Impacts of Phage Predation on Microbial Dynamics in Temperate Ecosystems

A thesis submitted in partial fulfillment of the requirement
for the degree of Bachelor of Science in Biology from
The College of William and Mary

by

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ABSTRACT

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in Temperate Ecosystems
(Under the direction of Kurt E. Williamson)

In marine ecosystems, virus-mediated microbial mortality results in cell lysis and release of cellular contents, freeing dissolved organic nutrients. This nutrient recycling facilitates competition within diverse bacterial assemblages. In contrast, little is known about the role of phage predation in terrestrial ecosystems. A single study done in Barrow, Alaska found phage predation to be a key factor controlling bacterial population dynamics. By applying the methods used in the Arctic experiment to a temperate environment, the goal of this study was to examine the impact of phage predation in temperate soil ecosystems. Upland and wetland field sites were established in the College Woods on the William & Mary campus and PVC soil collars were installed at each site. Five days post installment, each collar received one of four treatments, done in six replicates: acetate (C), nutrients (N, P, K), Tea extract + ferrous sulfate (antiphage, "TeaF"), or sterile water (control). Upon treatment, microbial respiration of each collar was measured for eight consecutive days using an infrared gas analyzer. At day three and day eight, selected collars were harvested for direct count analysis of microbial cells and virus particles, as well as microbial biomass carbon. At both the wetland and upland sites, treatment of soil with the antiphage solution had no significant impact on microbial respiration or abundance. Nutrient amendment also failed to significantly impact microbial dynamics. Acetate treatment at the upland site, however, resulted in a significant increase in microbial respiration, but not in microbial biomass carbon or abundance. Furthermore, TeaF did not appear to exhibit virucidal activity in temperate soils, as it has been documented to exert in Arctic soils. Two field tests examining the effects of varying volumes of TeaF treatment on autochthonous phage were

conducted to explore this disparity. Results indicated that TeaF lacks virucidal capacity in temperate soils and appears to facilitate a washout effect on soil viral populations that is also produced by water. Further studies are necessary to understand the impacts of phages on microbial dynamics within terrestrial soils. This early study neither defends nor rejects the idea that bacteriophage serve as significant drivers of bacterial mortality. Thus, despite their abundance in soils, impacts of phages on soil microbial dynamics remain unknown.

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I. INTRODUCTION

Bacteriophage Predation in Marine Ecosystems

Early studies of viruses in environmental systems relied on culture-dependent methods and counts of plaque forming units (Farrah, 1987; Moebus, 1987). These types of studies restricted detection of viruses to only those infecting lab-culturable hosts. With this narrow scope of detection, it was impossible to estimate the total viral abundance within a viral community of unknown composition, let alone the impacts of viral infection on microbial community structure and function. The vast global abundance of viruses had yet to be recognized and the idea that viruses were ecologically insignificant and uninteresting was widely accepted (Bergh et al., 1989). This notion was revised in 1989 when the use of transmission electron microscopy (TEM) revealed that viruses are present at high concentrations in marine environments. With virus particle abundance estimates reaching 2.5×10^{8} mL^{-1}, Bergh and colleagues saw that viral abundance exceeded bacterial abundance (0.2×10^{6} to 6.2×10^{6} bacterial cells mL^{-1}) in the marine pelagic and coastal environments sampled in their study (Berg et al., 1989). Since the initial application of TEM to the study of viral ecology, further methodological advances, specifically the use of epifluorescence microscopy (EFM) and flow cytometry, have made enumeration of viruses more efficient in terms of time and monetary investment. Subsequent studies have shown viruses to be present in large numbers in all aquatic environments, with viral abundances on the order of 4.8×10^{6} mL^{-1} in marine pelagic environments, 1.6×10^{7} mL^{-1} in coastal marine environments, and ranging from 4.2×10^{6} mL^{-1} to $9.6 \times ^{10}$ mL^{-1} across freshwater systems (Srinivasiah et al., 2008). With an estimated global abundance of 10^{31} total particles, today viruses are acknowledged as the most abundant biological entities on earth (Wommack, 2010; Chibani-Chennoufi et al., 2004).

Since the discovery of high viral abundance in aquatic ecosystems, there has been a concerted effort to discern the role of viruses within aquatic microbial communities. The majority of aquatic viruses appear to be bacteriophage, or viruses that infect bacterial cells (Cochlan et al., 1993; Weinbauer, 2004). Numerous studies support the hypothesis that bacteriophage predation represents a form of top-down control that limits the biomass of each susceptible species of bacteria and heavily contributes to the diversity of marine microbes (Bratbak et al., 1992; Sandaa et al., 2009; Thingstad, 2000). This hypothesis depends largely on two factors: a phage's host specificity and the probability that a phage will come into contact with a host cell. Viruses lack any means of self locomotion, and therefore rely solely on random diffusion to facilitate collisions (infections) with host cells. Additionally, each bacteriophage is generally believed to be capable of infecting only its specific host strain of bacteria (Holmfeldt et al., 2007). Some evidence suggests that there are bacteriophages with the ability to infect a broad range of hosts, but the prevalence of such phages and their ecological importance in nature is still unclear (Langley et al., 2003; Beumer and Robinson, 2005). Even so, a higher concentration of host cells within an environment increases the probability that a bacteriophage will collide with its host and infect the cell. These aspects of aquatic phage-host interactions have given rise to the "kill the winner" hypothesis, so called because the bacterial population that reaches high abundance in any environment is more likely to suffer from viral infection (Thingstad, 2000).

Viral control of bacterial activity differs from the long studied protistan grazer-bacterial prey interactions. Unlike grazer induced mortality, lytic viral infection results not only in cell death, but cell rupture, releasing progeny virions and cellular debris into the surrounding environment (Weinbauer and Höfle, 1998). Inactivated viruses and cellular debris are degraded in the environment, adding to the pool of dissolved organic nutrients and providing free

resources for competing microbes. In effect, top-down control by marine bacteriophages limits the success of any one bacterial species, but the recycling of dissolved organic nutrients through viral lysis can result in the temporal succession of many different dominant species within a nutrient-limited environment, and thus contributes to overall bacterial species richness (Thingstad, 2000; Miki et al., 2007).

Bacteriophage Predation in Terrestrial Ecosystems

Much like marine ecosystems, soils harbor vast numbers of viruses with abundances ranging from 1×10^9 gram dry weight $(gdw)^{-1}$ in agricultural soils to 1×10^5 gdw^{-1} in desert soils. However, while the role of bacteriophage in marine ecosystems has been an area of active research over the past two decades, there has been little investigation of the impacts of bacteriophage in terrestrial microbial ecosystems (Bratbak et al., 1992; Fuhrman, 1999; Thingstad, 2000). This lack of research and consequent void of knowledge pertaining to terrestrial viral ecology is most easily attributed to methodological problems in studying terrestrial viruses. To monitor phage-host dynamics requires the accurate quantification of changes in phage and host abundances over time. Reliable methods for extracting and enumerating whole viral assemblages from soil samples have only recently been developed (Williamson et al., 2003; Ashelford et al., 2003). As a result of these methodological challenges, few studies have focused on the interactions of autochthonous soil bacteriophages and their hosts.

A small number of published studies on this topic examined the dynamic interactions between the phages and bacteria colonizing *Beta vulgaris* (sugar beet) roots and leaves (Ashelford et al., 1999, Ashelford et al., 1999). These studies suggest that the abundance of

phage within the phytosphere of the sugar beet is not only subject to temporal variation that parallels changes in host density over time, but variation within individual phage populations infecting the same host (Ashelford et al., 1999). Studies such as these support the idea that bacteriophages are an integral part of microbial dynamics in soils at the single host level. There remains little direct evidence, however, that viruses exert significant selective pressure on terrestrial microbes at the community level or contribute to biogeochemical cycling on a global scale in terrestrial environments, as they are known to do in marine ecosystems.

The results of a microcosm experiment performed by Srinivasiah et al. (2008) provided the first evidence suggesting that viral assemblages within soil respond rapidly to environmental changes. In this study, the amendment of soil microcosms with yeast extract (C source) resulted in a significant increase in bacterial abundance one day after treatment, with a similar increase in viral abundance following two days later. Elevated levels of both bacteria and virus particles were sustained for the entirety of the 30 day experiment. These findings were contrary to the previous hypothesis that viral decay and production within soils is much slower than in marine ecosystems, further opening the door for the potentially significant role of bacteriophages in terrestrial microbial dynamics (Srinivasiah et al., 2008).

Shortly thereafter, a separate field study provided further evidence for a dynamic interplay between viruses and bacteria in terrestrial ecosystems, specifically the exertion of top-down control on Arctic soil microbes via phage predation (Allen et al., 2010). In this study, Allen and her colleagues demonstrated that reduction of native bacteriophage abundance by way of a novel virucidal agent, tea with ferrous sulfate (TeaF), resulted in an increase in both microbial respiration and abundance. Prior to the Allen et al. (2010) study, soil microbial biomass was thought to be dictated primarily by bottom-up controls such as labile carbon

concentration. The results of the Arctic study, however, found the most influential source of ecological pressure in this system was bacteriophage predation, rather than substrate limitation. These results provide the strongest evidence, to date, that phage predation may be an important mechanism for top-down control of microbial populations in terrestrial environments.

In addition to unearthing the importance of autochthonous phage in arctic soils, this study established a potential tool for investigating the role of phage predation in other terrestrial environments. The purpose of the research presented here was to assess the methods put forth by Allen et al. (2010) for application in temperate soils, with the overarching goal of expanding our knowledge of phage-host dynamics within temperate soils ecosystems. As the Arctic study was the first to apply TeaF as a general virucidal with documented anti-phage activity against only two *Salmonella* phages (de Siquiera et al., 2006), the first phase of the study consisted of a laboratory exploration of the virudical capacity of TeaF. This was done by testing TeaF against three phages (Bacteriophage T4, Mycobacteriophage Larva, Mycobacteriophage CrimD) in time-series phage attenuation assays.

The second phase of the study was aimed at investigating top-down control in temperate soil microbial ecosystems and entailed application of the methods used in the Arctic study (Allen et al., 2010) at two sites within the College Woods of William & Mary, in Williamsburg, Virginia. The final phase of this research concentrated on assessing the efficacy of TeaF as virucidal in temperate soils.

