

**Native soil virus abundance, composition, and mobility under
natural and managed agricultural conditions**

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Doctor of Philosophy

Degree

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DEDICATION

To my parents, Michael and Regina, and my brother Marshall, for always pushing me to aim higher, and to my beloved wife Kat, for your unwavering love and support.

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ABSTRACT

Soil viruses are ubiquitous in the environment and important for their influence on host community composition and function. Viral infection influences host evolution, metabolism, function, diversity, community evenness, and more. As host bacteria play critical roles in agricultural systems, including nutrient cycling and soil aggregate formation, the influence which soil viruses have on their hosts makes them key players in these systems. However, many important questions remain regarding virus abundance and distribution under human imposed and natural conditions in agricultural systems. Understanding how these conditions impact virus abundance, transport, and community structure is critical for a broader understanding of soil microbial functional diversity.

Here, we undertook two main studies aimed at understanding these conditions. First, we conducted a field-based study of virus and bacteria abundance, as well as viral reproductive strategy, under agricultural management practices including no-till, nitrogen fertilization, and cover crop rotations. We determined that viral abundance and lysogenic fraction were most significantly influenced by sampling date, rather than by the imposed management practices. Additionally, we determined that increased concentrations of nitrogen elevated virus abundance while lowering bacteria abundance and lysogenic fraction, potentially indicating a community wide prophage induction event.

To explore additional factors which might have influenced virus abundance between sampling dates in our field experiment, we conducted our second main study, which was a lab-based column experiment to mimic natural rainfall or irrigation patterns to determine the influence of interrupted flow patterns on the mobilization and community composition of viruses in agricultural soils. Additionally, we added

atmospheric nitrogen deposition levels of ammonium to our columns to determine if trace levels of nitrogen influenced viral transport and community composition. We determined that interrupted hydrologic flow has more influence on viral transport and abundance than the ammonium amendments. Additionally, we made novel discoveries regarding the differential transport of virus families with varying physiochemical (i.e. shape, size, and surface properties) properties.

In total, we filled important knowledge gaps about the abundance, diversity, and transport of native viruses in natural soil systems, which serves as a foundation for future studies in soil viral ecology in these economically important managed systems.

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CHAPTER 1: INTRODUCTION

BACKGROUND AND MOTIVATION

Viruses are the most abundant biological entities on earth. Bacteriophages (viruses which infect prokaryotic hosts) are ubiquitous within the soil environment. Myriad research has shown the importance of these bacteriophages on host function and diversity (Bi et al., 2021; Breitbart et al., 2018; Emerson et al., 2018; Liang et al., 2019a; Trubl et al., 2021). Viruses have been shown to exert top-down control of host population by lytic reproduction (Kuzakov & Mason-Jones, 2018). Additionally, they have been shown to influence host function through lysogenic reproduction (Ghosh et al., 2008; Liang et al., 2020b; K. E. Williamson et al., 2007). Viruses exist as an immense genetic reservoir and are integral to host evolution (K. E. Williamson et al., 2017). Studies from marine surface waters have also shown that when soil viruses lyse microbial hosts, the release of microbial necromass into the environment has a significant impact on nutrient cycling, with up to 50% of biomass produced per day returning to the dissolved pool (Suttle, 2005). The potential influences soil viruses have over microbial processes, nutrient cycling, and ecosystem services are only now becoming fully appreciated. Despite the increasing volume of research, knowledge regarding viruses' role in soil systems is in its infancy due to the complex nature of the soil system. Investigation into soil viral ecology must examine the abundance, distribution, community composition, and transport of viruses to fully understand the role of viruses in soil ecosystems.

The mechanisms by which viruses impact their microbial hosts and thereby the greater environment are largely dependent on the method of viral reproduction. Lytic reproduction, productive viral infection which results in the death of the host cell, and lysogenic reproduction, wherein the virus, in the form of a prophage, is replicated as the

host grows and divides, have differential impacts on host ecology (Erez et al., 2017). Some viruses, called temperate phage, can alternate between lysis and lysogeny, and have shown community wide shifts in reproduction strategy under varying environmental conditions (Liang et al., 2020b; Tan et al., 2020; Weinbauer et al., 2003; K. E. Williamson et al., 2007; S. J. Williamson et al., 2002). Much of our understanding of environmental viral ecology comes from aquatic studies. However, in natural soil systems, observing changes in viral reproduction is extremely complex due to the many chemical and physical factors which can influence virus-host interactions as well as impede microbial investigations. Furthermore, the diversity of soil viruses is so immense and the heterogeneity of soil so great that at any given moment in time an array of reproductive strategies is likely operative.

In order to infect hosts, temperate and virulent (obligate lytic) viruses must make contact with hosts within the soil system. As viruses are non-motile biological entities, phage-host contact is largely dependent on how soil physiochemical properties as well as hydrologic flow conditions influence soil virus transport, distribution, and retention in soil pores, as well as the ability of the virus to persist extracellularly in soil pores until contacting a new host. The movement of viruses in soil is influenced by the interactions among soil surface properties (such as organic matter or iron oxide coatings), virus properties (such as shape, size, and surface charges), and soil solution conditions (such as ionic strength, ion composition, pH, air bubbles, and flow rate) (Anders & Chrysikopoulos, 2009; Chu et al., 2000; Guan et al., 2003; Molnar et al., 2015; Sasidharan et al., 2016; Zhuang et al., 2009; Zhuang & Jin, 2003a). Understanding viral retention and transport supports predictions of viral function in the soil environment.

It is critically important to identify factors which impact the distribution and abundance of soil viruses under real soil conditions in order to understand how their replication might influence bacterial host community. Agricultural soils are some of the most widely studied given their importance on food security, economics, and environmental conservation. Sustainable agriculture practices have been suggested to enhance microbial abundance and diversity within soil systems; however, their impacts on soil viruses are largely unknown. As viruses are important players in the lives of the microbial hosts, studies must be done to examine how they fit into overall soil ecology, and must start by answering fundamental questions regarding viral abundance, community composition, and transport in natural systems.

To fill some of these existing knowledge gaps regarding viruses in natural systems, we undertook a series of experiments to observe the response of native soil viruses to changes in environmental conditions based on both human imposed, and natural processes related to sustainable agriculture. First, in a field-based experiment, we quantified soil virus and bacteria abundance in a long-term agriculturally managed system of continuous cotton production to investigate how management practices and seasonal changes impacted viral abundance and reproduction. Next, we conducted laboratory-scale soil column experiments to mimic irrigation events and atmospheric nitrogen deposition to understand viral movement and retention in a soil profile. Our goal was to identify key hydrological, geochemical, and biological factors which might determine the distribution, abundance, and replication strategies of native soil viruses in natural soil. Our specific objectives of the field experiment were to determine (1) changes in viral and bacterial abundance under varying agricultural management practices

including winter cover crop rotation, tillage management, and nitrogen fertilization, (2) how the viral and bacterial abundance changes over the course of a growing season, and (3) key factors which might impact viral reproduction on the field scale. Then, in our lab-scale soil column transport experiment, we aimed to determine (4) how interrupted flow events influence soil virus transport through a saturated soil column by mimicking repeated rainfall or irrigation events, (5) if trace levels of ammonium equal to that found in atmospheric N deposition had any effect on viral mobilization, and (6) if the soil virus abundance in the residual column soil was affected by the interrupted flow events and the ammonium additions. Finally, by performing next-generation sequencing of the soil column transport experiments, we aimed to determine (7) if interrupted flow differentially mobilized varying viruses or influenced the community composition of easily transported versus highly retained viruses, (8) the community composition of soil viruses and bacteria immobilized or retained in the soil column, (9) if nitrogen deposition impacts soil virus mobilization or production in the soil profile, and (10) if we could identify switches from lysogenic to lytic viral reproduction under saturated interrupted flow conditions and atmospheric deposition of nitrogen. In total, by observing virus and bacterial host abundance, mobility, community composition, and distribution within the soil profile, we expected to gain understanding of the varying responses of soil viral populations to various agricultural management practices.

VIRUS ABUNDANCE AND DISTRIBUTION

In the total biosphere, there are estimated to be 10^{31} viruses, with virus abundance in soil ranging from 10^7 to 10^9 virus like particles (VLPs) per gram of dry soil (K. E.

Williamson et al., 2003, 2017). The distribution and abundance of these viruses in the environment has been shown to be impacted by many factors, including nutrient concentrations, host abundances, pH, moisture content, season, and temperature (Almeida et al., 2015; Finke et al., 2017; Keshri et al., 2017; Narr et al., 2017; K. E. Williamson et al., 2005). In addition, the community composition of viral populations responds to environmental conditions. Soil moisture content, pH, depth, and nitrogen and carbon contents have been shown to cause shifts in viral community composition (Emerson et al., 2018; Liang et al., 2020b; Santos-Medellin et al., 2021; ter Horst et al., 2021). Higher viral abundances have correlated with higher host abundances, suggesting that much of viral abundance is more dependent on the response of host cells to environmental factors than on direct environmental impacts on viruses (Emerson et al., 2018; K. E. Williamson et al., 2007).

However, inferring viral behaviors and abundances from host data alone leaves gaps in our understanding of viral communities. Fewer studies have been undertaken to directly observe how environmental changes impact viral communities rather than host communities. Sustainable agriculture has been shown to influence the host populations in many ways, as detailed below. However, these same studies must be performed for virus populations to determine how sustainable agriculture practices directly impact the abundance of viruses and their mobility and distribution within the soil profile under varying agricultural management practices.

CHARACTERIZATION OF VIRAL COMMUNITIES

A major way to directly investigate viral communities is by sequencing their genetic material. In recent years, there have been major advances in the investigation of viral communities thanks to next-generation sequencing technologies. However, progress taxonomically identifying viruses is slow, due to the lack of a universal phylogenetic marker gene seen in bacteria (i.e. the 16S rRNA genes), and therefore viruses must be isolated and characterized and entered into a database for identification. Additionally, the functions of many viruses are unknown due to lack of characterization in isolates.

Therefore, there remain many “Unknown” viral families, whose DNA or RNA sequence has not been catalogued. Despite these challenges, metagenomic approaches for assessing viral diversity have rapidly expanded viral knowledge. For instance, in a recent review by Williamson et al. (2017) reported only ~2,000 complete bacterial virus genomes, and there were few non-bacterial viruses identified from soils, such as viruses of archaea, fungi, and protozoa (K. E. Williamson et al., 2017). In 2019 alone, 668 terrestrial soil metagenomes were used to search for and identify upwards of 24,000 soil viral sequences, which clustered into over 17,000 viral operational taxonomic units (Graham et al., 2019). The global knowledge base for viral taxonomic and auxiliary metabolic gene identification has rapidly expanded due to improvement of metagenomic capabilities.

While there are many methods used to examine viral genetic data, DNA sequencing is, as of yet, the most commonly used tool. One method, mentioned above, is mining viral genetic data from metagenomic data processed from whole-soil samples. While it is possible to acquire insight into the virus community using this metagenomic sequencing, higher resolution viral data can be recovered if viruses are first extracted and

purified from the soil complex in order to perform viral metagenomics, creating viromes (Santos-Medellin et al., 2021). However, generation of viromes relies upon first extracting soil viruses, which is difficult due to the heterogenous nature of soil systems. Soil virus purification can be achieved by extraction with a buffer and physical dispersion, followed by centrifugation to pellet soil and separate viruses from the soil complex, and filtration for purification (Liang et al., 2020b; K. E. Williamson et al., 2013). Once viruses are separated and purified from soil, DNA can be extracted using one of many commercial extraction kits, including Qiagen and MoBio. However, due to the purification limitations in soil systems, many samples require amplification before sequencing, which can introduce biases into sequencing data by preferentially amplifying single-stranded DNA viruses relative to double-stranded DNA viruses (Kim & Bae, 2011). Once virus DNA sequences are acquired, the general steps for bioinformatics processing include quality control of sequences, trimming of low quality and contaminated reads and adapters used for sequencing, assembly of sequences, viral genome identification, taxonomic annotation, functional annotation, and host prediction (Bi et al., 2021; Emerson et al., 2018; Hesse et al., 2017; Sorensen et al., 2021). Many tools are available to complete these processes, and more are being developed rapidly. In general, ensuring that returned sequences are of viral origin is a critical step, which can be performed with tools such as VirFinder, VirSorter, checkV, and many more, which are often combined to achieve the highest quality reads (Bi et al., 2021; Guo et al., 2021a; Nayfach, Pedro Camargo, et al., 2021; Ren et al., 2017; Roux et al., 2015).

In addition to DNA sequencing, RNA sequencing, termed RNAseq for short, is a rapidly advancing tool which employs the sequencing of RNA rather than DNA to study

transcriptomes, which allows for more detailed investigations of gene expression in a sample (Bang-Andreasen et al., 2020; Marguerat & Bähler, 2010). Overall, the investigation of virus communities has greatly advanced our understanding of viruses in the soil environment and will continue to rapidly expand our knowledge base for viral function in soil ecosystems.

VIRUS INFLUENCE ON HOSTS

The importance of microbial (i.e bacterial and fungal) communities in global ecosystems is well recognized, but the contributions of viruses to these same systems is largely ignored. Viruses are particularly important for the impact they have on their bacterial hosts, as they can regulate host community composition, influence host function, contribute to host evolution, and even mediate nutrient cycling through host infection. Viruses have been shown to moderate the evenness of the host community via the lytic cycle of host predation, which is based on a mechanism known as “kill the winner” (Thingstad & Lignell, 1997). This mechanism states that the “winning” bacteria species, which most successfully capitalizes on resource availability and out-competes the other species, may subsequently be the most common target for lytic viral infection, thereby controlling the population of that bacterial host through high infection rates. In this way, the viral infection causes a sharp collapse of the dominant bacterial phyla, giving way for resource capitalization by other bacterial community members. As such, viruses exert important controls on the evenness and diversity within a host community.

Viruses influence host function through expression of auxiliary metabolic genes (AMGs), and transcriptional regulators. AMGs encoded by the virus genome can

reprogram host metabolism (Breitbart et al., 2018). Many AMGs have been shown to encode metabolic pathways/components for photosynthesis, carbon metabolism, and metabolisms of nitrogen, sulfur, and phosphate, thereby boosting host metabolism and energy and enhancing production of viral progeny (Bi et al., 2021). Many of these AMGs are acquired during horizontal gene transfer, which also contributes to the co-evolution of viruses and bacteria (Suttle, 2007). Importantly, the regulation of host metabolism is one mechanism by which viruses impact biogeochemical cycling. Additionally, viruses can have a direct impact on nutrient cycling by releasing host intracellular material upon lysis. In surface oceans, viral lysis has been shown to re-cycle between 25-50% of biomass produced daily (Suttle, 2007). In these ways, viruses are important players in ecosystems, and the mechanisms by which they impact their hosts are largely dependent on their replication cycle. There exists a knowledge gap in soil microbiology regarding the mechanisms that control the switch between lysis and lysogeny. In order to understand how viruses might impact hosts, we must better understand what drives viral life strategy decisions not just for specific phage host pairs but on a community wide scale in soil ecosystems.

VIRUS REPRODUCTIVE STRATEGIES

As viruses are obligate parasites, their modes of reproduction are inherently of interest for their host ecology. Viral lysis and lysogeny affect the host in different ways, and as such it is critical to understand not only the mechanisms that cause the switch, but also overall ecological factors which might shift overall community viral production to one or the other. Lytic reproduction is the culmination of a productive viral infection,

wherein new viral progeny are produced and released into the environment, resulting in the death of the host cell (Weitz et al., 2019). Lysogenic reproduction involves viruses inserting their genetic material into a host, which integrates into the host DNA, and is replicated each time the host reproduces and divides (Erez et al., 2017). Some viruses are obligately lytic, known as virulent. Conversely, temperate phage can reproduce via both lysis or lysogeny, and they have been shown to change reproductive strategy on a community level in response to environmental conditions. The factors underlying a switch from lysis to lysogeny are not well understood, especially in soil systems, as most of our data comes from aquatic environments.

Studies regarding viral replication involve characterizing abundances of free viruses purified from the environment and lysogenic prophage contained within host cells. Free viruses in terrestrial and aquatic systems can be studied by purification and enumeration using epifluorescence microscopy or flow cytometry (Marie et al., 1999; K. E. Williamson et al., 2005). However, enumeration of lysogenic phage is more difficult and depends on triggering prophage induction and measuring the response in the form of new virus production. Agents which stress the host cell, including the antibiotic mitomycin-C (Mit-C) and ultraviolet (UV) light, have been used to activate the cell DNA repair mechanism, which signals the prophage to induce viral lytic cycle of the host cell, escaping as free viruses before host cell death (Jiang & Paul, 1998; K. E. Williamson et al., 2008). The inducible fraction is calculated as the number of hosts with prophage which can be induced to viral production, and has been shown to range from 0-84% in ocean waters and up to 85% in soil bacteria (Ghosh et al., 2008; Jiang & Paul, 1998). Such induction enumeration methods are limited by the inducing agent's ability to

activate lytic reproduction across a wide diversity of hosts and prophage. More recent investigative tools have been developed through metagenomic sequencing techniques, however these tools are also limited due to their overestimation of lytic viruses (Kieft et al., 2020a). Overall, while there are methods which can investigate prophage, it is extremely difficult to get an accurate picture of what percentage of the community is lytic or lysogenic due to the limitations of current tools.

Much of the research regarding lysis-lysogeny decisions in the environment has been based on the idea of viral “bet-hedging” in a concept taken from economics (Maslov & Sneppen, 2017). The aforementioned theory called “kill-the-winner” (KtW) describes not only host community regulation by viral predation, but also the prediction that lytic replication will be predominant when host abundance and activity is high, and lysogenic replication will be predominant when host abundance and activity are low (Ghosh et al., 2008; Liang et al., 2020a; Payet & Suttle, 2013; Weinbauer et al., 2003). In this way, viruses expand their populations rapidly via lytic reproduction during times of stable host reproduction and mitigate risks of population collapse during times unfavorable for productive hosts (Weitz et al., 2019). There are many studies from both aquatic and soil environments which support this theory. In a study of marine environments, a higher percentage of lysogens (i.e. bacteria infected with a prophage) were found in oligotrophic environments compared to coastal and estuarine environments, with higher nutrient availability (Jiang & Paul, 1998; Weinbauer et al., 2003). In soil systems, KtW draws support from studies showing prevalence of lysogeny deeper in the soil profile compared to surface soil (Liang et al., 2020b). Additional mechanistic studies have shown that quorum sensing molecules, produced by bacteria and used primarily for coordinating

virulence and biofilm behaviors, have been shown to induce viral lysis, suggesting that phage can sense host density and switch to lytic reproduction in order to capitalize on periods of high host activity (Liang et al., 2020a). In total, there is a vast body of evidence which supports the theory that viruses predominately replicate via lytic infection in the environment when resource availability is high and host activity and abundance are elevated.

In recent years, however, there has been growing support for a competing theory known as “piggyback-the-winner” (PtW). This theory states that the prevalence of lysogenic reproduction will increase at high microbial growth and abundance rates, whereby viruses existing as prophage will “piggyback” off the success of their host cell. Evidence supporting PtW can be found in aquatic, soil, and mechanistic studies. Knowles et al. (2016) conducted a meta-analysis of aquatic, terrestrial, and biological (i.e. human lungs) environments and showed that viral densities were more consistent with lysogeny than lysis at high microbial abundance (Knowles et al., 2016). A recent study of the gram-negative marine bacteria *V. anguillarum* showed that high prevalence of quorum sensing molecules actually suppressed viral induction, and that free phage were more prevalent at low host densities, suggesting that the bacteria evolved to maintain high lysogeny at high cell densities (Tan et al., 2020). In soil systems, higher rates of lysogeny were found in temperate Delaware soils than extreme Antarctic soils, but life cycle could not be correlated with nutrient status using Mit-C-based prophage induction assays (K. E. Williamson et al., 2007).

Overall, a compelling argument has been made for both KtW and PtW, and more research is needed to understand environmental conditions and microbial mechanisms

which drive these decisions in temperate phage at an ecosystem level. In soil systems, the virus to microbe ratio has been shown to range from 0.001 to >1000, which underscores the complexity of virus and host relationships in these heterogeneous systems (K. E. Williamson et al., 2011). Therefore, mechanisms which control lysis-lysogeny decision making in aquatic viruses may not be applicable to soil virus communities. The research undertaken in this dissertation aims to shed light on factors driving viral reproductive strategies in soil.

NATIVE VIRUS AND HOST COMMUNITIES UNDER AGRICULTURAL MANAGEMENT

Agriculturally managed soils are of great importance to food security, economics, and environmental sustainability. Many studies have been conducted regarding soil health and soil quality, citing soil microbes (i.e. bacteria and fungi) as key players in nutrient cycling, carbon sequestration, stable aggregate formation, and more (Dubey et al., 2019). As viruses greatly influence host abundance, diversity, and function, it is critical to understand the roles of viruses in these agricultural systems to gain a more complete understanding of soil ecology. As such, we chose a long-term (40+ years) experimental conservation management site in West Tennessee that produces cotton to investigate soil viral population dynamics in agricultural soils.

Long term agriculture has been shown to reduce overall soil fertility (i.e. optimal pH, soil organic matter, nutrients, and texture) (de Valença et al., 2017). Some sustainable agriculture methods, such as no-tillage and off-season winter cover crop implementation, have been shown to increase carbon sequestration, promote stable

aggregate formation, decrease erosion, and increase nitrogen concentrations, which have been linked to increased biomass carbon (Feng et al., 2003; Hobbs et al., 2008).

Understanding how these practices, and other common agricultural management practices, such as nitrogen fertilization and irrigation, influence both virus and host abundance and activity in soil systems will expand foundational knowledge of soil viruses in managed ecosystems.

Implementing no-till methods, in which the soil is not turned between plantings, increases nutrient concentrations, such as soil organic carbon and total nitrogen, increases microbial biomass carbon, and alters the community composition of soil microbes (Feng et al., 2003; Wang et al., 2012). Compared to no-tillage, soils which were tilled have been found to host low virus-like-particle abundance, likely due to the more homogenous structure of the soil post-tilling (Narr et al., 2017).

Cover crops, various crops planted in the off-season of growing primary crops, have been shown to benefit soil health by decreasing erosion, enhancing carbon sequestration, providing nitrogen fixation with legumes, providing protection from physical disruption (e.g., rain-drop impact), and promoting soil aggregate formation to improve soil structure (Hobbs et al., 2008). Additionally, the benefits of improved soil structure also bring more optimal balance between water-holding capacity and good aeration of soils. Soil water content has been shown to be significantly correlated with viral abundance, due to thicker water films on and between soil particles and smaller air-water interfaces, which are known to inactivate viruses (Thompson & Yates, 1999; K. E. Williamson et al., 2005). It is important to note here however that these results were obtained with model, non-indigenous phages introduced into soil. Thus, the ability of

native soil viruses to persist extracellularly in soils remains unknown. One prevailing hypothesis to explain the extraordinarily high virus-to-microbe ratios observed in some soils maintains that viruses native to soil ecosystems are inherently more stable in soil than viruses from aquatic or other environments such as GI tracts. Cover cropping has been shown to increase microbial abundance and diversity, due to the increased plant diversity providing a more diverse input of soil substrates, especially under leguminous species (Vukicevich et al., 2016). Direct assessment of cover cropping on viral abundance and diversity remains a knowledge gap to be filled, one of the stated goals of this research.

Nitrogen additions are important for agricultural crops but can have varying effects on the soil microbial community based on the time scales of nitrogen applications. As a rate-limiting nutrient for microbial growth, a dose of nitrogen can stimulate growth and activity in nitrogen-limited microbial environments (Geisseler & Scow, 2014). This suggests that nitrogen inputs would increase viral reproduction and, likely, host a higher percentage of viruses in the lytic rather than lysogenic phase (Narr et al., 2017). However, long-term inorganic nitrogen additions can acidify the soil because the process of nitrification produces two moles of hydrogen ions for each mole of ammonium oxidized which lowers soil pH (Brady & Weil, 2008). Nitrogen additions generally cause a decrease in bacterial species richness and a shift in the bacterial community structure to favor an active and copiotrophic community composition as compared to non-fertilized soils (Fierer et al., 2012; Zeng et al., 2016). So, while a short term or single dose of nitrogen can induce a growth response from the microbial community, long term applications can cause soil acidification, decreasing the overall microbial biomass carbon

and activity of that microbial community. Overall, the effects of nitrogen fertilization on the bacterial and by extension the viral community depend on the balance between stimulation of host growth and the negative consequences of acidification.

Naturally occurring nitrogen deposition may also be an important factor influencing bacterial hosts in the soil environment. Nitrogen deposition has been shown to occur in the southeastern United States at a rate of 7.6 kg/ha per year, 67% of which is in the form of reduced N (i.e. ammonia and ammonium) (Y. Li et al., 2016) Increasing atmospheric deposition has been shown to reduce microbial activity and alter bacterial composition (Zhou et al., 2018). However, these same impacts have yet to be observed in soil virus populations.

Irrigation is an important part of agriculture, and it is critical to limit water use while maintaining a sufficient soil moisture for crop growth. One method, called surge irrigation, uses periodic bursts of water into the soil, which reduces the advancement of the wetting front into deep soil profiles. This process occurs because dry soil infiltrates water more quickly than wet soil as wet soil creates seals as soil particles consolidate. So, after an interrupted flow, when moisture is re-introduced into wet soil, the water advances quickly through the wet soil, and then the dry soil at the wetting front slows the advance (Henry & Krutz, 2017). This process thereby maintains more moisture in the surface soil and reduces the overall amount of water lost to deep irrigation (Shock & Welch, 2011). This allows for less water to be used overall, contributing to the sustainability of the agriculture system. However, these wetting fronts and flow patterns could potentially influence virus and bacteria movement through the soil profile, causing alterations in the microbial community in the surface and deep soil profile.

Agriculturally managed systems are critical areas of study due to their importance to human survival as well as their impacts on environmental sustainability and climate change (Richards et al., 2018). As links between virus and host community ecological function become more established, research such as the experiments undertaken for this dissertation will provide a strong knowledge base for understanding abundance, community composition, and mobility of viruses in these critical agricultural systems.

VIRUS TRANSPORT

Wetting events with interrupted flow, such as multiple rainfall events or surge irrigation techniques, cause movement of soil solutes, clays, and microorganisms through the soil profile. It is thus important to study how nanosized viruses move under these natural and human imposed conditions as the transport of viruses from small pores to larger pores is likely pre-requisite to their impact on their micro-sized hosts. Regardless of soil chemical conditions, viruses and hosts must make contact for viral infection to occur. Therefore, identifying factors and understanding processes which affect the retention, distribution, and transport of viral particles is critical to predicting the roles of viruses in reshaping soil microbial communities. When outside the host cell, free extracellular viruses exist as passively transported colloidal particles, whose mobility and retention are controlled by the physical, chemical, and hydrodynamic forces in the soil environment.

Transport and retention of viruses in soil systems are controlled by interactions between the surfaces of the viruses and the soil and are affected by soil solution chemistry. These interactions are determined by electrostatic, van-der Waals,

hydrophobic, and capillary forces (Bradford et al., 2003). Soil particles have electric double layers which are responsible for the charges attracting or repelling soil viruses, and the properties of the electric double layers are subject to changes in the soil components, primarily by changes in soil surface chemistry. The double layer is comprised of an absorption layer, called the stern layer, and a diffuse layer called the Gouy-layer. Elements in the stern layer are strongly bound to the soil surface while elements in the diffuse layer are weakly bound to the soil surface (Figure 1.1) (Jin et al., 2020). The diffuse layer is comprised of oppositely charged ions adjacent to the soil surface (S. Li & Xu, 2008). The charge condition of that layer is the zeta potential, which is the electric potential of the surface and changes across the point of zero charge (PZC) (i.e., the pH at which the quantities of positive and negative variable charges are equal). The pH at which a colloid free of permanent charges (i.e., mineral structure-generated charges) reaches the PZC is known as the isoelectric point of the colloid. When soil solution pH is higher than the isoelectric point, the colloid becomes negatively charged, and when the pH is below the isoelectric point, the colloid becomes positively charged (Michen & Graule, 2010). In this way, the charges of soil and viral surfaces are largely dependent on soil pH. Additionally, the ionic strength and ion composition of the soil solution can greatly impact the electric double layer at liquid-solid interfaces. Increasing ionic strength compresses the diffuse layer, bringing viruses closer to soil surfaces and thereby increasing virus retention via Van Der Waals force (Lance & Gerba, 1984; Masciopinto & Visino, 2017a; Walshe et al., 2009). The calculation of the interaction

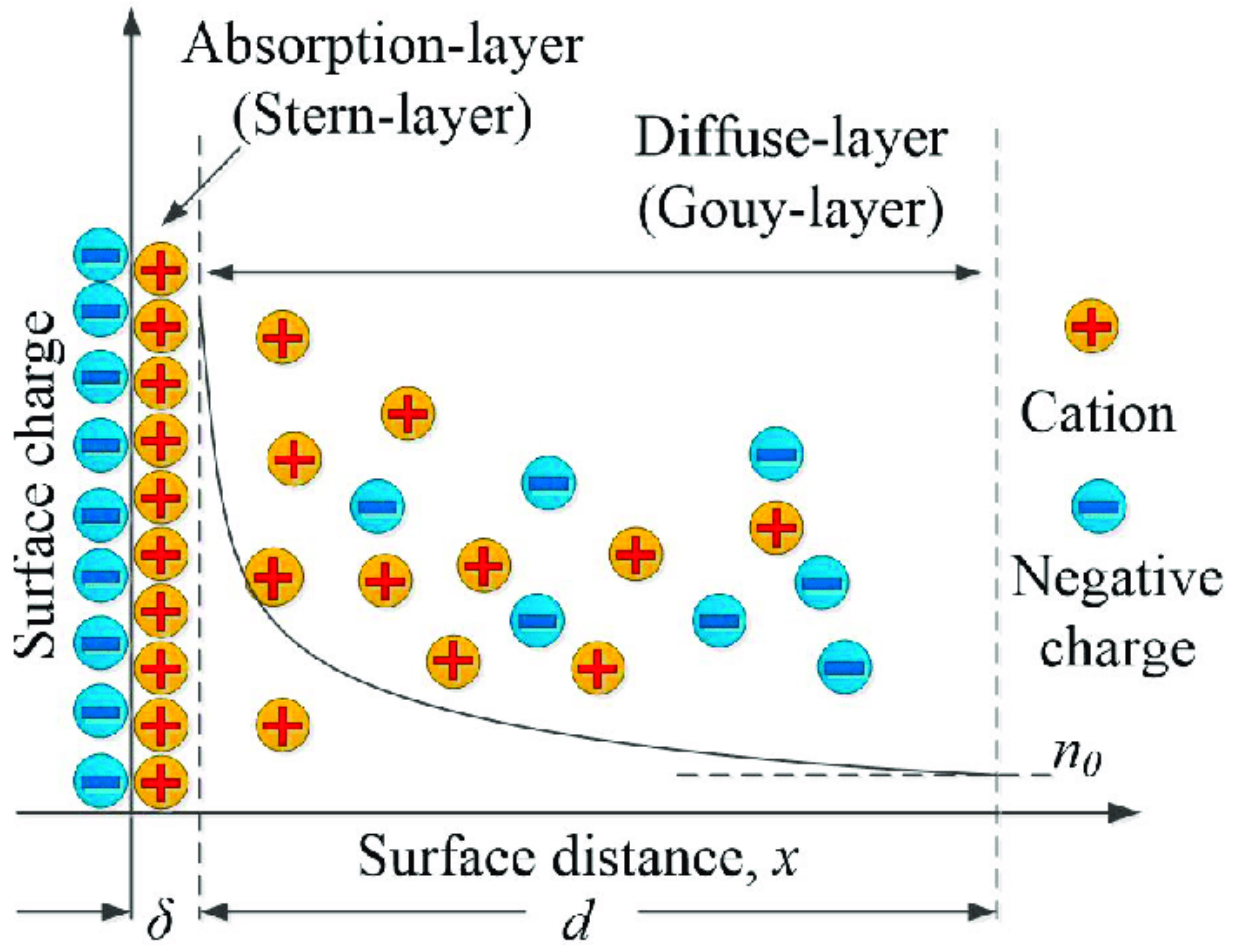


Figure 1.1. Depiction of electric double layer on soil surfaces. Image from “Analysis and Modeling of the Complex Dielectric Constant of Bound Water with Application in Soil Microwave Remote Sensing” by Jin et al, 2020.

energies between soils and viruses is usually performed using Deryaguin–Landau–Verwey–Overbeek (DLVO theory) theory (Bhattacharjee et al., 2000).

The composition of the soil solution also affects viral transport. Presence of clay colloids and dissolved organic matter (DOM) in the soil solution can influence soil virus transport. However, predicting these impacts is difficult due to varying chemical conditions during flow and differential effects at varying pore water velocities. For instance, it has been reported that at low pore-water velocity, the presence of clay colloids increases viral transport, but at high pore-water velocities clay colloids hindered viral transport (Syngouna & Chrysikopoulos, 2013). DOM in soil solution has been shown to, in general, decrease virus retention due to competition for attachment sites. However, this interaction varies with the surface properties of the viruses being studied (Schijven & Hassanisadeh, 2000; Zhuang & Jin, 2003a).

