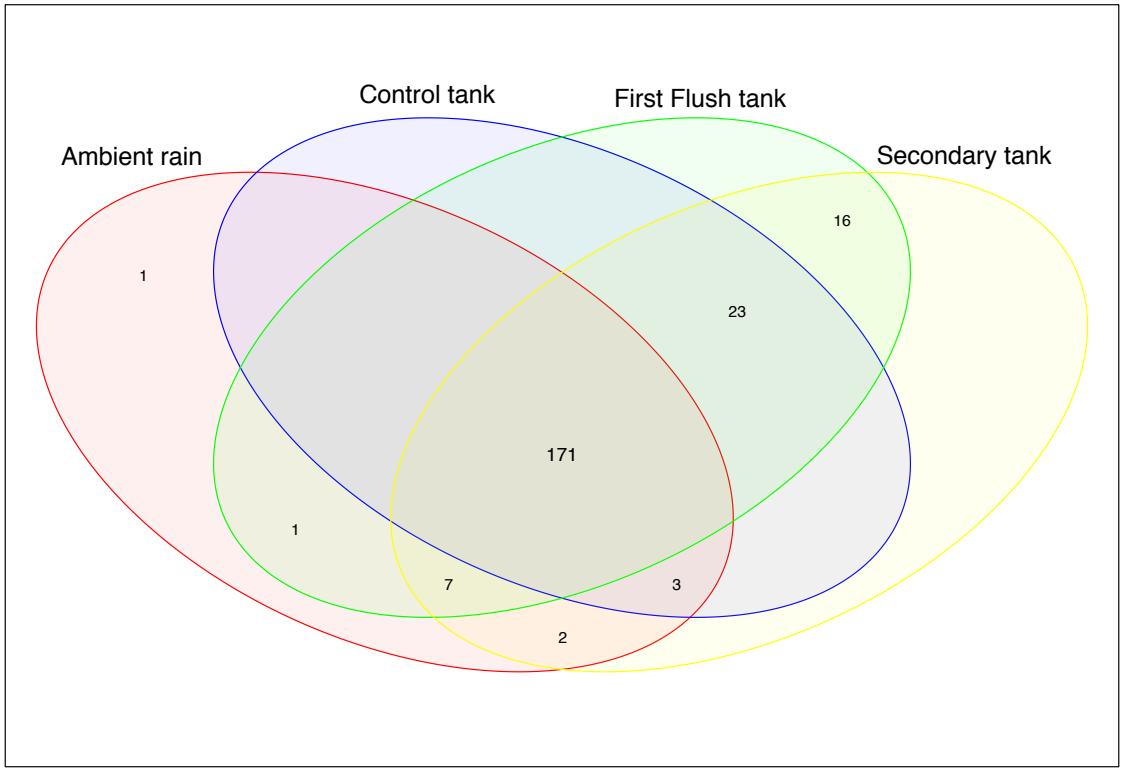
S3: Produce (Chard) core microbiome



S4: Soil core microbiome



S5: Water core microbiome



Chapter 6: Conclusions, Future Research and Public Health Significance

Conclusions and Future Research

Global climate change and population growth are key contributors to our existing freshwater crisis, which in turn is aggravating irrigation water scarcity and ultimately compromising food security and public health [189, 263]. A viable strategy by many countries to address irrigation water shortages is the use of nontraditional irrigation water sources such as advanced treated municipal wastewater, brackish water and rooftop harvested rainwater [50, 91, 264, 265]. Though multiple benefits have been addressed in the literature with regard to reusing nontraditional water sources, such as reducing pressures on overstressed aquifers [2, 266] and groundwater recharge [56], caution is needed concerning the use of these water sources for irrigation purposes due to the potential presence of both microorganisms (bacteria, virus, and protozoa) and chemicals (heavy metals, personal care products (PPCPs), pharmaceuticals, etc.). These contaminants could persist along the food production process resulting in foodborne illnesses and outbreaks and impacting public health and the economy [62, 75]. For example the 2011 multistate outbreak of Listeriosis due to consumption of cantaloupes from Jensen farms in Colorado, here the contamination was linked to equipment's at the packing facility and also contaminated water [267]. In 2018, agricultural water reservoir in Santa Maria farm in California was found to be the reason for the outbreak of *E. coli* O157:H7 infections linked to consuming romaine lettuce [261].