II. MATERIALS AND METHODS

Preparation of Virucidal Solution TeaF

The virucidal solution used in this study, TeaF, was a mixture of tea infusion and ferrous sulfate. One day before use in each of the experiments, tea infusion was prepared by bringing deionized water to a boil and steeping loose leaf tea (Twinings English Breakfast Tea, loose leaf, 7.5% w/v) for ten minutes. The infusion was then centrifuged for 20 minutes at 4750 rpm to pellet tea leaf debris. Following centrifugation, the infusion was autoclaved for sterilization, cooled, and stored overnight at 4°C. Directly before use in virucidal assays and field experiments TeaF was prepared by combining tea infusion with freshly prepared ferrous sulfate (4.3 mM $FeSO_4$). The final solution was 22% tea infusion and 68% ferrous sulfate (de Siquiera et al., 2006)

Phage Attenuation Assays

The virucidal activity of TeaF was tested against three bacteriophages, T4 (ATCC 11303-B4), Mycobacteriophage Larva, and Mycobacteriophage CrimD (both isolated from soil on campus by the College of William & Mary Phage Lab), via a time series phage attenuation assay. In each assay, 8 mL of phage stock of a known titer was exposed to 22 mL TeaF solution; for bacteriophage T4 controls, 22 mL SM buffer (100 mM NaCl; 8 mM $MgSO_4$•7 H_2O; 50 mM Tris-Cl) was used in place of TeaF; 22 mL 1X Phage Buffer (10 mM Tris stock; 10 mM $MgSO_4$; 140mM NaCl; 1 mM $CaCl_2$) was used for controls in both mycobacteriophage assays. Treatments and controls were done in duplicate. After the addition of phage to the TeaF or buffer solution, 1 mL samples from each tube were drawn, a 10-fold serial dilution was performed, and the dilutions were plated with host bacterium in a plaque assay for analysis of infectious titer

(plaque forming units or PFU). This was done in a time series fashion, with plaque assays performed using samples from each treatment and control at time 0, 1, 2, 3, 6, 12, 24, and 72 hours, and 1 week following the addition of TeaF. Bacteriophage T4 (100 µl) was plated on tryptic soy agar plates (per liter: 15 g agar, 15 g tryptic soy powder, 0.5 % NaCl) using *Escherichia coli* (100 µl; ATCC 11303) as host in tryptic soy top agar (per liter: 15 g tryptic soy powder, 7.5 g agar, 0.5% NaCl). Mycobacteriophage (Larva (100 µl) and CrimD (100 µl)) were mixed with *Mycobacterium smegmatis* (500 µl; ATCC 700084) in 1X top agar (per liter: 4.7 g 7H9 broth base; 7.0 g 7H10 agar base) and plated on Middlebrook 7H10 agar plates (per liter: 9.5 g 7H10 Agar base; 0.5% glycerol; 10% AD supplement (145 mM NaCl; 5.0% Albumin, Fraction V; 2.0% Dextrose); 50 µg mL^{-1} Carbenicillin; 10 µg mL^{-1} Cyclohexamide). All plates were counted for PFU data after incubation at 37°C for 24 hours (Wen et al., 2006).

In addition to infectious titer data, samples were taken from controls and treatments at each time point for viral abundance studies through epifluorescence microscopy (EFM). At each time point, 2 mL samples were drawn from each control and treatment, flash-frozen with liquid nitrogen, and stored at -80°C. Later samples were thawed, vacuum filtered through 0.02 µm Anodiscs (Millipore, Germany), stained with 400µm 2x SYBR Gold (Invitrogen, Eugene, OR) and visualized under an Olympus BX-51 microscope, using a FITC filter (Noble and Fuhrman, 1998).

Bactericidal Assays

For bactericidal assays, duplicate log phase cultures of *E. coli* (grown for 6 hours in TSB at 37°C with shaking at 250 rpm) and *M. smegmatis* (grown overnight in 7H9 medium *complete*

(per liter: 890 mL 7H9 liquid medium *neat* (0.5% 7H9 broth base; 0.2% glycerol); 100 mL AD supplement; 500μg mL^{-1} Carbenicillin; 100μg mL^{-1} Cycloheximide; 10 mL1mM $CaCl_2$) at 37°C with shaking at 250 rpm) were exposed to TeaF (final solution was 26% host cell liquid culture and 74% TeaF). After exposure for 0 and 24 hours, 10-fold serial dilutions of each exposure were plated (for *E.coli*, tryptic soy agar plates were used; for *M. Smegmattis* Middlebrook 7H10 agar plates were used), inverted, and incubated overnight at 37°C, after which plates were counted for colony forming units (CFU). To serve as control, duplicate *E. coli* cultures were grown in TSB and *M. smegmatis* cultures were grown in 7H9 medium *complete* for the 24 hour sampling period and plated for CFU as aforementioned for treatments.

Field Studies

Two sites of varying water content were chosen from within the College Woods (Oak-Hickory forest) on the campus of William & Mary in Williamsburg, VA. The "upland" site had an average gravimetric water content of 15%; the "wetland" site had an average gravimetric water content of 36% and was located beside a running stream. At each site, 24 polyvinyl chloride soil collars (10 cm diameter, 10 cm deep) were temporarily installed, allowing for six replicates of four treatments. Collars were spaced at intervals of roughly one meter and hammered into the ground, leaving a small lip exposed. After installation, vegetation was cleared from within each collar. This preparation of the field site was carried out five days prior to use in experiments to allow soils to recover from the disturbance (Allen et al., 2010).

On the sixth day after installation, each collar was injected with one of four treatments using a 60 mL sterile syringe. In replicates of six, collars received either carbon amendment (80 mL of 0.73 M ethyl acetate), virucidal treatment (80 mL TeaF), nutrient amendment [80 mL 5.3

mM KH_2PO_4 and 0.186 M NH_4Cl (50 %KH_2PO_4; 50% NH_4Cl)], or a control (80 mL sterile water) (Allen et al., 2010). All treatment solutions were prepared within one hour of application to soils. After treating collars the field site was covered with clear plastic dropcloths to prevent immediate washout from potential rainfall and remained covered between sampling times for the remainder of the experiment. Microbial respiration measurements were taken daily from each collar for eight days using an EGM-4 portable infrared gas analyzer (PP Systems).

On day three of the field experiment, three collars from each treatment were chosen at random and harvested (approx. 100g of soil were taken from each collar using a hand trowel and placed in sterile Whirl-pak bags) for analysis of bacterial cell and viral particle abundance, as well as microbial biomass carbon. The remaining twelve collars were harvested on day eight, after the final respiration reading was taken from each collar.

Bacterial Cell and Virus Particle Abundance via EFM

Soil samples from harvested collars were stored overnight at 10°C. The morning after harvesting, bacterial cell and viral extractions were performed. Bacterial cells were extracted by blending 10 g soil from each collar in 0.1% sodium deoxycholate followed by centrifugation of 20 mL of subsequent slurry in an 50 mL Oak Ridge tube containing 5mL Nycodenz (1.3 g mL^{-1}) for 20 minutes at 10,000 x g and 4°C in a Sorvall RC6 + centrifuge. After centrifugation, 1.5 mL samples of each extraction were dispensed into 2 mL cryotubes and stored at -80°C until processing for EFM (Williamson et al., 2011).

Viral particles were extracted from soil samples by suspending 5 g of soil in 15 mL of 1% potassium citrate and sonicating for three 1 minute intervals. Following sonication, samples were centrifuged in a Sorvall RC5B centrifuge for 20 minutes at 8000 rpm and 4°C. The

resulting supernatant was stored in 1.5 mL aliquots at -80°C for later use (Williamson et al, 2005).

For enumeration, Bacterial extracts were vacuum filtered onto 0.2 μm membrane filters (Millipore, Ireland), stained with 400μl 2x SYBR Gold (Invitrogen, Eugene, OR), and filters were mounted on a glass microscope slide with 20 μl of antifade solution (50% 1X phosphate-buffered saline, 50% glycerol, 0.01% *p*-phenlenediame). For enumeration of virus samples, extracts were thawed, vacuum filtered through 0.02 μm Anodiscs (Millipore, Germany), and stained and mounted to glass microscope slides as described above. Both bacterial and viral extraction slides were visualized under an Olympus BX-51 microscope, using a FITC filter. Ten to twenty fields per sample were photographed using a Hamamatsu C8484 digital camera and counted using MetaMorph Basic software (Olympus).

Microbial Biomass Carbon via Chloroform Fumigation

To gather microbial biomass carbon data, 10 g soil samples (three replicates of each treatment) were fumigated with ethanol-free chloroform in a vacuum desiccator by evacuating the chamber three times until chloroform boiled; after the third evacuation, the desiccator was left sealed and stored in the dark for five days to ensure the lysis of microbial cells. On the sixth day, the vacuum was released and carbon was extracted from soils by shaking for one hour in 0.5 M K_2SO_4. Each fumigated sample had an unfumigated counterpart that underwent shaking for one hour in 0.5 M K_2SO_4. After K_2SO_4 extraction, both fumigated and unfumigated samples were filtered through Whatman number one filter paper into 500 mL specimen cups and stored at -20°C until needed for processing (Vance et al., 1987). Dissolved organic carbon analysis was carried out at Chesapeake Bay Laboratories, Solomons, Maryland.

Impact of Treatment Volume

Results from the initial field studies showed that treatment of soils with 80 mL of TeaF did not significantly impact viral abundance. These results were different from those observed in the Arctic study, where treatment of soil with 80 mL TeaF resulted in a significant decrease in viruses per gram of dry soil. In an effort to discern if the lack of TeaF degradation of viral particles was due to inefficient volume, the upland field site was revisited in late July 2011 to test the impact of various volumes of TeaF on viruses and bacteria within the soil. Five days prior to the experiment, 18 polyvinyl chloride collars were installed as previously described. On day six after installation, triplicate collars were treated with either 80, 160, or 240 mL of TeaF or equivalent volumes of sterile water. After treatment, microbial respiration was monitored via a portable infrared gas analyzer and the site was covered in clear plastic. Environmental respiration readings were taken daily from each collar for three consecutive days. On the third and final day of the study all collars were harvested as previously described. Using soil from the harvested collars, bacterial and viral extractions were performed and abundance was measured as previously detailed in the upland and wetland experiments.

To test the hypothesis that liquid treatments were washing bacterial cells and viruses deeper into soil, a second experiment was performed to monitor the effect of increased TeaF treatment volumes on bacterial and viral populations within the upland soil in September 2011. As in the late July study, 18 collars were installed 5 days before use. On day 6 after installation, triplicate collars were treated with 80, 140, or 200 mL of TeaF or equivalent volumes of sterile water as control and covered in clear plastic after treatment. On the third day after treatment, all 18 collars were harvested by extracting one 6-inch deep, 2-inch diameter soil core from each

collar. Cores were capped at both ends and stored at -80°C until use in bacterial and viral extractions. After thawing, virus particle extractions were done separately for the top and bottom of each core using 5 g soil scraped from each end of the core. Bacterial cell extractions were also performed using 10 g soil from the top and bottom of each core. For both bacterial cell and virus extractions, soil samples were taken from within one inch of either the top or bottom of the core. Because bacterial and viral extractions were performed on the same day, there was some variation in the sequence with which the 5 g and 10 g samples were taken from the cores (virus extractions were not always performed first, thus the samples were not always taken from the extreme ends of the core). This sampling variation most likely increased the error in testing the washout hypothesis, as the level from which soil samples were taken had significant impacts on estimated viral and bacterial abundance.

Enzymatic Assays

An alkaline phosphatase assay was performed with soil samples from both the upland and wetland sites. Ten samples of approximately 25 g were taken from each site and 2 g portions of each sample were suspended in 5 mL 0.5 $CaCl_2$ in 15 mL screw-cap tubes. After the addition of 1 mL *p*-nitrophenyl phosphate liquid substrate system (MP Biomedicals, LLC), all tubes were incubated at 37°C for one hour. Following incubation, the absorbance of each sample was measured at 460nm using a Genesys 20 spectrophotometer (ThermoSpectronics, USA). The concentration of *p*-nitrophenol in each tube was then calculated by measurement against a standard curve of absorbance vs. concentration, generated from prepared standards of *p*-nitrophenol (0.0625, 0.125, 0.25, 0.5, 1.0, 2.0 mM).

A glucose oxidase assay was performed using soil samples from both upland and wetland sites to assess microbial glucose production, a measure of carbon utilization. Eight approximately 50 g samples of soil were taken from within each field site and homogenized in one bag for each respective site. Ten 5 g portions of homogenized soil from each field site were weighed into 50 mL conical tubes and suspended in 15 mL acetate buffer (1M, pH 5.0). Five samples from each field site were treated with carboxymethylcellulose (0.5% final concentration). The five samples that did not receive carboxymethylcellulose served as control. All samples were treated with toluene (2.7% final concentration) to inhibit microbial growth and incubated with shaking for 24 hours at 10°C. Colorimetric determination of glucose concentration was then carried out using a Glucose Assay Kit (Sigma-Aldrich, USA).Glucose concentration of carboxymethylcellulose treated samples and controls was determined by measuring absorbance with a Genesys 20 spectrophotometer (Thermo Spectronics, USA) and comparison to a standard curve constructed using known concentrations of glucose (0.11, 0.22, 0.33, 0.44 mM).