Physical factors controlling soil virus transport and retention include soil virus size and shape, soil pore structure, air-water interfaces, flow velocity, and soil water content. The physical sizes of virus particles and soil pores impacts viral mechanical straining. i.e. viruses removed from the solution and retained in soil micropores, wedges, or throats. Larger or aggregated soil viruses and smaller pore sizes are associated with increased straining as blocked pores inhibit virus movement in the soil profile (Bradford et al., 2003). In this way, soil texture greatly impacts soil virus transport, as soils higher in clay content have more micropores or aggregates. Additionally, the soil texture influences soil water retention, pore water connectivity, and ultimately virus movement (Rezanezhad et al., 2016). As discussed above, different soil surfaces or sites have varying charges, with positive charges on soil surfaces causing retardation of virus

transport through soil media. For example, metal oxide coatings on soil surfaces with pH dependent charges have been shown to reduce viral transport by providing positively charged attachment sites for negatively charged soil viruses (Zhuang & Jin, 2003b).

The natural system is much more complicated than can be recreated in a laboratory experiment, which uses model viruses and soil systems with known properties. For instance, one study has shown that when two viruses were investigated in a single transport experiment, the transport of both viruses was increased relative to when they migrated individually. This increase was attributed to mutual promotion of virus transport as a result of competition for limited attachment sites within the soil system (Xu et al., 2017). This study suggests that viruses in a natural system could interact with each other in ways that are, as of yet, unknown. While laboratory-based studies inform our understanding of the mechanisms driving virus movement in soil systems, it is difficult to apply these models to in-situ movement of diverse virus communities in soil profiles. As such, our investigation allows observation of natural soil virus communities in simulated natural conditions, thereby expanding our knowledge of real-world virus mobility.

IMPORTANCE AND GOALS

Much remains unknown about soil viruses in their natural habitat. As the world population grows and climate change intensifies, humans will become more reliant on their agricultural systems for reliable food production. Increased understanding of soil virus abundance, community composition, and mobility in these systems provides critical foundational knowledge for integrating soil viruses into studies of soil ecology, health, fertility, and productivity. Here we aimed to fill knowledge gaps regarding how

agricultural practices impact soil viruses, with particular emphasis on how these practices might impact lysis and lysogeny decisions in natural soil environments. Our specific goals were as follows:

Chapter 2

- 1) determine changes in viral abundance under varying agricultural management practices including winter cover crop rotation, tillage management, and nitrogen fertilization
- 2) determine how the viral and bacterial abundance changes over the course of a growing season
- 3) determine key factors which might impact viral reproduction at the field scale

Chapter 3

- 4) determine how interrupted flow events would impact native soil virus mobilization through a saturated soil column by mimicking repeated rainfall or irrigation events
- 5) determine if the trace levels of ammonium consistent with atmospheric deposition have any effect on viral mobilization
- 6) determine if the soil virus abundance in the residual column soil was affected by the interrupted flow events and the ammonium addition

Chapter 4:

- 7) determine if interrupted flow differentially mobilized varying viruses and influenced the community composition of easily transported versus highly retained viruses

- 8) determine the community composition of soil viruses and bacteria immobilized or retained in the soil column
- 9) determine if nitrogen deposition impacts soil virus mobilization or production in the soil profile
- 10) determine if prophage induction from lysogenic to lytic viral reproduction occurs under saturated interrupted flow conditions and atmospheric deposition of nitrogen

**CHAPTER 2: VIRAL AND BACTERIAL POPULATIONS RESPOND
DYNAMICALLY TO SHORT-TERM NUTRIENT FLUXES BUT
ARE INDEPENDENT OF LONG-TERM SOIL CONSERVATION
MANAGEMENT PRACTICES IN CONTINUOUS COTTON
PRODUCTION SYSTEMS**

A version of this chapter is in preparation for publication in *Applied Soil Ecology*, with authors Regan McDearis, Aubrey Fine, Xiaolong Liang, Ning Duan, Sean Schaeffer, Mark Radosevich, and Joe Zhuang.

My contribution to this work was sample collection, lab analysis of soil microbial abundance, statistical analysis, and writing of the manuscript.

ABSTRACT

Viruses are ubiquitous in soil systems, and critically important in shaping bacterial host communities and influencing nutrient cycling. When replicating via the lytic cycle, soil viruses can drive bacteria community composition via predation on hosts to produce viral progeny, also releasing host intracellular material into the soil environment. Conversely, during lysogenic replication, viruses can exist as prophage inserted within the host genome replicating only as the host cell grows and divides. While virulent phages can only replicate via the lytic cycle, temperate phages can revert from lysogenic to lytic replication in response to certain environmental signals that are not completely understood but may involve stress, resource availability, and host cell density. Few studies have examined the switch from lysogenic to lytic viral reproduction in native soil habitats. In model systems host stress, caused by exposure to antibiotics, or host growth, determined by prophage using quorum sensing signals, have been reported to cause prophage induction. As prophage induction can play an important role in nutrient transformation and host functioning, it is critically important to identify and understand factors which might influence phage reproductive decisions in viral ecology. In this study, we examined viral and bacterial abundance using epifluorescence microscopy, and calculated the lysogenic fraction in soils collected from a long-term experimental cotton field evaluating 12 different conservation management practices in Jackson Tennessee. Treatments of cover crop, tillage, and nitrogen fertilization were analyzed to determine if these factors affected viral population dynamics and, namely, the switch from lysogenic to lytic viral reproduction. We sampled in May 2018, May 2019, and June 2019. Mean viral abundance across all treatments was highest in June 2019, which also had the

highest mean nitrogen concentration of all sampling times. Conversely, bacterial abundance and lysogenic fraction were higher in the two May sampling times. The decrease in bacterial abundance coupled with an increase in viral abundance in June 2019 suggests that the elevated nitrogen concentration potentially triggered a community wide viral induction event due to the input of a growth-limiting nutrient. This is significant due to the impacts that released viral progeny as well as free bacterial necromass could have on soil nutrient cycling and bacterial host abundance. The long-term agricultural management treatments showed little impact on virus and bacteria abundance, likely due to the acclimation of microbial communities to imposed treatments over the 40+ year continuous management. While we were unable to directly link long-term treatments with microbial abundance via analysis of variance, we determined that treatments did significantly impact biogeochemical measurements, such as nitrogen and carbon content. These biogeochemical measurements in turn had significant correlations with virus and bacteria abundance. In this way, we conclude that the treatments can indirectly impact microbial abundance via influences in chemical concentrations. This research indicates that viral abundance on the field scale is more affected on the temporal scale of an annual cotton production cycle than by long term management practices.

INTRODUCTION

Soil bacteria communities in agriculturally managed systems have been shown to be greatly impacted by management practices (Feng et al., 2003; Geisseler & Scow, 2014; Narr et al., 2017; Wang et al., 2012). However, the impacts of these same practices on soil viruses remain largely unknown. Viruses which infect soil bacteria are extremely abundant and diverse, and play a role in shaping host community diversity and function (Allen et al., 2010; Breitbart et al., 2018; Emerson et al., 2018; K. E. Williamson et al., 2017). In fact, marine studies have revealed that viral lysis of host cells via lytic reproduction contributes to nutrient cycling through the release of host cell lysate, in what is known as the “viral shunt” (Suttle, 2007). It remains unclear, however, whether the viral shunt occurs in soil systems. To shed light on this topic, factors controlling virus abundance and viral life cycles, lytic and lysogenic reproduction, must be more fully understood.

Two general types of viral reproduction are lytic and lysogenic strategies. In the lytic reproductive cycle, a virus infects a host cell, injecting its genetic material into the host, and hijacking the host metabolism to produce new viruses. Once completed, the host cell is lysed and new viruses are released into the environment to find new hosts.

Alternatively, in the lysogenic cycle, a virus infects the host with its genetic material which then incorporates into the host genome and replicates as the host replicates. The viral genome in this integrated state is called a prophage and the host a lysogen.

Temperate viruses can switch between lytic and lysogenic strategies depending on the host and environmental conditions (Erez et al., 2017). In soil, lysogenic viruses may have an advantage over lytic viruses for surviving long periods of host inactivity, although the

ability of indigenous soil viruses to persist extracellularly has not been investigated (Marsh & Wellington, 1994). Lytic life strategies are common when system production, or bacterial growth, is high, while lysogenic life strategies are predominant when system production is low, indicating that not just abundance but more importantly host activity accounts for virus reproductive strategy (Ghosh et al., 2008; Liang et al., 2020b; Payet & Suttle, 2013). A lysogenic reproduction strategy may be favored during times of slow bacterial host growth, as shown by an increase of inducible viruses found in winter months (slow growth) as compared to other months of rapid host growth (S. J. Williamson et al., 2002). Lysogeny is prevalent at low host density and activity whereas lysis is prevalent at higher host density and activity (Weinbauer et al., 2003).

The switch from lysogenic to lytic reproduction can be caused by multiple factors. Bacterial communities have been shown to host a high proportion of lysogenic phages, which can be induced to the lytic cycle by stressing the bacterial hosts with the antibiotic mitomycin C (Mit-C) (Ghosh et al., 2008). The antibiotic stresses the host bacteria, activating the DNA repair system, which signals the virus to switch to the lytic cycle and produce progeny before the host cell dies. In the soil environment, many types of host stress are thought to cause the switch from lysogenic to lytic state, from dramatic temperature and water content changes to additions of toxic substances.

Conversely, “positive” signals can also trigger an induction response. It is possible for prophage to be induced into lytic reproduction via quorum sensing. Ghosh et al. (2009) showed that acyl-homoserine lactones (AHLs), quorum sensing molecules in gram-negative bacteria, can induce lytic virus production (Ghosh et al., 2009). This could mean

that the viruses have sensed high host density, therefore conditions are optimal to reproduce progeny that are likely to contact a new host (Liang et al., 2020a).

This pattern of viral replication, where lysis is favored at high host density, is known as the kill-the-winner theory (KtW). In KtW theory, high host cell density and activity promotes lytic viral infection, and the free viruses then most frequently infect the most productive and numerically abundant bacterial hosts, reducing their numbers and leading to greater host community evenness ((Liang et al., 2020a; Maslov & Sneppen, 2017) Additionally, KtW states that viral production is dependent on host activity, and will favor lytic reproduction under the most favorable biogeochemical conditions for host growth, and that when conditions are oligotrophic, lysogenic reproduction may become more prevalent (Payet & Suttle, 2013). Inversely, some recent studies have contradicted the KtW theory, reporting that prophage abundance is highest when bacterial abundance is highest, known as “Piggyback-the-winner,” wherein viruses are “piggybacking” off of host in times of high host activity (Knowles et al., 2016). In fact, a recent study showed that high host density and abundance of quorum sensing molecules repressed viral induction and favored lysogeny, supporting the “piggyback-the-winner” theory (Tan et al., 2020).

In-situ factors underlying this switch from lysogenic to lytic reproduction are of great interest due to the potential impacts on the host community and nutrient cycling. Here, we investigated changes in soil virus and bacterial abundance and estimated the lysogenic fraction in prophage induction assays using mitC amendment. A range of conservation agricultural management practices that included winter cover cropping, tillage, and

nitrogen fertilization in a long-term continuous cotton production system were examined to determine factors driving virus life strategy under a gradient of nutrient availability.

Soil viruses and bacteria have been shown to respond to changes in soil organic matter, nutrient availability, and water content (Chen et al., 2021; Feng et al., 2003; Payet & Suttle, 2013; Srinivasiah et al., 2015; Wang et al., 2012). Management practices, including winter cover cropping, tillage management, and nitrogen fertilization, impact soil microbial communities via changes in soil properties. Soil cover crops have been linked to higher soil microbial biomass due to increased soil organic carbon levels and cover from physical disturbance such as rain (Hobbs et al., 2008). Compared to conventional tillage methods, no-till management increases soil organic carbon, total nitrogen, and microbial biomass carbon, and the microbial community structure is known to shift under these management practices (Feng et al., 2003). No-till methods show higher levels of microbial biomass carbon, total nitrogen, microbial abundance, as well as differences in microbial community structure (Wang et al., 2012).

These changes to the bacterial community structure can also lead to apparent shifts in the composition of viral assemblages. Srinivasiah et al. (2015) showed that adding a carbon source to stimulate bacterial growth (and therefore abundance) increased viral abundance, and that varying carbon and nutrient sources stimulated varying members of the bacterial community at different rates (Srinivasiah et al., 2015). Nitrogen is an essential nutrient which can stimulate growth/activity in nitrogen-limited microbial environments. When limiting, the addition of nitrogen fertilizer can increase bacterial growth, but long-term applications can acidify soil, limiting nutrient availability and lowering the bacterial abundance, thereby lowering the virus populations as well (L.

Chen et al., 2014). Virus abundance has been shown to be positively correlated with soil pH (Narr et al., 2017).

Long-term imposition of these agricultural management practices creates a nutrient availability gradient ideal to study virus-host interactions. Agricultural management practices, such as no-till management and cover crop implementation which potentially support greater microbial activity, are predicted to have higher microbial abundance. However, their impact on virus life strategy, lytic or lysogenic reproduction, remains unknown. It is critically important to study viral replication as soil bacteria can be affected by top-down predation via soil viruses (Emerson et al., 2018; K. E. Williamson et al., 2007).

The objective of this study was to investigate viral replication strategies under a gradient of nutrient availability by measuring how virus abundance, bacterial abundance, and inducible fraction are altered due to long-term agricultural management practices of winter cover crop, tillage management, and nitrogen fertilization. We hypothesized that (1) soil bacterial and virus abundance would be highest under the management practices and within the sampling dates with the highest nutrient availability and water content, and (2) lysogenic viral reproduction would be higher under less favorable soil conditions. We extracted soil viruses and bacteria, and performed viral induction assays, which we quantified using epifluorescence microscopy. Multivariate analyses were performed to determine if imposed treatments had significant effects on microbial abundance, and correlation analyses were performed to determine which soil properties had the greatest influence on microbial abundance and lysogenic fraction. Our results indicated that nitrogen content had a significant influence on viral abundance, and that the largest shifts

in viral and bacterial abundance occurred between sampling dates rather than due to long-term management practices. Additionally, we present evidence that a viral induction event may have occurred between May and June of 2019. This research provides insight into the soil conditions which favor lytic versus lysogenic reproduction and provides directions for future studies of viral population dynamics in soil systems.

METHODS

Site Description and Sample Collection

Soil samples were collected from the West Tennessee Research and Education Center in Jackson Tennessee at three timepoints selected to coincide with pre- and post-addition of N-fertilizer, during May 2018, May 2019, and June 2019 from a Lexington silt loam (fine-silty, mixed, thermic, Ultic Hapludalf). Originally, this experiment aimed to cover the entire growing season of cotton from cover crop burn down in May, to post fertilization in June, to peak cotton biomass in October, and finally post-harvest in December. However, samples were lost from the 2019 October and December sampling times due to the failure of our -80°C when labs were locked down due to the coronavirus pandemic. As such, we used samples which were processed from May and June of 2019, and additionally included our data from the test sampling which occurred in May of 2018. This field site of continuous cotton production was established in 1981 as a long-term conservation management experiment. This field was treated with calcitic lime in 2015, therefore acidification of the soil due to N fertilization was not observed and on average soils had a pH of > 6 . The experiment had a randomized complete block design (RCBD) with a split-plot arrangement by four replications (Appendix Figure 2.5). The

main treatment plot consisted of two levels of nitrogen, ammonium nitrate (NH_4NO_3) applied at 0 or 67 kg/ha. The sub-plots include three levels of winter cover crop, with treatments of no cover, winter wheat (*Triticum aestivum*), and hairy vetch (*Vicia villosa* Roth). The subplots were split by tillage management (no till and conventionally tilled). Treatments are abbreviated by tillage, cover crop, and nitrogen application as T or N for till or no-till, N, V, or W for no cover, vetch, or wheat cover, and 0 or 67 for N application. So, NV67 refers to a plot with no-till, vetch cover, and 67 kg/ha N fertilizer applied, whereas NN0 would refer to no-till, no cover, and 0 kg nitrogen applied.

Soil cores were collected from 0-10 cm of the soil surface from 12+ random locations within each treatment plot using a 2.5 cm diameter soil core which was sterilized with an ethanol rinse between each plot. The soil cores from each plot were homogenized in a single bag and transported to the University of Tennessee, Knoxville on the same day of collection, and stored overnight at 4°C. The following day the samples were sieved at field moisture content through a 2-mm screen to remove rocks and plant roots in preparation for virus and bacterial extraction and quantification of soil properties.

Extraction and Enumeration of Soil Viruses and Bacteria

Immediately following the sieving, the soil was extracted for viruses and bacteria, as described in (Liang et al., 2019a). First, 30 g of soil and 100 mL of cold buffer (10 g potassium citrate, 1.44 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, and 0.24 g KH_2PO_4 , adjusted to pH 7; stored at 4°C) were combined in a glass mason jar and blended at high speed for three minutes (K. E. Williamson et al., 2005). To isolate soil viruses, 45 mL of the soil slurry was

centrifuged in 50-mL tubes at 5,000 rpm for 20 minutes at 4°C, then the supernatant was filtered through 0.22-µm Millex Durapore Filters (Merck Millipore Ltd., Tullagreen, Co. Cork, Ireland) to remove bacteria. The resulting filtrate was considered a virus-enriched extract (Liang et al., 2019a). Soil virus extracts were flash-frozen using liquid nitrogen and stored at -80°C until further use.

Soil bacteria were separated from soil mineral solids using a Nycodenz cushion; 9 mL of the blended soil slurry was gently pipetted onto 2 mL of 67% (w/v) Nycodenz solution (Accurate Chemical & Scientific, Westbury, NY) and centrifuged at 4,000 rpm for 20 minutes (Liang et al., 2019b). The solution from the interface was collected and mixed with sterile glycerol (to a final glycerol concentration of 30% for cryoprotectant). Two 2-mL tubes of bacterial extract were flash-frozen with liquid nitrogen and stored at -80°C until enumeration. The remaining bacterial extracts were used for induction assays.

To perform prophage induction assays, bacterial extracts were centrifuged at 5,000 rpm to pellet the bacteria, and the supernatant containing free viruses was discarded. The bacteria were then resuspended in the cold extraction buffer, and divided into “Control” and “Induced” tubes. In the induced treatment, Mitomycin-C ($1 \mu\text{g mL}^{-1}$) was added to stress the bacterial cells and stimulate prophage to the lytic mode. The assay was wrapped in aluminum foil and left for 18-h in the dark for incubation at room temperature. After incubation, bacteria and virus samples were collected from each control and induced tube; 2 mL of solution was collected for bacterial abundance and 2 mL of solution was filtered through 0.22-µm Millex Durapore Filters (Merck Millipore Ltd., Tullagreen, Co. Cork, Ireland) to isolate viruses. In total, each assay yielded a

control virus, control bacteria, induced virus, and induced bacteria sample to be used to calculate viruses produced under host stress. Samples were flash-frozen with liquid nitrogen and stored at -80°C until enumeration.

The extracted viruses and bacteria, as well as bacteria and viruses from the induction assay, were all quantified using epifluorescence microscopy, as described by (Liang et al., 2019a; K. E. Williamson et al., 2005). Frozen extracts were thawed and treated with Deoxyribonuclease (DNase) I enzyme to digest extracellular DNA. Treated viral extracts were then vacuum filtered on a Whatman Anodisc filter with a 0.02-µm pore size, stained with a 2X concentration of SYBR Gold nucleic acid gel stain, and examined using a Nikon Eclipse E660 epifluorescence microscope (Liang et al., 2019a). Enumerated virus-like-particles and bacteria particles are henceforth assumed to be viruses and bacteria and will be referred to as such.

Lysogenic fraction (LF) is the percentage of extracted bacteria whose prophage can be induced into lytic reproduction via antibiotic stress, thereby creating new free viruses and destroying the host cell. LF was calculated as $LF = (Viruses\ Induced * 100) / (Burst\ Size / Control\ Bacteria)$, where viruses induced was calculated as $VI = (Viruses\ from\ "Induced"\ Treatment - Viruses\ from\ "Control"\ Treatment)$, burst size was calculated as: $(Viruses\ from\ "Induced"\ Treatment / Bacteria\ Lysed)$, and bacteria lysed was calculated as $(Bacteria\ from\ "Control"\ Treatment - Bacteria\ from\ "Induced"\ Treatment)$. The calculated lysogenic fraction occasionally resulted in zero or negative values for viruses induced, or burst size, or both. In the case of a negative value of any of these measurements, that sample was recorded as a 0, and treated as if prophage induction was below detection.

Soil Biogeochemical Properties

Soil water content (GWC) was determined gravimetrically. Salt-extractable organic carbon (EOC) was measured using the chloroform slurry method (Fierer & Schimel, 2002). Salt-extractable nitrogen concentrations (NO_3^- and NH_4^+) were measured using microplate-based spectrophotometric determinations (Doane & Horwáth, 2003). The pH was determined using an electrode (UI-trabasic, Denver Instrument, Bohemia, NY, USA).

Statistical Analyses

A Mixed Model Analysis of Variance (ANOVA) was performed with the GLIMMIX procedure in SAS 9.4 (SAS Institute Inc, Cary, NC) to determine if our 12 treatments had statistically significant effects on microbial abundance, lysogenic fraction, and biogeochemical measurements. Normality, outliers, and equal variance were checked with the UNIVARIATE procedure, and the least squared means were determined with Fisher's Least Significant Difference at 5% significance level. Correlation analyses were performed via the PROCCORR procedure in SAS (SAS Institute Inc, Cary, NC) both for each individual sampling dates, as well as for all sampling times pooled together. This was done to examine relationships between microbial abundance and soil properties on the plot scale within sampling dates, as well as on the temporal scale across all sampling dates.

RESULTS

Temporal Effects

Virus abundance (VA), bacteria abundance (BA), and lysogenic fraction (LF) were more significantly impacted by sampling date than by the long-term agricultural management treatments (Figure 2.1 and 2.2, Appendix Figure 2.6-2.8). The VA, BA, and LF were thus averaged across all treatments within each sampling date, and these averages were significantly ($p < 0.0001$) different between sampling dates. While all three dates, May 2018, May 2019, and June 2019, had a significant variance in VA, BA, and LF according to ANOVA, the June 2019 sampling was most different from either of the May sampling dates. In June 2019, the VA was highest (7.38×10^9 viruses/ gram dry weight (gdw) soil), while the BA and LF were the lowest, at 2.81×10^8 bacteria/gdw soil and 15.9%, respectively. The average VA was 2.79×10^9 in May 2018 and 1.94×10^9 in May 2019. The average BA was 3.42×10^8 in May 2018 and 4.04×10^8 in May 2019. The LF average was 47.0 in May 2018 and 35.8 in May 2019 (Figure 2.1).

There were many significant differences in measured soil factors between sampling dates (Figure 2.3, Appendix Table 2.2). GWC, nitrogen concentrations (nitrate, ammonium, and total inorganic nitrogen), organic carbon (EOC), pH, and microbial respiration were all significantly different between sampling dates. GWC was highest in May 2019, nitrogen was highest in June 2019, and microbial respiration was highest in June 2019.

Mean VA, BA, and LF were correlated with biogeochemical properties across all sampling dates to show trends in microbial abundance across the measured nutrient

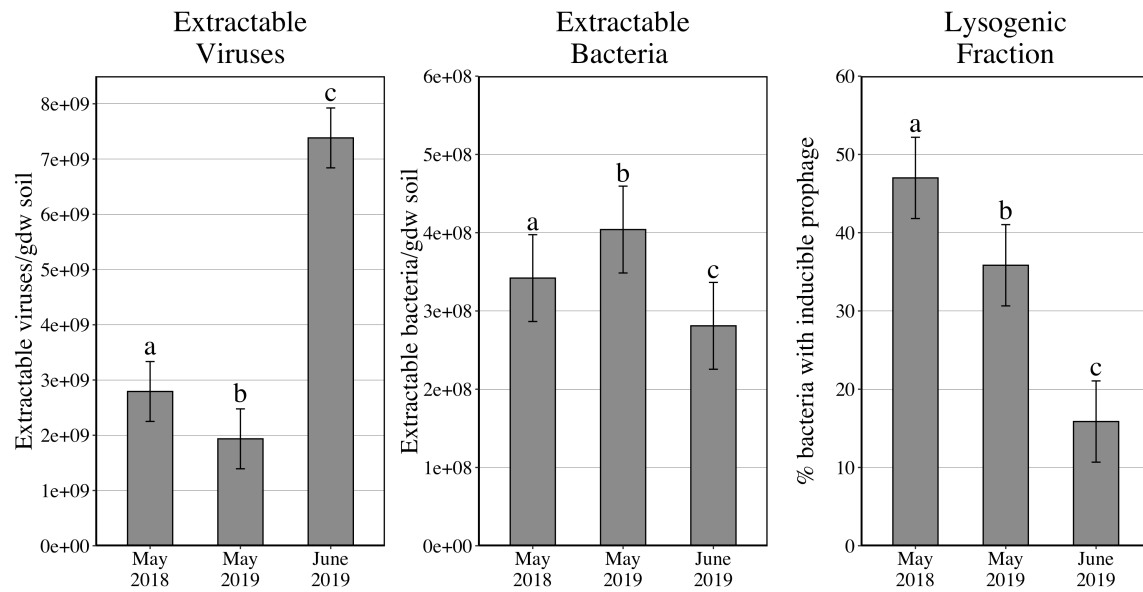


Figure 2.1. Mean extractable virus abundance at each sampling date (left). Mean bacteria abundance at each sampling date (middle). Mean lysogenic fraction at each sampling date (right). Superscript denotes significance at a level of ($p < 0.01$).

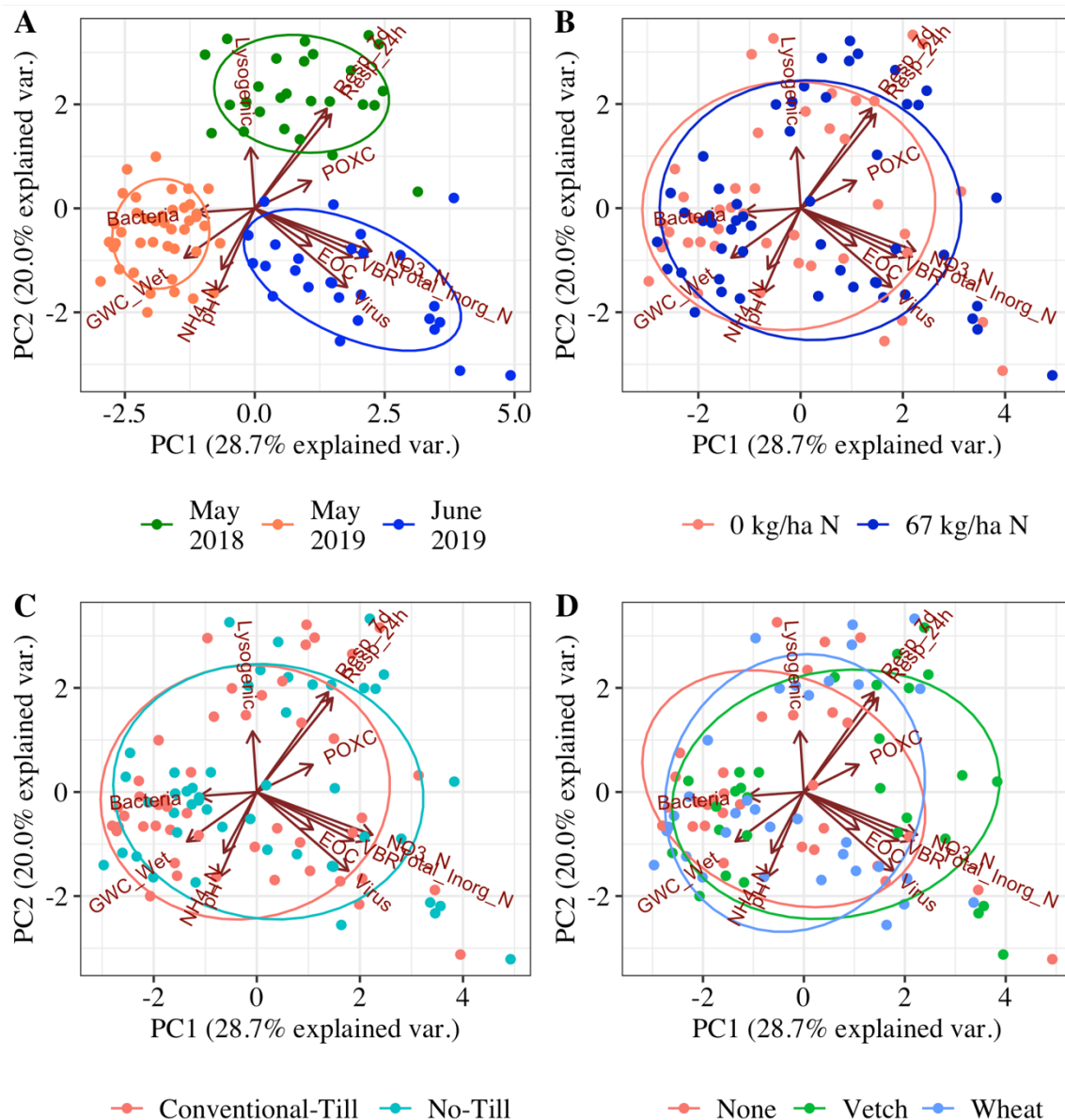


Figure 2.2. Principal component analysis (PCA) of soil samples were clustered by (A) Month, (B) nitrogen fertilization, (C) tillage, and (D) cover crop. The length of each arrow shows the importance of that factor to explain the difference between samples.

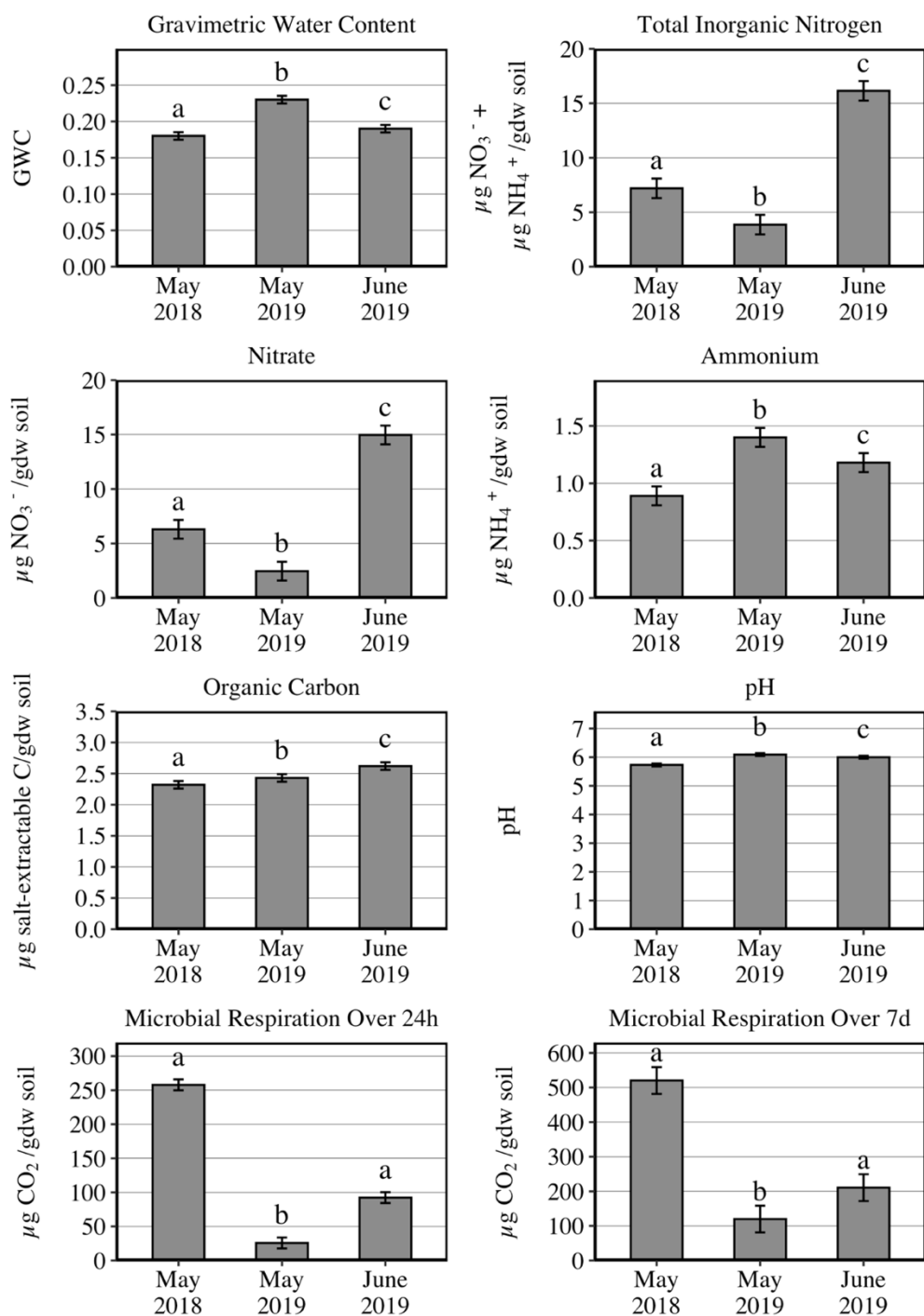


Figure 2.3. Biogeochemical measurements at each sampling time, averaged across all 48 samples and 12 treatments within that sampling time. Column labels of a, b, and c denote statistically significantly different measurements at a level of $p < 0.01$.

conditions. VA, BA, and LF significantly correlated with biogeochemical measurements, including GWC, nitrogen, EOC, and microbial respiration (Table 2.1). VA was lower and BA was elevated when GWC of samples was higher ($p=0.0002$). VA was highest when, in June 2019, the nitrogen concentration was highest. VA was positively correlated ($p<0.0001$) with both nitrate and total inorganic nitrogen concentrations across sampling dates (Figure 2.4). Conversely, LF was lowest when nitrogen content was elevated, negatively correlating with nitrate ($p=0.0012$) and total inorganic nitrogen ($p=0.0001$). VA increased with EOC ($p=0.0002$). BA increased with pH ($p=0.035$), while LF decreased ($p=0.0009$). Finally, LF was elevated when microbial respiration was higher, both for the 24-h respiration test ($p=0.0108$) and the seven-day respiration test ($p=0.0016$). Overall, when nitrogen and carbon concentrations were elevated, VA increased while BA and LF decreased.

