Effects of experimentally elevated virus abundance on soil carbon cycling across varying ecosystem types

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Abstract

Viruses are abundant and diverse members of soil communities, but their influences on soil biogeochemical cycling are poorly understood. To assess the potential for viruses to influence soil carbon (C) cycling in varying environmental contexts, we sampled soils from four contrasting ecosystem types across the continental United States: conifer forest, broadleaf deciduous forest, tallgrass prairie, and agricultural cropland. We then experimentally increased virus abundance in the soils by inoculating microcosms with virus concentrates isolated from the same original soils and incubated the soils for 14 days. The virus-treated conifer forest and prairie soils respired significantly less C (14 µg and 10 µg less C per gram of soil, respectively) over the course of the 14-day incubation compared with control soils, though the effects were proportionally small in magnitude (3% and 6% reductions in cumulative respiration, respectively). Following the initial 14-day incubation, we conducted a ^{13}C-glucose tracer incubation. In contrast to the initial incubation, after glucose addition we observed effects on respiration only in the agricultural soil, where respiration of soil organic matter-derived C nearly doubled in the virus-treated soils compared with control soils. We also observed overall reduced incorporation of ^{13}C into microbial biomass (*i.e.*, lower growth yield) and lower carbon use efficiency on average in all virus-treated soils. These results demonstrate that viruses can influence overall microbial metabolism but with different aggregate effects on soil C balance across soil types depending on soil physicochemical properties. Overall, our study demonstrates that viral influences on soil microorganisms can manifest in altered fates of soil C, with either increased or decreased respiratory C loss depending on ecosystem type.

1. Introduction

Viruses are ubiquitous inhabitants of all natural ecosystems and are the most abundant biological entities on the planet (Kuzyakov and Mason-Jones, 2018). Natural virus populations perform many ecological functions; they are obligate intracellular parasites and therefore regulate the abundance and diversity of their hosts (Weinbauer and Rassoulzadegan, 2004; Barnett and Buckley, 2023; Quirós et al., 2023) as well as rates of host-driven biogeochemical cycles such as the carbon (C) cycle (Kuzyakov and Mason-Jones, 2018). The ecological role of viruses is particularly well-resolved in marine ecosystems, where phages (viruses that infect prokaryotes) kill 20 – 40% of bacteria daily, returning massive amounts of dissolved organic matter (DOM) to the water column (Suttle, 2007). This phenomenon, known as the 'viral shunt', is acknowledged as a major player in marine C and nutrient cycling. Similar mechanisms likely exist in other environments like soils, though the extent to which viruses influence soil biogeochemical cycling is unknown.

Though the influences of viruses on soil biogeochemistry are currently unclear, there is growing interest in the potential ecological roles played by viruses in terrestrial ecosystems (Williamson et al., 2017; Kuzyakov and Mason-Jones, 2018; Emerson, 2019; Sokol et al., 2022). Indeed, many studies have emerged in recent years that investigate the abundance, diversity, and composition of virus communities in various soil contexts. These studies have demonstrated that both the abundance of viruses and the frequency of phage infection of bacterial cells are very high in soil, often exceeding that observed in aquatic environments. For example, phages typically outnumber prokaryotes more than 10-fold in soils (Cobián Güemes et al., 2016), while 30-100% of prokaryotic cells in soils are infected with phages at any given time, depending on the environment (Bowatte et al., 2010; Takahashi et al., 2011). Other studies have demonstrated

that numerous environmental factors can influence the structure of soil virus communities, including soil pH (Lee et al., 2022), soil depth (Liang et al., 2019, 2020), soil moisture (Wu et al., 2021), vegetation cover (Narr et al., 2017; Roy et al., 2020), and agricultural management regime (Duan et al., 2022). However, most of these existing studies are observational in nature and focus on *responses* of virus communities to changing soils rather than the *effects* of viruses on soil functions (*e.g.*, Williamson et al., 2005; Emerson et al., 2018; Trubl et al., 2018; Santos-Medellín et al., 2023). As our knowledge of the structure of soil virus communities increases rapidly, we need more studies that quantify the implications of these changes for soil biogeochemical fluxes.