Statistical Analyses

All statistical analyses were conducted using Prism version 5.03 (GraphPad Software, Inc.). Phage counts from phage attenuation assays were evaluated using a paired t-test. Bacterial and phage counts from field studies were evaluated using ANOVA with subsequent Tukey's multiple comparison tests. Longitudinal respiration data were analyzed with repeated measures ANOVA and Dunnett's multiple comparison tests to determine deviation of treatments from control. Significance is quoted as 95% percent confidence interval ($p < 0.05$).

III. RESULTS AND DISCUSSION

Effect of TeaF in Phage Attenuation Assays

TeaF has been documented to have potent virucidal activity against two bacteriophages that infect *Salmonella typhimurium*. As such, TeaF was declared an appropriate reagent in the deactivation step of a phage amplification assay used to determine the presence of *Salmonella typhimurium*, a potential food contaminant (de Siqueira et al., 2006). The application of TeaF in the field studies of Allen et al. (2010) represents the only other documented use of TeaF since it was proven to deactivate the two *Salmonella* phages. Before applying TeaF to studies of terrestrial microbial communities, the virucidal efficacy of TeaF was tested against three bacteriophages: T4 (a tailed virus of the *Myoviridae* family which infects *Escherichia coli*), Larva and CrimD (both of which are mycobacteriophages of the *Siphoviridae* family that infect *Mycobacterium smegmatis*).

Bacteriophage T4 was an appropriate starting point for the study because it is easily propagated in the lab. After the initial test against T4, the virucidal capacity of TeaF was tested against Mycobacteriophages Larva and CrimD. Since Larva and CrimD were recently isolated from environmental soil samples from the College of William & Mary campus, where proposed field studies would make use of TeaF in monitoring microbial dynamics within soil, these phages were particularly attractive candidates for use in this study. The goal of this phase of research was to shed light on possible mechanisms of TeaF virucidal action, determine the spectrum of such virucidal activity, and to produce results supporting or discouraging application of TeaF in the field.

Exposure to TeaF rendered each of the three bacteriophages tested incapable of infecting host cells immediately, as shown by the lack of plaques from TeaF-treated phage stock (Table 1). Phage attenuation assays included examination of infectivity of bacteriophage following exposure to TeaF over the course of 24 hours (T4), three days (CrimD), and one week (Larva). Prior investigation of TeaF virucidal activity did not monitor the effects of TeaF exposure that exceeded twenty minutes (de Siqueria et al., 2006). Later time points were included in the current study to allow for the possibility of transient effects of TeaF on phage infectivity. Complete loss of phage infectivity occurred upon contact with TeaF and all phage particles remained non-infectious for the entirety of each individual assay (Table 1). These results suggest that TeaF had an irreversible effect on phage particles that prevented infection or interfered with adsorption of otherwise infectious particles.

Analysis of phage attenuation assay exposures via EFM revealed that TeaF actively degraded virus particles, in addition rendering them non-infective. In the TeaF x T4 phage attenuation assay (Figure 1), untreated controls averaged 6.47×10^7 viruses mL^{-1} and did not vary significantly over the 24 hour sampling period (Two-way ANOVA, $p = 0.7302$). The total virus particle count from TeaF-treated stock decreased to 9.0×10^6 mL^{-1}at t_0. After further exposure for two hours, the T4 phage population underwent a second decrease, dropping to 5.32×10^6 mL^{-1}. Degradation of phage particles ceased after two hours of exposure to TeaF and the phage particle count stayed at an average of 3.7×10^6 mL^{-1} for the remainder of the 24 hour

Table 1. Sensitivity of Phages to TeaF

	Surviving numbers of bacteriophage (PFU ml^{-1})											
	a. Bacteriophage T4				b. Mycobacteriophage CrimD				c. Mycobacteriophage Larva			
	T1	T2	C1	C2	T1	T2	C1	C2	T2	T2	C1	C2
Time (hr)												
0	0	0	$4.5x10^8$	$1.0x10^9$	0	0	2.5×10^8	3.1×10^8	0	0	4.7×10^8	5.0×10^8
1	0	0	$2.9x10^8$	$3.2x10^8$	0	0	1.2×10^8	4.0×10^8	0	0	3.2×10^8	2.0×10^8
2	0	0	$4.5x10^8$	$3.6x10^8$	0	0	2.4×10^8	2.3×10^8	0	0	5.7×10^8	5.1×10^8
6	0	0	$4.7x10^8$	$5.3x10^8$	0	0	3.0×10^8	2.1×10^8	0	0	ND	5.2×10^8
12	0	0	$2.9x10^8$	$2.7x10^8$	ND	ND	ND	ND	0	0	6.6×10^8	6.9×10^8
24	0	0	$3.1x10^8$	$2.7x10^8$	0	0	2.0×10^8	1.7×10^8	0	0	5.3×10^8	6.8×10^8
72	ND	ND	ND	ND	0	0	2.1×10^8	1.5×10^8	0	0	4.3×10^8	4.6×10^8
105	ND	ND	ND	ND	ND	ND	ND	ND	0	0	4.4×10^8	5.4×10^8

a. Bacteriophage T4 samples in SM buffer were exposed to TeaF [75% TeaF (v/v)]. At each time point, dilutions were made using samples from each control and treatment tube. Aliquots of each dilution were mixed with *E. coli* and plated to enumerate the number of surviving viable phage (PFU).

b. Mycobacteriophage CrimD samples in 1X Phage Buffer were exposed to TeaF [75% TeaF (v/v)]. At each time point, dilutions were made using samples from each control and treatment tube. Aliquots of each dilution were mixed with *M. Smegmatis* and plated to enumerate the number of surviving viable phage (PFU).

c. Mycobacteriophage Larva samples in 1X Phage Buffer were exposed to TeaF as described above with phage CrimD.

ND = not determined

Figure 1. Bacteriophage T4 x TeaF Attenuation Assay Abundance

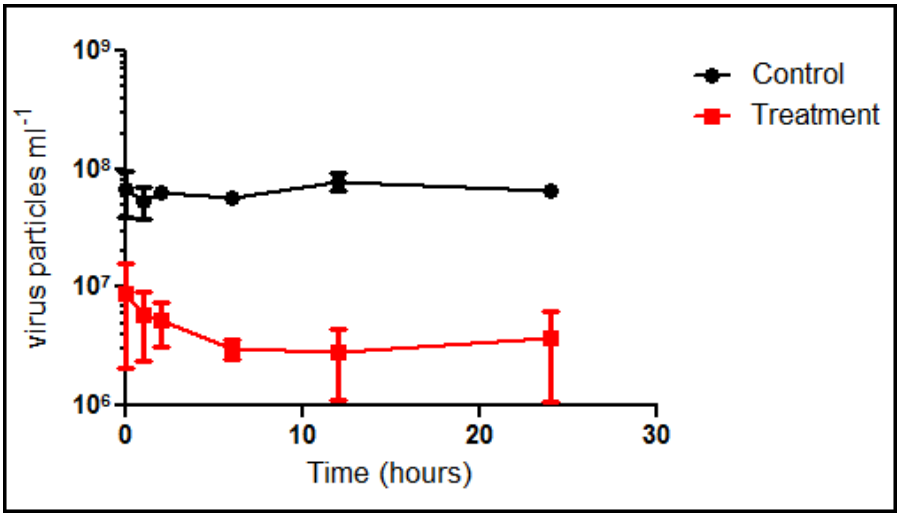

Fig. 1. Average counts of phage particles from EFM. Each point represents the average of two measurements with the standard deviation as indicated. Comparison of treatments and controls with a paired t-test indicated that the effect of TeaF treatment was significant ($p = 0.002$).

Figure 2. Mycobacteriophage Larva x TeaF Phage Attenuation Assay Abundance

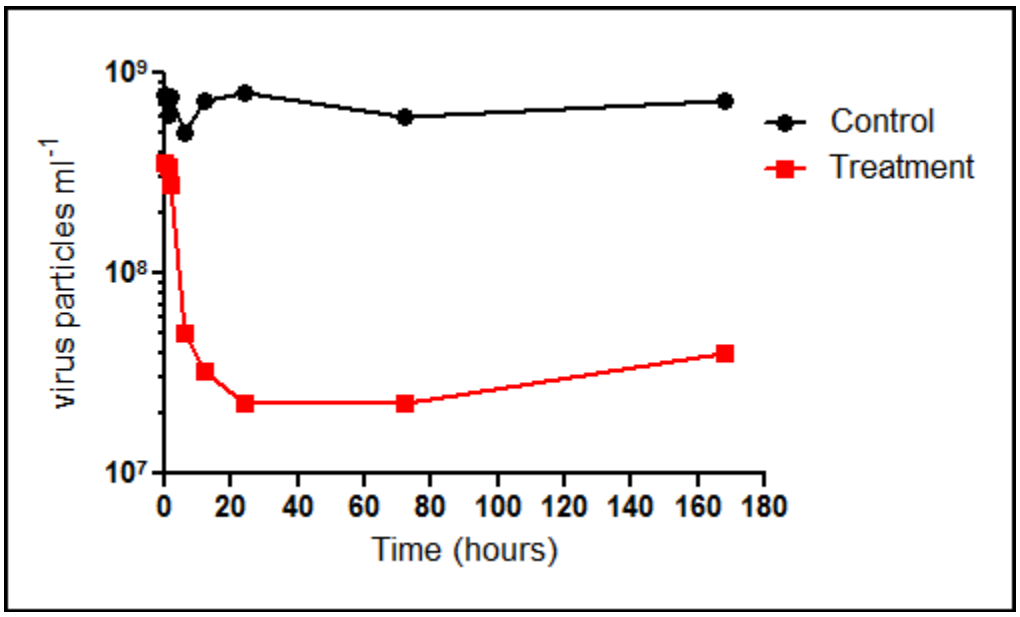

Fig. 2. Average counts of phage particles from EFM. Each point represents the average of two measurements with the standard deviation as indicated. A paired t-test indicated that the effect of TeaF treatment was highly significant ($p < 0.0001$).

study. Overall, TeaF decreased the number of bacteriophage T4 particles in solution by over 10-fold within two hours of exposure (Paired t-test, $p = 0.0020$).

Similarly, TeaF degraded particles of Mycobacteriophage Larva. Results showed significantly fewer countable Mycobacteriophage Larva particles immediately after treatment with TeaF, when compared to controls (Paired t-test, $p < 0.0001$) (Figure 2). Viral abundance of controls averaged 6.89×10^8 mL^{-1} and did not change significantly over the course of the seven day experiment (Two-way ANOVA, $p = 0.3815$). Treatment with TeaF resulted in an immediate decrease in Mycobacteriophage Larva particles: at t_0 there were 3.93×10^8 mL^{-1}. After six hours of exposure to TeaF, the number of countable particles decreased to about 5×10^7 mL^{-1}. For the remaining five sampling points, viral abundance varied marginally, with an average of 3.93×10^7 viruses mL^{-1}. As in the TeaF x T4 phage attenuation assay, TeaF was shown to actively degrade Mycobacteriophage Larva particles in a rapid fashion. It should be noted that while TeaF effectively degraded both T4 and Larva particles, after 24 hours of exposure Larva particles had undergone roughly a 100-fold decrease, whereas TeaF only reduced T4 particles by about 10-fold. This evidence suggests that the virucidal impact of TeaF may not be uniform across all families of bacteriophage.