Agricultural management effects on viral and bacterial abundance

The long-term conservation management treatments did not have statistically significant effects on VA, BA, or LF according to ANOVA. However, treatments had some statistically significant impacts on biogeochemical measurements. GWC, EOC, nitrate, and microbial respiration over 24-h were all highest under the vetch treatment ($p<0.01$), and EOC was highest under the no-till treatment ($p<0.01$). (Appendix Figures 2.9-2.10). While VA, BA, and LF could not be directly linked to the long-term conservation management treatments via ANOVA (Appendix Figures 2.6-2.8), measured VA, BA, and LF did correlate with GWC, EOC, and pH, which were significantly impacted by cover crop and tillage management.

Table 2.1. Correlations between virus abundance, bacteria abundance, Virus to bacteria (V:B) ratio, and lysogenic fraction and biogeochemical properties. Values indicate the strength and direction of the correlation, * indicates significance at $p<0.05$, ** indicates significance at $p<0.03$.

	Virus Abundance	Bacteria Abundance	V:B Ratio	Inducible Fraction
Field Moisture (GWC)	-0.24359**	0.30579**	-0.27243**	-0.01988
Ammonium ($\mu\text{g NH}_4^+$ /gdw soil)	-0.01059	0.0897	-0.09802	-0.09729
Nitrate ($\mu\text{g NO}_3^-$ /gdw soil)	0.66538**	-0.14355	0.47462**	-0.26682**
Total Inorganic N ($\mu\text{g NO}_3^- + \text{NH}_4^+$ /gdw soil)	0.65747**	-0.13468	0.46159**	-0.2719**
Labile Carbon (mg POXC/gdw soil)	0.04451	-0.00181	0.11177	-0.11247
Organic Carbon (mg EOC/gdw soil)	0.3312**	-0.14762	0.24625**	-0.06307
pH	0.12835	0.17924*	0.01818	-0.22132**
Microbial Respiration (24h)	-0.02351	-0.00494	0.0327	0.21635
Microbial Respiration (7 days)	0.00735	0.02602	0.01857	0.26695**

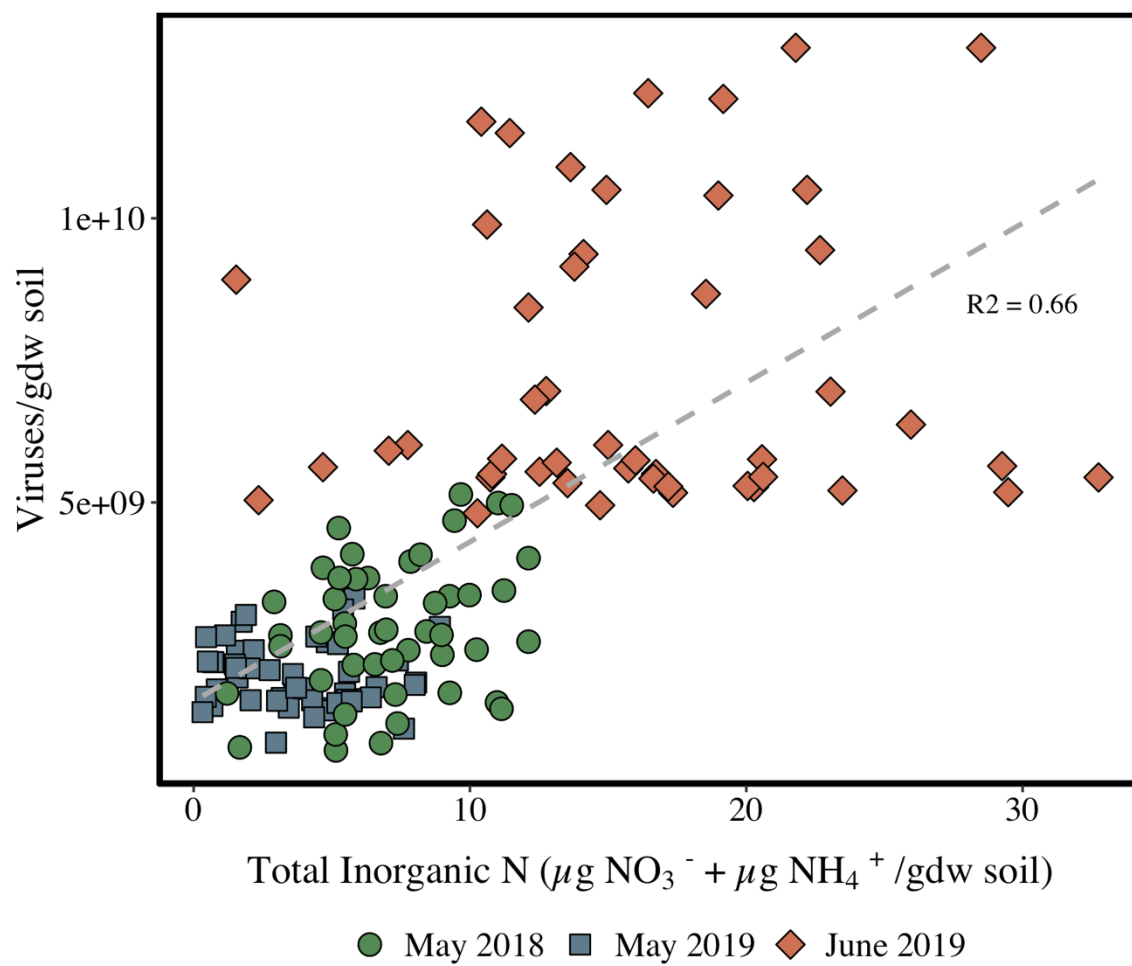


Figure 2.4. Virus abundance correlated positively ($p < 0.001$) with the increase in total inorganic nitrogen concentration across sampling times.

Correlations were performed between VA, BA, and LF within individual sampling dates to determine how these measurements vary between plots rather than between sampling dates. VA was positively correlated with GWC at all sampling times, and this correlation was significant in May 2018 and May 2019 ($p < 0.05$) (Appendix Figures 2.11-2.13). BA increased with GWC at every sampling date ($p < 0.01$) (Appendix Figures 2.14-2.16). VA also increased with EOC and was significantly correlated in May 2019 ($p < 0.01$) (Appendix Figures 2.17-2.19). VA positively ($p < 0.01$) correlated with nitrate and total inorganic nitrogen in May 2018. BA increased with pH at all sampling times and was significant ($p < 0.01$) in June 2019. VA increased with microbial respiration in the May sampling dates but decreased with respiration in June 2019. The correlations between VA and microbial respiration measured after twenty-four hours and seven days were significant ($p < 0.05$) in May 2018. These measured soil properties which influenced VA, BA, and LF were influenced by long-term management practices.

DISCUSSION

Overview

Investigating soil microbial dynamics in field-based systems allows us to understand how in-situ conditions might affect virus and bacteria reproduction. Here, the differences observed between long-term agricultural management practices over a growing season provided a gradient of nutrient availability. In June 2019, when nutrient availability was at its peak, with the highest levels of nitrate, ammonium, total inorganic nitrogen, and EOC, virus abundance was the highest. Interestingly, there seemed to be an optimal range for water content for viral abundance; when water content was at its

highest in May 2019, virus abundance as well as all biogeochemical factors were at their lowest levels, suggesting that overall elevated field moisture was not optimal for viral abundance. Overall, these changes across sampling dates proved to be more predictive of virus abundance, bacteria abundance, and lysogenic fraction than the long-term agricultural management practices. Indeed, the fact that the agricultural treatments have been ongoing for upwards of forty years may well be the reason that there are very few observable differences in VA, BA and LF between management practices; the microbial community has acclimated to the management practice and is now rather stable. Instead, seasonal changes, such as changes in rainfall or temperature, might be a better explanation for the changes we observed between our sampling dates.

Temporal shifts in virus abundance indicate an induction event

The time scales in which we observe VA, BA, and LF play an important role in shaping our understanding of microbial abundance. We observed that the short-term (i.e., within growing season) time scale was the more significant in impacting microbial population dynamics rather than the long-term management practices of cover crop, tillage, or nitrogen fertilization treatments which have been in place for 40+ years at this site (Figure 2.2). Virus and bacteria abundance, and lysogenic fraction significantly changed between the May and June sampling dates in 2019 (Figure 2.1). In June 2019, there was a considerable increase in extractable viruses compared to the previous month, while extractable bacteria and lysogenic fraction were significantly lower. This is consistent with an event or change in field condition just prior to the June 2019 sampling that may have triggered a prophage induction event or increase in viral production,

thereby increasing the VA and lowering the BA and LF through prophage induction and host lysis.

In order to understand which factors might be influencing viral abundance and production in agricultural soils, we examined biogeochemical measurements as well as precipitation data. The increase in mean virus abundance in June 2019 was positively correlated with conditions which are typically considered optimal for host growth, including higher nitrate concentration, total inorganic nitrogen, and organic carbon concentration (Figure 2.3). Surprisingly, however, we observed that June 2019 had the lowest host abundance and lysogenic fraction. This significant increase in viral abundance, coupled with the decrease in bacterial host abundance and lysogenic fraction, suggests that there was potentially a prophage induction event just prior to sampling in June 2019. In this scenario, some events may have triggered prophage to the lytic cycle, thereby lysing hosts and increasing viral abundance, along with releasing host intracellular material into the nutrient pool, while decreasing host abundance and thereby decreasing the lysogenic fraction. Indeed, the elevated EOC concentrations in June 2019 could be considered evidence of an induction event. Potentially, if there was a shift to lytic replication followed by the release of microbial necromass from the lysed hosts cells, it would result in the release of labile organic materials to the dissolved organic carbon pool in the soil. As prophage induction can be in response to both positive and negative signals to prophage, we aimed to determine which factors could potentially induce viral lytic reproduction in the June 2019 sampling.

Nitrogen fertilization

Nitrogen is an essential macronutrient which can stimulate growth/activity in nitrogen-limited microbial environments (Geisseler & Scow, 2014). This suggests that the short-term increased nitrogen availability that occurred following N-fertilization between the May and June sampling dates may have promoted viral reproduction and, likely, a higher percentage of temperate phages in the lytic rather than lysogenic phase (Narr et al., 2017). An influx of nitrogen fertilizer before the June 2019 sampling could have stimulated host growth, alerting the viral prophage to optimal lytic reproduction conditions, inducing the switch from lysogenic to lytic reproduction, and accounting for the increase in free viruses and decrease in lysogenic fraction and bacteria abundance.

Water content

Interestingly, overall virus abundance was *negatively* correlated with GWC; when the mean GWC was highest in May 2019, the mean VA was lowest. This relation suggests that there might be an optimal range of water content for soil virus abundance; when the water content was extremely high as a stress in May 2019, the viral abundance dropped significantly. Alternatively, the high rainfall immediately preceding the sampling on this date may have resulted in the leaching of free extracellular viruses downward with infiltration temporarily reducing the overall viral abundance.

Precipitation data 10 days prior to sampling shows that pre-sampling conditions were very different for each date (Appendix Figure 2.20). Prior to the May 2018 sampling, there was little precipitation. June 2019, there was much more rainfall prior to the sampling event. These data suggest that the May 2018 samples, upon analysis,

underwent a large rewetting effect, accounting for the large increase seen in microbial respiration relative to the other sampling dates which were not as dry (Figure 2.3). Therefore, the microbial respiration data appeared to have more to do with the precipitation than the biological factors themselves.

Microbial respiration and water content are important factors to consider when characterizing an induction event. If microbial respiration is high, and therefore host activity is high, one would assume that conditions are optimal for viral production. However, we observed that the viral abundance was highest in June 2019, when microbial respiration and water content were in between that of the two May values (Figure 2.3 A, G, &H). We propose that this elevated microbial respiration in May 2018 was due to a wetting effect from the drier May 2018 samples; the respiration was elevated because the hosts experienced a re-wetting effect upon sample processing, and may not have been indicative of true microbial respiration in the field conditions. The increased microbial respiration in June 2019 compared to May 2019 supports our theory that host activity may have driven a viral induction event. Although it is impossible to directly link GWC as a cause of our induction event, we showed that optimal moisture conditions in June 2019 may be a factor driving host activity and thereby viral production.

Treatment effects on prophage induction

We hypothesize that a broad-based prophage induction event occurred just prior to the June sampling and determined that the most likely influencing factor was the increase in nitrogen content. These population patterns support the KtW theory of viral

infection, which states that viral production is dependent on host activity, and will favor lytic reproduction under the most favorable biogeochemical conditions for host growth, and that when conditions trend oligotrophic, viruses favor lysogenic reproduction (Liang et al., 2020a; Maslov & Sneppen, 2017; Payet & Suttle, 2013; K. E. Williamson et al., 2007; S. J. Williamson et al., 2002). As nitrogen is a limiting factor to bacterial growth, the increase in nitrogen content in June 2019 could have triggered an increase in host productivity. Indeed, the microbial respiration was higher in June 2019 than in May 2019, consistent with was an increase in microbial activity. Studies have shown that viruses use acyl-homoserine lactones (AHLs) to sense host activity and density (Ghosh et al., 2009; Liang et al., 2020a). Potentially, in June 2019, the elevated VA could be attributed to a viral induction event based on an increase in host activity, stemming from increase in nitrogen availability.

The May and June sampling dates covered pre- and post-fertilization with ammonium nitrate to half of the sampled plots. Puzzlingly, while we observed changes in the viral abundance which associated with nitrogen content, this nitrogen content did not associate with the nitrogen treated plots, which prompts speculation as to the origin of the increased nitrogen. Potentially, there may have been some drift in N fertilizer application which caused inconsistent correlations between measured and applied nitrogen. Alternatively, the cover crop burn-down and integration into the soil mass may have increased soil N. Overall, while we showed that there was an association between nitrogen content and viral abundance, and perhaps viral reproduction, we cannot be certain that the management practice is what caused the shifts.

Plot management effects on virus and bacteria abundance

Our investigation of this long-term conservation management experimental site in Jackson, Tennessee revealed that variability in microbial abundance could not be directly associated with long-term agricultural management practices. There could be several reasons why management practices were difficult to link to microbial abundance, including pore-scale heterogeneity and physics, and the difficulty of obtaining a representative sample across a large plot when microbial abundance and populations can change over the short spatial scale of centimeters or less. Our analysis revealed that viral abundance, bacterial abundance, and lysogenic fraction could not be directly significantly linked to our long-term agricultural management practices. However, we were able to directly link these practices to biogeochemical measurements, such as elevated water content, organic carbon, and nitrate levels under the vetch cover crop treatment. In turn, water content, organic carbon, and nitrate could be correlated to viral abundance. In this way, the various management practices may have had an effect on virus and bacteria abundance due to their impacts on the soil biogeochemical conditions.

CONCLUSIONS

Overall, we hypothesized that (1) soil bacterial and virus abundance would be highest under the management practices and sampling dates with the highest nutrient availability and water content and (2) soil lysogenic fraction would be higher under less favorable soil environmental conditions. Indeed, the virus abundance was highest when nitrogen and carbon concentrations were higher in June 2019, but bacteria abundance was lower. This contradicts previous studies, which have shown that viral abundance and

bacterial abundance are positively correlated. A compelling explanation for this would be a viral induction event; elevated virus abundance may have been a result of productive viral infections which lead to the lysis and death of bacteria hosts, driving viral abundance counts up and bacterial abundance counts down. Additionally, viral abundance was not highest in soil with the highest water content, suggesting there may be optimal moisture content less than saturation which supports the greatest lytic production of viruses in soils. Furthermore, for hypothesis (2), lysogenic fraction was highest in May 2018, which we conclude was the sampling date with the least favorable environmental conditions in soil at the experimental site. Although May 2018 had a higher nitrate concentration than May 2019, the soil was very dry, and the ground was hard-packed, which was likely a non-ideal environment for soil microbial activity. In total, increased viral abundance and decreased lysogenic fraction correlated strongly with nitrogen concentration, suggesting that fertilization and viral activity played a role in shaping soil microbial abundance. Overall, we conclude that viral abundance on the field scale is more affected on the temporal scale than by long term management practices.

In general, soil viral induction is still an intriguing area for future research, and many field and lab-based experiments will be needed to investigate additional factors which can induce viral lytic reproduction in soil habitats. Originally, we had additional samples from October 2019 and December 2019 which were compromised and could not be evaluated, which would have helped us understand more clearly how soil viruses and bacteria respond to changes in temperature, moisture, nutrient status, and more in an annual cycle. As it stands, our three sampling dates, as well as our plot-to-plot investigation of management practices within sampling dates, provided a good gradient

of nutrient availability. We have shown that at high resource (C, N, and moisture) availability, virus abundance was elevated while inducible fraction was low, which may be consistent with prophage induction to the lytic cycle. While this work represents an initial investigation for in-situ prophage induction, future studies are needed to quantify factors that impact the switch from lysogenic to lytic reproduction such as host cell density, diversity, stressors, and other edaphic variables.

APPENDIX

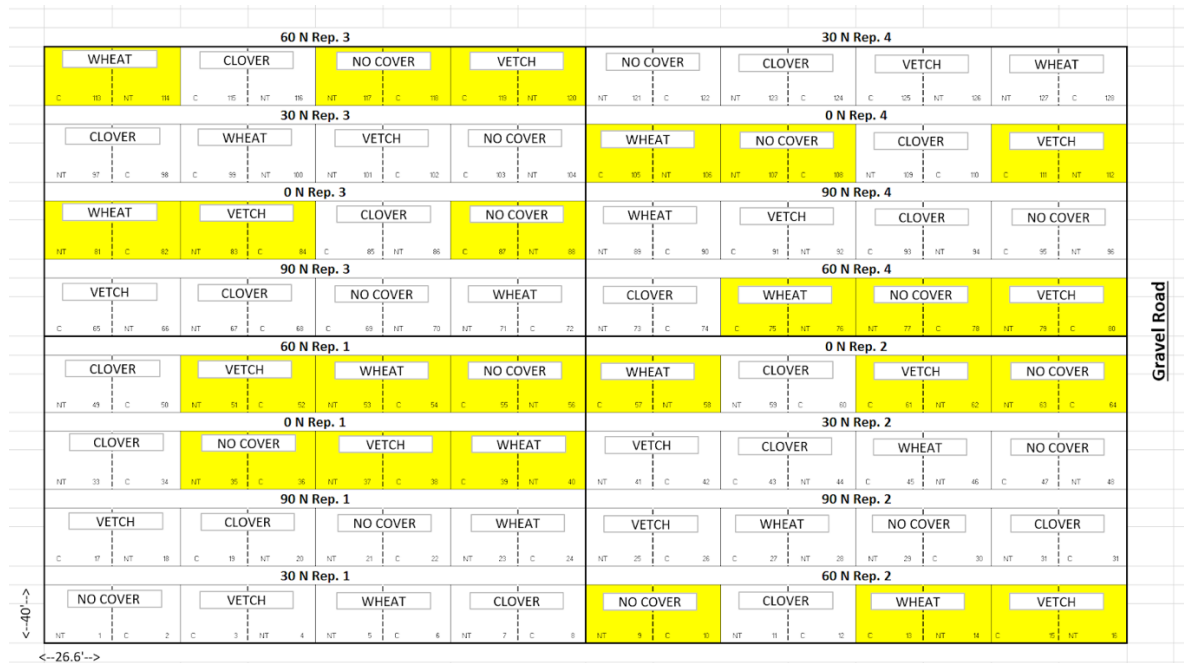


Figure 2.5. Layout of RCBD split-plot treatments. Plots highlighted in yellow were sampled.

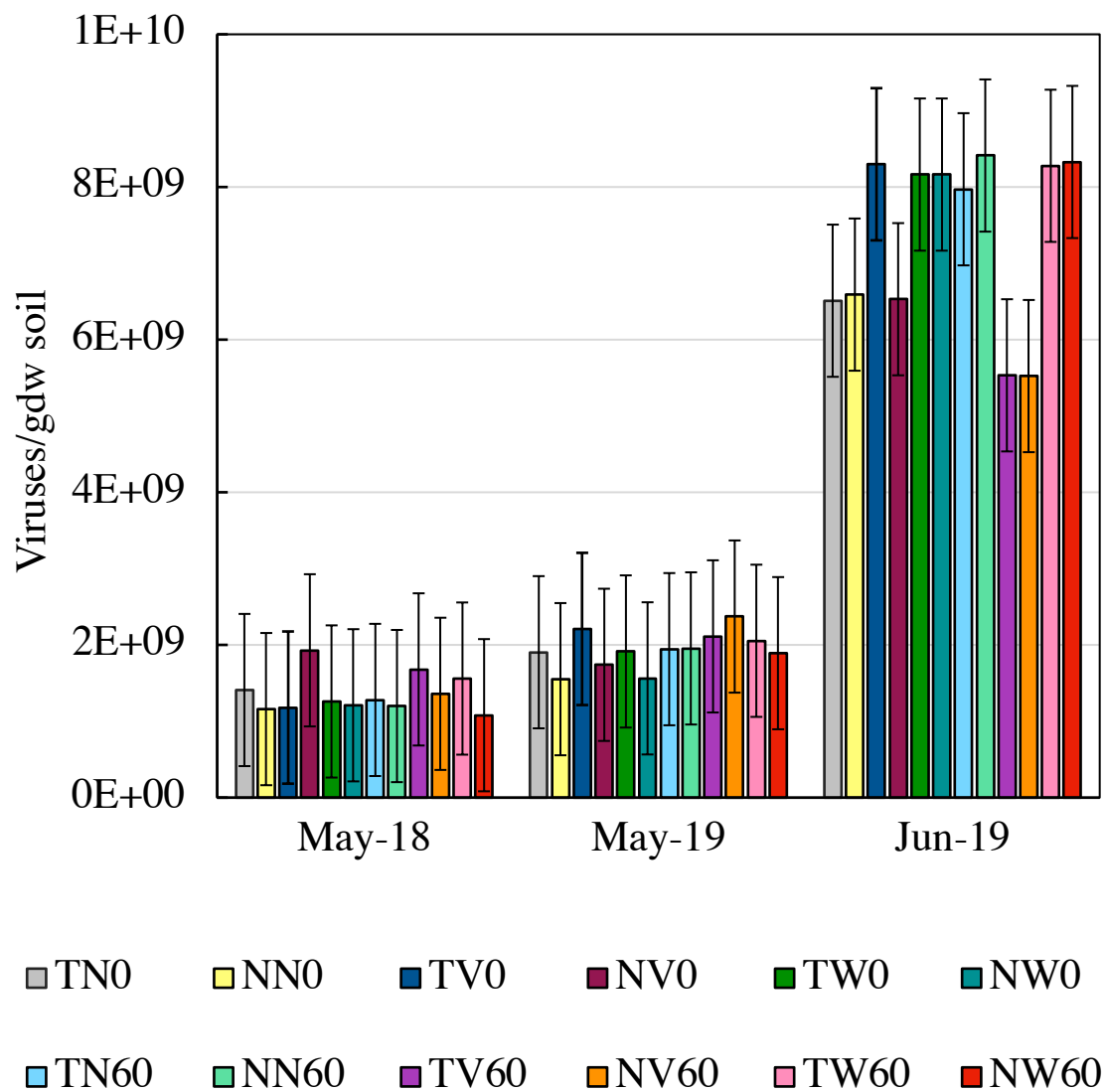


Figure 2.6. Virus abundance under each treatment at each sampling time.

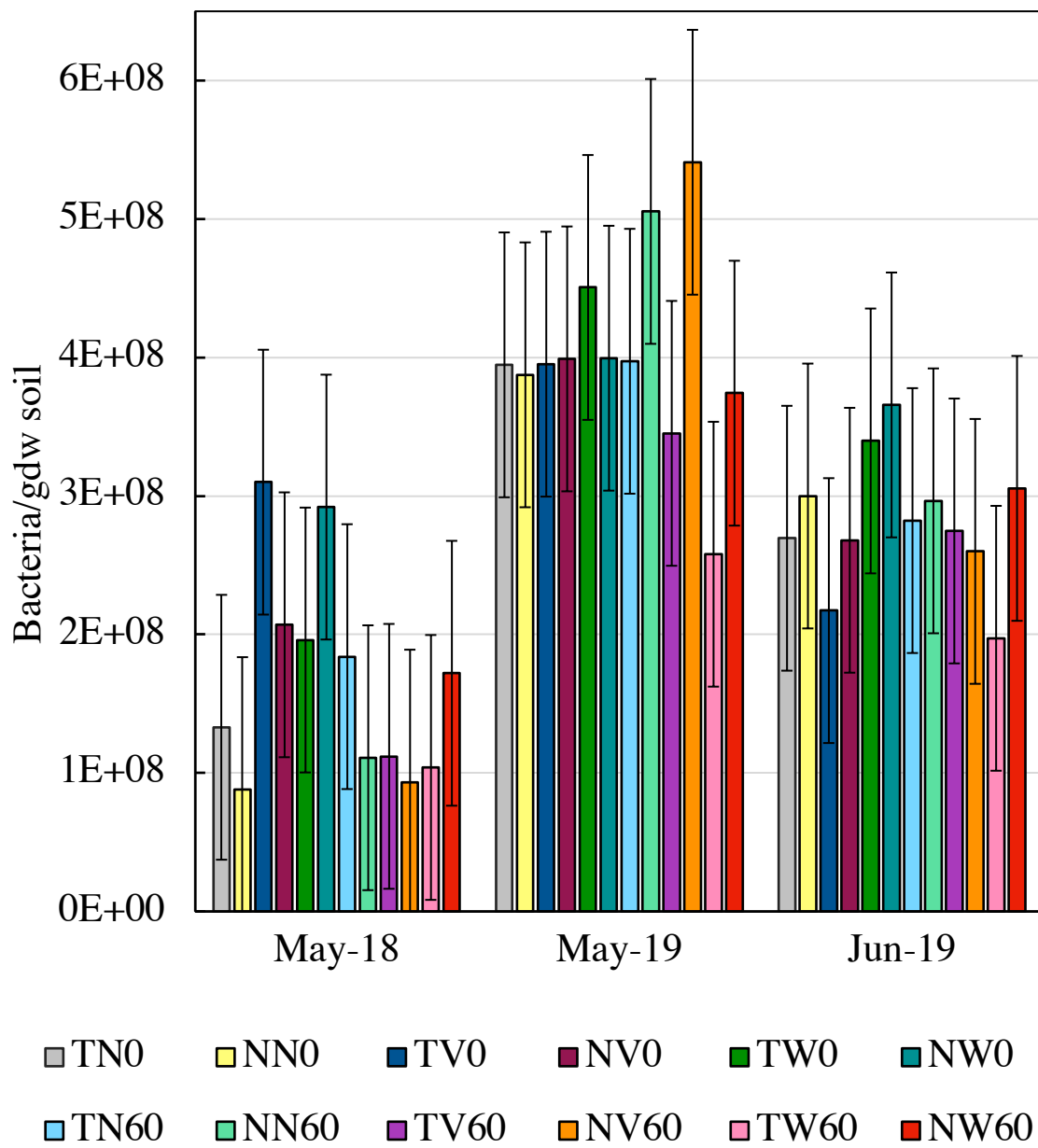


Figure 2.7. Bacteria abundance under each treatment at each sampling time.

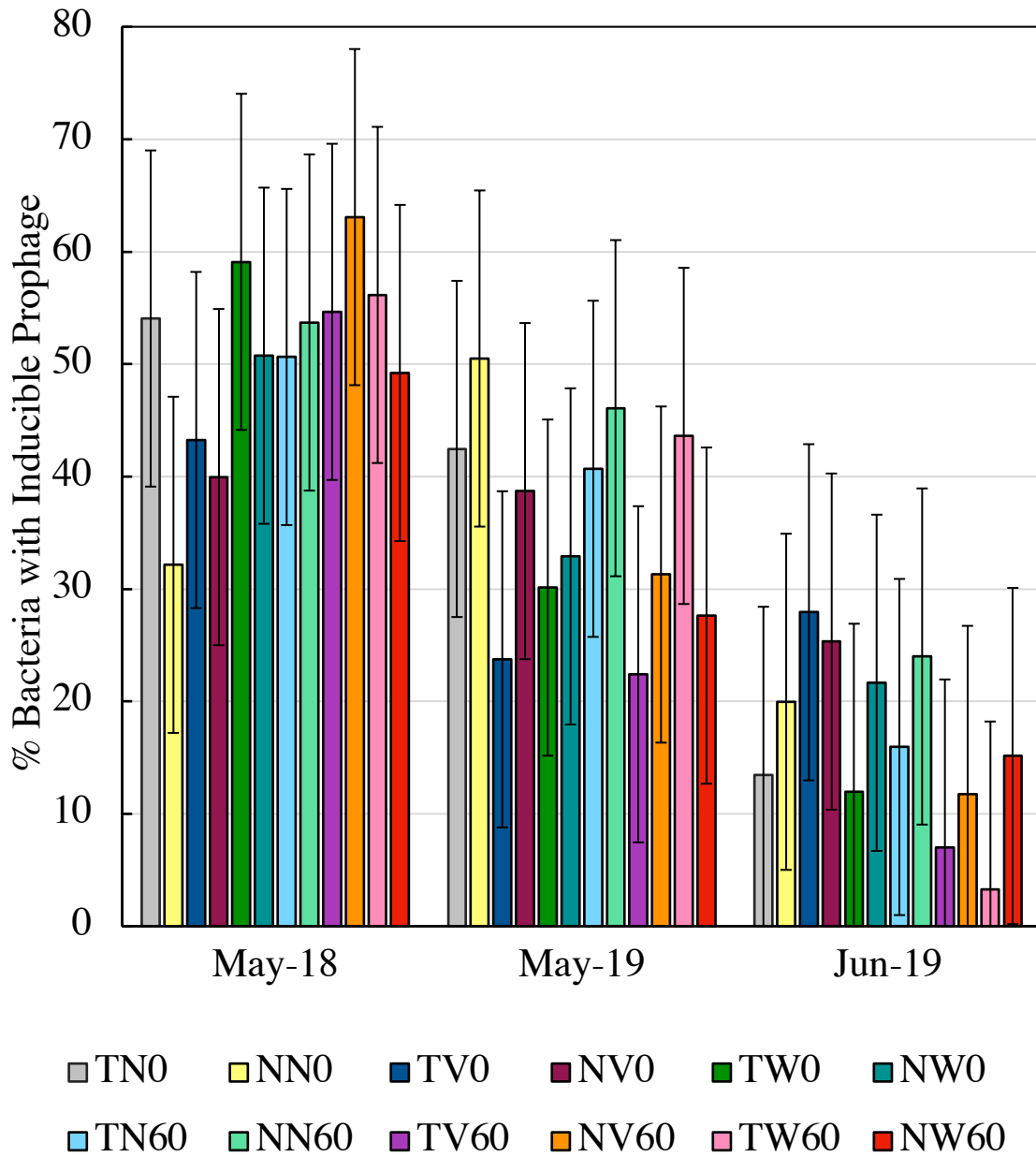


Figure 2.8. Lysogenic Fraction under each treatment at each sampling time.

Table 2.2. Mean of biogeochemical measurements taken at each sampling time.

Superscripts denote differences between sampling dates at a significance of $p < 0.03$.