The high abundance and infection rates of viruses commonly observed in soil highlight the potential for viruses to influence microbial host populations in soil, with implications for host-driven biogeochemical fluxes. However, the type and magnitude of the effects may depend upon the proportion of soil viruses in different virus life cycles. For example many soil viruses have been shown to be in the lysogenic cycle, where phages replicate by incorporating into the host's genome (Williamson et al., 2007). Phages in the lysogenic cycle do not kill their host, but they do induce stress responses upon infection and can alter host metabolism by incorporating phage-encoded genes into their host's genome (*i.e.*, phage conversion) (Emerson et al., 2018; Trubl et al., 2018). Viruses can also lyse hosts via lytic replication, preventing further host metabolism and releasing the host's cytoplasmic contents into the soil matrix. Phages in the lysogenic cycle can also produce this effect if changing environmental conditions (*e.g.*, soil rewetting or resource pulses) trigger them to enter the lytic cycle (Van Goethem et al., 2019). Studies using stable isotope probing have demonstrated transfer of host-derived C into soil viral populations, providing direct evidence that viral lysis occurs in soil communities (Starr et al., 2021; Barnett

and Buckley, 2023). These virus life cycles, in turn, represent specific mechanisms by which viruses might influence soil biogeochemical cycling, though it remains unclear whether viruses detectably impact pools and fluxes of soil C and nutrients.

Direct evidence of virus influences on soil biogeochemical cycling will require experimental manipulation of rates of viral infection in soil. A few studies have attempted to do this, though with contrasting results. These studies experimentally manipulated virus abundance via inoculation of soils with concentrated natural virus communities and found that increased viral abundance in soil can alter the structure of bacterial communities (Braga et al., 2020) and can stimulate microbial respiration (Albright et al., 2022; Tong et al., 2023). In contrast, other studies found that higher virus abundance can suppress microbial activity (Wei et al., 2021; Liang et al., 2024), which has also been observed in studies where viral lysis was chemically induced in soils (Heffner et al., 2023). These results demonstrate that virus effects can be qualitatively different among studies, potentially related to the differences in soil properties or among experimental conditions. Further, most of the prior experimental studies manipulate virus abundance using sterilization-inoculation procedures (Braga et al., 2020; Albright et al., 2022; Tong et al., 2023), and these highly artificial systems may not function comparably to natural soils. Thus, the presence, direction, and magnitude of virus effects on biogeochemical cycling in natural soil ecosystems all remain unresolved.

The goal of this study was to investigate the influences of viruses on C cycling in intact soils from four contrasting ecosystem types: conifer forest, broadleaf deciduous forest, tallgrass prairie, and agricultural cropland. We assessed the potential for viruses to influence C cycling in those soils by increasing virus abundance in the soils via inoculation with virus concentrates extracted from the same original soils. After adding the concentrates, we measured respiratory

loss of C from the soils for 14 days and then conducted a ^{13}C-glucose tracer addition and incubation to assess microbial-mediated cycling of labile C. We hypothesized that 1) the virus addition treatments would result in reduced microbial carbon use efficiency due to stress responses induced in microbial hosts, 2) that reduced microbial carbon use efficiency would increase respiratory losses of C from the soils, and 3) that changes in microbial C metabolism would be accompanied by community-level changes in soil microbiota. Specifically, we predicted that the virus additions would increase community evenness due to disproportionately large effects of viruses on the most abundant microbial community members (Van Goethem et al., 2019).

2. Materials and Methods

2.1 Soil sampling and characterization

Soils were collected from four locations across the continental United States at sites representing wide variation in vegetation types and soil properties. Soil from a conifer forest ecosystem dominated by Douglas fir (*Pseudotsuga menziesii*) and western red cedar (*Thuja plicata*) was collected from the University of Idaho experimental forest in northern Idaho. Soil from a tallgrass prairie ecosystem dominated by native, perennial warm-season grasses, *e.g.*, big bluestem (*Andropogon gerardii*) and Indiangrass (*Sorghastrum nutans*), was collected at the Konza Prairie Biological Station near Manhattan, Kansas. Soil from a mixed deciduous forest ecosystem dominated by red maple (*Acer rubrum*) and red oak (*Quercus rubra*) was collected in southwest Virginia. Agricultural cropland soil was collected from experimental plots at the Kellog Biological Station in Michigan with a corn-soybean-wheat crop rotation and conventional tillage and fertilizer inputs. All site and soil information are provided in Supplementary Table S1. In the two forest sites, O-horizon material was removed from the soil surface prior to sampling.

In each site, soil was collected to 10 cm depth from three locations with representative vegetation (three replicate plots in the case of the agricultural site) and then shipped on ice to the University of Idaho. The soils from each site were combined into a single composite sample, sieved at 2 mm, homogenized, and stored at 4°C for 4 – 8 weeks prior to the start of the experiment. The use of a single composite sample per site allowed us to apply a single virus inoculum to every replicate experimental unit from each site as opposed to generating a unique virus inoculum for each experimental unit, which would not have been feasible with our low-throughout virus concentration method (see experimental design below).