In the third and final phage attenuation assay, TeaF significantly decreased the number of Mycobacteriophage CrimD particles in solution (Paired t-test, $p = 0.0002$) (Figure 3). Immediately after exposure, TeaF reduced the number of phage particles in solution by roughly 5-fold relative to untreated controls. After this initial drop, phage particle abundance from TeaF treated samples did not vary significantly for the remainder of the three day experiment and averaged 7.64×10^7 ml^{-1} (Two-way ANOVA, $p = 0.4373$). The average abundance for control

Figure 3. Mycobacteriophage CrimD x TeaF Phage Attenuation Assay Abundance

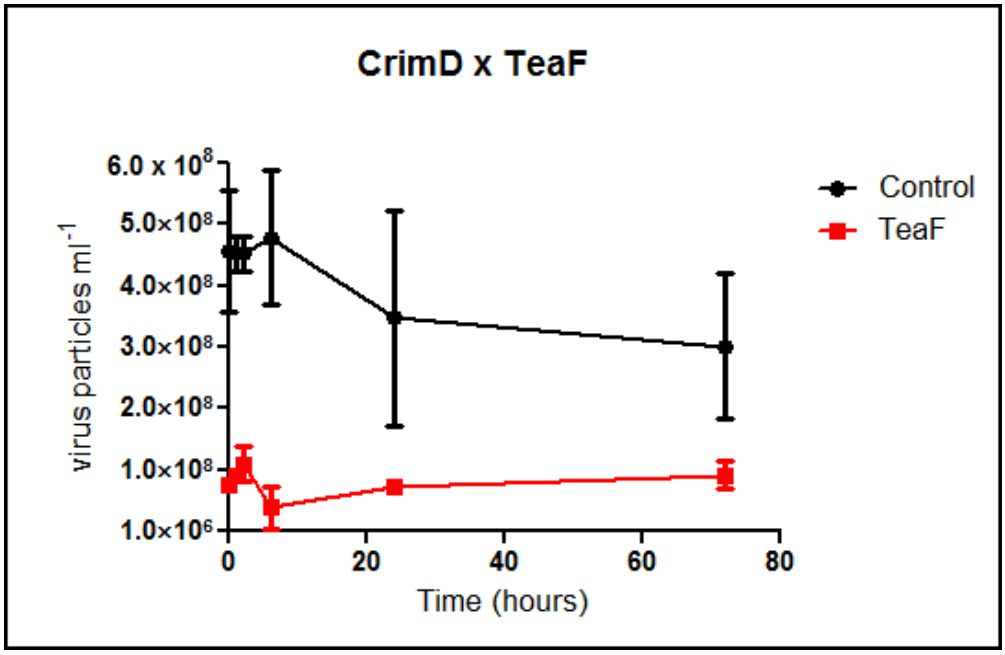

Fig. 3. Average counts of phage particles from EFM. Each point represents the average of two independent experiments with standard deviation as indicated. A paired t-test found that the effect of TeaF treatment was significant (p = 0.0002).

Figure 4. Sensitivity of *Escherichia coli* to TeaF

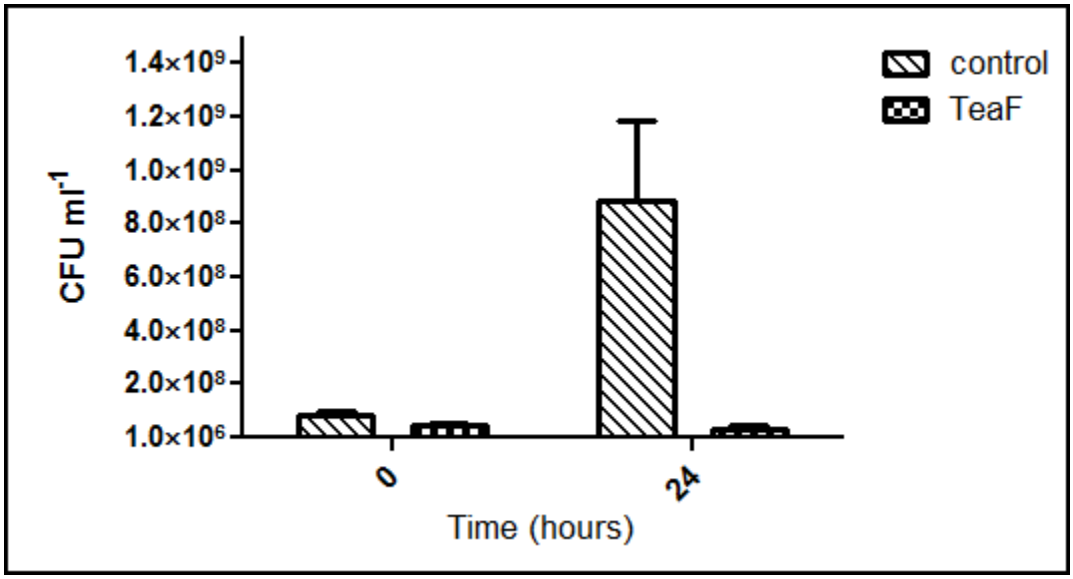

Fig. 4. Effects of TeaF on *E. coli* were considered significant (Two-way ANOVA, p = 0.0167). While untreated controls increased in CFU over the 24 hour period incubation, it should be noted that TeaF did not have a marked negative impact on *E. coli.*

samples was 4.14 x 10^8 ml^{-1} and did not fluctuate significantly during the assay (Two-way ANOVA, $p = 0.8558$).

While these studies fall short of proving that TeaF is a broad-spectrum virucidal, they do expand our knowledge of TeaF virucidal activity. TeaF rapidly inactivated and degraded T4, Mycobacteriophage Larva, and CrimD. This provides evidence that TeaF can inactivate phages of two different families and certainly does not rule out the possibility that TeaF is capable of inactivating a large number of bacteriophage types. Additionally, while the exact mechanism by which TeaF exerts its virucidal activity is unknown, the results of this study show that TeaF degrades phage particles. It is, however, highly unlikely that degradation is the sole means by which TeaF renders bacteriophages noninfectious. At any given time point in the phage attenuation assays, bacteriophage abundance within treatments, as determined by EFM direct counting, was above 10^6 mL^{-1} and exceeded the limit of detection by plaque assays for the phage-host systems used here. Thus, TeaF immediately inactivated or interfered with phage adsorption of all particles and rapidly degraded susceptible subpopulations of virions for each of the phage strains tested. Furthermore, the rapid inactivation of Mycobacteriophages Larva and CrimD, which were native to the sites of prospective field studies, supported the idea that TeaF could be successfully deployed at the field scale for studying top-down control by phages in local soil microbial communities.

Bactericidal Assays

One of the overarching goals of the phage attenuation assays was to assess the virucidal efficacy of TeaF before potential use in studies of bacteriophage predation in terrestrial ecosystems. It would be counterproductive to have a virucidal agent that provided any direct

benefit or harm to the microbial population, as this would make it impossible to distinguish between these direct effects of TeaF on the microbial population and any indirect effects of TeaF via alleviation of bacteriophage predation. Thus, bactericidal assays were a necessary check before applying TeaF in the field. When exposed to TeaF, cultures of host bacteria *Escherichia coli* (T4 host) failed to grow over the course of the 24 hour experiment and analysis of CFU data via two-way ANOVA confirmed that TeaF had a significant inhibitory effect on *E. coli* growth ($p = 0.0167$) (Figure 4). While TeaF-treated *E. coli* cultures failed to match the growth of the control cultures, it should be noted that TeaF did not have a clear bactericidal effect. In contrast to the inhibitory effects of TeaF on *E. coli*, *Mycobacterium smegmatis* (Larva and CrimD host) did not appear to be negatively or positively impacted by treatment with TeaF (two-way ANOVA, $p = 0.7584$) (Figure 5). Thus, it seemed reasonable to conclude that any bacterial response to TeaF treatment in the field would be due to alleviation of top-down control by phages and not due to direct impact of TeaF on bacterial metabolism.

Enzymatic Assays and Assessment of Bottom-Up Control in Soil Microbial Communities

Enzymatic assays performed on the Arctic soil ecosystem showed that microbes at the field sites were not substrate limited and that these soils were rich in available nutrients (Allen et al., 2010). Accordingly, nutrient and substrate amendments did not produce a significant increase in microbial abundance or respiration at the Arctic site. The reduction of autochthonous virus abundance, however, did produce a significant increase in both microbial respiration and abundance. Thus, in the absence of bottom-up controls, the growth and activity of bacterial species living the Arctic system appear to be primarily limited by top-down controls via phage predation. Similar enzymatic assays performed at the two temperate sites chosen for the present

study revealed that, unlike the Arctic soils, both temperate soils were both carbon and phosphorus limited (Table 2).

Microbial glucose production increased significantly when upland soils were treated with carboxymethylcellulose, a soluble cellulose analog (Unpaired t-test, $p = 0.04$) (Table 2). Likewise, treatment of wetland soils with carboxymethylcellulose resulted in an increase in microbial glucose production, although not significant ($p = 0.48$). Thus, at both the upland and wetland sites, microbial metabolism was limited by substrate availability (as indicated by the increase in glucose production upon the addition of carboxymethylcellulose). The upland site exhibited statistically significant carbon deficiency.

Measurable levels of alkaline phosphatase within both the wetland and upland soils indicated that soil bacteria were actively scavenging for phosphorus (Table 2). Similar to the results of the glucose assay, upland site phosphorus deficiency exceeded that of the wetland site, as indicated by the higher activity of alkaline phosphatase within upland soils.

These indications of substrate and nutrient limitations bring to light the fact that microbial dynamics within the temperate soils examined in this study are potentially more complex than those of the Arctic soil system studied by Allen et al. (2010). As displayed by the enzymatic assays, microbes within the temperate soils are most likely limited by substrate availability, a bottom-up control. Thus, it may be more difficult to detect and quantify the impacts of bacteriophage predation against the background of substrate and nutrient limitations presented by the temperate soils of this study.

Figure 5. Sensitivity of *Mycobacterium smegmatis* to TeaF

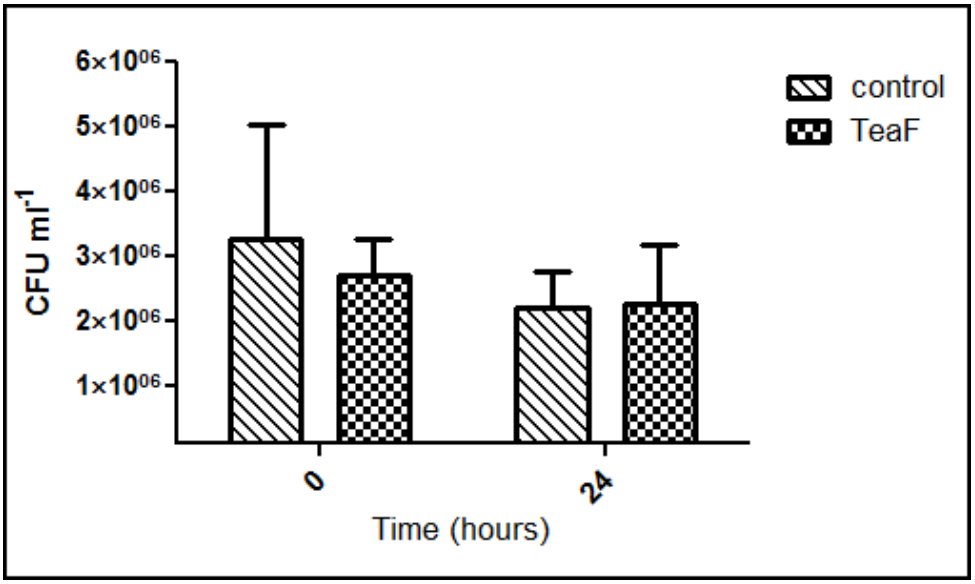

Fig. 5. Effects of TeaF on *M. smegmatis* were not considered significant (Two-way ANOVA, $p = 0.7584$)

Table 2. Enzymatic Assays

	Upland	Wetland
Glucose production (µg C g^{-1} h^{-1})		
(-) carboxymethylcellulose	45.78	6.81
(+) carboxymethylcellulose	58.77	8.51
ratio	0.78	0.80
p-value[a]	0.04	0.48
Alkaline phosphatase activity[b] (pmol mL^{-1} h^{-1})	592.18	307.26

a. Statistical significance was determined by testing differences between amended and non-amended samples with an unpaired t-test (n=5 in each group).

b. Alkaline phosphatase activity was measured through colorimetric determination after reacting with *p*-Nitrophenyl Phosphate for one hour. Measurable levels of alkaline phosphatase indicate that soil microbes are limited by phosphorus.