Detection of these bacterial communities is still performed via culture dependent methods, which is time consuming and intensive and sometimes fail to capture viable-but-non-culturable (VBNC) bacteria. With the introduction of newer culture independent methods like multiplex PCRs, next generation sequencing techniques the identification of non-culturable bacteria in food and water sources has drastically improved. Yet, since sequencing techniques are nucleic acid based, they are unable to differentiate live and metabolically-active versus those that are represented by free, relic DNA, not resent in viable cells.

My dissertation chapters heavily focused on the characterization of bacterial communities in nontraditional water sources including reclaimed water (Chapter 3 and 4), agriculture ponds (Chapter 3 and 4), tidal brackish creeks (Chapter 4), non-tidal freshwater creeks (Chapter 4) and rooftop harvested rainwater (Chapter 5). My main goal was to identify metabolically-active bacteria in these water sources via coupling DNA labeling and sequencing techniques. In all three manuscript chapters, we presented data characterizing both total and metabolically active bacterial communities in multiple nontraditional irrigation water sources.

My first manuscript entitled **"Characterizing metabolically-active bacteria in reclaimed water and ponds using bromodeoxyuridine DNA labeling coupled with 16S rRNA and shotgun sequencing"** characterized live (metabolically-active) and total bacterial communities in two nontraditional irrigation water sources (reclaimed water and ponds) collected over a year from the Mid-Atlantic region. Bacterial communities in both waters were diverse and we were able to identify the presence of bacterial genera and/or species (*Actinobacterium* spp., *Flavobacterium*

spp., *Aeromonas media*, *Aeromonas hydophila*, *Propioniobacterium* spp., *Pseudomonas fluorescens* and *Arcobacter* spp.) of importance to both human and/or animal health. Additionally, our 16S rRNA sequencing data revealed the presence of currently non-culturable members of the phylum *Actinobacterium* (*Candidatus* Aquiluna*, Canditatus* Rhodoluna*, Candidatus* Planktoluna and *Candidatus* Planktophila*)* in BrdU-treated and non-BrdU-treated water samples. These bacterial species that have been previously identified in water sources via sequencing studies would have gone undetected in a culture-based study. These data have enabled us to demonstrate, for the first time, that these organisms appear to be metabolically-active in both reclaimed water and ponds.

Interestingly, we also detected diverse antimicrobial resistance (AMR) and virulence genes in both reclaimed and pond water. A large body of research across different regions of the world shows the presence of AMR and virulence genes in surface waters and reclaimed waters [76, 77, 268–271]. For example, presence of multidrug resistant *E. coli* have been observed in Dutch surface water and wastewater [272]. Similarly, Goldstein et al. (2012 and 2014) detected the presence of methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci in both influent and effluent samples recovered from four U.S. wastewater treatment plants [76, 77]. Detection of these antibiotic resistant bacteria generally involved either plating different samples on non-selective or antibiotic selective agar plates, purifying the bacterial colonies and using different methods to determine the minimum inhibitory concentration (MIC) for a wide array of antibiotics [188] or using new molecular techniques. But, using all of the above-mentioned

techniques, there is lack of knowledge whether the metabolically-active bacterial fraction of an environmental sample actually carries these genes or not. Using our novel approach, identified presence of AMR and virulence genes and was found more commonly in non-BrdU treated water samples compared to BrdU-treated samples, indicating that these genes may be less associated with the viable, metabolically-active organisms.

A highlight of this study was the coupling of BrdU labeling and DNA sequencing to identify metabolically-active bacteria, AMR genes and virulence genes in alternative irrigation water sources in the United States. The knowledge gained from this study will help advance research regarding mitigation strategies to remove pathogenic bacteria, such that these nontraditional water sources can be used for irrigation purposes. Additionally, this study identified new metabolically-active bacteria in water sources that have previously gone unnoticed in culture-based studies. Further studies of these newly identified bacteria will enhance our knowledge of the bacterial communities in these water bodies.