	May 2018	May 2019	June 2019
Field Moisture (GWC)	0.18 ^a	0.23 ^b	0.19 ^c
Ammonium ($\mu\text{g NH}_4^+/\text{gdw soil}$)	0.89 ^a	1.4 ^b	1.18 ^c
Nitrate ($\mu\text{g NO}_3^-/\text{gdw soil}$)	6.3 ^a	2.46 ^b	14.96 ^a
Total Inorganic Nitrogen ($\mu\text{g NO}_3^- + \text{NH}_4^+/\text{gdw soil}$)	7.19 ^a	3.86 ^b	16.14 ^c
Labile Carbon (mg C/gdw soil)	271.04 ^a	207.1 ^b	234.19 ^c
Organic Carbon (mg C/gdw soil)	2.32 ^a	2.43 ^b	2.62 ^c
pH	5.73 ^a	6.09 ^b	6.00 ^c
Microbial Respiration (24h)	257.89 ^a	25.68 ^b	92.27 ^a
Microbial Respiration (7 days)	520.27 ^a	119.66 ^b	210.52 ^a

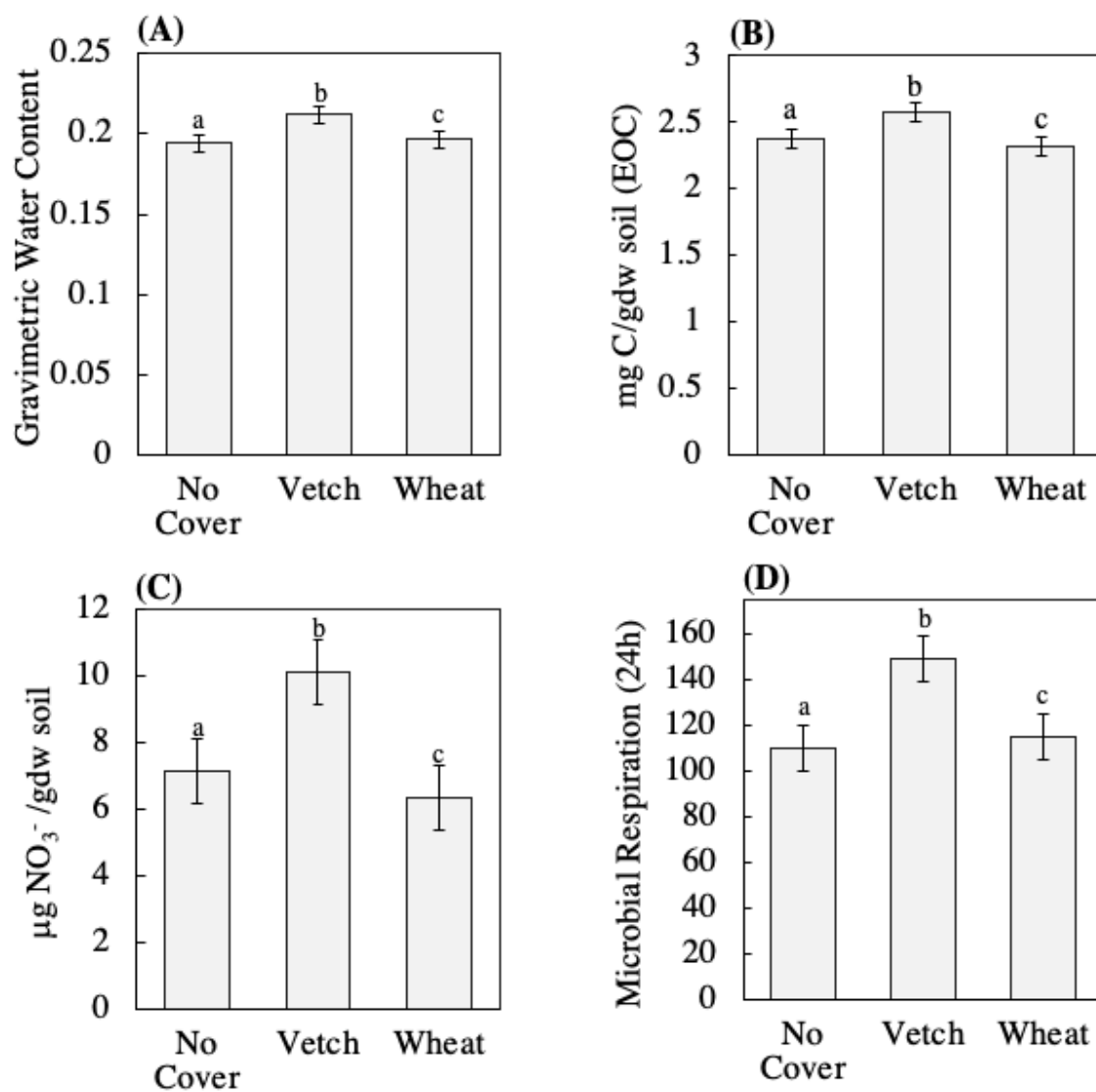


Figure 2.9. Biogeochemical measurements which were significantly ($p < 0.05$) different between cover crop treatments, as denoted by superscripts.

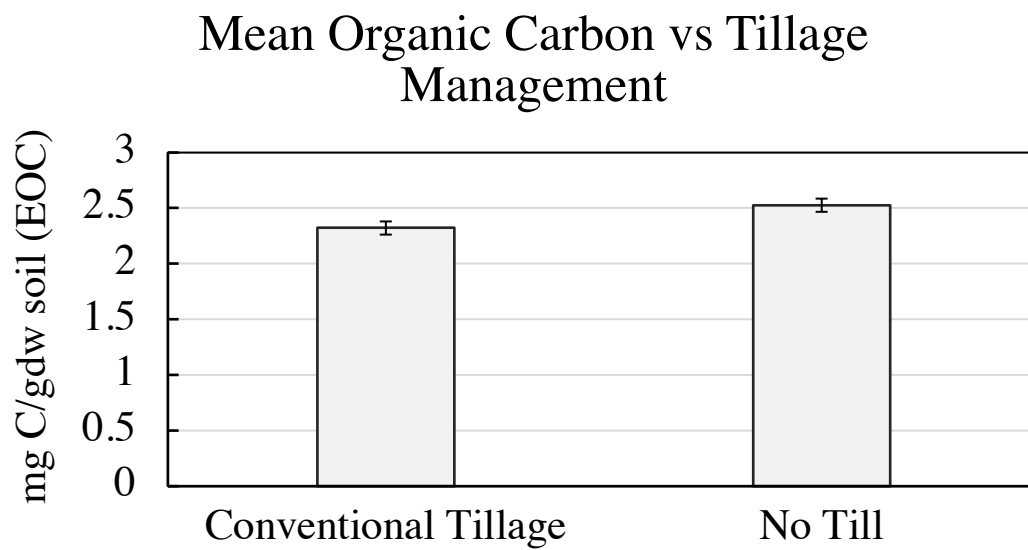


Figure 2.10. Organic carbon was significantly influenced by tillage management at a level of $p < 0.01$.

GWC and Viral Abundance May 2018 Samples

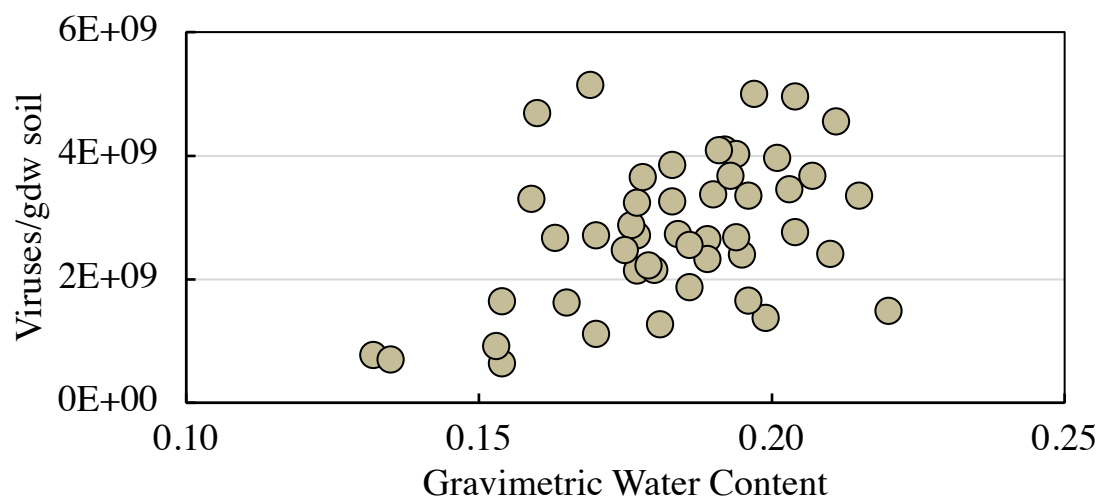


Figure 2.11. Virus abundance significnatly ($p < 0.05$) correlated with gravimetric water content in May 2018.

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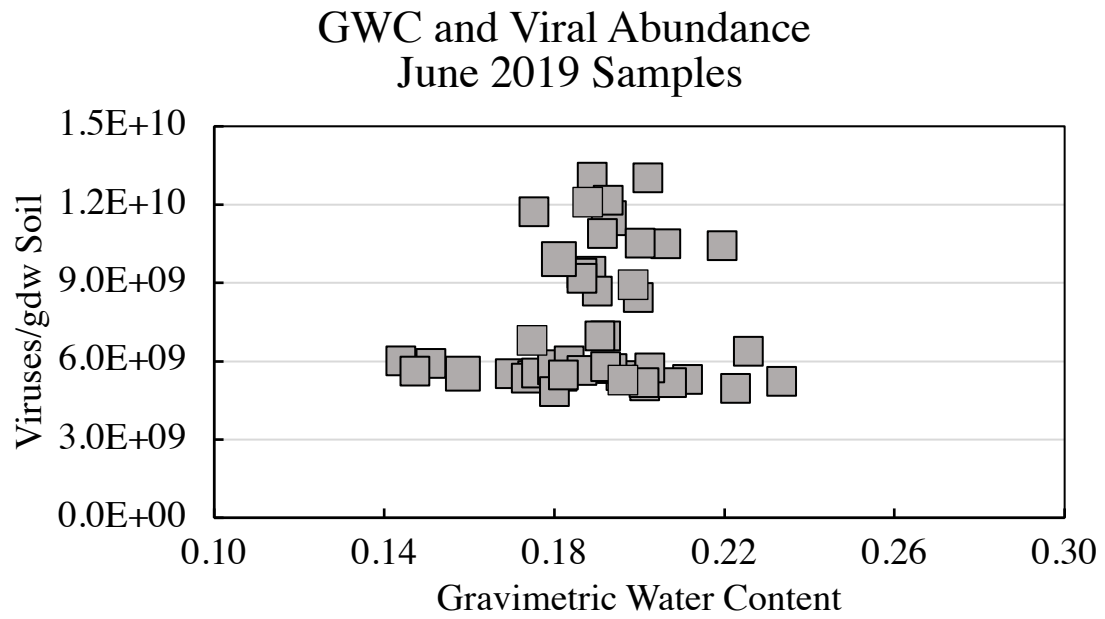


Figure 2.13. Virus abundance significnatly ($p < 0.05$) correlated with gravimetric water content in June 2019.

GWC and Bacteria Abundance May 2018 Samples

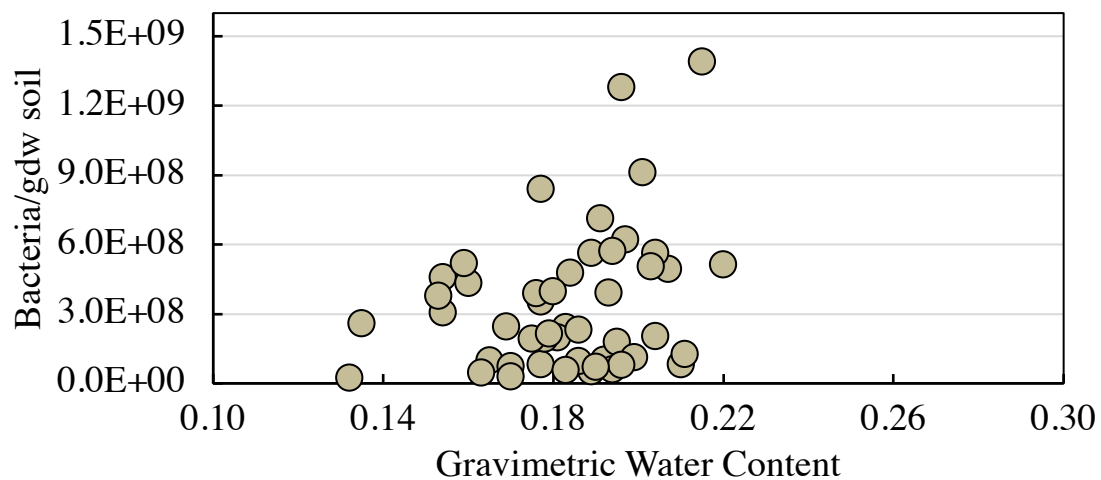


Figure 2.14. Bacteria abundance significantly ($p < 0.05$) correlated with gravimetric water content in May 2018.

GWC and Bacteria Abundance May 2019 Samples

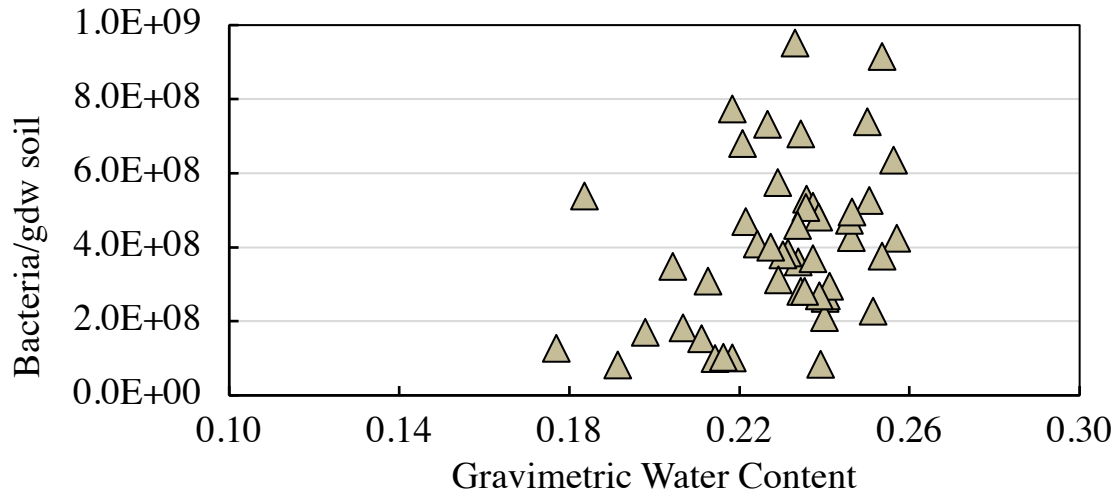


Figure 2.15. Bacteria abundance significantly ($p < 0.05$) correlated with gravimetric water content in May 2019.

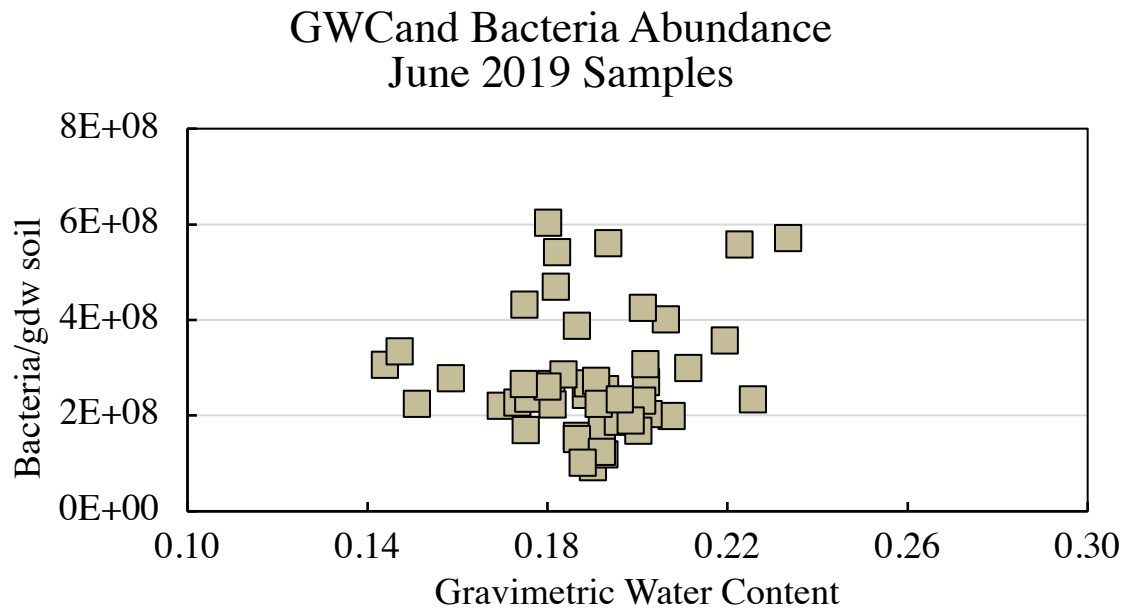


Figure 2.16. Bacteria abundance significantly ($p < 0.05$) correlated with gravimetric water content in June 2019.

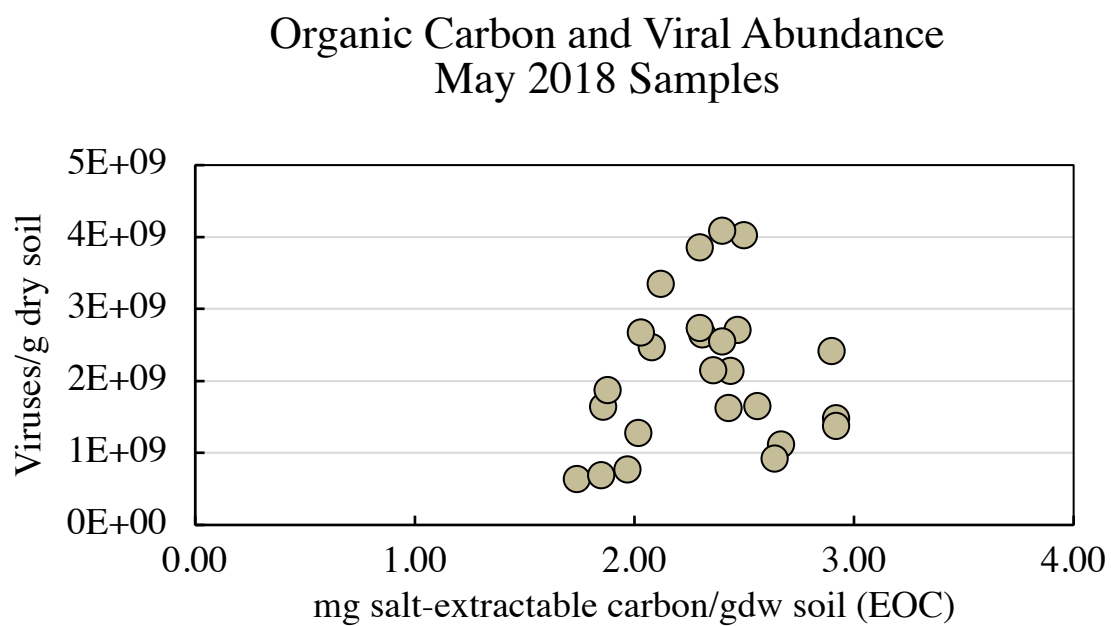


Figure 2.17. Virus abundance significnatly ($p<0.05$) correlated with organic carbon (EOC) content in May 2018.

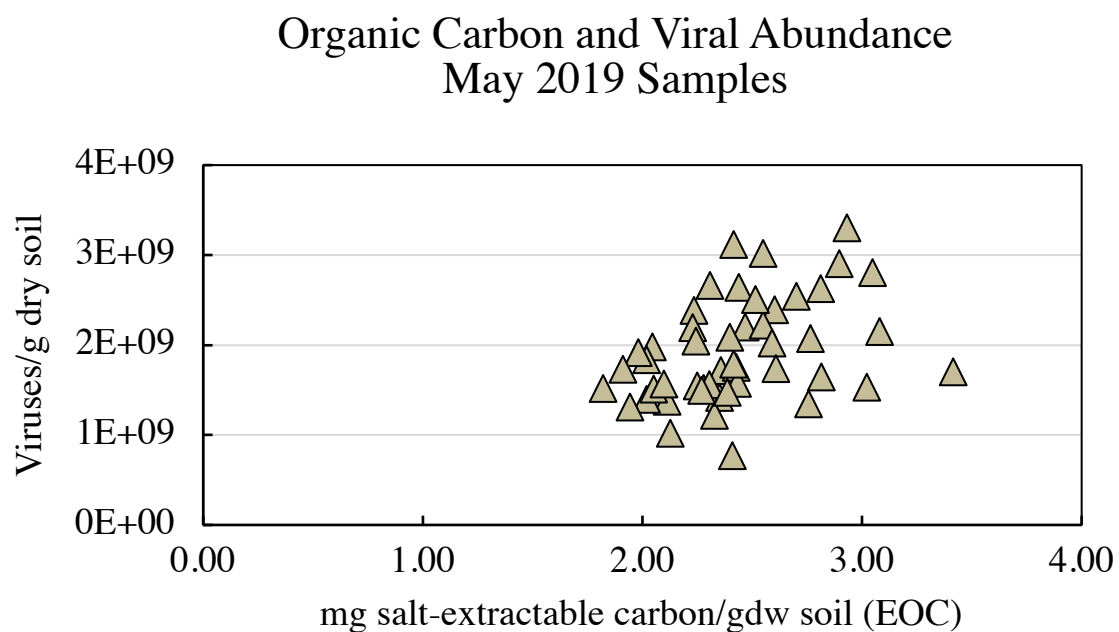


Figure 2.18. Virus abundance significnatly ($p<0.05$) correlated with organic carbon (EOC) content in May 2019.

Organic Carbon and Viral Abundance June 2019 Samples

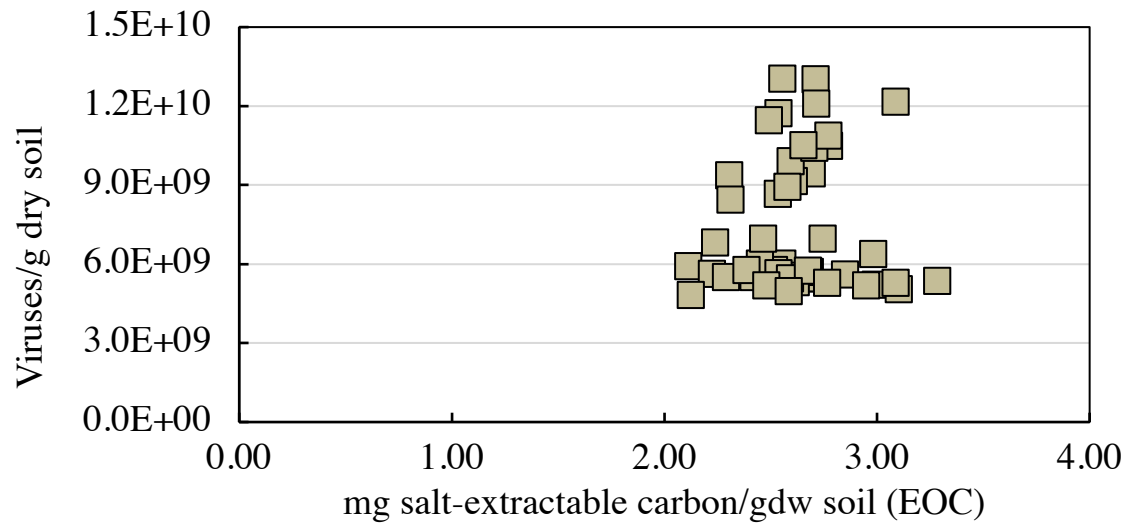


Figure 2.19. Virus abundance significantly ($p < 0.05$) correlated with organic carbon (EOC) content in June 2019.

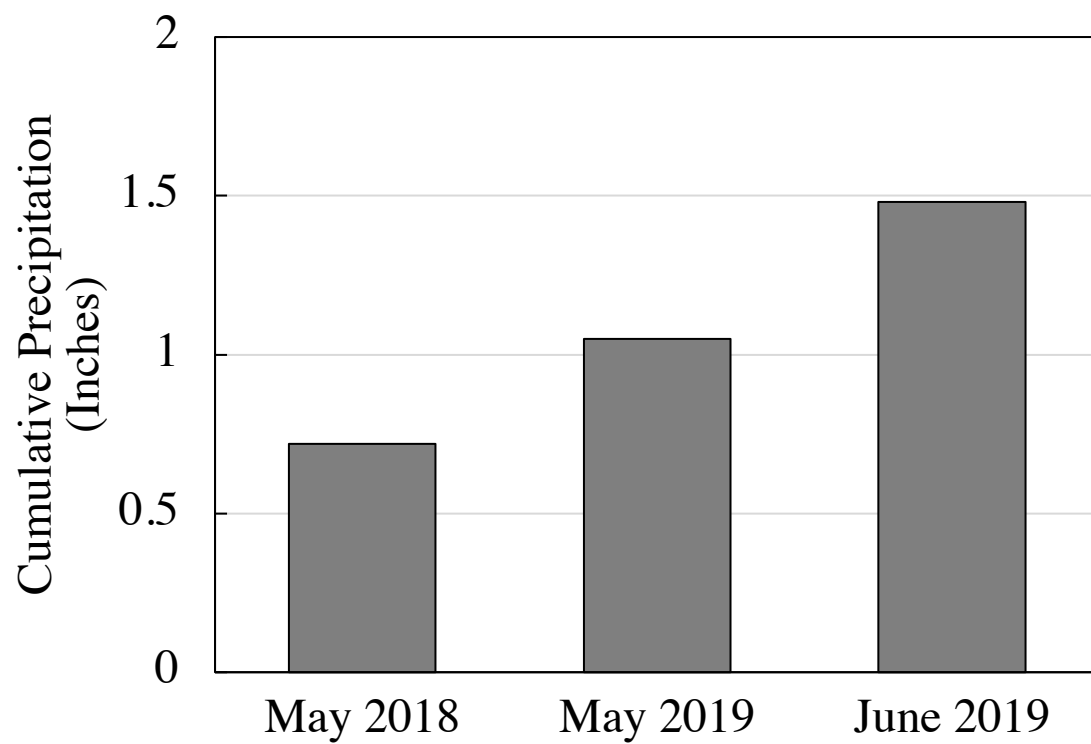


Figure 2.20. Cumulative precipitation ten days prior to each sampling date.

CHAPTER 3: DYNAMICS OF MOBILE AND IMMOBILE NATIVE SOIL VIRUSES UNDER INTERRUPTED FLOW CONDITIONS

My contribution to this work was laboratory work including soil collection, column experiments, and virus enumerations. Samples from this experiment were sent to the University of Tennessee Water Quality Core facility for analyses of chemical concentrations. I performed all statistical analyses, prepared the visualizations, and wrote this manuscript.

ABSTRACT

Repeated wetting events such as high-frequency rainfall or irrigation influence soil viruses' mobility, which affects their eventual contact with and potential impacts on their microbial hosts. Water flowing through soil pores can differentially mobilize soil viruses based on the chemical and physical conditions of soil solution, soil surface and pore structure, and virus size and surface properties. Trace amounts of chemicals, which are introduced in rainwater, could potentially impact soil virus movement and/or reproduction in natural soil systems.

Here, we aimed to understand how reoccurring wetting events which mimic repeated rainfall or irrigation events would impact soil virus mobilization through a saturated soil column. In addition, we wanted to understand if the trace levels of ammonium, which can be found in rainwater, have any effect on viral mobilization. Lastly, we wanted to determine if the soil virus abundance in the residual column soil was affected by the interrupted flow events or the ammonium additions. To achieve these objectives, we set up soil column experiments wherein we repacked agricultural soil into the columns and subjected them to daytime flow followed by overnight flow interruption, where there was no influent or effluent. From the effluent, we collected soil virus abundance and chemical concentration data. Following the five daily interrupted flows, we deconstructed the soil columns and sampled the soil for viral abundance at three depths along the soil column: the inlet (0-5 cm), the middle (10-15 cm), and the outlet (20-25 cm) of the column.

We determined that soil viral abundance was significantly impacted by interruptions in hydrologic flow. Following each flow interruption, there was a spike in

viral abundance in the effluent when the flow was resumed. The largest viral abundance spike occurred in pore volume 2. We attributed the abundance spike to either the release of easily-detachable viruses from soil surfaces into soil solution or biological production of viruses during the overnight flow interruption. The residual soil viruses remaining in the columns were evenly distributed across soil depths, indicating that the viral transport mechanisms were consistent across the soil column, and that there was no reattachment of soil viruses along the column. This result suggests that the viruses could have potentially travelled farther than the length of our soil columns. Finally, we found that inputs of trace levels of ammonium consistent with ammonium atmospheric deposition did not impact virus abundance within the effluent pore volumes or in the residual soil columns. Overall, this work greatly enhances our understanding of viral movement and retention in simulated natural conditions, giving insight into native viral transport patterns into soil depths as a result of repeated wetting events.

INTRODUCTION

Soil microbial communities are frequently subject to the impacts of natural hydrological conditions such as rainfall and irrigation events and drying/wetting cycles. Water flow through soil pores influences not only microbial habitats but also distributions of microfauna, dissolved carbon, nutrients, oxygen, and enzymes. In addition to hydrological conditions, chemical conditions of the influent water, such as nitrogen in rainwater might also exert an influence on soil bacterial and virus communities during rainfall events.

Soil virus transport under varying environmental conditions have long been addressed due to its relevance to water quality, pathogen movement, and soil hydrology (Kenst et al., 2008; Xu et al., 2017). Previous studies have shown that transport and retention of viruses are impacted by many physiochemical conditions, such as ionic strength, flow rate, ion composition, and pH of soil solution (Masciopinto & Visino, 2017b; Walshe et al., 2009; Zhuang & Jin, 2003b, 2003a, 2008). Such previous studies used model systems to identify key drivers and mechanisms that control movement behaviors of viruses. However, studies which more closely simulate environmental conditions are equally if not more important for understanding viral chemistry and physics in natural systems. As such, it is important to study native soil viruses under conditions which simulate natural events, such as varying rainfall patterns or natural environmental chemical changes. One of many natural environmental changes is atmospheric nitrogen deposition, which can be wet or dry and occurs in the southeastern United States at a rate of 7.6 kg/ha per year, 67% of which is in the form of reduced nitrogen species, such as ammonium or ammonia (Y. Li et al., 2016). As a result,

atmospheric nitrogen deposition increases the nitrogen content of soil and has been shown to impact soil microbial community (Zhou et al., 2018), thereby changing viral host availability (Avrahami et al., 2002).

In this study, we aimed to understand how native viral mobilization might change under simulated environmental conditions, such as repeated rainfall events and atmospheric nitrogen deposition. A specific objective of the study was to determine how interrupted hydrologic flow patterns influence mobile and residual soil virus abundance under trace ammonium concentrations. We hypothesized that the interrupted flow conditions would allow for an increase in viral abundance between disconnected elution periods (i.e., flow interruption period) by allowing viruses to disperse out of micropores and into the mobile liquid phase. We predicted that overnight flow interruption would increase soil virus recovery, but there would be extractable residual soil viruses which remain in the column at the conclusion of the experiment. Additionally, we hypothesized that the ammonium concentrations at trace levels would not have significant impacts on virus transport.

To test these hypotheses, we conducted column-scale experiments by repacking native field soil into columns and introducing ammonium-containing ($2 \mu\text{g NH}_4^+/\text{g}$ column soil) solution into the vertical soil columns from the bottom of the column under saturated flow conditions. The input was ceased between each pore volume to create overnight flow interruption. We enumerated viruses and conducted chemical analyses on the effluent liquid and, at the end of the experiment, the soil column was sectioned to collect soil samples from different depths for enumeration of residual soil viruses.

Multivariate and correlation analyses were performed to analyze the relationships among

flow dynamics, chemical conditions (including added ammonium), and virus abundance in the effluent and the soil.

Results indicated that virus abundance in the effluent increased in the second pore volume, and then decreased with each subsequent pore volume of the injected liquid and that the interrupted flow increased viral recovery. However, the trace ammonium did not significantly impact viral transport and retention. These results provide valuable insights into the potential impacts of sequential rainfall/irrigation events and atmospheric nitrogen deposition on soil virus transport and retention in the soil profile. This virus-focused study furthers our understanding of soil microbial responses to environmental dynamics.

METHODS

Soil Collection and Preparation

Soil was collected at the East Tennessee Research and Education Center in Knoxville, TN from an Ultisol soil classified as a fine-loamy, mixed, subactive, thermic Typic Hapludult. Soil sampled from the top 10 cm was transported immediately to the University of Tennessee, Knoxville, where it was air-dried for 48 hours and sieved through a 2 mm screen to remove plant roots. The air-dried soil was stored in a bag at room temperature ($21\pm 2^{\circ}\text{C}$) to create the most stable conditions for the soil microbial community prior to the column experiments and to facilitate column packing.