Upland Field Study

Treatment of upland soils with TeaF did not significantly impact microbial respiration (Figure 6). Respiration within soil collars that were nutrient (NPK) amended was similarly unaffected. Collars that received extra carbon in the form of ethyl acetate, however, displayed significantly higher respiration than control collars over the course of the eight day experiment

Figure 6. Upland Soil Microbial Respiration

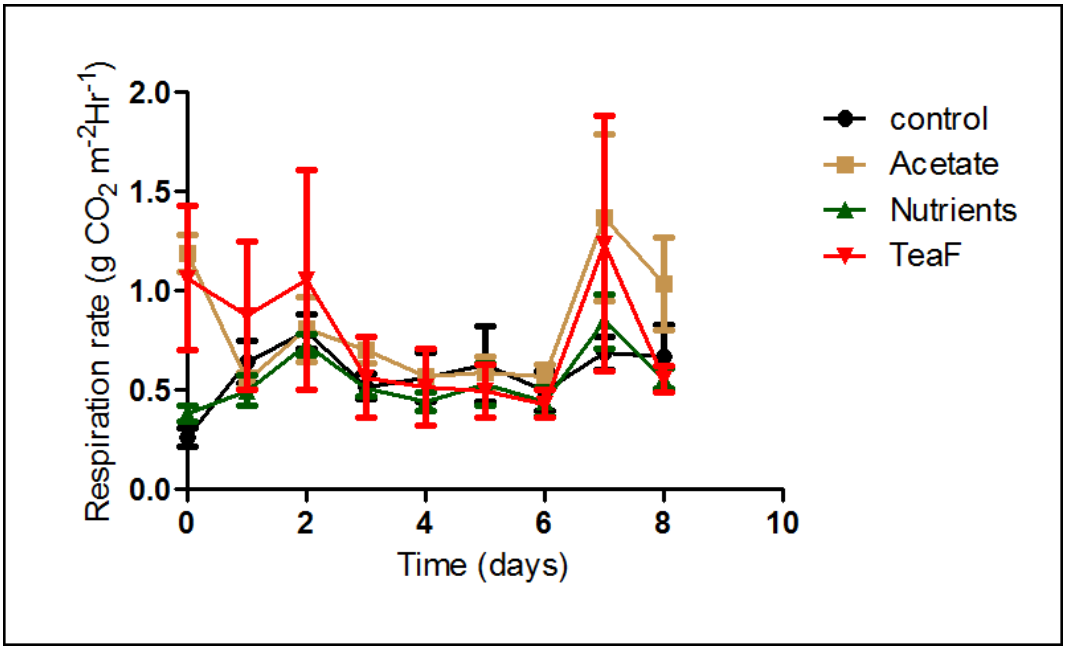

Fig. 6. Respiration rates in upland field site experiment for different treatments over eight days. Each point before day four of the study represents the average of six independent experiments. Due to the harvesting of half of the soil collars for abundance studies at day three, points representing data from days four through eight of the experiment are the average of three independent experiments. Error bars indicate standard deviation. Repeated measures ANOVA revealed that treatments had a significant effect on microbial respiration (p = 0.0148). Subsequent Dunnett's multiple comparison test showed that only acetate collars varied significantly from the control.

(Repeated measures ANOVA, $p = 0.0148$ with Dunnett's multiple comparison test). These results suggest that soil microbes at the upland site are significantly limited by carbon substrate availability. Additionally, directly after administration of treatments, TeaF and acetate treated collar respiration rates were significantly higher than the rates observed in nutrient and water amended collars (One-way ANOVA of day 0 data, $p < 0.0001$). This early elevated respiration in TeaF collars may be telling of an immediate microbial response to alleviation of phage predation that was then obscured by the dynamic nature of phage-host interactions. If TeaF inactivated phage immediately upon application to the upland soil, the immediate increase in microbial respiration suggests that phage predation is a significant source of control over the soil microbes.

The fact that microbial respiration did not remain elevated in TeaF-treated collars could be indication of finely-tuned viral response to host availability. It is possible that, after the initial reduction of infective phage particles via TeaF, soil microbes were under fewer constraints and grew (as indicated by elevated respiration), therefore increasing host availability for the infectious phage that remained in the soil after TeaF treatment, enabling the reestablishment of phage-mediated control over microbes by day three of the experiment, when abundance samples were taken. Such a hypothesis would have been strengthened by the observation of similar trends in microbial respiration at the wetland site, but this was not the case. At the wetland site, TeaF did not produce a significant increase in microbial respiration on day 0. On day seven respiration rates in all collars increased, with the greatest increase occurring in TeaF and acetate treated collars. The general increase in respiration that was seen in all collars was most likely in response to the rainfall occurring between respiration sampling on day six and before the elevated measurements taken on day seven. It is also likely that the exaggerated increase in microbial respiration within TeaF and acetate treated collars was indicative of a state of

metabolic fitness that was achieved due to the alleviation of top-down control by phages and bottom-up control from substrate limitations.

Direct counts of viral and bacterial abundance were performed via EFM with samples taken from soil collars on days three and eight of the experiment. One-way ANOVA of viral abundance data revealed that viral abundance was not significantly different across treatments, including the putative virucidal, TeaF ($p = 0.5821$) (Figure 7). Similarly, treatments had no significant effects on bacterial abundance (Figure 8). These results starkly contrasted those documented by Allen et al. (2010) in the Arctic study, suggesting that either none of the treatments induced changes in concentrations of phage, nutrients, or carbon, or that none of these factors play a significant role in determining microbial dynamics.

In addition to measures of respiration and direct enumeration, measures of microbial biomass carbon taken from day eight of the upland study show that TeaF did not have a significant impact on the growth of soil microbes (Figure 9). Microbial biomass carbon was not found to be significantly different across treatments (One-way ANOVA, $p = 0.0176$).
In the Arctic study, TeaF treatment effectively reduced soil bacteriophage abundance. This reduction in autochthonous phage was paralleled by an increase in microbial respiration and bacterial cell abundance, implying that phages exert significant control over microbial dynamics in the Arctic soil ecosystem (Allen et al., 2010). In the temperate upland soil study presented here, TeaF failed to significantly decrease native bacteriophage populations and had no impact on the microbial respiration (barring a fleeting effect on day 0) or abundance, nor any impact on the concentration of microbial biomass carbon.

One of the major differences in soil characteristics between these two studies is soil water content and saturation. It is possible that the main confounding factor between these experiments

is the saturation level of the soils. Bacteriophages depend on random collisions with host cells to propagate infections. In an aqueous setting, this random diffusion is an effective means of transport because viruses are suspended in solution. In porous media such as soils and sediments, however, movement of phages within soil depends on pore space and the saturation level of the soil, with saturation increasing viral mobility (Shein and Devin, 2006). The Arctic permafrost

Figure 7. Upland Soil Virus Particle Abundance

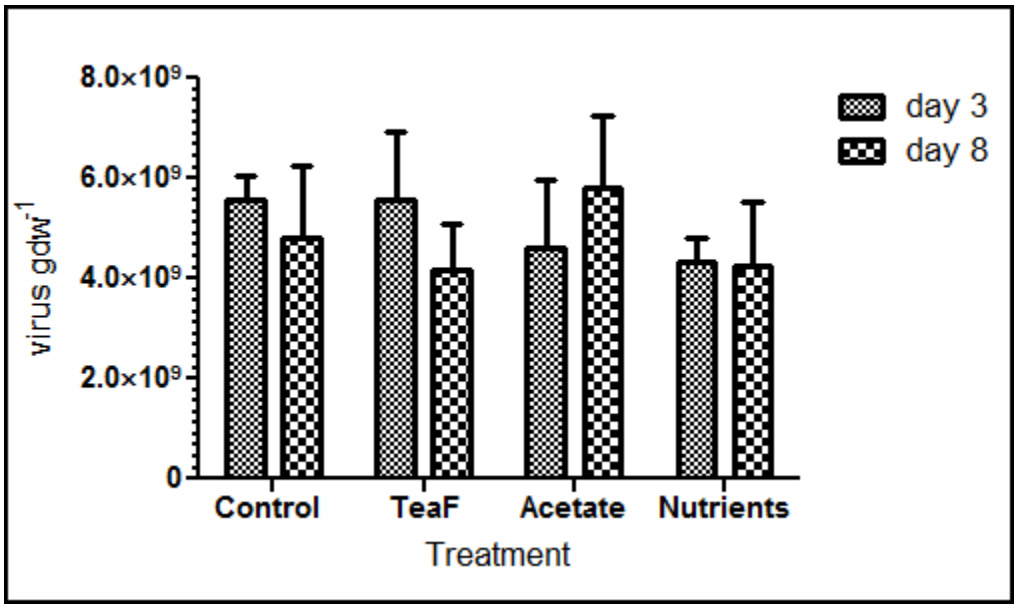

Fig. 7. Average counts of phage by EFM from upland soil collars after treatment. Each bar represents the average of three measurements with standard error as indicated. Differences in viral abundance across treatments was not significant by one-way ANOVA (p = 0.5821).

Figure 8. Upland Soil Bacterial Abundance

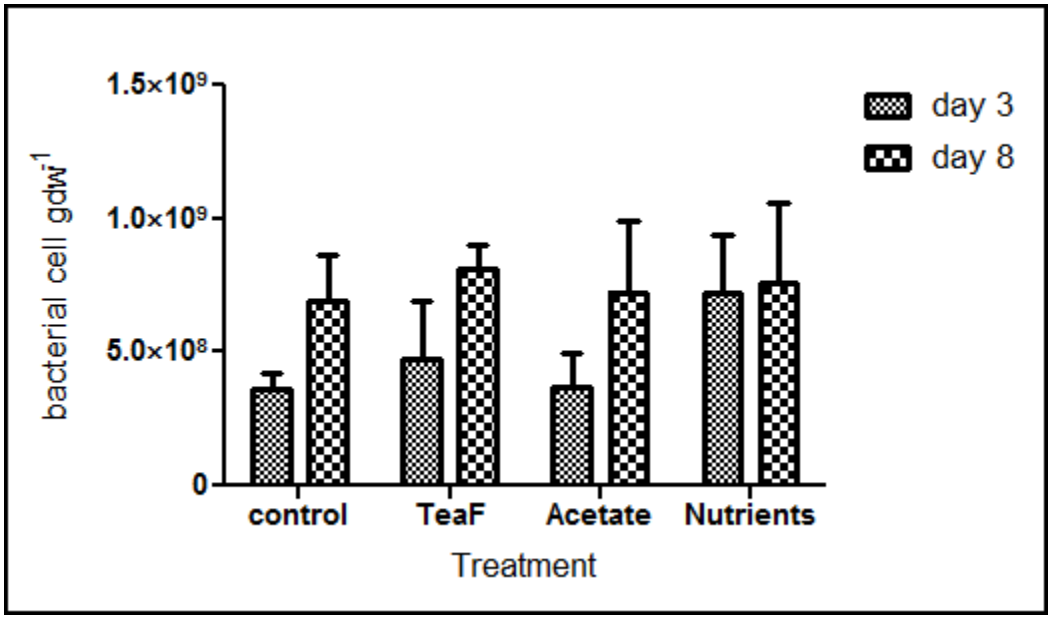

Fig. 8. Average counts of bacterial cells by EFM from upland soil collars after treatment. Each bar represents the average of three measurements with standard error as indicated. Differences in bacterial abundance across treatments was not significant by one-way ANOVA (p = 0.7373).