Prior to the start of the experiment, we measured several physicochemical properties of the four soils. We measured the moisture content by mass loss after drying at 105°C and determined water-holding capacity (WHC) by wetting the soils to field capacity and then drying at 105°C. Soil pH was determined from 10 g of soil in a 1:1 soil:deionized water slurry with a Mettler Toledo sevencompact pH meter (Metter Toledo, Columbus, OH,USA). We determined the extractable C and N content of the soils by extracting 6 g of soil with 0.05M K_2SO_4 (1:5 soil:solution ratio) while microbial biomass C was determined using a liquid chloroform extraction (Fierer and Schimel, 2002). Extracts were analyzed for dissolved organic C and total dissolved N using a Shimadzu TOC/TNM-L (Shimadzu Corporation, Kyoto, Japan).

2.2 Experimental Design

We experimentally increased viral abundance in the soil communities by inoculating them with virus concentrates extracted from the same original soils. We extracted the viruses by adding 500 mL of autoclaved and chilled (4° C) saline magnesium buffer (10 mM $MgSO_4$, 100 mM NaCl, and 50 mM Tris-Cl, pH 7.5) to 500 g fresh soil and shaking on a reciprocal shaker on low speed for 30 minutes. We used a saline magnesium buffer because these solutions are

commonly used in culture-based virology studies for the purpose of maintaining the viability of phage particles. We centrifuged the slurries for 20 min at 4,000 rpm in a 4°C centrifuge to pellet soil particles and then decanted the supernatant containing microbial cells and virus particles. To maximize recovery of virus particles from the soils, we re-extracted the soil pellets twice for a total of three extractions (Williamson et al., 2005), resulting in a total of ~1.5 L of extract per soil. To remove remaining particulates from the extracts, we poured them through a sterilized 53 µm sieve. We then isolated and concentrated the virus particles in the extracts using a two-step tangential-flow filtration (TFF) method, similar to prior studies (Braga et al., 2020; Tong et al., 2023). First, microbial cells were removed by circulating the extracts through a Pellicon® XL50 capsule with a 0.22 µm pore-size membrane (MilliporeSigma, Burlington, MA, USA). Then, virus-sized particles were concentrated in the filtrate using a Pellicon® XL50 capsule with a PES 100 kDa membrane. We recirculated the extracts through the capsules until 100 mL remained in the first retentate fraction (containing concentrated microbial cells) and 50 mL remained in the second retentate fraction (containing concentrated virus-sized particles). The filtration apparatus was maintained at < 10 psi for the duration of the process to maintain the integrity of the virus particles. The TFF capsules were sanitized according to the manufacturer's instructions between samples. The virion concentrates were stored at 4°C and applied to soil microcosms within 72 h of the initial extraction. Prior to applying the concentrates to soil microcosms, we screened them for bacterial contamination using two methods, both of which showed no evidence of contamination in the concentrates. First, we visually examined the virus inocula using epifluorescence microscopy (with the main purpose of quantifying the virus-like particles, see below) and observed no evidence of intact cells. Second, we plated the inocula onto LB agar and observed no microbial growth.

For the experiment, twelve microcosms of each soil type were established by adding 30 g dry weight equivalent of soil to autoclaved 473 ml glass mason jars. We adjusted the soils to 65% WHC with sterile water, loosely capped the jars, and pre-incubated the soils in the dark at 20°C for one week prior to adding treatments. Approximately 48 hours prior to establishing the experimental treatments, we removed the lids from the microcosms and allowed them to dry down such that the added treatments would return the soils to 65% WHC. Microcosms were randomly assigned to receive either the live virion concentrate treatment (hereafter, the 'virus' treatment) or the control treatment. To apply the virus treatment, we added 3 mL of the virion concentrate originating from the same soil to the microcosms, which approximately doubled the abundance of extractable virions in the 30 g of soil: the initial 1:3 soil:buffer extract was concentrated 30-fold by TFF (1500 mL/50 mL = 30), therefore each 1 mL of concentrate contained the extractable virion-equivalent of 10 g of soil (1 g : 3 mL × 30 = 10 g/mL). We used epifluorescence microscopy to quantify the virus-like particle (VLP) concentration of the virion inoculum from each soil (see section 2.4 below). VLP concentrations in the inocula varied widely among the four soils and were as follows: 8.84×10^7 VLPs mL^{-1} (deciduous forest), 3.42×10^8 VLPs mL^{-1} (agricultural soil), 7.44×10^8 VLPs mL^{-1} (prairie soil), and 6.24×10^9 VLPs mL^{-1} (conifer forest). This variation reflects differences in the abundance and/or extractability of viruses among the different soil types (Williamson et al., 2013).