Soil Column Experiment

All experiments were conducted in plexiglass (acrylic) columns (25 cm in length and 3.8 in inner diameter). The input solution was introduced via a multi-channel

peristaltic pump from the bottom of the column (200 $\mu\text{L}/\text{minute}$) while effluent was continuously collected from the top into 20-mL glass tubes using Retriever II fraction collectors (Figure 3.1). Each tube collected 9.5-mL of the effluent, representing one sample fraction that was equivalent to one-sixth pore volume. Teflon tubing or stainless-steel tubing was used throughout the experimental system. For virus enumeration and chemical measurements, the six effluent fractions of each pore volume of the outflow were pooled into two samples, with fractions 1-3 becoming “Sample A” and fractions 4-6 becoming “Sample B”. So, each column had samples 1A, 1B, 2A, 2B, 3A, 3B, 4A, 4B, 5A, and 5B, and the time from “B” to “A” represented 16-h no-flow period (e.g., the flow was interrupted between 1B and 2A).

Three replicate experiments were conducted under the saturated flow conditions with overnight flow interruption. To begin the experiment, the air-dried soil was dry-packed into columns, and then one pore volume of input solution was introduced at 200 $\mu\text{L}/\text{minute}$ until column saturation, at which point the pump was turned off and the column was left overnight for 16 hours. The next day, the pump was turned on for 8 hours to inject a new pore volume of the input solution at flow rate of 200 $\mu\text{L}/\text{minute}$ while the effluent was collected. Then, the pump was shut off again for 16 hours of flow interruption. This pattern continued for five times to collect a total of 5 pore volumes of the effluent (Table 1). At the conclusion of the experiment, the columns were

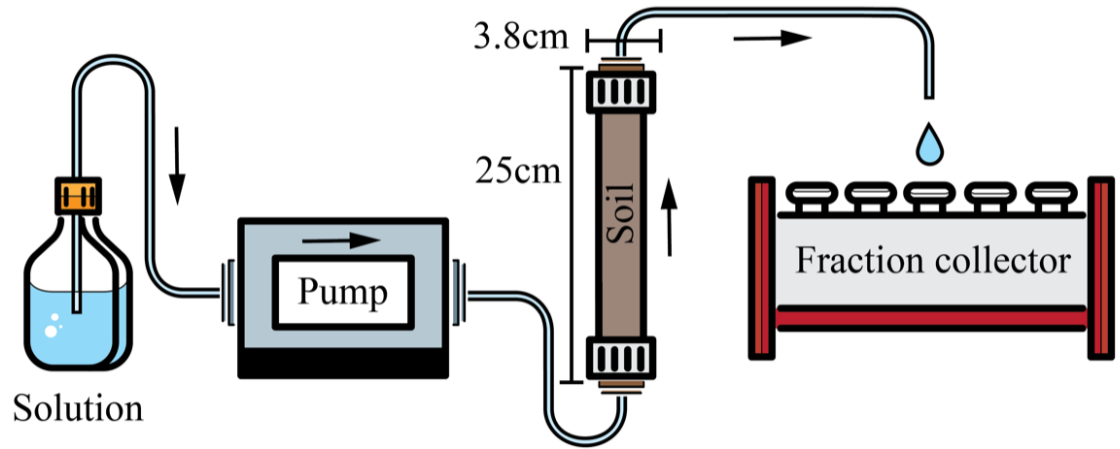


Figure 3.1. Schematic of column transport system.

deconstructed, and the column soil was segmented into five 5-cm sections along the depth of the soil column, with Depth 1 at the bottom (closest to the inlet) and Depth 5 at the top (closest to the outlet) (Figure 3.2). Measurements were taken from Depth 1 (“inlet”), 3 (“middle”), and 5 (“outlet”) for virus abundance and chemical concentrations (Figure 3.2).

Treatments consisted of varying input solutions to mimic atmospheric N deposition at an amount of 6.5 kg/ha. This level of nitrogen is consistent with the level of ammonium/ammonia which would be received over the course of one year in the southeastern United States if the total amount of nitrogen per year were 7.6 kg/ha. Two application methods of ammonium input were used. In the “single-N addition” (SN) column, 22.7 mg/L NH_4Cl was used in the first pore volume, followed by four pore volumes of the nitrogen-free control solution. In the “multiple-N addition” (MN) columns, 4.45 mg/L NH_4Cl was administered to each of the five pore volumes of input solution. In total, roughly 2 micrograms of NH_4^+ were applied to each gram of soil in the ammonium-amended columns, either in the first pore volume (SN) or equally across 5 pore volumes (MN) (Figure 3.3). The control (C) solution included calcium chloride (CaCl_2) at a concentration of 273.1 mg/L. Additional calcium chloride was added to each input solution to bring each solution to a uniform ionic strength of 10 mM.

Virus Extraction and Enumeration

For virus isolation of column effluent liquid, each half pore volume sample (A and B) were filtered through 0.22- μm Millex Durapore Filters (Merck Millipore Ltd., Tullagreen, Co. Cork, Ireland). To extract soil viruses from deconstructed column soil,

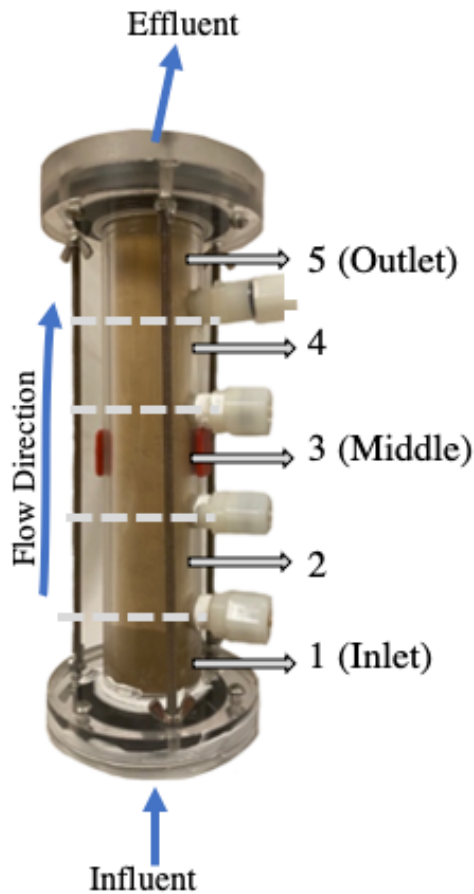


Figure 3.2. Soil column used for experiment, shown in the process of being saturated. The division of segments used for soil sampling upon column destruction are numbered 1-5. Input solution flows from the bottom to the top of the column. Outlets on the side of the column were sealed and not used for this experiment. Samples were taken from the inlet, middle, and outlet depths for analysis.

	Day 1 Saturate Column with Pore Volume 1		Day 2 Collect Pore Volume 1		Day 3 Collect Pore Volume 2		Day 4 Collect Pore Volume 3		Day 5 Collect Pore Volume 4		Day 6 Collect Pore Volume 5		Day 7 Collect soil from Inlet (0-5cm), Middle (10-15 cm), & Outlet (20-25 cm)
Control	0 μg NH_4^+ /g soil	⊗	0 μg NH_4^+ /g soil	⊗	0 μg NH_4^+ /g soil	⊗	0 μg NH_4^+ /g soil	⊗	0 μg NH_4^+ /g soil	⊗	0 μg NH_4^+ /g soil	⊗	Destructively Sample Soil Columns
SN	6.4 μg NH_4^+ /g soil	⊗	0 μg NH_4^+ /g soil	⊗	0 μg NH_4^+ /g soil	⊗	0 μg NH_4^+ /g soil	⊗	0 μg NH_4^+ /g soil	⊗	0 μg NH_4^+ /g soil	⊗	
MN	1.07 μg NH_4^+ /g soil	⊗	1.07 μg NH_4^+ /g soil	⊗	1.07 μg NH_4^+ /g soil	⊗	1.07 μg NH_4^+ /g soil	⊗	1.07 μg NH_4^+ /g soil	⊗	1.07 μg NH_4^+ /g soil	⊗	

Figure 3.3. Flow interruption schematic. Each day flow occurred for 8 hours at a rate of 200 $\mu\text{L}/\text{min}$, followed by a 16 hour flow interruption, shown by the red Xs. Input solutions included Control, Single-N addition (SN), and Multiple-N additions (MN).

70g of soil was blended on high with 233.3 mL of cold buffer (50 mM phosphate buffer containing 1% potassium citrate, w/v, pH 7) for 3 minutes (K. E. Williamson et al., 2005). To isolate viruses, 45 mL of soil slurry was centrifuged in 50-mL tubes at 5,000 rpm for 20 minutes at 4°C, then the supernatant was filtered through 0.22- μ m Millex Durapore Filters (Liang et al., 2019a). All viral extracts were flash-frozen and stored at -80°C until enumeration.

Both the effluent viruses and the extracted viruses from residual column soil were quantified via epifluorescence microscopy (K. E. Williamson et al., 2005). Viral extracts were thawed and treated with Deoxyribonuclease (DNase) I enzyme to digest extracellular DNA, and then vacuum filtered on a Whatman Anodisc filter with a 0.02- μ m pore size to capture viruses between 20 and 220 nm in diameter (Liang et al., 2019a; Liang et al., 2019b). Filters were stained with a 3X concentration of SYBR Gold nucleic acid gel stain. The dyed filters were examined using a Nikon Eclipse E670 epifluorescence microscope with FITC excitation (467–498 nm wavelength) and emission (513–556 nm) filter set Using images of viruses taken with a Retiga Ex-i CCD camera (Qimaging, BC, Canada), virus-like-particles (VLP) were enumerated using IPlab software (BD Biosciences, Massachusetts, MA) (Liang et al., 2019a).

Chemical Measurement

Concentrations of ions of interest were determined at the University of Tennessee Water Quality Core Facility (<https://cee.utk.edu/water-quality-core-facility>) via ion chromatography on an ICS-1100 system with background suppression to achieve low detection limits. Concentrations of chloride (Cl^-), nitrate (NO_3^-), nitrite (NO_2^-), hydrogen

phosphate (HPO_4^{2-}), sulfate (SO_4^{2-}), fluoride (F^-), and ammonium (NH_4^+) were determined for effluent liquid and deconstructed soil column samples. Due to interference with the phosphate-based buffer, measurements could not be determined for hydrogen phosphate and ammonium for the deconstructed column soil samples.

Statistical Analyses

To evaluate eluted viral abundance and solution chemical conditions, a one-way ANOVA with repeated measures model was used, where input solution (control, single-N addition, or multiple-N additions) was the factor, and pore volumes (1A-5B) were the repeated measures. To evaluate the viral abundance and chemical measurements from the deconstructed soil columns, a one-way ANOVA with a split-plot design was used, where input solution (control, SN, or MN) was the factor and column depth (inlet, middle, and outlet) were the sub-factors. These ANOVA were performed by the *lme4* and *multcomp* packages in R (Douglas et al., 2015; Hothorn et al., 2008). Correlation analyses were performed based on Pearson's product-moment correlation ($p < 0.05$) in R to determine relationships between chemical measurements and virus abundance.

RESULTS

Hydrological Effects on Eluted Viral Abundance

In the effluent samples collected from the column during flow, virus abundance varied significantly ($p < 0.05$) with pore volume according to the one-way ANOVA with repeated measures. Viral abundance increased from pore volume 1 to pore volume 2, and subsequently began declining again in following pore volumes (Figure 3.4). Viral

abundance was greater in the first half of each pore volume (i.e. 1A, 2A, 3A, 4A, 5A) than the second half (i.e. 1B, 2B, 3B, 4B, 5B) (Figure 3.5). Additionally, following the 16-hour flow interruption between each pore volume, there was a spike in viral abundance. The spikes, meaning that each A sample was larger than the B sample of the preceding pore volume, indicate a very significant impact of overnight flow interruption on soil virus abundance in the effluent samples.

Spatial Distribution of Residual Soil Viruses

The residual soil viruses, viruses remaining in the soil at the completion of the flow experiment, were collected from three depths within the columns: the inlet (0-5 cm), the middle (10-15 cm), and the outlet (20-25 cm). Viral abundance was measured on the three soil samples. One way ANOVA with split plot design revealed no significant differences in viral abundance at different soil depths (Figure 3.5). This result indicates that mobilized viruses from the inlet soil area were not retained or accumulated in the outlet soil area via a certain mechanism (e.g., mechanical straining or re-attachment) and that the viruses collected before each overnight flow interruption represented the portion of mobile viruses that were dispersed from micropores and/or produced overnight before they were mobilized the next day by a new pore volume of elution.

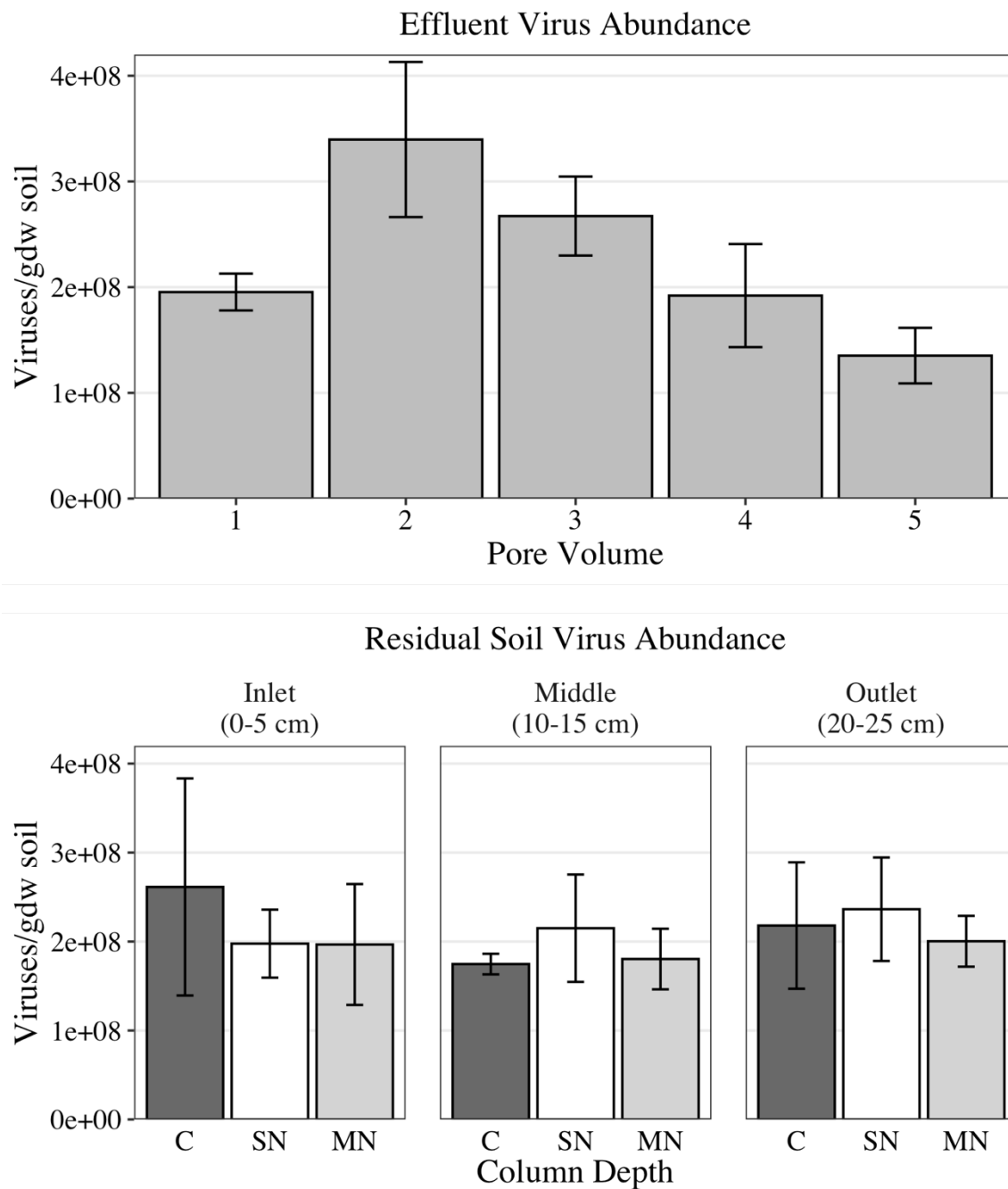


Figure 3.4. Virus abundance calculated per gram dry weight (gdw) of soil for effluent pore volumes (top) and residual soil (bottom). Input solutions included Control, Single-N addition (SN), and Multiple-N additions (MN).

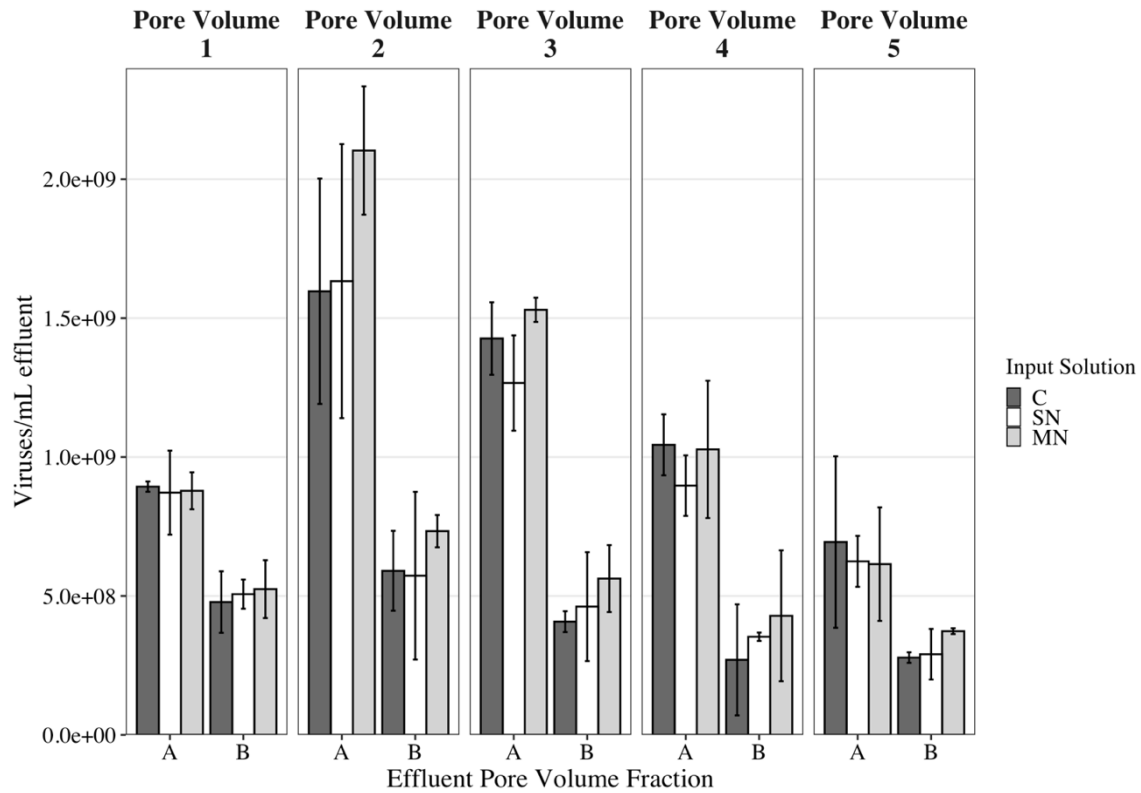


Figure 3.5. Viral abundance at each half pore volume. Fraction “A” is the combination of fractions 1-3 and fraction “B” is the combination of fractions 4-6 of each pore volume. Between each pore volume, the pump was turned off and there was no flow for 16 hours. Input solutions included Control, Single-N addition (SN), and Multiple-N additions (MN).

Relation Between Viral Abundance and Chemical Conditions

We measured the concentrations of various ions in the effluent solutions in order to determine relationships between solution chemistry and viral abundance. It is important to note that there were no differences in the effluent concentrations of ions between the columns treated with ammonium inputs and the control columns (Figures 3.6 and 3.7). All columns had the same concentrations of ions in the effluent, regardless of input solution. For all columns, eluted ammonium concentration was significantly negatively correlated with viral abundance ($p < 0.03$) (Figures 3.4 and 3.6). For example, in pore volume 2A, when viral abundance (viruses/mL effluent) reached its peak, NH_4^+ concentration (mg/mL) was at its lowest. As virus abundance declined in subsequent pore volumes, effluent ammonium concentration increased. No other chemical measurements were significantly correlated with eluted viral abundance. There were no correlations between viral abundance and chemical measurements from the residual column soil; however, ammonium and hydrogen phosphate in the soils were not measured due to buffer interference.

The ionic strength of measured ions in the effluent liquid was significantly different ($p < 0.03$) between pore volume 1A and subsequent pore volumes. Pore volume 1A had significantly higher ionic strength than subsequent fractions 1B-5B (Figure 3.7). In order to visualize native ionic strength independent of the background electrolyte (i.e. CaCl_2) in the input solution, chloride was subtracted from the total ionic strength. Ammonium was not subtracted from the overall ionic strength because the influent level

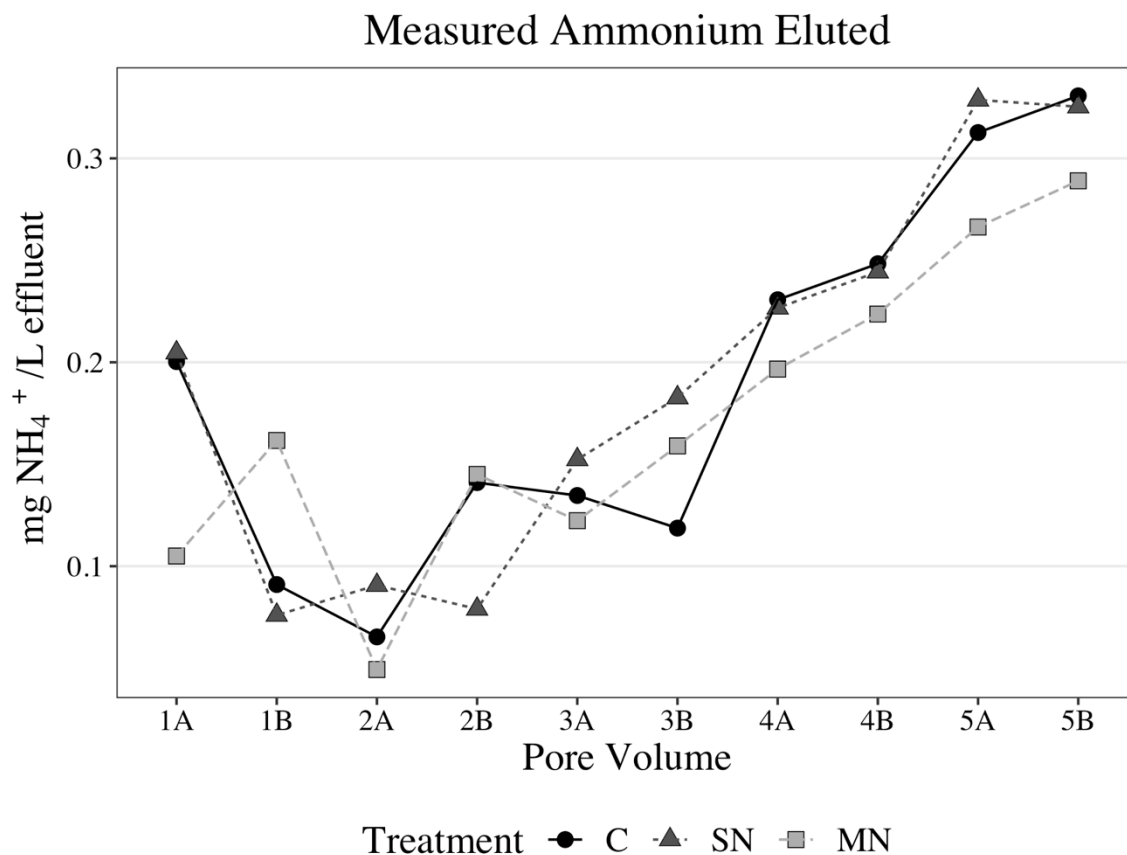


Figure 3.6. Concentration of NH₄⁺ across the duration of the flow experiment.

Ammonium is measured in mg/L effluent liquid. Input solutions included Control, Single-N addition (SN), and Multiple-N additions (MN).

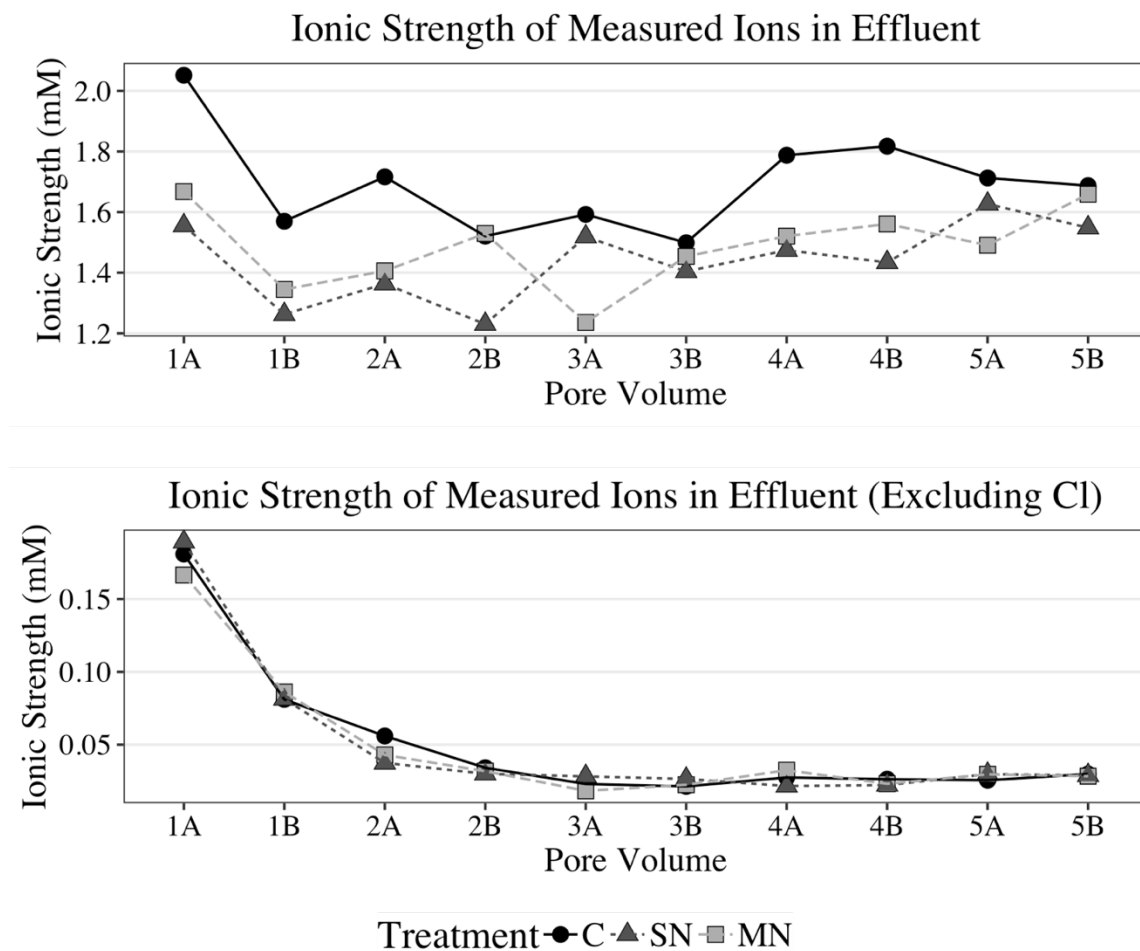


Figure 3.7. Calculated ionic strength (mM) of the sum of the measured ions in the effluent samples including chlorine (Cl^-), nitrate (NO_3^-), nitrite (NO_2^-), hydrogen phosphate (HPO_4^{2-}), sulfate (SO_4^{2-}), fluoride (F^-), and ammonium (NH_4^+). In the second plot, chlorine is excluded from the sum to show native ionic strength without the influence of the input solution, whose ionic strength was brought to 10mM with CaCl_2 . Input solutions included Control, Single-N addition (SN), and Multiple-N additions (MN).

was too low to influence the effluent concentration of ammonium. In this calculation, fractions 1A, 1B, and 2A had statistically significantly higher ionic strength than the remaining fractions 2B-5B (Figure 3.7).

Chemical Effects in Ammonium-treated Columns

Despite inputs of ammonium in columns with SN and MN input patterns, there were no significant differences in chemical measurements between the treatment columns and the control. In the SN and MN columns, ammonium levels dipped in pore volume 2A and rose in subsequent pore volumes in line with the control columns (Figure 3.6). In the residual column soil, N-treatment (i.e. SN or MN) was not a factor in viral abundance, and did not correlate with any chemical measurements. Ionic strength for ammonium-treated columns followed the same pattern as the control, with significant differences between pore volume 1A ($p < 0.01$) but not between treatments (Figure 3.7).

DISCUSSION

Overview

Viruses exist in soil pore water or attached on soil surfaces via electrostatic, hydrophobic, and water-film capillary forces. The viruses suspended in soil solution are easily mobilized by water movement during rainfall or irrigation events. Subsequently, soil-attached viruses might be detached into the flowing aqueous phase (i.e., pore water) if the chemical equilibrium at soil-water interfaces changes due to changes in solution chemistry (ionic strength, pH, ionic composition, or dissolved organic carbon). Under natural flow conditions in the environment, ion concentration and composition in both

soil surface and soil water vary with flow processes and dynamics, thus influencing virus detachment, re-attachment, and mobility. In our experiments, the spike in viral abundance in pore volume 2 (Figure 3.4) may have been due to the 16-hour flow interruption, which changed chemical conditions (e.g., ionic strength, dissolved oxygen concentration) and/or biological conditions (e.g., bacterial host activity and abundance) in soil pores.

Chemically, a decrease in ionic strength or viral abundance in the liquid fraction may have allowed virus diffusion from micro-pores into the mobile phase. Biologically, new viruses may have been produced by bacterial hosts during the overnight 16-hour flow interruption, which were then eluted upon the resumption of the flow conditions. No matter the mechanism, this result implies that the no-flow interval was necessary for increased recovery of mobile virus in the flowing soil solution.

Virus Recovery during Flow Interruption

Hydrologic flow has been shown to have a significant impact on virus release and transport. Virus release is kinetic, meaning that it is time dependent. Viruses with varying surface properties have varying release rates from soil surfaces, depending on the strength of the virus attachment to the soil surface. In pore volume 1, eluted viruses likely represent the viruses in the liquid phase, which can be mobilized very easily. After pore volume 1 was eluted, there was a 16-hour flow interruption overnight. This no-flow incubation may have allowed for viral diffusion. The diffusion allowed time for viruses easily-detached and/or viruses released from micropores to move into the bulk liquid phase. As a result, these viruses were eluted in pore volume 2, resulting in the large spike

in viral abundance. This increase in virus in the effluent in pore volume 2 was potentially explained by virus detachment mechanisms.

In subsequent pore volumes, the process repeated; each overnight incubation allowed for viral release from soil surface and micropores into the soil solution, where they could be subsequently eluted with the next pore volume when flow was restored. In other words, there was a spike of viral abundance following each overnight flow interruption. Likely, the viral abundance is much lower in the following pore volumes compared to pore volume 2 because there were fewer easily-detachable viruses which moved into the bulk soil solution. However, it is important to recognize that there were increases of elutable viruses which occurred due to the flow interruption. The peaks in each pore volume reflect change in the magnitude of fractions of mobile and attached viruses in response to flow processes and chemical dynamics. The results suggest that viral community requires time to recover after periods of extended flow, and that short flow periods might not have a large impact on soil viral movement.

Chemical Perturbation Facilitated Release of Soil Viruses

The chemical conditions of the columns changed during the course of the experiment. The total ionic strength of measured ions significantly decreased after pore volume 1A ($p < 0.03$) (Figure 3.7). A decrease in ionic strength has been associated with increase in the size of the diffuse electric double layer surrounding virus particles and soil surfaces, creating conditions less favorable for viral attachment and more favorable for viral transport when both viruses and surfaces are negatively charged (Masciopinto & Visino, 2017a; Zhuang & Jin, 2003b). Conversely, when viruses and surfaces are

oppositely charged, such as when there is a metal oxide coating, increased ionic strength promotes virus retention in the column (Chu et al., 2000). Virus transport is also affected by the ion composition of the transport solution. Bivalent cations, such as Ca^{2+} in our input solution, have been shown to increase negatively-charged virus transport in metal-oxide coated sand columns by partially screening the negative charges on viruses, allowing them to break free of the positively charged oxide coated surfaces (Zhuang & Jin, 2003b). The increase in calcium in the input solution may have allowed some viruses to detach from soil surfaces. Importantly, information about viral transport under varying ionic strength and ion composition is largely based on soil column experiments in which the charges and isoelectric points of both the viral particles and the soil surface are known. We used native soil and naturally occurring indigenous viruses, and therefore cannot make definite claims about the attachment and detachment mechanisms of the broad array of soil viral populations under varying ionic strength or ion composition as the surface properties of our viruses are unknown.