Figure 9. Upland Soil Microbial Biomass Carbon

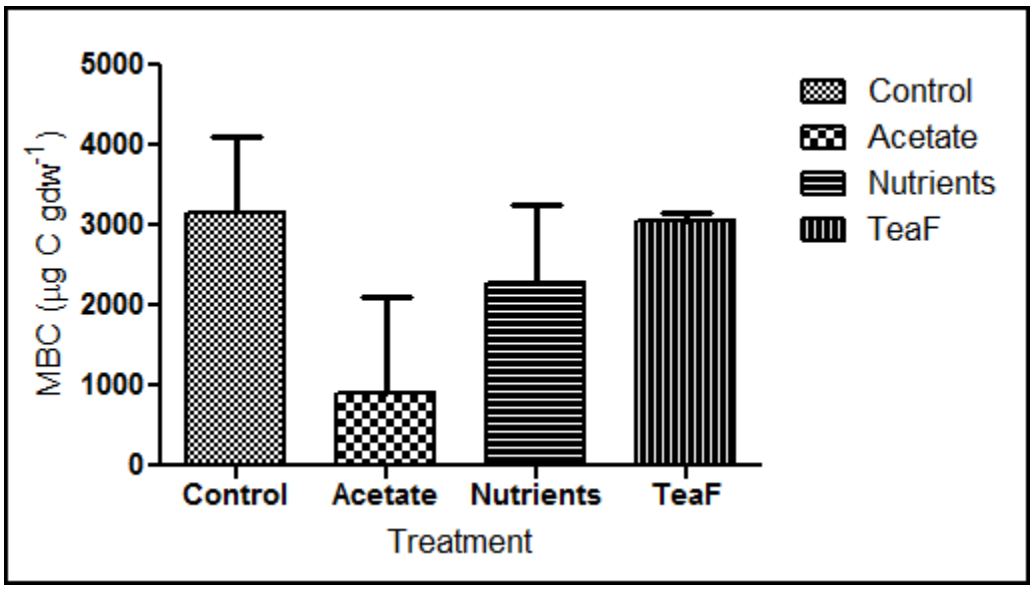

Fig. 9. Microbial biomass carbon measurements from chloroform fumigation. Each bar represents the average of three independent measurements with standard error as indicated. Differences in MBC across treatments was not significant in one-way ANOVA (p = 0.0725).

studied by Allen et al. (2010) was a saturated system with water content reaching 1000%, while average water content of the soil at the upland site was 15%. Thus, the exceedingly high water content of the Arctic permafrost soil potentially facilitated a greater rate of bacteriophage transport and infection, as well as a system in which treatments contacted their target (phages or microbes) for a period of time that was sufficient to effect change. By contrast, the lower water content of the upland soil likely impeded phage transport and contact with host bacteria. Furthermore, the porous sandy soil allowed for liquid treatments to quickly pass through surface layers – a phenomenon that was explored in detail later in this study.

Wetland Field Study

In an effort to test the hypothesis that soil water content is a key factor in controlling bacteriophage dynamics and impacting the efficacy of TeaF treatments, the upland study was duplicated at a wetland site (average water content 36%).

Although analysis of respiration data with repeated measures ANOVA indicated that treatments had a significant impact on microbial respiration ($p = 0.0008$), a subsequent Dunnett's multiple comparison test revealed treatments did not vary significantly from the control (Figure 10). Therefore TeaF did not facilitate an increase in microbial respiration. In contrast to the upland respiration results, treatment of wetland soils with acetate did not result in significant increase in microbial respiration. These results fell in-line with the findings of the enzymatic assays which suggested that the wetland site was less substrate-limited than the upland site.

Viral abundance studies performed on day three and day eight of the wetland experiment showed that treatment of soil with TeaF failed to significantly affect viral abundance. Additionally, acetate and nutrient treatments did not have any significant impact on viral

abundance (one-way ANOVA, $p = 0.2235$) (Figure 11). As in the upland experiment, treatment of soils with TeaF, acetate, and nutrients did not impact microbial abundance in any way that resulted in significant deviation from the control addition of sterile water (one-way ANOVA, $p = 0.6351$) (Figure 12).

Figure 10. Wetland Soil Microbial Respiration

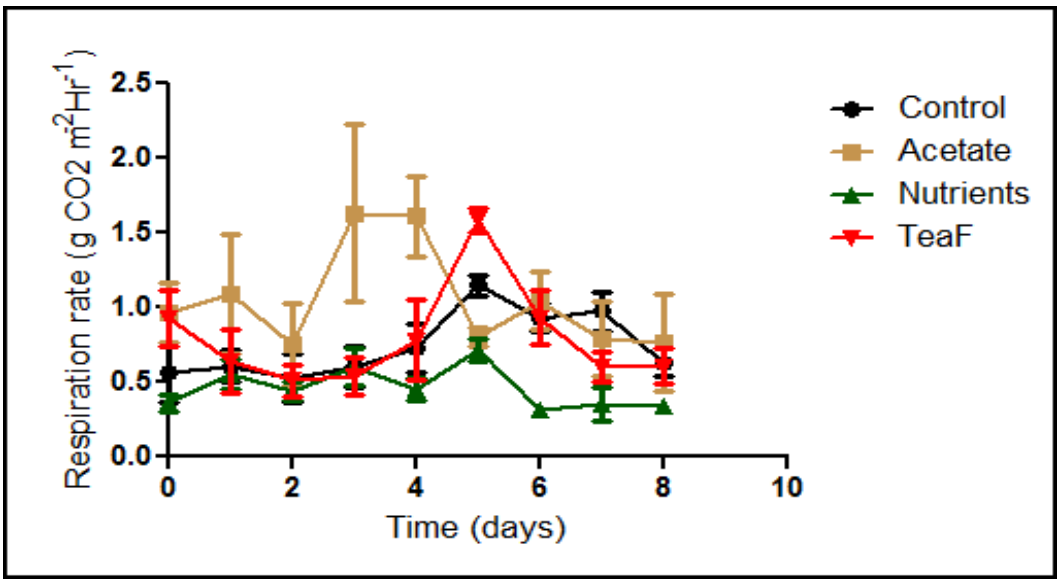

Fig. 10. Respiration rates in wetland field site experiment for different treatments over eight days. Each point before day four of the study represents the average of six independent experiments. Due to the harvesting of half of the soil collars for abundance studies at day three, points representing data from days four through eight of the experiment are the average of three independent experiments. Error bars indicate standard deviation. The effect of all treatments was considered significant by repeated measures ANOVA ($p = 0.0008$). Subsequent analysis of data with Dunnett's multiple comparison revealed that no treatment varied significantly from the control.

Figure 11. Wetland Soil Virus Particle Abundance

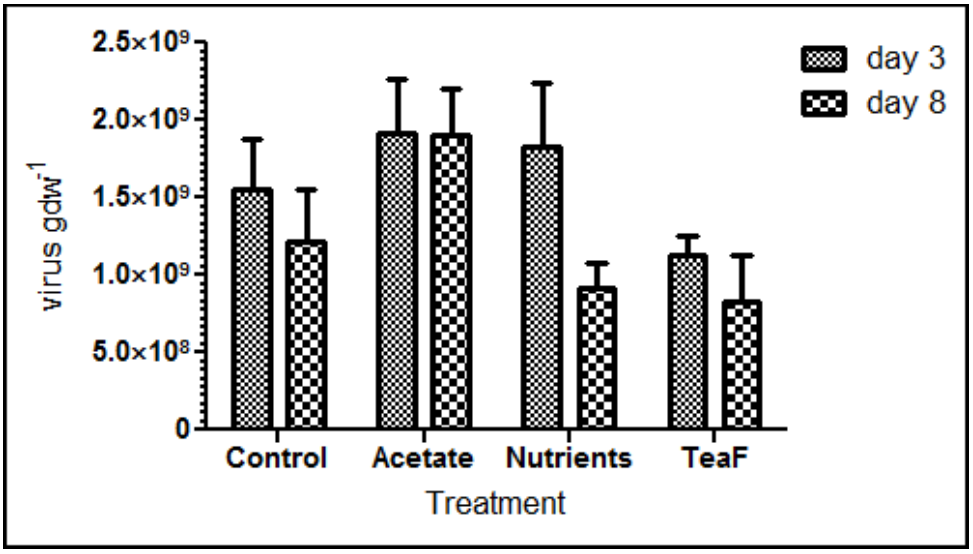

Fig. 11. Average counts of virus particles by EFM from wetland soil collars after treatment. Each bar represents the average of three measurements with standard error as indicated. Differences in viral abundance across treatments was not significant by one-way ANOVA (p = 0.2235).

Figure 12. Wetland Soil Bacterial Abundance

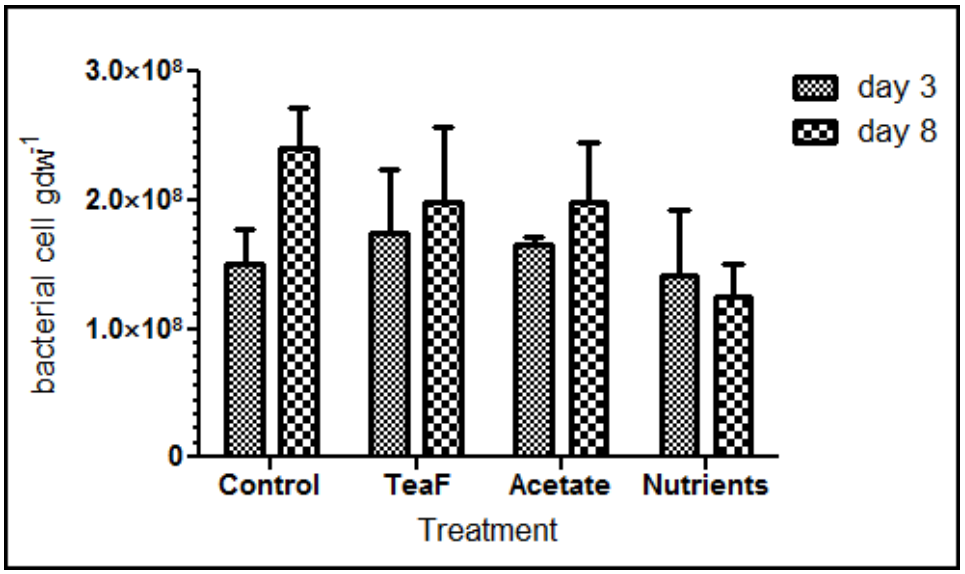

Fig. 12. Average counts of bacterial cells by EFM from wetland soil samples after treatment. Each bar represents the average of three independent measurements with standard deviation as indicated. Differences in bacterial abundance across treatments were not significant by one-way ANOVA (p = 0.6351).