For control microcosms, we added 3 mL of virion concentrate that had been autoclaved twice for 30 min at 121°C to destroy the viruses. In a preliminary trial, we also included soils that received sterile saline magnesium buffer, which we found to be indistinguishable from soils that received the autoclaved control inocula (Supplementary Fig. S1). Therefore, for the main experiment reported here, we only included the autoclaved controls, which are more directly

comparable to the live virus-treated soils. All treatments were added dropwise to the surface of the soil. Each treatment was replicated 6 times for a total of 48 microcosms (2 virus treatments × 4 soil types × 6 replicates = 48). Microcosms were incubated at 65% WHC at 20°C for 14 days. This short time frame was intended to assess immediate effects of the virus additions. On days 1, 2, 3, 5, 7, 10, and 14, we measured respiration in each microcosm using a static incubation technique (Bradford et al., 2008). Briefly, on each measurement day we capped the jars, flushed CO_2 from the jars' headspace using CO_2-free air and then measured CO_2 concentration in the headspace gas after incubating for 24 hours using a LI-7000 infrared gas analyzer (LI-COR Biosciences, Lincoln, NE, USA). Cumulative respiration for each microcosm was calculated by integrating under the time series curves.

2.3 ^{13}C-glucose tracing

After the initial 14-day incubation, we assessed microbial C cycling in the microcosms using ^{13}C-glucose tracing. We added a 99 atom% glucose-C tracer (Sigma-Aldrich product no. 660663) to the microcosms at a rate of 5 μg C per gram of soil, which was calculated to be less than 20% of the microbial biomass C pool across all soils (Supplementary Table S1) and was intended to minimize effects of the tracer on microbial activity. We added the ^{13}C glucose in 2 mL of solution and gently mixed the microcosms with a metal spatula. ^{13}C glucose was added to five replicates from each treatment, with one replicate designated as a natural abundance control, which received 2 mL of sterile water. After adding the tracer, we immediately capped the jars, flushed the headspace with CO_2-free air, and incubated the microcosms at 20°C for 24 h. After 24 h we measured CO_2 concentration in the headspace of the jars (see above) and stored a sample of headspace gas in pre-evacuated exetainer vials, which were later analyzed for $^{13}CO_2$-C on a

229 Thermo Scientific GasBench II coupled to a Thermo Finnigan Delta Plus XL isotope-ratio mass
230 spectrometer at the UC Davis stable isotope facility.
231 After gas sampling, we immediately froze a subsample of each microcosm soil at -80°C for
232 later microbial and viral analyses. With the remaining soil from each microcosm, we extracted
233 and analyzed extractable C and N and microbial biomass C as described in section 2.1 above. For
234 ^{13}C analysis of the extracts, we acidified the extracts with HCl to remove inorganic C,
235 concentrated the liquid down to 1 mL in glass vials in a 60°C oven, and then pipetted 500 µL of
236 the concentrated samples into an Ag capsule in four successive 125 µL aliquots, drying at 60°C
237 between aliquots. We then secondarily encapsulated the samples in Sn and measured them for
238 ^{13}C on and Elementar vario MICRO cube elemental analyzer (Elementar Analysensysteme
239 GmbH, Langenselbold, Germany) interfaced to a Sercon Europa 20-20 IRMS (Sercon Ltd.,
240 Cheshire, United Kingdom) at the UC Davis stable isotope facility. We used the $^{13}CO_2$-C data to
241 partition respired C between the glucose tracer and native soil organic matter using a mass
242 balance equation (Morrissey et al., 2017). Isotope composition of the dried extracts was used to
243 calculate ^{13}C incorporation into microbial biomass (*i.e.*, microbial growth yield), carbon use
244 efficiency (CUE) of the glucose tracer and biomass turnover rates using previously published
245 methods (Hagerty et al., 2014; Geyer et al., 2019). All equations for the ^{13}C analyses are
246 provided in the Supplementary Information.
247 2.4 Microbial and viral analyses
248 DNA was extracted from 0.25 g soil using the Qiagen Powersoil Pro kit (Qiagen Company,
249 Hilden, Germany) and quantified using a Qubit fluorometer (Thermo Scientific Inc., Waltham,
250 MA, USA). We measured total prokaryote abundance in the soils by qPCR amplification of the
251 16S rRNA gene using the 338F/518R primer pair (Fierer et al., 2005) and characterized