The second manuscript described diverse bacterial communities in four irrigation water sources in the Chesapeake Bay watershed sampled from May to September 2018 and is entitled **"Coupled DNA-labeling and sequencing approach enables the detection of viable-but-non-culturable *Vibrio* spp. in irrigation water sources in the Chesapeake Bay watershed"**. This study focused mainly on enumerating viable-but-non-culturable (VBNC) *Vibrio* spp. in nontraditional irrigation water sources with the help of culture-dependent and -independent methods. These VBNC *Vibrio* spp. often go undetected in culture-based studies, and

while they may be detected through sequencing approaches, most sequencing methods do not provide information on viability. However, through our novel approach of coupling BrdU labeling with DNA sequencing we were able to identify the presence of both pathogenic (*V. cholerae, V. parahaemolyticus* and *V. vulnificus*) and non-pathogenic *Vibrio* spp. in all water samples. Three water sites (tidal brackish creek, reclaimed water and non-tidal freshwater creek) showed the presence of *Vibrio* spp. through culture-dependent methods, while all four water sites showed the presence of *Vibrio* spp. in both BrdU-treated and non-BrdU treated water samples via sequencing methods. Through our culture-based method we identified 115 presumptive *Vibrio* isolates of which only 28 isolates were confirmed via multiplex PCR as *Vibrio'*s (*V. cholerae* (n=16)*, V. parahaemolyticus* (n=11) and *V. vulnificus* (n=1)). Our findings corroborate with other studies that have looked for *Vibrio* isolates in different water sources like reclaimed water and estuaries throughout the world [43, 44, 201, 203–206, 273–277]. Most of these studies used culture dependent – enrichment and plating on special media like thiocitrate bile salt agar (TCBS), Chromagar, etc to isolate *Vibrio*'s from the environment and then confirmed using qPCR, multiplex PCR techniques. In contrast, our innovative labeling/sequencing method helped us to identify *Vibrio* spp. in non-enriched BrdU-treated water samples, implying that, to detect VBNC *Vibrio* spp., we may be able to rely on culture-independent methods that incorporate both DNA labeling and sequencing.

Additionally, we identified the presence of other human pathogens in these water sources: *Clostridium bifermentans*, *Enterobacter cloacae*, *Plesiomonas shigelloides*, and *Bacillus cereus* in the BrdU treated water samples. These human

pathogens have been reported in association with foodborne illness and outbreaks [165, 216, 218–221, 223, 225, 227]. Additionally, some of these are opportunistic and multi-drug resistant which makes it even harder to be treated [225, 227, 278]. Overall, this study helped in identifying VBNC *Vibrio* spp. and other potential human pathogens in the tested nontraditional water sources.

Future studies building of this work should focus on: 1) identifying virulence and AMR genes in these water sources via shotgun sequencing is important as *Vibrios* have a tendency to acquire virulence genes from phages or through undefined horizontal gene transfer events, for example the cholera toxin gene was acquired from the CTX phage [279]; 2) testing antimicrobial susceptibility of the *Vibrio* isolates that were recovered via our culture-based study; 3) quantifying *Vibrio* spp. using quantitative PCR; and lastly 4) applying our novel DNA-labeling and sequencing technique to enumerate other foodborne VBNC bacteria (*Campylobacter* spp. , *E. coli*, *Salmonella* spp. *Listeria* spp., *Shigella* spp., *Enterobacter* spp., etc.) present in water sources that are potentially harmful to humans.

Our last manuscript was a field-based study to track bacteria from rooftop harvested rainwater (RHRW) to irrigated soil and produce and is entitled **"Source tracking microbial communities from rooftop harvested rainwater to irrigated soil and produce"**. For this study, we had a total of 186 samples (irrigation water=36, soil=90 and produce=60) that were recovered from a vegetable raingarden in Maryland, USA from June to August 2018. In this study, besides using BrdU labeling, we also utilized a second labeling technique, employing propidium monoazide (PMA) which has been used previously in tandem with sequencing [240].