Biological Effects on Eluted Viral Abundance

The increase in viral abundance in pore volume 2 could also be due to a prophage induction event, in which prophage within host bacteria switch from lysogenic to lytic reproduction, hijacking the host metabolism to produce new viral progeny. Some temperate viruses existing as prophage have been shown to move from the lysogenic to lytic life cycle in response to bacterial host stress, such as exposure to antibiotics (K. E. Williamson et al., 2007). Other studies have shown that prophage induction can occur in response to bacterial host quorum sensing where bacterial host density is high and

conditions favor free virus-host contact outside the cell, prompting prophage to switch from the lysogenic to the lytic phase (Liang et al., 2020a).

In our study, the sudden wetting of the dry-packed soil column could have created a bacterial host population surge due to high nutrient availability resulting from high soil moisture and pore connectivity. If bacteria activity was high during the 16-hour overnight incubation between pore volume 1 and pore volume 2, prophage may have been induced to lysis in order to contact new abundant hosts. Additionally, this peak may have occurred in pore volume 2 and not in subsequent pore volumes because the dissolved carbon and nutrient resources available to support viral reproduction may have been leached out of soil in the first two pore volumes. In fact, the native soil ionic strength decline in the first 1.5 pore volumes is consistent with the leaching process of soil solutes as usually expected (e.g., bromide) (Dai et al., 2020; Xing et al., 2016; You et al., 2003). Alternatively, prophage can be induced to lytic reproduction by sensing host stress signals, and a sudden re-wetting event can place osmotic stress on host cells. Host cell stress has been shown to cause induction of prophage reverting from lysogenic to lytic life cycles to avoid death within the host cell.

Interestingly, ammonium is the only measured ion to increase in concentration (mg/L) over the 5 pore volume course of the experiment (Figure 3.6). The pattern of ammonium increase was significantly negatively correlated to viral abundance ($p < 0.03$); when viral abundance was highest in pore volume 2A, ammonium concentration was lowest, and as viral abundance decreased, ammonium increased (Figures 3.4 and 3.6). Ammonium can be produced via ammonification, wherein microbes mineralize organic molecules containing amide groups to produce NH_4^+ ions. One major potential source of

organic molecules in soil systems is bacterial necromass, the remains of lysed bacterial cells mediated by viral infection. Bacterial necromass is released upon viral lysis, wherein the infected host cells burst open releasing free viral progeny. The increase in ammonium concentration in eluted pore volumes is consistent with the release of soluble organic-N due to viral-mediated cell lysis followed by mineralization to ammonium by surviving microbes. As such, this pattern of ammonium elution supports the theory that a viral induction event occurred in pore volume 2, which would account for both the spike in viral abundance and the gradual increase in eluted ammonium.

Uniformity of Residual Soil Virus Abundance

At the conclusion of the flow experiments, residual soil viruses remaining in the columns were extracted from three depths along the soil profile upon column deconstruction. The residual viral abundance in these soil samples was, on average 2.09×10^8 viruses per gram dry weight (gdw) of soil, which increased from the recoverable abundance of viruses in pore volume 5B (Figure 3.4). This means that there were residual soil viruses which were unable to be eluted because they were strongly attached or entrapped in pores under the flow conditions but still extractable under our extraction conditions. The solution used to extract residual soil viruses was 1% K-citrate in phosphate buffer. The use of this buffer, combined with physical disturbance in the form of high-speed blending, extracted viruses which were immobile under the flow conditions.

Interestingly, residual viral abundance in deconstructed soil columns was not statistically significantly different between column depths (Figure 3.4). This suggests that

the mobilized viruses from the inlet soil area were not re-attached in the outlet area via mechanical straining or surface attachment. Additionally, the similar residual virus abundances along the column implies that those mobile viruses might be able to travel a longer distance through soil profile depending on the maximum depth of water infiltration during rainfall or irrigation events. Finally, this uniformity suggests relatively stable physical and chemical conditions along the length of the soil profile.

Effect of Trace Ammonium Addition

Trace ammonium was added to simulate natural low-level ammonium which can occur in rainfall or irrigation in order to determine if these micro-perturbations in nitrogen concentration can impact mobile and immobile soil virus abundances. Our results show that the eluted viral abundance was not affected by trace ammonium additions (Figure 3.5). Specifically, the SN and MN treatments did not impact the viral abundance across pore volumes, and the release of viruses was similar in the ammonium treated columns and the control. This result suggests that the levels at which ammonium exists in wet deposition were not high enough to affect release of soil mobile viruses on a short time scale. Indeed, the chemical measurements showed that there was no difference in the levels of ammonium released from the control column and the ammonium-treated columns (Figure 3.6). Additionally, the residual soil virus abundance, which was extracted along three depths within the soil column, was not impacted by the trace ammonium inputs (Figure 3.4).

The accumulation of ammonium from deposition occurs over long-time spans in natural environments. However, the amount of ammonium deposited over the course of

one year, as we replicated in these columns, was not enough to influence virus movement and seemingly had little or no impact on new virus production. In the future, if the effects of ammonium additions were to be studied, the dose of ammonium should be significantly increased to reflect commonly used in N-fertilizer applications.

CONCLUSIONS

Soil virus transport and retention are complex processes with myriad influences, including flow conditions, solution chemistry, and virus surface properties. In our experiment, we aimed to determine how natural hydrological conditions, such as rainfall and irrigation events and drying/wetting, and trace levels of ammonium impacted native virus mobilization and retention. We hypothesized that interrupted-flow conditions would allow for an increase in viral abundance between elution periods by either allowing viruses to move out of micropores and into the mobile phase and/or to increase in abundance via reproduction through bacterial hosts. We observed that a significant difference in viral abundance occurred between pore volumes, specifically that there was a large spike in viral abundance from pore volume 1 to pore volume 2, indicating that the flow interruption increased soil virus recovery at least in soil pore water. In addition to this hydrological influence, alternative explanations for observed increases in eluted viral abundance between pore volumes include chemical changes, such as the changes in ionic strength and ammonium concentrations across pore volumes, or associated bacterial community influences, wherein new viruses may have been produced from bacterial hosts between pore volumes during the overnight flow interruption period.

Residual or immobile soil viruses extracted at the conclusion of the flow experiment indicated that there were viruses whose attachment could not be overcome by the experimental flow conditions alone. The uniformity of the residual viral abundance across the column soil profile suggests that there was little to no re-attachment of soil viruses along the travel distance in the soil. This result suggests that the eluted viruses could potentially travel distances greater than that of the length of our columns.

The trace levels of ammonium applied were meant to simulate the extremely low levels of ammonium which can occur in rainfall or irrigation events. The levels of ammonium were not high enough to influence virus transport, suggesting that there was no differential transport mechanisms in the ammonium treated columns versus the control. This is unsurprising, given the extremely low concentration (22.7 mg/L NH_4Cl) at which the ammonium was applied.

In total, this study shows that soil viruses have a distinct transport response to interrupted flow. The soil virus community can be categorized into two groups: mobile and immobile viruses. The mobile viruses are mostly subject to hydrological and biological impacts, while the immobile viruses are predominantly influenced by physiochemical factors. In order to determine the interactions between the mobile and immobile viruses and their roles in reshaping soil viral and bacterial communities, future research must be done. For example, this experimental study could be repeated under the same flow and ionic strength conditions for the control column, with the addition of sodium azide (NaN_3) to the input solution to inhibit bacterial host growth and in theory biological virus production. If the column with the inhibitor has the same viral elution pattern as the control, it could be determined that the process was physiochemical and not

biological. If the inhibited column did not show the viral spike, then biological replication of viruses might likely be responsible for the viral abundance increase.

The results are important for our understanding of how viruses in natural field soils respond to rainfall or irrigation events. Considering sequential flows under saturated conditions could occur under patterns of daily rain or irrigation in tillage/rooting depth of soil, it is important to know how soil viruses might respond to hydrological perturbations. From an ecological perspective these results may also be useful for interpreting the diversity patterns of soil viruses in surface and subsurface soil as the subsurface diversity may be influenced by the migration of surface soil viruses to considerable soil depths. Here, we have shown that a large fraction of soil viruses can be mobilized via sequential flow patterns, and that pausing flow results in a great increase in eluted viral abundance. Additionally, we have shown that, at levels on-par with atmospheric nitrogen deposition, ammonium does not affect the mobilization of native soil viruses. This research provides many valuable insights into the response of native soil viruses to changes in field conditions. The obtained results establish a scientific foundation for future studies on the influence of climate change on soil viral community as well as soil viral resilience to environmental perturbations.

**CHAPTER 4: DYNAMICS OF VIRAL AND BACTERIAL
COMMUNITY COMPOSITION DURING INTERRUPTED
HYDROLOGIC FLOW CONDITIONS IN SOIL COLUMNS**

My contributions to this work included soil virus and bacteria and extraction, concentration, and preparation for sequencing. Samples were sent to the Maryland Institute of Genomics for sequencing. Sequenced viral samples were bioinformatically processed to completed taxonomical and functional characterization by Xiaofen Wu, who also prepared a small portion of the “Viral Metagenome Extraction, Sequencing and Bioinformatics” section. I performed all statistical analyses, interpretation, and visualization of sequencing data, and wrote the manuscript.

ABSTRACT

The mobilization of soil viruses in natural systems during wetting events is potentially impacted by the surface properties of the soil complex, the physical properties of the soil structure, the surface properties of the virus, or the chemical conditions of the soil solution. Repeated wetting events, such as high-frequency rainfall or irrigation, have the potential to transport viruses downward in the soil profile. Additionally, trace levels of nitrogen occurring in wet atmospheric nitrogen deposition or irrigation waters may potentially impact viral transport by impacting the chemical conditions of the soil solution or the biological functioning of the host community. As such, it is critically important to study the differential mobilization of viruses through soil profiles in order to predict their influences on soil ecology.

Here, we used next-generation high throughput sequencing techniques to investigate the virus community composition of mobile and immobile viruses in an interrupted-flow soil column experiment. We aimed to characterize the sequential and variable mobilization of differing viral families and draw conclusions about the mechanisms controlling their fate and transport. We hypothesized that varying viral families, with varying surface properties, would elute from the soil column at different times as a mechanism of kinetic release. Additionally, we hypothesized that the additions of ammonium would have little effect on virus transport but alter the composition of the mobile viruses.

We determined that viruses do in fact become mobile at different rates, with Mimiviridae being eluted first, followed by the viruses in the order Caudovirales. We suggest that the icosahedral shape, large size, and enveloped capsid of the Mimiviridae

versus the head-tail shape, smaller, non-enveloped capsid of the Caudovirales contributed to their differences in mobilization. Additionally, we recognize that some changes in viral community composition may be driven by production of new viral progeny during the course of the flow interruption. Finally, we showed that there exists diversity in the residual soil columns which could not be removed during the elution conditions, but which could subsequently be removed by the extraction conditions. In total, this work revealed differential mobilization of varying viral families, and has significant implications for understanding the structure and function of vadose zone virosphere. The results will also further development of strategies for obtaining more representative virion samples for ecological studies in soil virology.

INTRODUCTION

Many wetting processes affect soil microbial attachment and transport in the environment, including natural events such as rainfall, or human-imposed processes such as irrigation. These wetting events differentially mobilize soil viruses and bacteria through the soil, with the magnitude of impact varying with soil properties, including soil surface charges, metal oxide and organic matter contents, soil texture, water content, and soil pore connectivity. Additionally, the chemical conditions of soil solution can affect soil viral transport through changes in ionic strength, pH, ion composition, and hydrophobicity (Masciopinto & Visino, 2017; Walshe et al., 2009; Zhuang & Jin, 2003). These factors are coupled to determine the behaviors of soil viruses, whose surface properties (e.g., isoelectric point and hydrophobicity), shapes, and sizes are widely varied. In total, the myriad factors influencing the attachment and mobilization of soil viruses are likely to impact not only mobilization of soil viruses but also the manner in which they interact with bacterial host communities.

Previous studies on soil virus transport aimed to quantify how model viruses respond to known physical and chemical conditions of soils. However, little is known about the retention and mobilization of native soil viruses under repeated wetting events such as repeated rainfall or surge irrigation. Surge irrigation is the intermittent application of water to fields under relatively short time spans in order to save water and increase wetting efficiency (Humpherys, 1989). This on/off cycle of wetting has been shown to increase water retention in surface soils. Additionally, it remains unclear how natural atmospheric wet nitrogen deposition can influence soil virus transport during rainfall wetting events. Here, we aimed to understand how interrupted flow conditions impacted

the community composition of mobile or releasable soil viruses. Further, we wanted to determine if atmospheric nitrogen deposition, a process known to impact viral host activity, influences the viral community during water infiltration. Finally, we aimed to determine the impacts of the nitrogen additions and hydrologic flow conditions on the residual viruses and their bacterial host communities at varying depths along the soil column. To accomplish this, we conducted flow-through experiments in repacked native soil columns under the conditions of interrupted flow and varying ammonium chloride amendments. We performed metagenomic analyses of virus-enriched samples of column effluent across five pore volumes, as well as of residual viral communities in column soil upon column deconstruction. Additionally, we conducted 16S rRNA gene amplicon sequencing of bacterial communities in the residual soil upon column deconstruction. Multivariate analyses were used to determine microbial community changes during the flow regime and at different depths of the column soil at the conclusion of the experiment. We hypothesized that soil viral community composition would vary with pore volumes of effluent flowing through the column, and that virus mobilization would be minimally impacted by changes in trace levels of nitrogen. Our results showed that repeated interrupted flow patterns significantly influenced virus mobilization, resulting in differential elution of viral families. These results provide insight into mobile and immobile virus communities and their potential for downward migration under saturated transient flow conditions in native soil systems.

METHODS

Soil Collection and Preparation

Soil was collected at the East Tennessee Research and Education Center in Knoxville, TN from an Ultisol soil classified as a fine-loamy, mixed, subactive, thermic Typic Hapludult. Soil sampled from the top 10 cm was transported immediately to the University of Tennessee, Knoxville, where it was air-dried for 48 hours and sieved through a 2 mm screen to remove plant roots. The air-dried soil was stored in a bag at room temperature ($21\pm 2^{\circ}\text{C}$) to create the most stable conditions for the soil microbial community prior to the column experiments.

Soil Column Experimental Design

All experiments were conducted in plexiglass (acrylic) columns (25 cm in length and 3.8 in inner diameter). The input solutions were introduced via a multi-channel peristaltic pump from the bottom of the column while effluent was continuously collected from the top into 20-mL glass tubes using Retriever II fraction collectors. Each tube collected 9.5-mL of the effluent, representing one sample fraction that was equivalent to one-sixth pore volume. Teflon tubing or stainless-steel tubing was used throughout the experimental system. For virome preparation, the six effluent fractions of each pore volume of the outflow were combined to form one pore-volume sample.

Three replicate experiments were conducted under the saturated flow conditions with overnight flow interruption (i.e., no inflow or outflow). To begin the experiment, the air-dried soil was dry-packed into columns, and then one pore volume of the input solution was introduced at $200\ \mu\text{L}/\text{minute}$ until column saturation, at which point the

pump was turned off and the column was left overnight for 16 hours. The next day, the pump was turned on for 8 hours to inject a new pore volume of the input solution at flow rate of 200 $\mu\text{L}/\text{minute}$ while the effluent was collected. Then, the pump was shut off again for 16 hours of flow interruption. This pattern continued for five times to collect a total of 5 pore volumes of the effluent. At the conclusion of the experiment, the columns were deconstructed, and soil was sampled in five 5-cm sections along the depth of the soil column, with Depth 1 at the bottom (closest to the inlet) and Depth 5 at the top (closest to the outlet). Measurements were taken from Depth 1 (“inlet”), 3 (“middle”), and 5 (“outlet”) for viral and bacterial DNA sequencing.

Treatments consisted of varying ammonium additions in the input solutions to mimic atmospheric N deposition at an amount of 6.5 kg/ha. This level of nitrogen is consistent with the level of ammonium/ammonia which would be received over the course of year in the southeast United States. Two application methods of ammonium input were used. In the “single-N addition” (SN) column, 22.7 mg/L NH_4Cl was used in the first pore volume, followed by four pore volumes of the nitrogen-free control solution. In the “multiple-N addition” (MN) columns, 4.45 mg/L NH_4Cl was contained in each of the five pore volumes, so that there were ammonium inputs in each pore volume. In total, roughly 2 micrograms of NH_4^+ were applied to each gram of soil in the ammonium-amended columns, either in the first pore volume (SN) or equally across 5 pore volumes (MN). The control (C) solution included calcium chloride (CaCl_2) at a concentration of 273.1 mg/L. Additional calcium chloride was added to each ammonium amended treatment solution to bring each solution to a uniform ionic strength of 10 mM.

Viral Metagenome Extraction, Sequencing and Bioinformatics

Mobilized viruses were isolated from effluent by filtration through 0.22- μ m Millex Durapore Filters (Merck Millipore Ltd., Tullagreen, Co. Cork, Ireland). Residual viruses were isolated from three depths (inlet, middle, and outlet) of column soil by extraction. To extract soil viruses from deconstructed column soil, 70 g of soil was blended on high with 233.3 mL of cold buffer (50 mM phosphate buffer containing 1% potassium citrate, w/v, pH 7) for 3 minutes (K. E. Williamson et al., 2005). To isolate viruses, 45 mL of soil slurry was centrifuged in 50-mL tubes at 5,000 rpm for 20 minutes at 4°C before they were filtered through 0.22- μ m Millex Durapore Filters (Liang et al., 2019a). Both effluent and residual soil viruses were concentrated for DNA extraction using our previous method (Liang et al., 2019a). From each sample, 75 mL of effluent was concentrated by centrifugation at 5,000 g for 20 minutes (F13S-14x50cy rotor, Sorval RC-6plus, Thermo Scientific) to a final volume of 200 μ L using Amicon centrifugal filters (100 K, Merck Millipore Ltd., Tullagreen, Ireland). Total genomic DNA was extracted from these virus-enriched concentrates using the AllPrep PowerViral DNA/RNA Kit (catalog no. 28000-50, Qiagen, Hilden, Germany). The quality and quantity of extracted DNA was verified with a Nanodrop (Thermo Scientific NanoDrop ND-3300 Fluorospectrometer 83060-50). Extracted and purified viral DNA was sent to Maryland Genomics (<https://marylandgenomics.com>) for library preparation and Illumina NovaSeq 6000 sequencing. Upon quality control examination, it was determined that the quantity of DNA in the effluent of 4th and 5th pore volumes of each column was too low to proceed to sequencing. Thus, the effluents of these two pore volumes were combined

into one sample for sequencing. The resulting virus-enriched metagenomes, hereafter “viromes” were analyzed using the following workflow.

FastQC v0.11.5 was used to check the quality of virome raw reads before removing adapters and low-quality reads with Trimmomatic v0.39 (Bolger et al., 2014). The trimmed reads were individually assembled using Megahit v1.2.9 with meta-large preset (Li et al., 2015), followed by quality assessment of assembled contigs using QUAST v5.0.2 (Gurevich et al., 2013).

Viral contigs were recovered using DeepVirFinder v1.0 (-l 5000) (Ren et al., 2020) and VirSorter2 v2.2.3 (--keep-original-seq --include-groups dsDNAphage,ssDNA --min-length 5000 --min-score 0.5) (Guo et al., 2021b). The quality of viral genomes identified by VirSorter2 was evaluated with CheckV v0.8.1 (Nayfach, Camargo, et al., 2021). Three criteria were used to identify viral contigs: (a) DeepVirFinder: score ≥ 0.9 and p-value ≤ 0.5 ; (b) VirSorter2: score ≥ 0.95 or hallmark genes > 2 ; (c) CheckV: number of viral gene > 0 . Contigs were at least 5 kb in length. Contigs had to satisfy at least one of the three criteria to be considered viral. All identified viral contigs were clustered at 95% average nucleotide identity over 85% alignment fraction using scripts `anicalc.py` and `aniclust.py` from CheckV suite, generating 22258 viral operational taxonomic units (vOTUs).

BBDMap (Bushnell, 2014) and Samtools v1.10 (H. Li et al., 2009) were used to map trimmed reads back to vOTUs. Coverage of each vOTU was obtained with CoverM v0.3.2 (--min-read-aligned-percent 0.75 --min-read-percent-identity 0.95 -m mean, (<https://github.com/wwood/CoverM>) using the bam file generated from read mapping. Relative abundance of each vOTU was calculated by dividing its coverage by the total

coverage of all vOTUs from the corresponding sample. Genes of vOTUs were predicted using Prodigal v2.6.3 (-p meta) (Hyatt et al., 2010). Taxonomic classification on vOTUs was performed using vConTACT2 v0.9.19 (--rel-mode Diamond --db ProkaryoticViralRefSeq94-Merged --pcs-mode MCL --vcs-mode ClusterONE) (Bin Jang et al., 2019) and the DemoVir scripts against the TrEMBL database (<https://github.com/feargalr/Demovir>) with a modification that BLASTp was used for the alignment (e-value $\leq 10^{-5}$). All predicted proteins were also functionally annotated with kofamscan v1.2.0 against the KEGG database (-f mapper) (Aramaki et al., 2020), dbCAN2 against the carbohydrate-active enzymes database (CAZy) (Zhang et al., 2018), and hmmsearch against the PFAM and Resfams databases (e-value $\leq 10^{-5}$) (Gibson et al., 2015; Mistry et al., 2021). VIBRANT v1.2.0 (Kieft et al., 2020b) was used to annotate auxiliary metabolic genes (AMG) and determine viral lifestyle (lysogenic or lytic). Potential hosts for each vOTU were predicted using SpacePHARER (Zhang et al., 2021) and the CRISPR spacer set (Dion et al., 2021).

Bacterial Amplicon Sequencing and Bioinformatics

Small subunit rRNA gene amplicon sequencing was performed on the soil from the deconstructed column sections. The DNeasy PowerSoil Pro kit (catalog no. 47016, Qiagen, Hilden, Germany) was used to extract DNA from 27 soil samples along three depths within the column profile including the inlet (0-5 cm), the middle (10-15 cm), and the outlet (20-25 cm) of each column. Extracted DNA was quantified using a Nanodrop (Thermo Scientific NanoDrop ND-3300 Fluorospectrometer 83060-50) and sent to Maryland Genomics (<https://marylandgenomics.com>) for amplification and sequencing.

The V3-V4 region of 16S rRNA genes were amplified and sequenced on an Illumina MiSeq using paired-end 300 bp reads. Sequence data was quality filtered via FastQC (Andrews, 2010) and the filtered sequence data was processed using DADA2 (Callahan et al., 2016). Generated amplicon sequence variants (ASVs) were assigned taxonomy via the SILVA bacterial reference database (v.132). Results were statistically analyzed and visualized in R (v 4.1.2) via RStudio (Team, 2021), using packages phyloseq (v 1.38.0) and vegan (v 2.5.7). To control for differences in sequence coverage, all samples were rarefied to the minimum number of sequences before analyses and visualization (Cao et al., 2022).

Data Analyses

Viromes and amplicon data were analyzed for differences in alpha diversity based on the Shannon diversity index in the vegan package in R (Oksanen et al., 2013). Values of Shannon α -diversity were compared for significance by one-way ANOVA using the lme4 and multcomp packages in R. Principle Coordinate Analysis (PCA) was conducted to examine differences in beta diversity. Permutational multivariate analysis of variance (PERMANOVA) was performed using the adonis function to determine significance of treatments, pore volumes, or column depth on community structure, also using the vegan package in R.

RESULTS

Virome Taxonomy

To assess the diversity of the viral community, a total of 160,110 viral contigs (>5 kb) were obtained using metagenomic sequencing. These contigs clustered into 22,258 viral operational taxonomic units (vOTUs). The composition of viral communities changed significantly across the duration of the flow experiment (Figure 4.1). The viral community which was eluted under flow conditions (i.e. the “pore volume” samples) was overall significantly different from the community of viruses which could be extracted from the residual soil. Additionally, the eluted viromes (average $n = 3906$) had three times the recoverable viral contigs per sample compared to the residual soil viromes (average $n = 1301$).

Principle component analysis was used to confirm which factors were most significant for shaping the viral community in the eluted fraction and the residual soil viromes. As stated, the differences between the effluent and residual soil viromes were significant (Figure 4.2 G). In the eluted soil viromes, the most significant factor was pore volume (Figure 4.2 H). In the residual soil viromes, the PCA suggested that soil column depth had an effect on viral community composition, however analysis of variance statistical analyses revealed that this difference was not significant (Figure 4.2 I). Additional PCA analyses (Figure 4.2 A-F) show that ammonium-containing input solution and replicate were not significant factors shaping virus community composition.

The community composition of viromes was separated by viral families to more clearly visualize changes across the duration of the flow experiment and into the residual

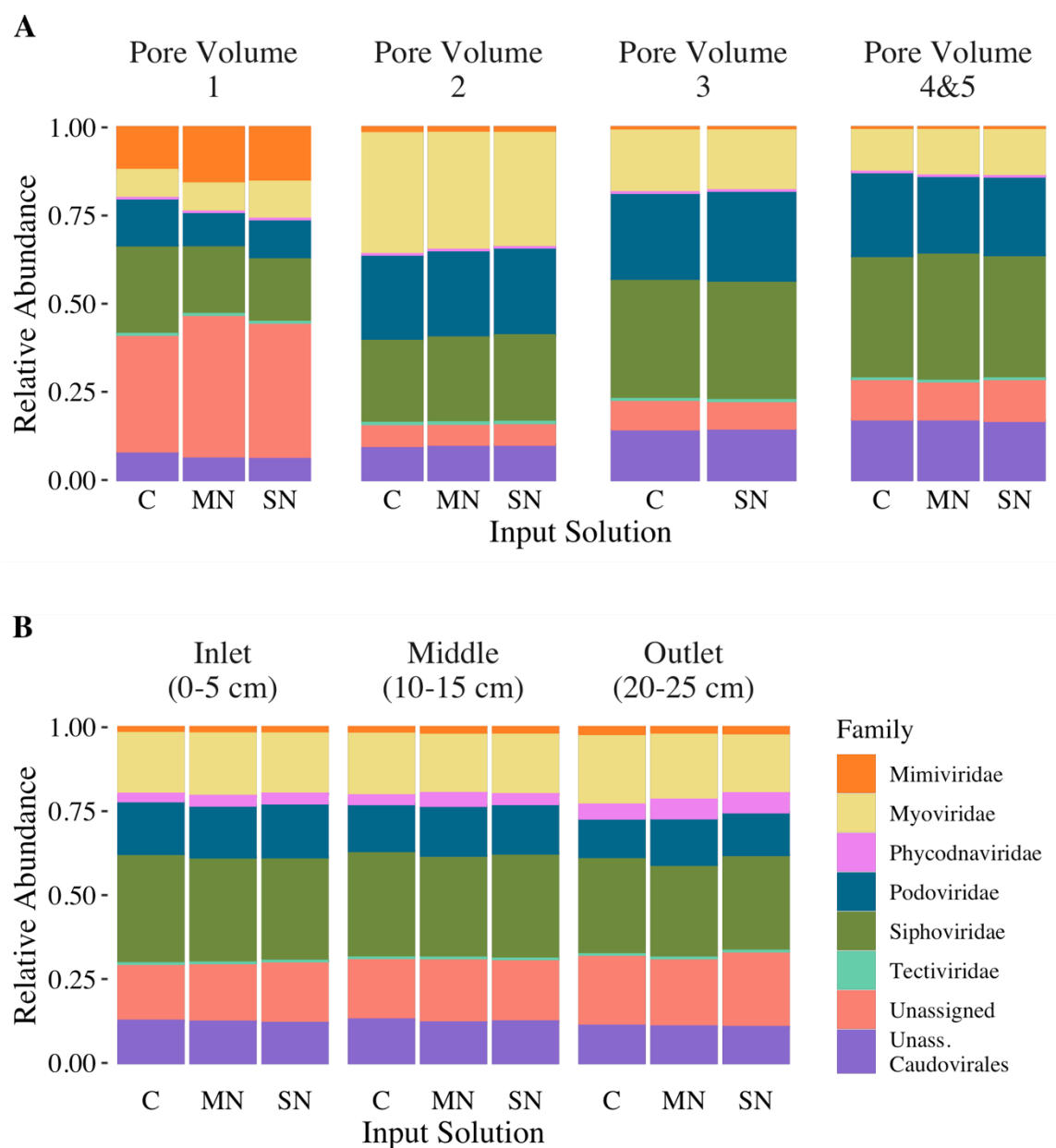


Figure 4.1. Family level abundance of soil viromes from (A) column effluent and (B) residual column soil destruction. Input solutions included Control, Single-N addition (SN), and Multiple-N additions (MN).

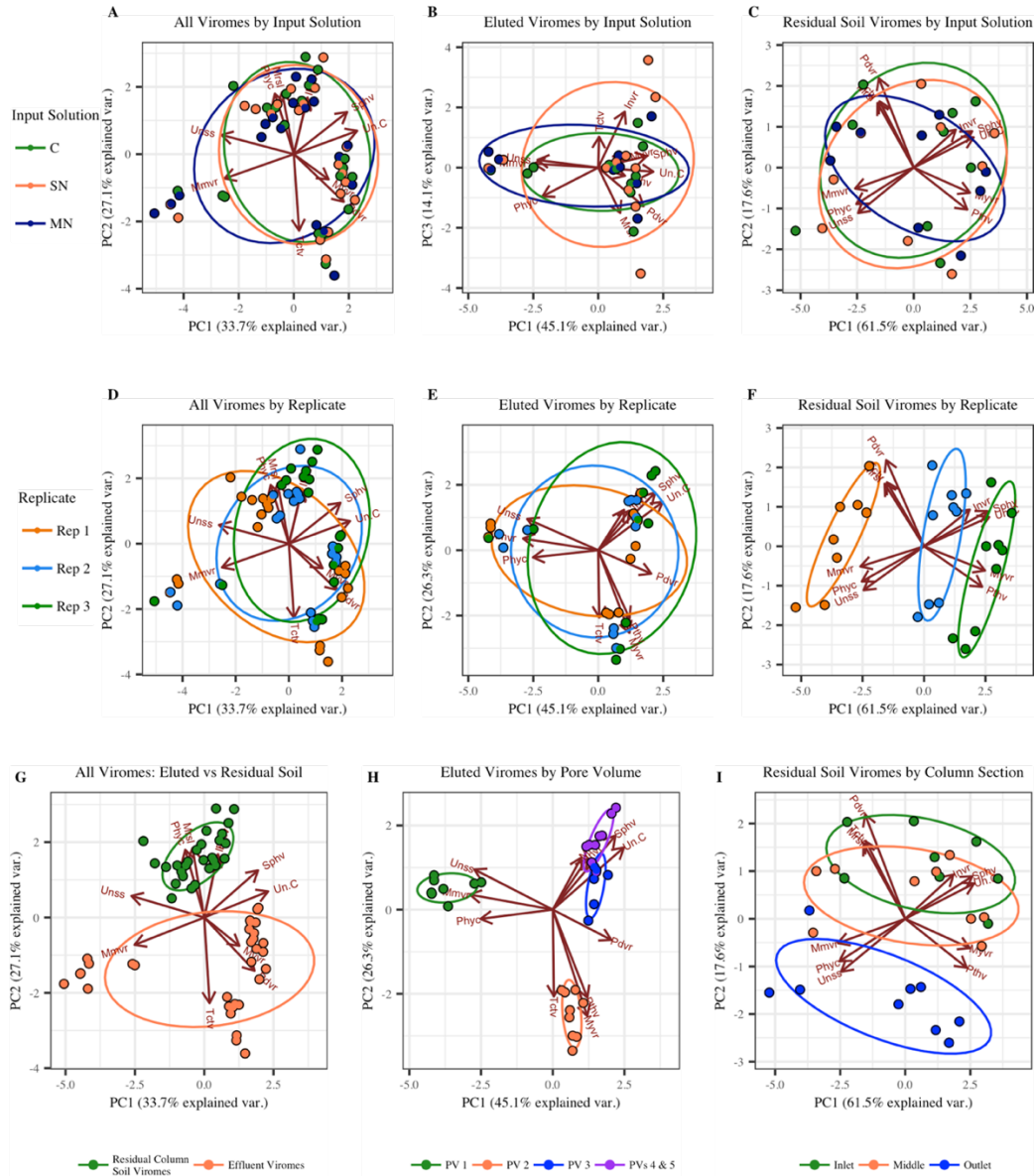


Figure 4.2. Principal coordinate analysis (PCA) of viromes. Plots A, B, and C examined the effects of input solutions including Control, Single-N addition (SN), and Multiple-N additions (MN). Plots D, E, and F were clustered by replicate. Plot G clustered samples into effluent and residual. Plot H clustered effluent samples less by pore volume. Plot I clustered residual soil samples by column depth.

soil (Figure 4.3). The first pore volume was dominated by unassigned viral families, whose abundance significantly ($p < 0.01$) decreased in the second pore volume. Additionally in pore volume 1, Mimiviridae were significantly ($p < 0.01$) more abundant than in subsequent pore volumes. There was a significant ($p < 0.01$) increase in the relative abundance of Myoviridae from pore volume 1 to pore volume 2, after which the abundance decreased and remained relatively stable. Podoviridae were enriched in pore volumes 2-5 ($p < 0.01$). Siphoviridae were highest in pore volumes 3-5 ($p < 0.01$). The evenness of the viral community increased as the flow experiment progressed, according to the alpha diversity based on the Shannon Index (H') ($p < 0.01$) (Figure 4.4).