Analysis of microbial biomass carbon from soil samples harvested on day eight of the wetland experiment provided further evidence that TeaF did not relieve any top-down control of phages over bacteria, as the addition of TeaF to wetland soils resulted in no marked change in carbon incorporated into microbial biomass (Figure 13). Other treatments (acetate and nutrients) also failed to facilitate significant changes in microbial biomass.

Consideration of both the upland and wetland experimental results raises the possibility that TeaF, although capable of rapidly inactivating and degrading phage particles in the lab and in Arctic soils, may not exhibit virucidal properties when applied to temperate soils. Based on EFM data, exposure of soil viruses to TeaF at both of the field sites caused no significant decrease in total viral abundance. The failure of TeaF to degrade virus particles in the temperate soils tested may be a result of insufficient treatment volume, insufficient contact time between soil viruses and TeaF, or perhaps viral resistance to the as of yet unknown virucidal mechanism of TeaF. Importantly, it is feasible that TeaF rendered phages within the soil non-infective, despite the solution's failure to degrade phage particles in wetland and upland soils. If the latter scenario is correct, the results suggest that phage predation is not a significant means of control within the terrestrial microbial communities monitored. In an effort to further assess the impacts of TeaF application to temperate soils, the upland field site was revisited for a more extensive assessment of TeaF effects on terrestrial bacteriophages and microbes.

Experimental Application of TeaF in Varied Volumes to Upland Soils

After conducting the initial field studies, a second field experiment was carried out in late July 2011 at the upland site in which eighteen soil collars were treated with various volumes of TeaF or sterile water. Collars were treated in triplicate with either 80, 160, or 240 mL of TeaF or

Figure 13. Wetland Soil Microbial Biomass Carbon

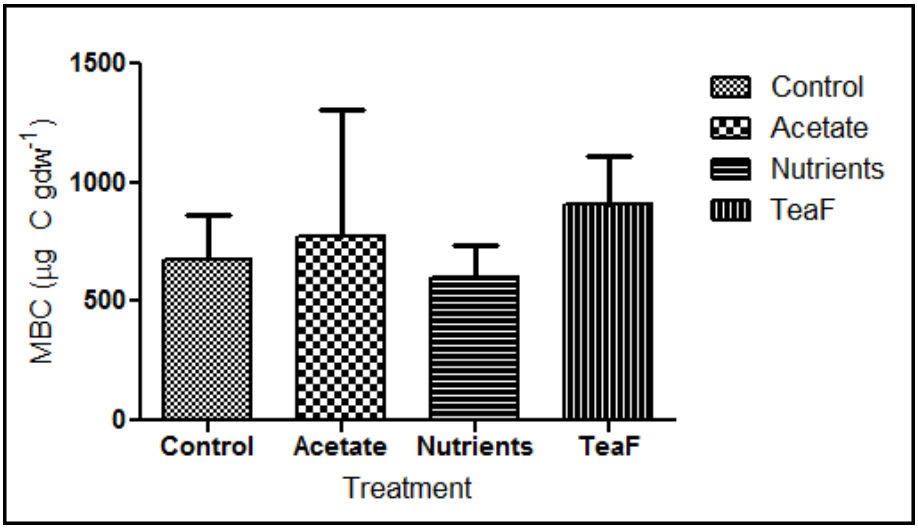

Fig. 13. Microbial biomass carbon measurements from chloroform fumigation. Each bar represents the average of three independent experiments with standard deviation as indicated. A one-way ANOVA found the differences in MBC across treatments were not significant (p = 0.7048).

Figure 14. Upland Soil Varied Volumes Experiment Viral Abundance

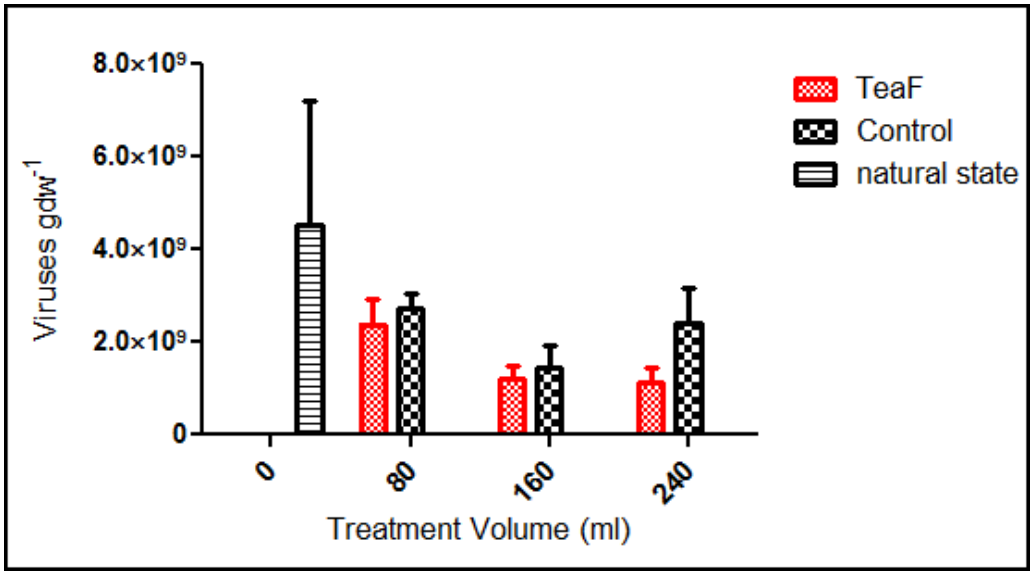

Fig. 14. Average counts of virus particles via EFM from upland soils after three days of incubation with treatments of various volumes. Each bar represents the average of three separate experiments with standard error as indicated. Differences in viral abundance across treatments were not significant by two-way ANOVA (p = 0.4353), while the volume of liquid added to the collars was significant (p = 0.0053).

equivalent volumes of sterile water. All treatments were rapidly absorbed by the soil, with 80 mL treatments barely moistening the entirety of the soil surface exposed within the collar.

Respiration measurements were taken immediately after treatment of collars and at 24 hour intervals for three days following. Due to a mishap with storage of data on the internal memory of the portable infrared gas analyzer, this respiration data was lost. After three days of incubation, soil collars were harvested for enumeration of bacterial cells and virus particles. Viral abundance decreased significantly as treatment volume increased (Two-way ANOVA, p = 0.0053). However, this decrease was a function only of increasing volume, and not significantly linked to treatment with TeaF (p = 0.4353) (Figure 14). Additionally, bacterial abundance was not significantly affected by treatment with TeaF (p = 0.1168) (Figure 15). These results suggest that, regardless of chemical composition, addition of liquid to soil collars may have washed phage particles out of surface soil that was sampled for abundance analysis.

A second investigation of the impacts of TeaF on bacterial and phage populations was conducted at the upland site in early September 2011. This experiment was altered slightly from the previous varied volumes experiment conducted in late July 2011. On the third and final day of the experiment, a soil core was taken from each of the collars, rather than sampling surface soil with a trowel. Coring allowed for preservation of the soil structure and enabled the collection of abundance data from samples at the ground surface as well as six inches below the surface.

Comparisons of viral and bacterial abundance from surface soils with bacterial and viral abundance six inches below the surface provided evidence for the washout hypothesis (Figures 16 and 17). Three untreated soil cores taken from within the field site showed that before treatment, surface soil viral abundance averaged 6.9×10^{13} gdw^{-1}. Six inches below the surface, viral abundance averaged 5.58×10^{12} gdw^{-1}. Treatment of soil with either 80, 140, or 200 mL of

Figure 15. Upland Soil Varied Volumes Experiment Bacterial Abundance

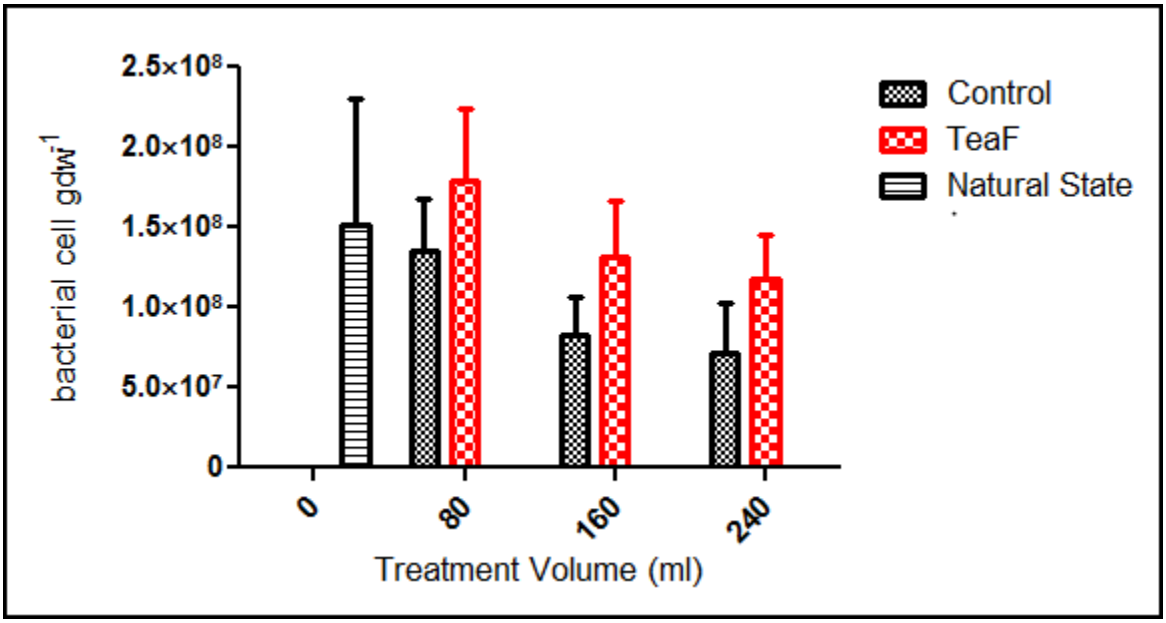

Fig. 15. Average counts of bacterial cells via EFM from upland soils after three days of incubation with treatments of various volumes. Each bar represents the average of three independent experiments with standard deviation as indicated. Two-way ANOVA found no significant differences across treatments on the abundance of bacterial cells (p = 0.1168).