prokaryotic communities by amplicon sequencing of the V4 region of the 16S rRNA gene using the 515F/806R primer pair (Apprill et al., 2015; Parada et al., 2016). Complete information on qPCR and PCR protocols is provided in the Supplementary Information. Amplicons were sequenced on the Illumina MiSeq platform with 2 × 250 bp paired end reads. Raw sequences are deposited under NCBI accession number PRJNA1077929. For all downstream analyses, we only used the higher quality forward reads with no truncation of the reads. We processed the raw sequences using the DADA2 pipeline (Callahan et al., 2016) using the default parameters except that we increased the 'maxEE' parameter to 5 to increase the number of sequences that passed through the initial quality filter. We assigned taxonomy to the resulting amplicon sequence variants (ASVs) using the IDTAXA algorithm (Murali et al., 2018) trained on the SILVA database (version 138.1) (Quast et al., 2013). We filtered out ASVs only occurring in one sample and rarefied to 5722 sequences per sample to account for variation in sequence depth among samples. While this sequence depth is lower than ideal, rarefaction curves indicated that species accumulation in all samples had plateaued by that point, indicating that the communities were reasonably well sampled (Supplementary Fig. S2). As metrics of prokaryotic alpha diversity, we used the Shannon, Simpson, and Pielou's evenness indices.

We enumerated the abundance of virus-like particles (VLPs) in the soils using epifluorescence microscopy. We first weighed 0.5 g soil into 2 mL tubes, added 1.5 mL of sterile 4°C saline magnesium buffer, and vortexed the tubes on high speed for 20 minutes. Tubes were then centrifuged at 5000 rpm for 20 minutes to pellet soil particles. We filtered the supernatants through 0.22 µM PES syringe filters to remove microbial cells and then 1:10 diluted the samples with sterile water. 1 mL of diluted sample was then filtered through a 25 mm diameter 0.02 µM pore-size Whatman anodisc filter and stained in the dark for 15 minutes with 2× SYBR gold

(Invitrogen, Waltham, MA, USA). Stained filters were mounted onto glass slides with antifade solution and visualized with a Nikon Ti-E confocal microscope at 1000× magnification using an excitation wavelength of 488 nm. We captured 10 digital images of each filter and enumerated VLPs in each image using FIJI (Schindelin et al., 2012). For enumeration of VLPs in the initial virus concentrates, we 1:100 diluted the concentrates in sterile water and then 0.02 μM-filtered, stained, and visualized the samples as described above.

2.5 Statistical analyses

Statistical analyses were performed in R (R Core Development Team, 2019). To assess the respiratory responses to the virus treatments among the soils we used linear mixed models in the lme4 R package (Bates et al., 2019) with 'virus treatment', 'soil type', and 'measurement day' as fixed effects and 'jar' as a random effect, which accounts for repeated measurement of the same jars over time. To assess pairwise differences between virus treatments within each soil type on each measurement day, we used the 'contrast' function (Tukey method) in the emmeans R package (Lenth et al., 2019). We assessed assumptions of normality of the residuals of the models using Shapiro-Wilk tests and when deviation from normality was observed, we used generalized linear mixed models with gamma distribution and log-link function. For variables measured at the end of the experiment, we used linear models with 'virus treatment' and 'soil type' as factors. We assessed the normality of the residuals of the models and conducted pairwise comparisons within soil types as described above. To determine effects of the treatments on bacterial ASV composition, we used PERMANOVA in the vegan R package (Oksanen et al., 2019) with Bray-Curtis dissimilarities. To identify specific bacterial ASVs within each soil type that responded to the virus treatments, we conducted differential abundance analysis using

297 Deseq2 (Love et al., 2014). All data and analysis scripts are available on figshare:
298 https://doi.org/10.6084/m9.figshare.25723785.v1.

299 3. Results

300 3.1 Soil physicochemical properties

301 The four soils used for the microcosm experiment had dramatically different
302 physicochemical properties (Supplementary Table S1). For example, the deciduous forest soil
303 substantially more extractable organic C (49% – 374% higher) and total extractable N (46% –
304 149% higher) than any of the other soils. However, the deciduous forest soil was also highly
305 acidic (pH 3.76), while the other soils had pH values of 5.93 (agricultural soil), 6.2 (conifer
306 forest), and 7.34 (prairie soil). Another notable difference was that the agricultural soil had more
307 than 3-fold lower microbial biomass C than any of the other soils. All physicochemical data is
308 reported on Supplementary Table S1.