The taxonomy of the residual soil viromes more closely resembled the 4th and 5th pore volumes than the first three pore volumes (Figure 4.1 & 4.3). Gene sharing networks confirmed that pore volume changes were the most predictive of viral taxonomy, and showed that the residual soil viral community most closely shared genes with pore volumes 4 and 5 (Figure 4.5). Compared to the eluted viromes, the residual soil viromes had elevated abundance ($p < 0.01$) of Unassigned viruses as well as Phycodnaviridae, while abundance of Podoviridae, Siphoviridae, and Unassigned Caudovirales decreased ($p < 0.01$) (Figure 4.3). There were observable differences in viral community composition across the three column depths (inlet, middle, and outlet), however these differences were not statistically significant.

Ammonium additions in the SN and MN experiment did not cause significant differences in viral community composition compared to the control experiment (Figure 4.2 A-C, Appendix Figure 4.12 & 4.13). However, there were more recoverable viral

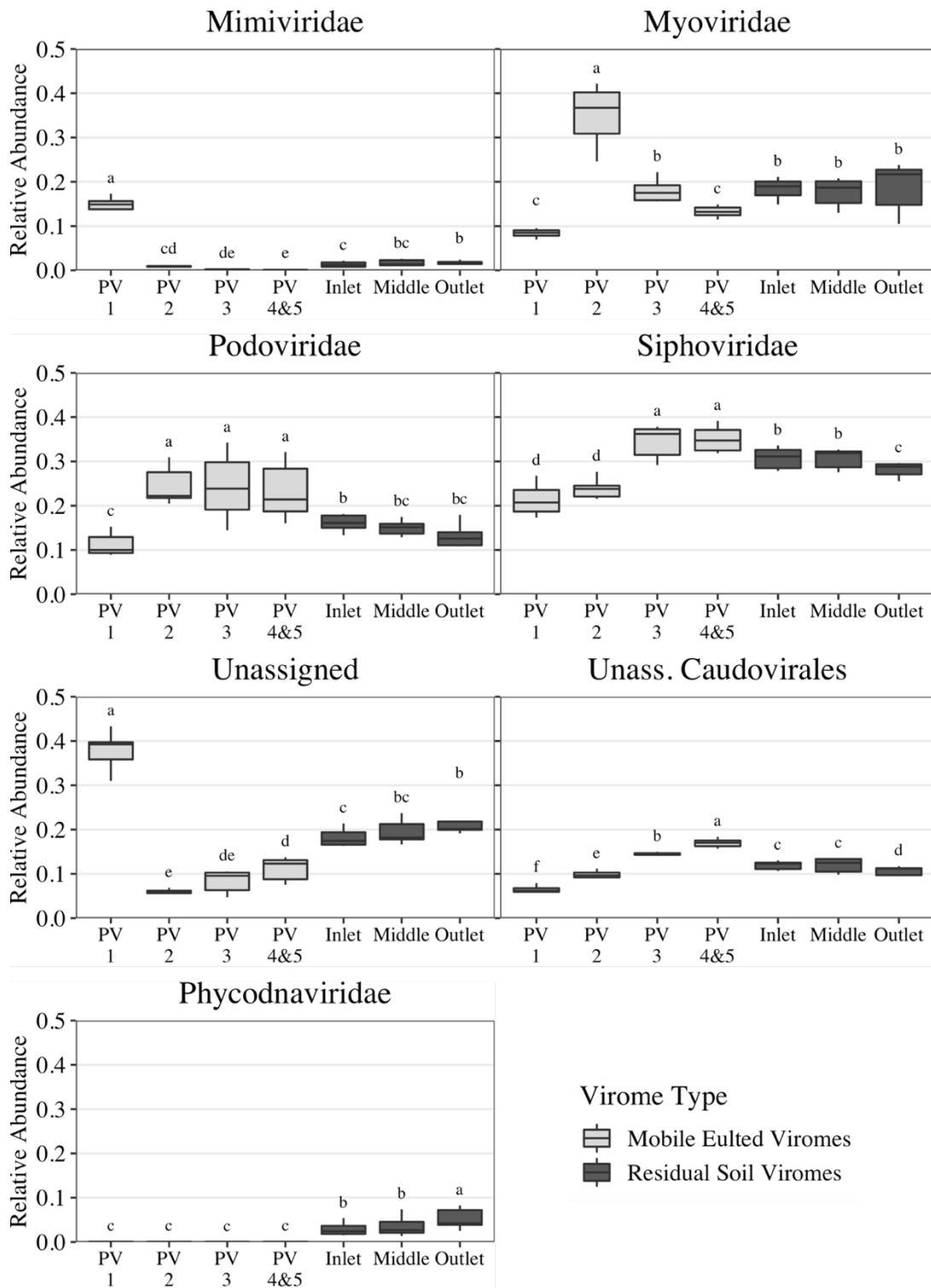


Figure 4.3. Mean relative abundance of families across pore volume fractions and residual soil. Letters denote significance at a level of $p < 0.01$.

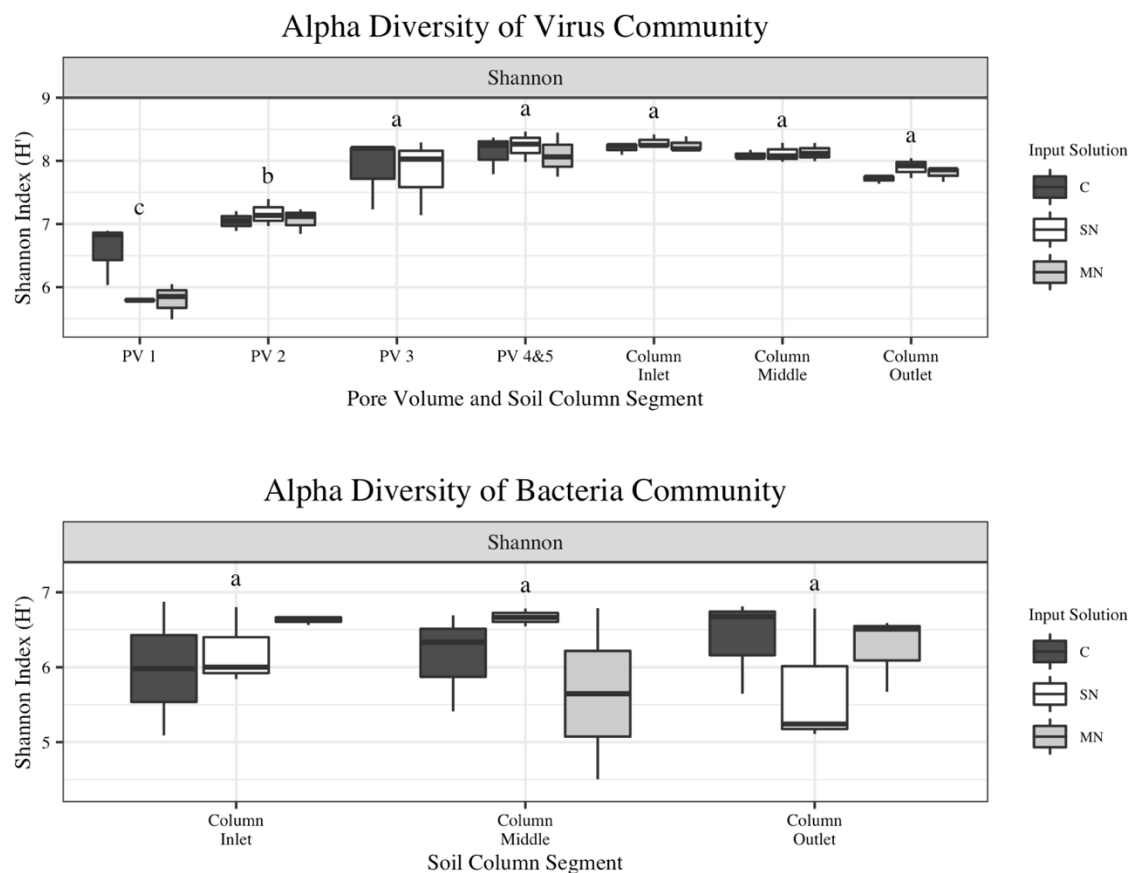


Figure 4.4. Alpha diversity of virus and bacteria communities based on the Shannon Index (H'). Alpha diversity was significantly different between pore volumes, as well as between eluted and residual viruses, as denoted by superscripts, at a level of ($p < 0.01$). Superscripts apply to all treatments within each pore volume or column segment. There were no statistically significant differences in alpha diversity in the bacterial community. Input solutions included Control, Single-N addition (SN), and Multiple-N additions (MN).

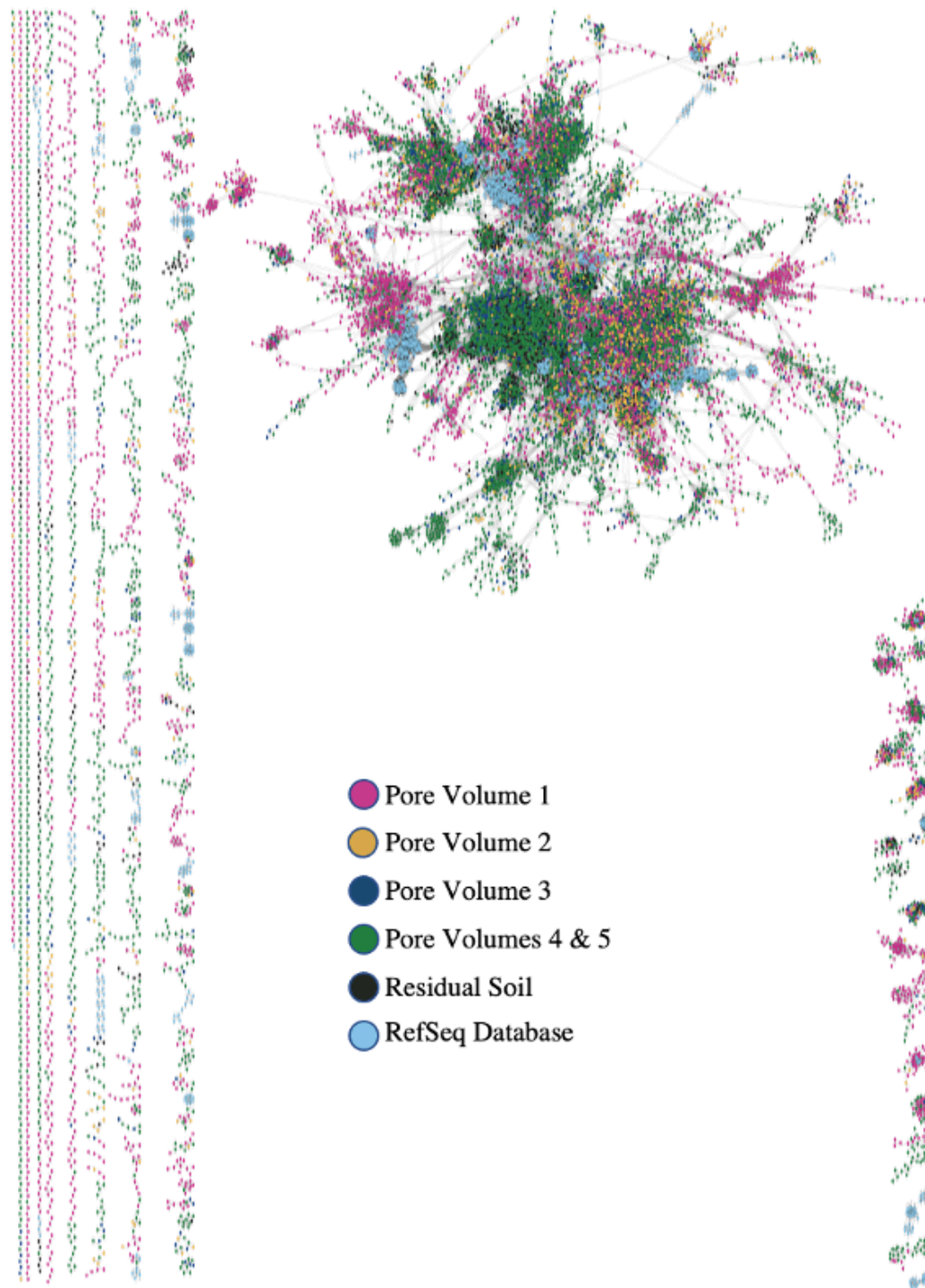


Figure 4.5. A gene-sharing network for viral contigs isolated from pore volume 1 (pink), 2 (Yellow), 3 (dark blue), 4&5 (green), and residual soil (black), and the NCBI RefSeq database (light blue).

contigs in the control experiments (n=61,173) than in the SN (n=57,690) or MN (n=41,247) experiments.

Viral Replication

Virus life cycle strategy assessments revealed that, of recoverable complete circular genomes, obligate lytic vOTUs were predominant (Figure 4.6). Averaged across all viromes, 90% of vOTUs were lytic while 10% were lysogenic. The incidence of lysogenic vOTUs increased slightly after the first pore volume of effluent. The highest incidence of lysogenic vOTUs was found at the soil column outlet, where 15% of recoverable viral genomes were from temperate phage. However, it must be noted that this method of discovery of viral replication cycle is inherently biased towards discovery of lytic viruses due to the way that lysogenic viruses are identified. VIBRANT identifies lysogenic viruses by searching for viral sequences in data excised as “bacteria-like” due to low v-scores (i.e. the sequence was identified as likely bacterial in origin, then analyzed again to determine viral prophage). Additionally, any virus that encodes an integrase is identified as lysogenic. However, lysogenic viruses which are only a partial sequence without an integrase will be misidentified as lytic. Therefore, our results showed some changes in identifiable viral replication cycles but should not be taken to completely represent the true distribution of viral replication strategies in the viromes reported here.

Functional Annotation

The functional potential of viruses was determined by examining auxiliary metabolic genes (AMGs) from generated vOTUs. KEGG annotation revealed

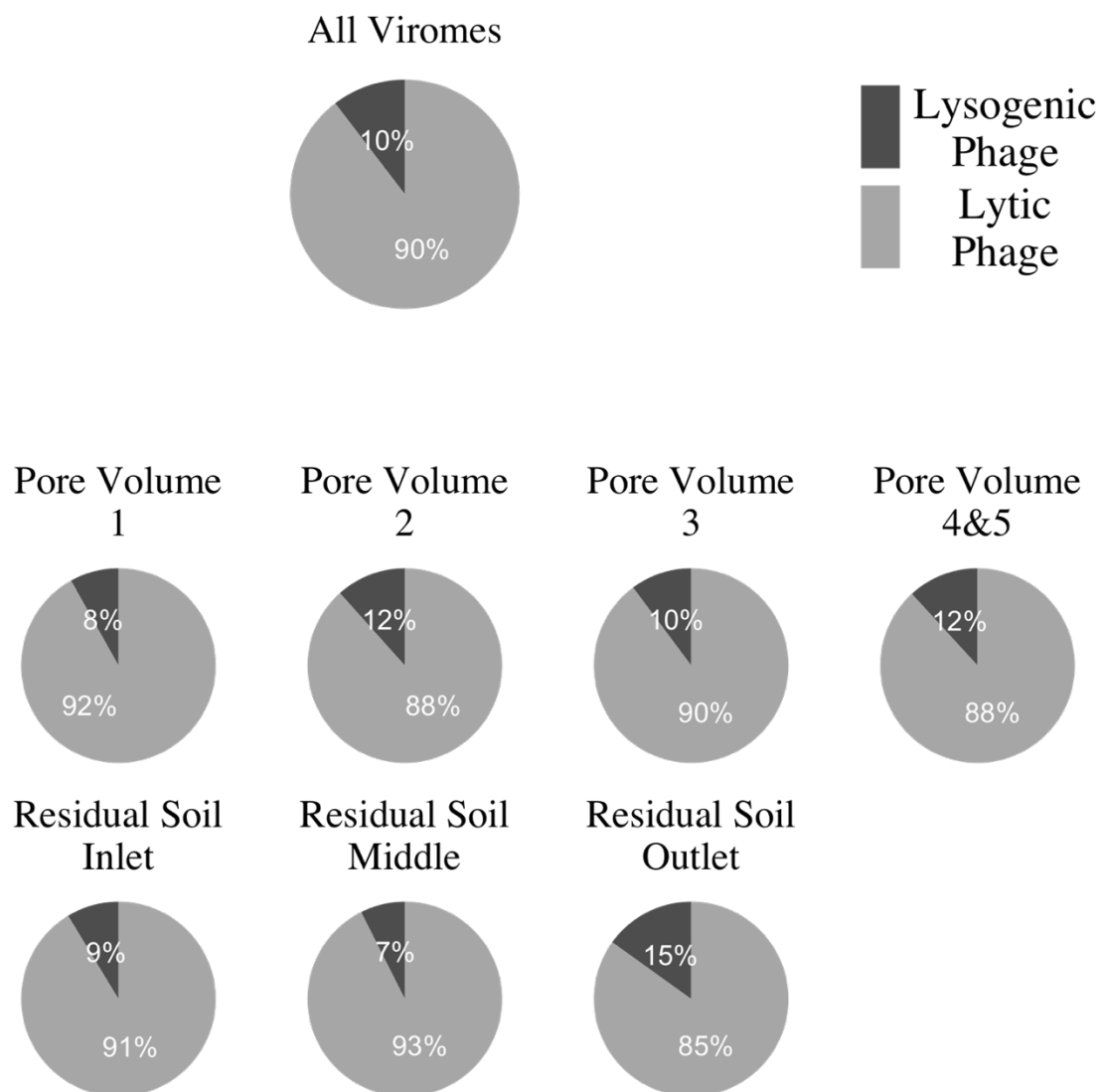


Figure 4.6. Predicted lytic vs lysogenic vOTUs using VIBRANT averaged for all viromes (top), across pore volumes (middle), and across column depths (bottom). Results were averaged across all input solutions at each pore volume and column depth.

that the distribution of functional genes was relatively similar across all pore volumes and into the residual soil (Figure 4.7), however the functional potential of pore volume 1 more closely related to that of the residual soil viromes than to other eluted fractions. The predicted functional genes were mainly annotated under “genetic information processing,” “replication and repair,” and “cell growth and death.” Some AMGs predicted functions for “metabolism,” which indicates potential viral impact on bacterial metabolism. Virus encoded carbohydrate active enzymes (CAZymes) which might contribute to bacterial metabolism were analyzed using the CAZy database (Figure 4.8). The major CAZyme family predicted for all samples was glycoside hydrolase (GH), whose distribution was relatively constant across all samples. The middle depth of the residual soil along with pore volume one were enriched in carbohydrate-binding (CB) module enzymes, while the inlet of the soil column was enriched in polysaccharide lyase (PL) (Figure 4.8).

While the ammonium additions in the SN and MN treatments did not have significant effects on the distribution of AMGs or CAZymes, there was an important difference in the number of annotated functional genes. Overall, the control group had the highest total (effluent and residual samples) number of AMGs ($n=19,563$) and CAZymes ($n=7515$) compared to SN (n AMGs = 10,755, n CAZymes = 4826) or MN (n AMGs = 8,917, n CAZymes = 3127). Genes which might have functional relevance to nitrogen metabolism were investigated to further examine potential impacts of ammonium additions. Interestingly, the only four AMGs discovered were all *nirK*, found in the control column second pore volume (1), fourth and fifth pore volumes (2), and the MN second pore volume (1).



Figure 4.7. Functional annotation of virus samples using the KEGG database, averaged for all input solutions at each pore volume and column depth.

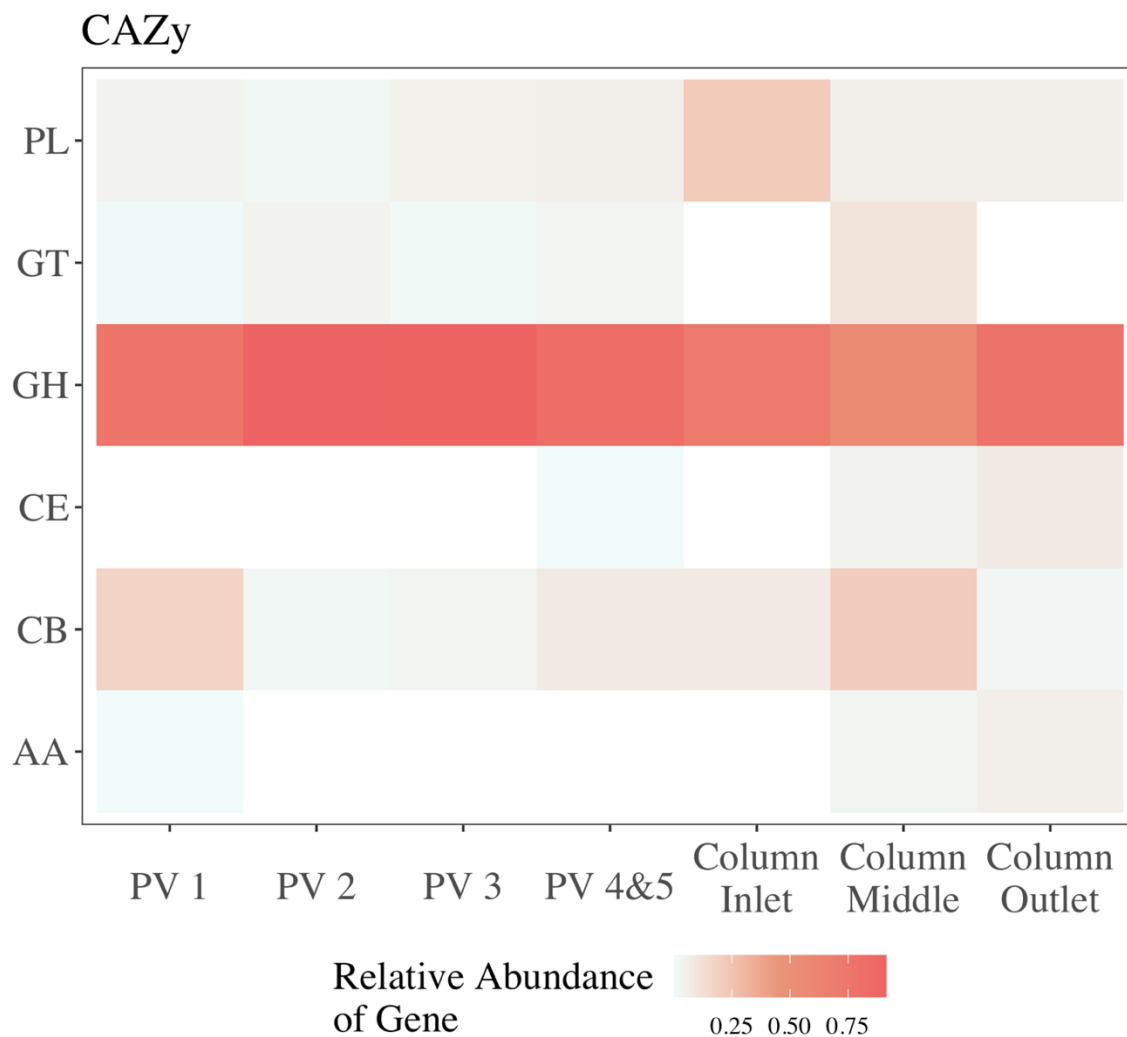


Figure 4.8. Predicted carbohydrate-active enzymes using CAZy database, averaged for all input solutions at each pore volume and column depth. Enzyme families include polysaccharide lysases (PL), Glycosyl Transferases (GT), Glycoside Hydrolases (GH), carbohydrate esterases (CE), carbohydrate-binding molecules (CB), and auxiliary activities (AA).

Virus Host Prediction

Viruses were linked to putative hosts (Figure 4.9). Host frequency was normalized to pore volumes in order to visualize changes in host prediction during the course of the flow experiment. In general, pore volume 1 and pore volumes 4&5 had a higher number of predicted hosts. Examining the relative abundance of hosts across pore volumes rather than the absolute abundance allows observations of shifting host prediction across the duration of the experiment. Proteobacteria was overwhelmingly the most abundant predicted host, and its distribution was stable across all pore volumes and depths in the soil column except the outlet. According to an ANOVA, the relative frequency of Actinobacteria, Bacteroidetes, and Firmicutes was elevated in the outlet section of the soil column as compared to the eluted fractions ($p < 0.01$). Siphoviridae had the most predicted hosts, which consisted mainly of Proteobacteria and Actinobacteria. Myoviridae was mostly linked to Proteobacterial hosts.

Bacterial Community Composition

The taxonomic composition of the bacterial community was determined from the column soil at three depths: the inlet (0-5 cm), the middle (10-15 cm), and the outlet (20-25 cm) at the Phylum level (Figure 4.10). The dominant bacterial phyla were Actinobacteria and Proteobacteria. The community composition was very similar across all sampled depths, as there were not significant differences between community structure determined at the inlet, middle, and outlet of the columns. There were more recovered amplicon sequence variants (ASVs) in the control ($n = 338,524$) compared to the SN ($n = 285,141$) and MN ($n = 293,128$) treatments. However, the alpha diversity

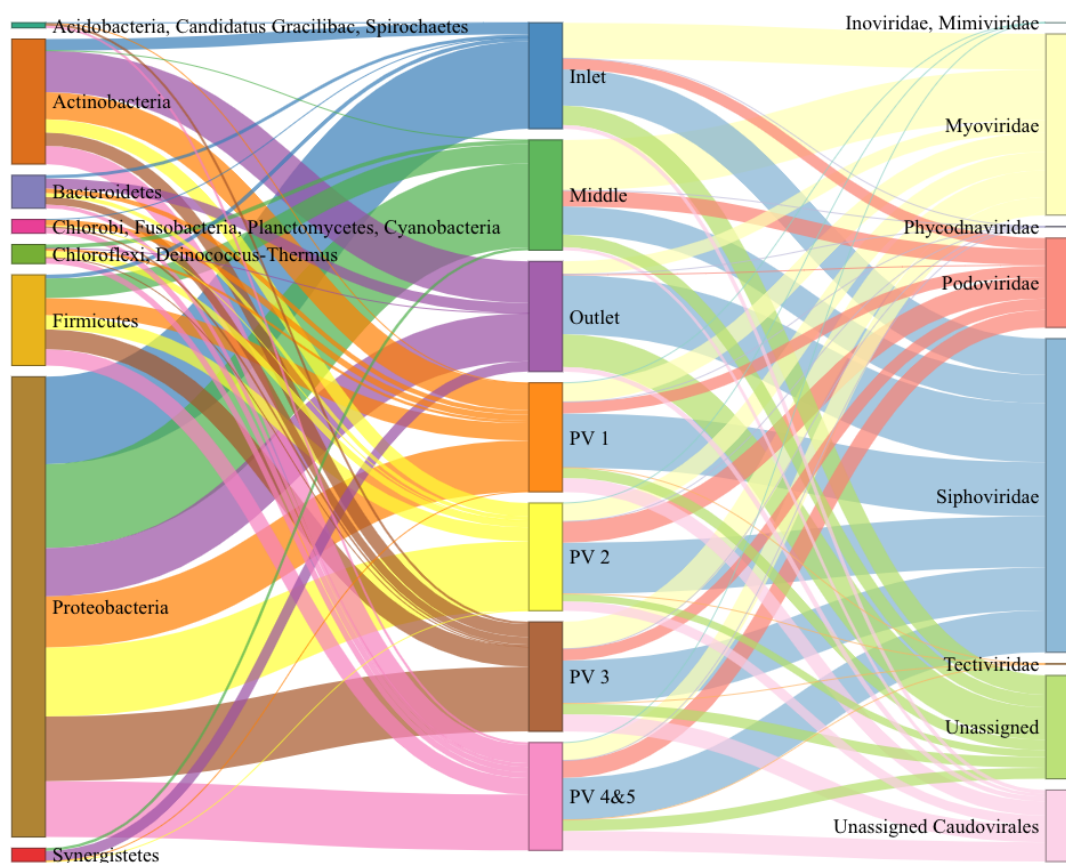


Figure 4.9. Potential host linkages based on CRISPR shpacepharer set Abundance of predicted hosts was normalized across pore volumes to visualize changes in host community across the duration of the flow experiment. “Inlet,” “Middle,” and “Outlet” refer to the three depths at which soil was collected upon column destruction, and PV1-5 refer to pore volumes 1-5 of effluent.

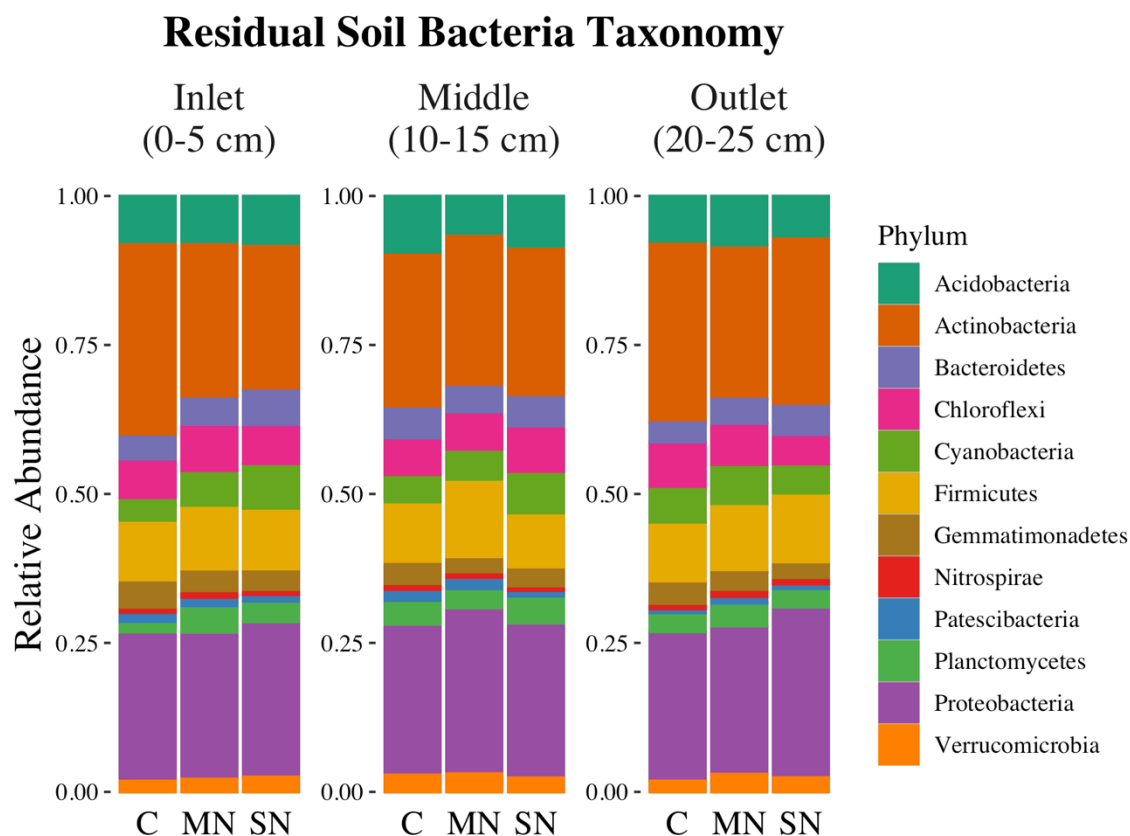


Figure 4.10. Community composition of the top 12 most abundant bacterial phylum for taken across three column depths (inlet, middle and outlet) for the control (C), single-N addition (SN), and multiple-N addition (MN) treatments. Communities consist of phylum-level relative abundance of rarefied amplicon sequences.

based on the Shannon Index (H') did not significantly differ with ammonium additions or soil column depth according to a one-way ANOVA with a split-plot design (Figure 4.2 B). A principle component analysis was used to determine any differences in community structure among the N-amendment regimes, soil depth, and replicate experiments (Figure 4.11). The relative abundance of Actinobacteria was elevated in the control column, but this was not a statistically significant difference ($p < 0.08$) (Figure 4.11). To ensure detection of ammonium input effects, bacterial taxonomy was searched for nitrogen transforming bacteria. The only ammonium oxidizing bacteria identifiable in the community data was *Nitrospira*, which showed no significant differences in abundance across ammonium additions or depth within the columns (Appendix Figure 4.14). Overall, bacterial communities were nearly identical between all columns, regardless of N-amendment regime, column depth, or replicate experiment.