Figure 16. Upland Soil Coring Experiment Viral Abundance

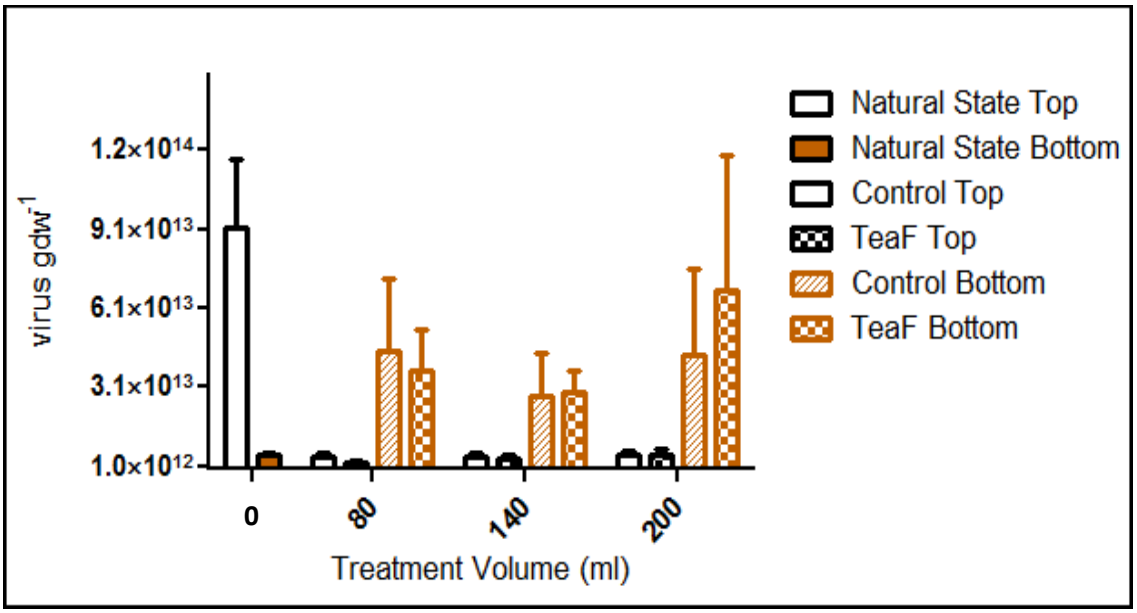

Fig. 16. Average counts of viral abundance via EFM from the top and bottom of upland soil cores harvested after a three day incubation with varying volumes of treatment. Each bar represents the average of three independent experiments, with standard deviation as indicated. Analysis of data via two-way ANOVA revealed a highly significant interaction between treatment (TeaF or sterile water) and treatment volume ($p = 0.0003$). Bonferroni posttests indicate that all treatments (TeaF and sterile water at all volumes) resulted in a significant reduction in phage abundance in the top of the core ($p < 0.01$) in comparison to abundance in the top of untreated cores.

Figure 17. Upland Soil Coring Experiment Bacterial Abundance

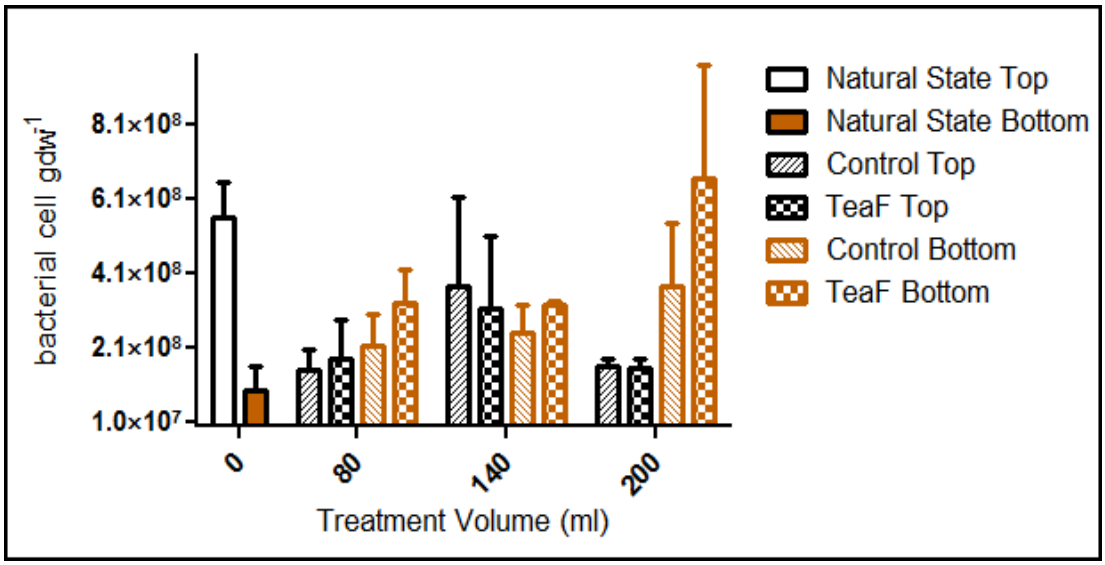

Fig. 17 Average counts of bacterial abundance via EFM from the top and bottom of soil cores harvested after a three day incubation with varying volumes of treatment. Each bar represents the average of three independent experiments, with standard deviation as indicated. Analysis of bacterial abundance data via two-way ANOVA revealed a highly significant interaction between treatment volume and treatment ($p = 0.0002$). Subsequent Bonferroni posttests showed that 80 mL treatments resulted in a significant reduction in bacterial abundance ($p < 0.01$ for 80 mL control top and $p < 0.05$ for 80mL TeaF), 140 mL treatments did not significantly impact bacterial abundance ($p > 0.05$), and 200 mL treatments resulted in significant decrease in bacterial abundance in the top of cores ($p < 0.01$ for 200 mL sterile water control and 200 mL TeaF). For TeaF treated collars, the significant decrease in phage in the top of the core was paired with a significant increase in bacterial abundance in the bottom of the core ($p < 0.001$).

TeaF or sterile water resulted in a significant decrease in the number of virus within surface soil (Two-way ANOVA with Bonferroni posttests, $p = 0.0003$). The decrease of virus particles in the top of soil cores was paralleled by an increase in viral abundance at the bottom of cores that was proportional to the volume of treatment added (Figure 16). Similarly, 80 and 200 mL treatments of both TeaF and sterile water significantly decreased the amount of bacteria within surface soil samples (Two-way ANOVA with Bonferroni posttest, $p = 0.0002$) (Figure 17). The lack of a significant impact of 140 mL treatments is most likely due to sampling error associated with sampling from slightly different levels within in each core for extraction procedures, as discussed earlier in the methods. Much like the trend seen in viral abundance, the decrease in bacterial cells at the top of the core was paralleled by increased bacterial abundance at the bottom of the core. This increase was most apparent when soil was treated with 200 mL of TeaF or sterile water (the maximum volume tested in this study).

The viral abundance data presented here further indicates that TeaF is ineffective at degrading virus particles within soils. Rather than degrading viruses as in the laboratory assays, TeaF appears to simply fill pore space and facilitate downward mobility of viruses (and bacteria) in the field. These results stand in stark contrast to those published by Allen et al. (2010) which showed that TeaF significantly decreased the total number of virus particles within Arctic soils. A host of factors could contribute to the discrepancy between the effectiveness of TeaF in Arctic versus temperate soils. The most obvious confounding factor, as aforementioned, is the differing levels of saturation between the temperate soils of this study and the Arctic soil. The relatively low saturation and high sand content of the wetland and upland sites facilitated flow-through of treatments into soils, carrying phages and bacteria deeper into the soil. It is possible that the flow-through was so rapid in these temperate soils that the virucidal mechanism of TeaF, which

remains unknown at this point, did not have sufficient time to interact with and degrade virus particles. Although the phage attenuation assays showed inactivation of phage stock upon exposure to TeaF at t_0, it is important to remember that there was a small unavoidable time lag between when samples were taken from exposures and when they were plated. This small window of time (ten minutes or less), may have inadvertently served as the crucial incubation time for TeaF to degrade and deactivate phage. Thus, the porosity of the soil may have prevented TeaF from interacting with bacteriophages for long enough to exert virucidal effects.

In short, treatment of soils with acetate, nutrients, TeaF, or sterile water did not give rise to significant changes in microbial abundance, respiration, or biomass within the two temperate soils tested in this study. These results are somewhat surprising, as a previous study showed that temperate soil bacteriophages and microbes are dynamic and respond rapidly to environmental changes such as substrate availability (Srinivasiah et al., 2008). It is likely that the results presented here are largely an artifact of methods that failed to deliver treatments in a fashion appropriate for unsaturated soils. If such is the case, then these results put us no closer to understanding the dynamics of temperate soil microbial communities.

As aforementioned, enzymatic assays performed on the two temperate soils examined in this study indicated that both soils were somewhat limited in carbon and phosphorus, with the upland site being limited to a greater degree than the wetland site (Table 2). In contrast, the Arctic soils studied by Allen et al. (2010) were not substrate deficient, which resulted in a lack of bottom-up controls over soil microbes. In the absence of bottom-up controls, phage predation served as the main factor of limitation over microbial growth. Since the temperate soils of this study are characterized by low concentrations of labile carbon and phosphorus, it is likely that bacterial growth is limited by the availability of nutrients and carbon. The liable presence of

bottom-up controls does not, however, necessarily come at the exclusion of top-down controls exerted by phage predation. However, the simultaneous presence of both types of controls would likely create difficulties in specifically parsing out top-down control by phage predation. It seems plausible that microbes within temperate terrestrial environments such as those of this study are subject to pressure from both bottom-up and top- down sources. Further investigation is necessary to fully explain the interplay between soil microbes, the bacteriophages infecting them, and the bottom-up controls presented by the environment.

Future Considerations

Thus far, TeaF has proven to be an ineffective virucidal in temperate soils, but effective at degrading phages within Arctic soils and several phages in laboratory assays. One hypothesis put forth in this paper for this ineffectiveness was the low saturation of the temperate soils. A microcosm experiment in which a portion of soil from each site is flooded with sterile water to saturation and then treated with TeaF would allow for exploration of this hypothesis. If the low saturation (open pore space) of temperate soils is to blame for TeaF ineffectiveness, then one would expect to see TeaF decrease viral abundance within microcosms that were flooded. Furthermore, one could test this hypothesis by seeding sterile soil with T4 (one of the bacteriophages against which TeaF has shown virucidal activity in the laboratory) and then monitoring abundance of T4 after treatment with TeaF.

The failure of TeaF to degrade virus particles in temperate soils may not be fully explained by low soil saturation. It is possible that TeaF contacted autochthonous viruses for a period of time that was sufficient to degrade virus particles, but failed to so because viruses were resistant to the TeaF virucidal mechanism. As aforementioned, the virucidal mechanism of TeaF

is unknown. If the key virucidal component of the TeaF concoction is the tea, rather than the ferrous sulfate, the fact that the soils used in this study were from oak-hickory forests may be of significance. Oaks are known to produce high levels of tannins, chemical compounds that are also present within tea leaves. Therefore tea-derived tannins are an integral part of the TeaF virucidal mechanism, it is possible that TeaF failed to degrade viruses because the viruses within the oak-hickory forest soil are adapted to withstand high tannin exposure. One might begin to explore this hypothesis by testing the virucidal capacity of tea infusion alone, followed by a test of the virucidal capacity of ferrous sulfate alone. This could be done through application of the phage attenuation assay methods developed earlier in this study for testing the efficacy of TeaF as a laboratory virucidal.

Finally, in an effort to further explore the incongruity of TeaF virucidal activity in the lab versus the temperate field setting, performing a series of consecutive phage attenuation assays with progressively lower concentrations of TeaF would be a worthwhile endeavor. In the laboratory, TeaF immediately inactivated the three bacteriophages tested and degraded a subpopulation of each phage. The exposure solution was 75% TeaF and 25% phage stock. It seems unlikely that such a high concentration of TeaF could be mimicked in a temperate field system in which TeaF treatment rapidly moves through soils. Thus, exploration of the lower limit of TeaF concentration that maintains virucidal activity could provide some insight into the feasibility of using TeaF in temperate soils. It is possible that TeaF works only at concentrations that are unattainable in temperate soils, where treatments freely flow through open soil pore space.

Development of a virucidal that is effective in temperate soils is necessary if we wish to further our knowledge about the interaction of terrestrial bacteriophages and their host microbes

by way of the current approach (the only approach to date). As the results of this study have shown, such a task may be arduous. The virucidal that is effective in one environment may prove ineffective in another. Until we can develop a virucidal that is applicable in temperate field studies, the impact of bacteriophages on the microbes within these environments will remain elusive.

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APPENDIX A

Field Site Soil Characteristics

	% sand	% silt	% clay	% organic matter	pH	% w
Upland	75.2	18.8	6	4.4	4.1	15
Wetland	75.2	16	8.8	5.8	7.6	37