Additionally, we also tracked bacterial communities from the irrigation water source (rainwater) to irrigated soil and produce using the SourceTracker software. Our study was able to identify the likely transfer of bacterial communities from rooftop harvested rainwater to irrigated produce. Hence, our data provided evidence that potentially harmful bacteria could reach food crops irrigated with rooftop harvested rainwater, and when these products are consumed raw, this could potentially result in harmful effects on consumers. Specifically, we observed the presence of *Pseudomonas* spp., *Aeromonas* spp., and fecal indicators (*Escherichia*, *Citrobacter*, *Enterobacter* and *Enterococcus*) in all samples collected. Detections of these bacteria is in tandem with other studies that have looked at roof-top harvested rainwater. Most of these studies have either used culture-based methods in combination with either PCRs, or 16S rRNA sequencing techniques to detect these bacteria. Our study was able to tease out the metabolically-active bacteria from the relic DNA in not only the rooftop harvested rain water but also from the irrigated soil and produce. On evaluating the two labeling techniques, PMA-treated samples seemed to exaggerate viability of bacterial communities when compared to BrdU-treated samples and hence BrdU seems to be a more promising labeling technique to be coupled along with DNA sequencing methods to detect metabolically-active bacteria.

Future studies to further this work could involve: 1) more sampling time points to investigate whether rain events play a key role in dispersing bacterial communities from rainwater to irrigated crops; 2) mitigation strategies like installing a cost-effective filter system to remove or reduce bacterial communities before the

harvested rainwater is applied on food crops; and lastly 3) an evaluation of the persistence of rainwater- transferred bacteria in the irrigated food crop.

Public Health Significance

Fresh produce comes in contact with water during various phases of food crop production including but not restricted to chemical application, irrigation, worker hygiene and food processing [280]. With limited access to good quality freshwater and increasing reliance on nontraditional irrigation water sources knowledge of the microbial community, chemicals and physical contaminants in these water sources is an absolute necessity to reduce the risk of foodborne illnesses and outbreaks in the future. The US Food and Drug Administration (FDA) with the implementation of the Food Safety Modernization Act (FSMA) [16, 17] is striving to shift from responding to foodborne outbreaks to preventing them by introducing the Produce Safety Rule (PSR, 21 CFR 112) [18] (discussed in detail above). In brief, the PSR requires irrigation water quality to meet *E. coli* standards that are deemed safe in order for the water to be applied on produce. With stricter irrigation quality standards and limited access to good quality irrigation water sources farmers are pressured to do regular water quality testings. Hence, there is a great demand for rapid, sensitive, specific and accurate methods to detect bacterial communities. Conventional culture-dependent methods have been the gold standard in detecting foodborne pathogens [281]; however, recovery of VBNC bacteria is a key limitation of these methods [9]. Though molecular techniques have helped to characterize and enumerate VBNC bacteria

[282, 283], the methods lack the ability to differentiate between dead and viable cells as DNA is very persistent in nature [118]. Viability of a pathogen is vital information for farmers, regulatory agencies and consumers. Hence, my doctoral research added important new knowledge focused on the characterization of metabolically-active bacteria in nontraditional water sources via coupling DNA labelling and sequencing techniques. This innovative technique can provide rapid, sensitive and accurate detection of bacterial communities in these water sources.

The overall impact of my research is that, with the use of the novel methods developed herein, researchers are better able to inform farmers about the potential microbial risks of using nontraditional water sources for agricultural irrigation. Indirectly or directly, my research goals address the following:

1) Use of alternative water sources will require a series of treatments before the water is released into the environment, supporting **a healthier ecosystem**. Additionally, water recycling can decrease diversion of fresh water from sensitive ecosystems and provide long-term sustainability of our water supplies.

2) Our knowledge of the prevalence of viable pathogenic bacterial communities like *Vibrios, Aeromonas, Escherichia, Enterobacter, Pseudomonas, Sphingomonas, Arthrobacter* and *Flavobacterium* in water sources from the Mid-Atlantic region can inform future efforts to improve **sustainable agriculture** and also prevent foodborne illness and future food and water related outbreaks.

3) Our overall findings are directly applicable to key stakeholders in the region: farmers who need to develop mitigation strategies to ensure **resilient**

agricultural communities and sustainable food production in the face of ongoing climate variability.

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