DISCUSSION

Viruses in natural soils are extremely diverse, with varying surface properties, size, and reproduction strategies. Viral movement in soil can be controlled by a number of processes, including attachment and detachment from soil surfaces due to electrostatic, hydrophobic, and water film capillary forces, or release from soil pores due to dispersion. Here, we found that the community composition of eluted viruses changes under simulated natural soil flow conditions over time. Additionally, we found that the residual viruses remaining in the soil after the leaching were significantly different from the eluted viruses. Interestingly, the bacterial hosts of these viruses remained relatively constant despite differences in ammonium additions.

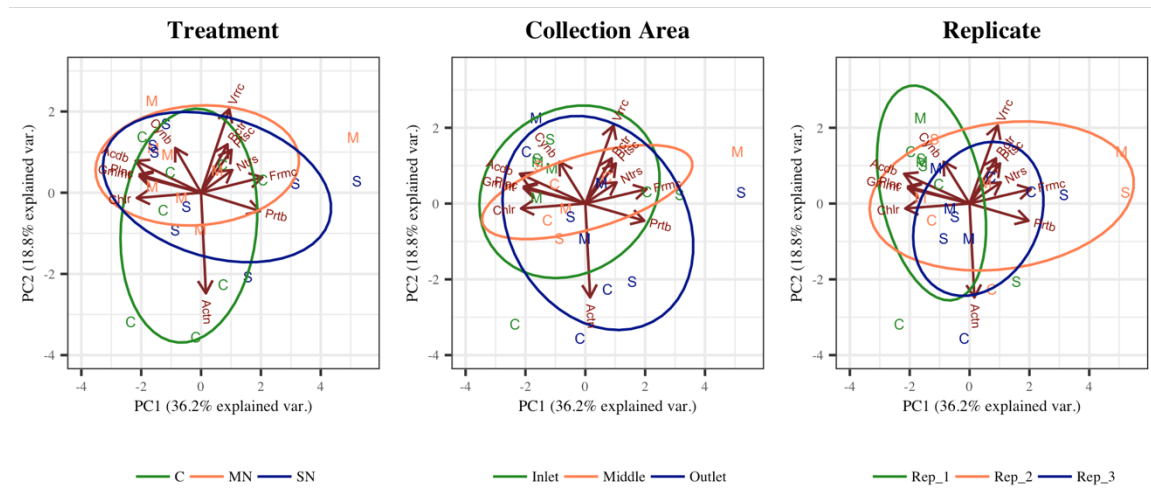
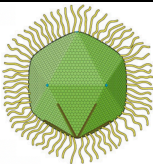
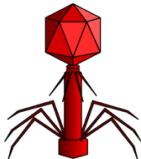
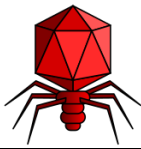
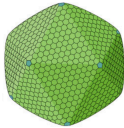
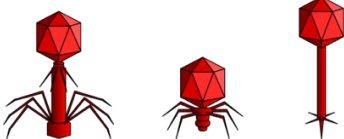


Figure 4.11. Principle component analysis (PCA) of soil bacterial communities including *Acidobacteria* (Acdb), *Actinobacteria* (Actn), *Bacteroidetes* (Bctr), *Chloroflexi* (Chlr), *Cyanobacteria* (Cynb), *Firmicutes* (Frmc), *Gemmatimonadetes* (Gmmt), *Nitrospirae* (Ntrs), *Patescibacteria* (Ptsc), *Planctomycetes* (Plnc), *Proteobacteria* (Prtb), and *Verrucomicrobia* (Vrrc). Sample points are represented by their treatments of control (C), SN (S), and MN (M). The samples were clustered by treatment (left), collection area (center), and replicate (right). The length of each arrow shows the importance of that phylum to explain the overall variance in the bacterial community between samples.

Virus elution as predicted by structure

The observed shifts in viral community composition across pore volumes eluted (Figures 4.1. & 4.4) could have been caused by many physiochemical factors, such as soil and virus surface properties, or shape and/or size of virus particles. Each family of viruses taxonomically identified has characteristic shapes and surface properties. Almost all of the eluted Mimiviridae family was eluted within the first pore volume (Figure 4.4). Mimiviridae was physically the largest (e.g., capsid diameter) viral family eluted, belonging to the nucleocytoplasmic large DNA virus clade (NCLDV) (Walker et al., 2020). In general, Mimiviridae have icosahedral geometries, and are enveloped viruses (Table 4.1). The extent to which Mimiviridae's size and surface properties accounted for their early elution from the columns is unclear. It is also possible that new viral progeny of the Mimiviridae family were produced during the first flow interruption between column saturation with pore volume 1 and the subsequent elution of pore volume 1 after the 16 hour flow interruption, and that these new viruses were readily eluted when the flow resumed. In the second pore volume, the relative abundance of Myoviridae was greatest compared to all other pore volumes (Figure 4.4). Myoviridae are bacterial phages with a non-enveloped head-tail shapes (Walker et al., 2020). The tails are intermediate in length and can be either contractile or non-contractile. Podoviridae and Siphoviridae are both non-enveloped head-tail viral families (Walker et al., 2020). Interestingly, while Podoviridae have a short tail and Siphoviridae have a long tail, both of these head-tail viruses with similar capsid diameters were eluted in similar patterns, with relative abundance increasing through the second to fifth pore volumes (Figure 4.4).

Table 4.1. Images and morphological images of identified virus families. Images labeled *a* are by user Ninjaticoshell (2014). *Viruses*, retrieved from <https://commons.wikimedia.org/wiki/User:Ninjaticoshell#Microorganisms> under a Creative Commons Attribution-ShareAlike 4.0 International license, and those labeled *b* are from ViralZone, SIB Swiss Institute of Bioinformatics under a Creative Commons Attribution 4.0 International License (Hulo, et al. 2020).

Mimiviridae	 <i>b</i>	Icosahedral Diameter = 750 nm Enveloped
Myoviridae	 <i>a</i>	Head-Tail Head size = 111x78 nm Contractile tail size = 113x16 nm Non-enveloped
Podoviridae	 <i>a</i>	Head-tail Head diameter = 60 nm Non-contractile tail size = 20x8 nm Non-enveloped
Siphoviridae	 <i>a</i>	Head-tail Head diameter = 60 nm Non-contractile tail size = 150x8 nm Non-enveloped
Phycodnaviridae	 <i>b</i>	Icosahedral Diameter = 120-200 nm Enveloped
Caudovirales	 <i>a</i>	Order of head-tail viruses Head diameter = 45-170 nm Tail size = 3-825 nm Non-Enveloped

Without knowing the exact surface properties of each individual virus, it is impossible to clarify the role of virus surface properties in the differential elution of viruses from the soils. Mimiviridae was the largest virus eluted, which could have been an important factor in influencing their mobilization in the first pore volume; their large size may have preclude them from entering micro-pore spaces, making them more likely to have existed in the mobile liquid fraction. Interestingly, the predominant viral family in the second pore volume was the second largest viral family, Myoviridae, and the predominant viral families in the third to fifth pore volumes were smaller, Podoviridae and Siphoviridae (Figure 4.4). These results suggest that virus size might play a role in determining virus mobilization from soil under saturated flow.

Another factor potentially affecting virus movement was the shape of the viruses. Mimiviridae are icosahedral enveloped viruses, while Myoviridae, Podoviridae and Siphoviridae are all head-tail non-enveloped viruses. Additionally, Unassigned Caudovirales is an order of viruses which are all non-enveloped tailed viruses, and the relative abundance of this Order increased with each pore volume (Figure 4.4). Based on these observations, it is possible that the surface properties of the non-enveloped capsid or head-tail shape contributed to increased attachment, while the Mimiviridae's icosahedral enveloped capsids promoted transport. Additionally, the phages may have influenced each other, as it has been shown that sometimes presence of multiple viruses can promote co-transport of viruses (Xu et al., 2017). Overall, based on the changes seen in taxonomy after each flow interruption, we can determine that hydrologic flow patterns had a significant influence on virus release and elution from the soil column.

Viral production across pore volumes

Production of new viral progeny may have been an important contribution to the changes in viral community structure with increasing pore volumes. Enumeration of the total number of viruses eluted over time revealed an increase in overall abundance of viruses in the second pore volume (see Chapter 3, Figure 3.4), which could have potentially been due to production of new viruses, rather than only from viral release from the soil column. After the overnight (16 hour) flow interruption, Myoviridae experienced the largest increase in relative abundance in the effluent of the second pore volume (Figure 4.4), which contains some temperate and virulent species (Walker et al., 2020). Potentially, temperate Myoviridae could have been induced to lytic reproduction from a lysogenic cycle, causing the increase in Myoviridae identified.

Prophage induction can occur in response to host stress, as shown by studies which have subjected hosts to the antibiotic Mitomycin-C, causing prophage to switch to lytic reproduction to escape the stressed/DNA-damaged host cell through cell lysis and release of viral progeny (K. E. Williamson et al., 2007). As shown in previous examinations of these columns, measured ions in the effluent decreased significantly between the first and second pore volumes (see Chapter 3, Figure 3.7). Based on this data, if we assume that the nutrient concentration decreased significantly between the first and second pore volumes, this lack of nutrients in the soil solution may have potentially stressed bacterial hosts, thereby inducing viral induction.

An alternative biologically based explanation for the shifts in virus community across pore volumes is competition for hosts. Potentially, after the relative abundance of many unknown viral species was eluted in pore volume 1, Myoviridae were able to

capitalize on predation of hosts with less competition. In this way, the changes in eluted viral abundance may have been be a reflection of competitive predator-prey dynamics shifting within the soil columns. Overall, whether through lytic or lysogenic cycles, production of new viruses throughout the duration of the five pore volumes of the flow-through experiment could have contributed to the changes in relative abundance of viral families.

Uniformity of Residual Soil Virus Community Composition

The community composition of viromes extracted from residual column soil after the flow experiment were significantly different ($p < 0.001$) than that of the eluted viruses (Figure 4.3G). Much of the difference was associated with the presence of Phycodnaviridae, which were present at significantly higher relative abundances in the residual soil viromes and nearly absent in the eluted viromes (Figures 4.1 & 4.4). Phycodnaviridae are icosahedral enveloped viruses (Walker et al., 2020). The release of Phycodnaviridae in the residual soil viromes could be due to interactions with the extraction buffer chemistry or the physical dispersion caused by blending soil samples. There were also a greater number of unassigned viruses in the extracted viromes than in the final pore volume of the eluted viromes. It is very likely that these viruses have some surface property which enables them to attach more strongly to soil surfaces but detach in the presence of chemical conditions that disrupt the adsorptive forces binding them to solid surfaces and/or physical dispersion of aggregates that may have entrapped the virus in small pores.

The community composition of the viruses remaining in the soil was similar across the three sampled columns depths (Figure 4.1). This suggests that there was no redistribution of viruses along the flow path as a result of reattachment and/or entrapment deeper in the soil column. In general, the difference in viral community composition between the eluted and residual soil viromes shows that residual viral families, which were immobile during elution but subsequently removed during destructive extraction, likely had varying characteristics of surface charge and/or shape, allowing them to remain in the soil column until physiochemical conditions drastically changed upon column deconstruction. An alternative explanation is that the viruses eluted during column flow were new viruses produced during the flow-interruption intervals between pore volumes. Further experimentation is needed to determine if either or both mechanisms account for the virome composition patterns observed in this study.

Viral host prediction differs from observed host taxonomy

Virus host prediction gives insight into factors driving changes in the viral community across the flow experiment. The relatively stable host predictions across pore volumes might support the theory that the changes in viral community composition are driven by hydrogeologic factors rather than biological ones. For instance, if there were large changes seen in host prediction across pore volumes, that could be evidence that the host community was shifting due to viral predation. Interestingly, while Proteobacteria was the most abundantly predicted host, it was not the most abundant bacteria phylum found using amplicon sequencing (Figures 4.9 & 4.10). Actinobacteria and Proteobacteria have very similar true abundances (i.e. the relative abundance determined

by amplicon sequencing rather than by host prediction), but Actinobacteria was predicted as a host far less frequently than Proteobacteria. The high frequency of Proteobacteria as a predicted host is mostly due to the high abundance of Siphoviridae, but might also suggest that Proteobacteria host a wider range of viruses than Actinobacteria.

Bacterial Community Composition Uniformity Across Column Depth

Bacteria taxonomy derived from 16S rRNA gene amplicon sequences from three depths within the soil column showed that bacteria community composition was dominated by Actinobacteria and Proteobacteria (Figure 4.10). Along the depth of the soil column, the community composition of bacteria was similar, suggesting that the conditions for bacterial host development were similar along the column. Additionally, this suggests that there was no significant phylum-specific migration/accumulation of certain of bacteria along the column, or potentially that there was little to no movement of these phyla at all. Furthermore, this result suggests that there was no major build-up of bacterial cells at any depth due to straining or blockage of the soil pores. Potentially, this was a result of the soil texture; the soil used in this experiment was a sandy loam, whose pores would be larger than a clayey soil, thereby causing less restriction of bacteria movement based on pore size.

The similarity of the bacterial host community in the columns at various depths suggests that perhaps bacterial communities are not as differentially affected by hydrologic flow interruptions as viral communities. Future studies which are able to obtain soil bacterial community composition data from effluent flow might shed light on the changes of bacterial host composition due to hydrologic flow.

Impacts of trace ammonium on viral and bacterial community

Previous research has indicated that atmospheric nitrogen deposition had significant impacts on bacterial community activity and diversity (Zhou et al., 2018). Atmospheric N deposition over 10 months showed decreased microbial activity and shifts in diversity. The application rate of nitrogen in our experiments was 6.5 kg/ha applied over one week. This rate was meant to mimic the amount of nitrogen received in a season via atmospheric N deposition. Interestingly, fewer viral contigs and bacterial ASVs were recovered from ammonium amended columns than from the control columns. The recovered vOTU AMGs and CAZymes were also fewer in the ammonium treated columns (SN and MN) compared to the control, suggesting decreased activity in these columns. In this way, our results support the theory that nitrogen deposition might limit bacterial activity.

However, the bacterial community composition and alpha diversity did not vary with ammonium treatment in these experiments (Figure 4.10 & 4.4). Even when searching for bacteria relevant to nitrogen cycling, the ammonium treated columns showed no evidence of increased populations of nitrogen-relevant taxa. Overall, there was a decrease in vOTUs, bacterial ASVs, AMGs, and enzyme recovery in ammonium amended columns which, coupled with recent findings regarding decreased bacterial activity under nitrogen amendments, suggests that nitrogen deposition might play a larger role than previously thought in regulating bacterial activity in soil systems.

CONCLUSIONS

The transport of native soil viruses is complex and reliant on many factors and conditions. In this study, we aimed to observe the changes in viral and bacterial community composition under interrupted flow conditions that simulate natural wetting events. We found that viral community composition shifted as the flow experiment progressed and that viruses with varying surface properties were released from soil attachment at different rates. We suggest that a contributing factor to this differential elution of viral families was virus particle size and dispersion. Likely, smaller viruses, which are contained in micropores, dispersed into the mobile phase as each pore volume eluted the liquid viral fraction. Alternatively, some viral production could have occurred between flows during the overnight incubation. AMGs discovered in each virome show slight differences in genetic potential of viral communities between pore volumes. Importantly, there are differences in the virus community composition between the eluted liquid samples and the residual soil samples, indicating that the viruses remaining in the soil column at the conclusion of the flow experiment could not be eluted by the flow conditions but could be detached by the extraction process.

Overall, this study shows that the transport of native soil viruses is highly affected by hydrological processes, and that viral families respond differently to interrupted flow conditions. We conclude that the most likely explanations for the observed variable mobilization of viral families involves the physiochemical properties of the viral families, including size, capsid envelopment, and shape and chemical surface properties. Levels of ammonium equal to nitrogen deposition did not have a meaningful impact on viral or bacterial community composition. In the future, the biological response to nitrogen

additions would need to be measured by adding much greater quantities of ammonium for host stimulation to examine the specific responses of viruses infecting bacteria stimulated by N-addition.

The results are very important for our understanding of the transport and retention of natural viral communities under rainfall or irrigation events. We have shown that virus transport may be size dependent, which is very important for predicting the evolution and spatial distribution of the diversity and abundance of native soil viruses under field conditions. Overall, this study provides important insights into the impacts of natural flow conditions on the mobilization of soil viral communities.

APPENDIX

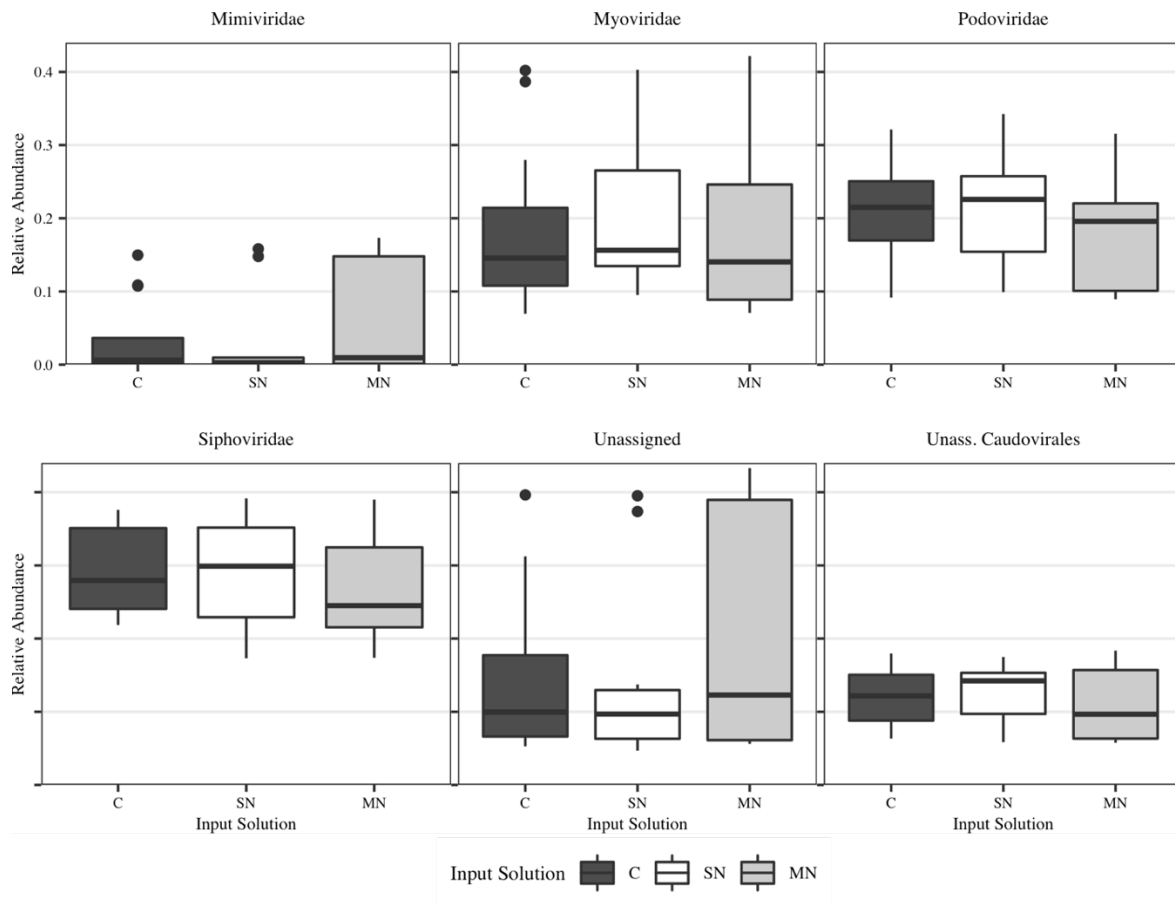


Figure 4.12. Relative abundance of viral families averaged by input solution, including control (C), single-N additions (SN), and multiple N additions (MN).

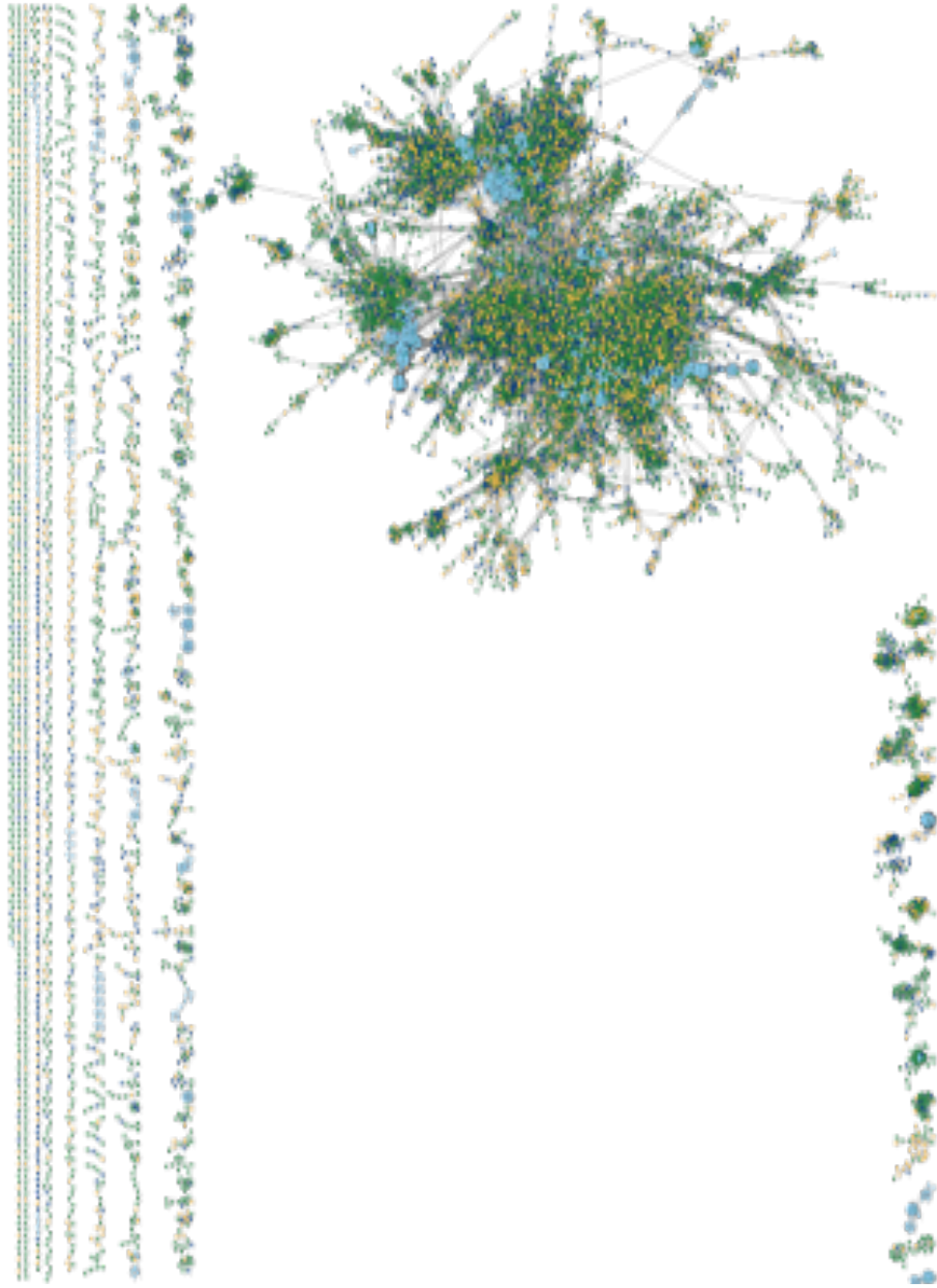


Figure 4.13. A gene-sharing network for viral contigs isolated from the Control (green), SN (yellow), and MN (dark blue) treatments, and the NCBI RefSeq database (light blue).

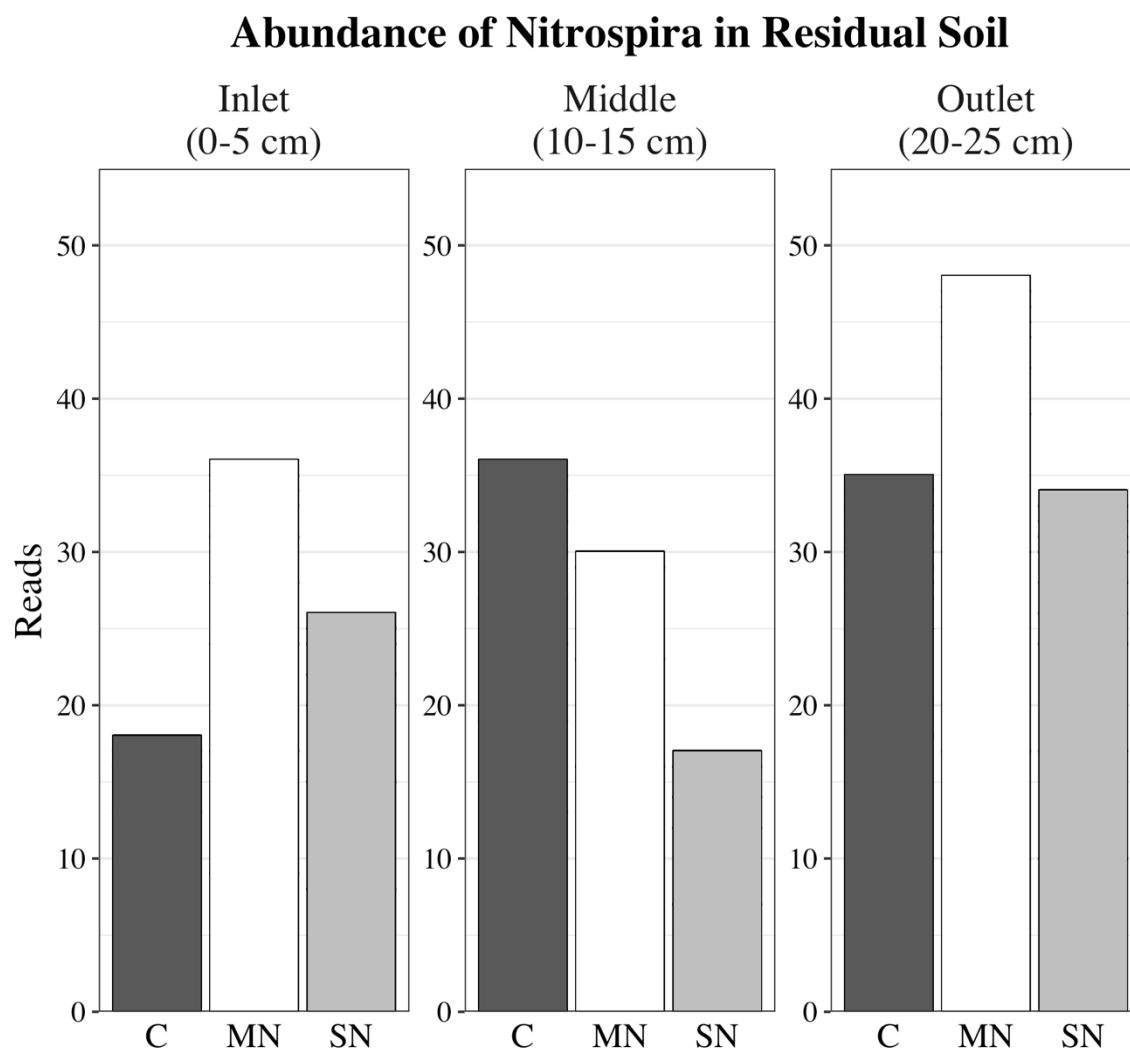


Figure 4.14. Abundance of Nitrospira found at each column depth was not significantly different between depths (inlet, middle and outlet) and ammonium input solutions (SN and MN).

CHAPTER 5: CONCLUSIONS

Soil viruses are critical players in ecosystem functioning due to their influence on the abundance, diversity, evolution, and function of bacterial hosts through both lytic and lysogenic infection. Viruses have substantial influences on biogeochemical cycling of carbon and nutrients, thereby largely determining soil productivity and health. As such, soil viruses are of great economic and environmental interest in agricultural systems. However, research into soil viruses lags behind that of the aquatic ecosystems due to the difficulties of extraction and isolation of soil viruses. In recent years, rapid progress has been made in soil viral ecology due to the advancements of molecular techniques such as DNA and RNA sequencing. Unfortunately, these methods alone are not enough to fully characterize the complexities of viruses in native soil systems. Therefore, we performed both field and laboratory based investigations utilizing an array of techniques such as epifluorescence microscopy, flow-through column techniques, hi-throughput sequencing, and bioinformatics to elucidate the abundance, community composition, and mobility of viruses in natural and simulated soil systems. The novelty of this dissertation research lies in the investigation of natural soil virus communities by flow-through and metagenomics techniques. Simulating the transport of natural soil virus communities provides valuable insights into real-world virus transport under periods of repeated rainfall events, which could increase with worsening climate change. The findings of the research performed for this dissertation are summarized as follows.

1. Viral abundance, bacterial abundance, and lysogenic fraction are more sensitive to short term temporal changes (i.e. across a growing season) than long term agricultural management practices (e.g., 40+ years of tillage, fertilization, or cover crop rotations).

2. Virus abundance in agricultural soil systems increases with increasing nitrate and organic carbon concentrations in the soil. Increased nitrogen was associated with decreased lysogenic fraction, suggesting that increased application of nitrogen to agricultural field systems may trigger soil viral induction.
3. Periods of interrupted flow, such as would be found in sequential rainfall or irrigation events, increased the transport of viruses compared to continuous flow conditions.
4. The mobility of soil viruses is not impacted by wet atmospheric deposition of ammonium. On a short time scale (e.g., one week) atmospheric nitrogen deposition of ammonium is not significant enough to change soil viral or bacterial community composition under low concentrations of nitrogen deposition, which occurs in regions such as the southeast United States.
5. Viruses are differentially eluted from soil profiles according to viral family. Initial flow is dominated by Mimiviridae, a giant virus. Subsequent flow saw increases in Caudovirales viruses including Myoviridae, Siphoviridae, and Podoviridae. Importantly, some viruses which remained immobile under elution conditions were subsequently releasable under extraction conditions, including Phycodnaviridae.
6. Residual soil virus and bacteria abundance and community composition were uniform across soil depths, suggesting that viruses and bacteria could potentially be transported greater distances than that of our soil column before experiencing reattachment to soil surfaces.

Research on soil viruses is still in a fundamental stage, despite the increased interest and explosion of information due to next-generation sequencing. Much remains to be discovered regarding virus abundance, distribution, and function in soil ecosystems. Phenomena which are well characterized in aquatic systems, such as the re-cycling of microbial necromass via viral lysis (i.e. the viral shunt), remain unproven in soil systems. Investigations of viruses in their native environments, such as field studies, give insight into their true distribution and community composition in native systems. However, these types of investigations alone do not suffice, and must be coupled with laboratory-based experiments which are able to distinguish relative contributions of various factors and mechanisms as well as answer fundamental questions about soil viral ecology. These knowledge gaps, including drivers of virus abundance and community composition, factors influencing viral induction, links between viral ecology and biogeochemical cycling, remain to be filled. The rapid development of metagenomic tools go a long way into unravelling the viral community complexity in natural systems, but must be coupled with more fundamental research regarding virus life cycle strategies in order to truly understand viral ecology in soil systems.

Soil virus ecology is a rapidly expanding research field with many intriguing scientific issues for novel discoveries. The dedicated characterization of the world's virosphere will generate important information for disentangling the key links between environmental viruses and ecosystem level ecology which will help scientists to better understand the biosphere as a whole. Research works such as the dissertation presented here shed light on the hard-to-characterize world of native soil viruses, and help lay the groundwork for future investigations into viral ecology.

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VITA

Regan McDearis was born in Fairfax, Virginia in 1994, where she lived with her parents and brother. Inspired by her grandmother's love of gardening and the outdoors, Regan pursued environmental education through NSF summer programs and environmental sciences classes in high school. Regan transferred this love of the outdoors to her higher education, when she attended Virginia Tech for a degree in Environmental Science. During her college summers, Regan worked in the environmental field, both at the Virginia Office of Environmental Quality, and working an eco-tourism job in Alaska on the Kenai River. Trips to such beautiful places, as well as University-led trips to Costa Rica and Ecuador, deepened Regan's passion for the natural world. While at Virginia Tech, Regan's interest in soil science was sparked by working in the Environmental Microbiology lab as an undergraduate student researcher.

After graduating from Virginia Tech magna cum laude and as an Honor's Scholar with a Bachelors' Degree in 2016, Regan then moved with her now-wife to Knoxville, Tennessee to pursue her Masters in Soil Science at the University of Tennessee, specifically to join the Soil Microbiology lab. In the first year of her Masters' studies, Regan applied and was accepted to transfer her program to a Ph.D., where earned straight A's while she studied soil viral abundance, communities, and transport.