309 3.2 Effects on soil respiration and soil C and N pools

310 To determine the effects of the virus treatments on soil respiratory C loss, we measured
311 respiration for 14 days after adding the virus concentrates to the microcosms. Respiration
312 generally declined over the course of the incubation and was dramatically different among the
313 different soils, with the highest respiration rates observed in the two forest soils and the lowest in
314 the agricultural soil (Fig. 1). We also observed effects of the virus treatments on soil respiration
315 dynamics in some but not all soils, resulting in a significant soil type × virus treatment
316 interaction ($\chi^2 = 7.9$, $p = 0.048$, Fig. 1). The agricultural and deciduous forest soils exhibited no
317 detectable respiratory responses to the virus treatments during the initial 14-day incubation,
318 while the virus-treated conifer forest and prairie soils had small but significant reductions in
319 respiration compared with controls during the middle and late stages of the incubation (Fig. 1).

Specifically, on days 5 and 10, the virus-treated prairie soils respired 1 – 1.3 μg less C per gram of soil compared with control soils (~10% reduction in respiration, Fig. 1) while on days 7 and 10 of the incubation, the virus-treated conifer forest soils respired 1.7 – 2 μg less C per gram of soil compared with control soils (~5% reduction in respiration, Fig. 1). The results for cumulative respiration were similar, with an average of 10 μg and 14 μg less respired C per gram of soil for the virus-treated prairie and conifer forest soils, respectively (Supplementary Fig. S3). In contrast to the respiration results, we did not observe significant effects of the virus treatments on soil microbial biomass C, extractable organic C, or total extractable N pools measured at the end of the experiment in any of the soils (Supplementary Table S2).

3.3 ^{13}C tracer incubation

To assess the effects of the virus treatments on the cycling of labile C, we conducted a ^{13}C glucose tracing incubation. Addition of the tracer stimulated microbial activity in all soils (Fig. 1), possibly due to the added glucose and/or to the added moisture and physical mixing. In the prairie, conifer forest, and deciduous forest soils, respiration was not affected by the virus treatments following addition of the glucose tracer (Fig. 1). In contrast, in the agricultural soil, we observed 1.8-fold higher total respiration in the virus-treated soils compared with the control soils after adding the tracer (Fig. 1). Analysis of the $^{13}CO_2$-C data indicated that >90% of the additional respired C in those soils originated from native soil organic matter (SOM) (Fig. 2), though respiration of glucose-C was also significantly higher in those soils (Supplementary Fig. S4). This indicates that priming of SOM decomposition was accelerated in the virus-treated agricultural soils after the glucose addition.

Analysis of ^{13}C in the soil extracts showed no differences in ^{13}C in the extractable organic C pool (Supplementary Fig. S5) but showed a clear main effect of virus treatment on ^{13}C

accumulation in microbial biomass, *i.e.*, microbial growth yield (Fig. 3). Specifically, microbial growth yield was 20% lower on average in the virus-treated soils ($\chi^2 = 11.7$, $p < 0.001$), though pairwise differences were only significant for the conifer forest soil (Fig. 3). Similarly, CUE exhibited a clear main effect of virus treatment, where CUE was ~6% lower in the virus-treated soils on average ($F = 17.4$, $p < 0.001$), with significant pairwise differences in both the conifer forest and agricultural soils (Fig. 4). CUE was generally very high in our experiment (though notably different among soil types, Fig. 4), which reflects the easily assimilable nature of glucose and the small quantity of tracer we added (Islam et al., 2023). We used the respiration, CUE, and microbial biomass C values to estimate microbial biomass turnover rates and observed a significant soil type × virus treatment interaction ($\chi^2 = 20.4$, $p < 0.001$), where the virus-treated agricultural soils had ~2.3-fold higher estimated biomass turnover and no significant differences for any of the other soils (Supplementary Fig. S6).

3.4 Viral and microbial analyses

To identify effects of the virus treatments on soil bacterial communities we conducted qPCR and amplicon sequencing. We did not detect effects of the virus treatments on 16S rRNA gene abundance in any of the soils (Supplementary Fig. S7). The 16S rRNA gene amplicon sequencing data revealed that prokaryotic ASV community composition was dramatically different among the four soils (Fig. 5), as were the relative abundances of the dominant prokaryotic phyla (Supplementary Fig. S8), though all communities were generally dominated by Proteobacteria (30% of all sequences), Actinobacteriota (26% of all sequences), Acidobacteriota (8% of all sequences), Verrucomicrobiota (7% of all sequences) and Firmicutes (6% of all sequences). However, there were no effects of the virus treatments on prokaryotic ASV community composition (Fig. 5). This held true when analyzing all samples from all soil

366 types together (Fig. 5) and when analyzing the four soils separately (Supplementary Fig. S9). In
367 line with the community-wide analyses, Deseq2 did not reveal any 16S rRNA gene ASVs that
368 were differentially abundant between the virus treatments in any of the soils. We also did not
369 observe effects of the viruses on alpha diversity metrics (Shannon, Simpson, Pielou's evenness)
370 in any of the soils (Supplementary Table 3).

371 In contrast to the bacterial community results, we did observe effects of the virus treatments
372 on VLP abundance. Specifically, we detected a significant main effect of virus treatment (χ^2 =
373 5.5, p = 0.019, Fig. 6), with microcosms receiving the virus concentrates having ~18% higher
374 VLP abundance on average across all soils. However, we did not detect pairwise differences in
375 VLP abundance between the virus treatments within any individual soils (all $p > 0.05$, Fig. 6).
376 Treatment effects were stronger when considering virus:prokaryote abundance ratios, i.e., ratios
377 of VLP abundance to 16S rRNA gene abundance. While these ratios cannot be interpreted as true
378 virus:prokaryote ratios due to the different enumeration methods (microscopy vs. qPCR), they do
379 provide a relative metric of the responses of viral vs. microbial abundance to our treatments. We
380 detected a significant main effect of the virus treatments on VLP:16S ratios (χ^2 = 8.3, p = 0.003,
381 Supplementary Fig. S10), with virus-treated microcosms having 24% higher VLP:16S ratios
382 across all soils. Further, we detected significant pairwise differences in VLP:16S between virus
383 treatments in both the agricultural soil (25% higher VLP:16S in the virus-treated microcosms)
384 and in the conifer forest soil (31% higher VLP:16S in the virus-treated microcosms)
385 (Supplementary Fig. S10). The same trend was evident in the prairie soils, though the difference
386 was not statistically significant (Supplementary Fig. S10).

387 4. Discussion

Given the spectacularly high abundance of soil viruses, the dearth of knowledge regarding their ecological roles represents a fundamental gap in our understanding of soil ecology (Kuzyakov and Mason-Jones, 2018). Though our knowledge of soil virus ecology is limited, attention being paid to soil viruses is growing (Williamson et al., 2017; Emerson, 2019) and emerging research demonstrates the potential for viruses to have ecosystem-scale effects on soils (Braga et al., 2020; Albright et al., 2022; Tong et al., 2023; Liang et al., 2024). Our study contributes to this growing body of research by providing experimental evidence that viruses can alter soil microbial community metabolism, including reductions in both microbial growth yield and carbon use efficiency. Further, we show that those virus-mediated changes in microbial metabolism can manifest in changes to the aggregate C balance of soils, though the respiratory responses to increased viral loads were sometimes small in magnitude and the direction of the responses varied among soil types.

Here, we increased virus abundance in intact soils above background levels by adding natural virion concentrates to the soils. After entering the soil, there are multiple possible fates of the added virions. Added virions may simply decompose if they do not encounter a host or if they were damaged in the extraction/filtration process. Alternatively, virions might infect a new host, which will likely result in increased physiological stress in soil microorganisms (Flores-Kim and Darwin, 2016). We hypothesized that increased stress, in turn, would manifest in reduced CUE of the microbial communities. Supporting this hypothesis, we observed a consistent reduction in microbial growth yield and CUE in the virus-treated soils. The reduced microbial growth yield and CUE we observed is consistent with work from marine ecosystems, which has demonstrated reduced bacterial growth efficiency and growth yield in association with the marine viral shunt (Bonilla-Findji et al., 2008; Motegi et al., 2009). However, our results are notably different from

411 another study in soils that also investigated microbial CUE following virus additions, which
412 found that virus additions increased CUE (Tong et al., 2023). That study, however, measured
413 CUE using an extracellular enzyme stoichiometry method, which is not comparable to the
414 isotope tracing method used in this study (Schimel et al., 2022). Because of this difference, the
415 studies might be difficult to compare directly, though this does highlight an important
416 inconsistency that should be addressed by future research. One possible reason for the
417 discrepancy is that, unlike Tong et al., our study measured CUE following addition of a labile
418 resource (glucose), which might have triggered viral processes that do not occur as frequently
419 under baseline conditions, *e.g.*, lytic replication. Alternatively, it is possible that viral lysis could,
420 in fact, increase CUE in some situations, potentially due to the increased availability of high-
421 quality C from newly lysed microbial necromass. Studies that use ^{18}O isotope tracing to measure
422 microbial efficiency both with and without labile C additions could potentially resolve this
423 discrepancy (Geyer et al., 2019).
424 We also hypothesized that the physiological responses of microbial communities would be
425 accompanied by shifts in microbial communities, but we did not observe any changes in 16S
426 rRNA gene amplicon profiles of the soils. No evident community response could be due to relic
427 DNA in the soil, which can obscure detection of microbial responses over short time scales
428 (Carini et al., 2020), especially if effect sizes are small (Kittredge et al., 2021). This suggests that
429 any effects of our virus treatments on microbial community composition, if present, may have
430 been small in magnitude. If true, this would also indicate that the changes in soil C cycling we
431 observed are probably attributed to changes in the physiological state of the soil microorganisms
432 rather than large changes in the composition of the communities. Alternatively, because rates of
433 microbial mortality may have been high in our experiment (with proportionally high

accumulations of relic DNA), it is also possible that our treatments could have obscured larger-magnitude changes in community composition compared with other studies. Indeed, prior studies have linked increased phage abundance to higher proportions of relic DNA in soil (Wei et al., 2021). Future studies that use relic DNA removal methods prior to characterizing communities could assess these possibilities. We also note that our study only would have detected responses of abundant taxa, given the somewhat limited sequence depth in our analysis. While our dataset is likely missing many rare taxa, we reasoned that since our study was focused on broad C cycle processes, any effects that were related to changes in microbial community composition would be attributed to taxa that are abundant and would probably have been detected.

Our other hypothesis was that reductions in microbial CUE would result in increased respiratory loss of C from the soils. We observed support for this hypothesis only in the agricultural soil, and, intriguingly, the increase in respiration only occurred after addition of the glucose tracer. Though we initially designed the tracer addition to minimize effects on microbial activity, we hypothesize that the response of this soil was, in fact, related to the pulse of C. It is possible, for example, that microbial growth responses to the pulse of labile C triggered lytic replication in some of the new viral infections, delivering new substrate to nearby unaffected populations, *i.e.*, a viral shunt. This may have occurred in the agricultural soil and not the others because the glucose addition represented a proportionally much larger resource input to that soil, as agricultural soils are generally more C-limited than other soils. Indeed, the glucose C addition represented ~12% of the standing microbial biomass C in that soil versus 1 – 4 % in the others. Future studies could evaluate this by performing similar experiments but with varying rates and timings of C addition to soils with varying C pool sizes. Overall, the reduced growth yield, reduced CUE, and increased respiration we observed in the agricultural soil is very similar to

457 what occurs in the marine viral shunt (Suttle, 2007; Bonilla-Findji et al., 2008; Motegi et al.,
458 2009) and suggests that a similar mechanism may operate in some soils. Viral mechanisms might
459 be involved in other key soil processes that involve resource pulses such as rhizosphere priming.
460 Indeed, most of the additional respired C in the agricultural soil was the result of priming, with
461 the virus additions approximately doubling the priming effect in those soils. In line with this
462 result, we also observed greater microbial biomass turnover rates in the agricultural soil, which
463 suggests the possibility that viral shunts in soil could contribute to mineral stabilization of C
464 (Cotrufo et al., 2013), despite the greater respiratory C loss.
465 It is interesting that the other three soils (conifer forest, deciduous forest, and prairie) also
466 exhibited generally lower growth yield and CUE despite there being no increases in respiration
467 in those soils. The reduced accrual of ^{13}C in microbial biomass in those soils cannot be accounted
468 for by ^{13}C in the extractable organic C pools, which were not different between the control and
469 virus-treated soils. The reduced ^{13}C in microbial biomass without an increase in respiration
470 suggests that there must be non-respiratory fates of the unaccounted for ^{13}C in those soils that
471 would explain the reduced growth yield and CUE. One possibility is that the C became mineral-
472 bound following microbial death, which may have occurred at higher rates in the virus-treated
473 soils. This speculation is supported by the fact that the non-agricultural soils were finer-textured
474 (Supplementary Table S1), which might have provided more surface area for mineral
475 associations to occur. It is also interesting that during the initial 14-day incubation the virus
476 treatments either had no effect on respiration or slightly reduced respiration, in contrast to the
477 glucose tracer incubation. Taken together, the range of respiratory responses we observed
478 represents the varying and potentially countervailing influences that viral infection might have
479 on overall microbial activity and soil C balance. For example, while increased availability of

lysed biomass C could increase overall respiration (as in a viral shunt), reduced respiration could occur if stressed microbes redirect resources away from energy metabolism or if rates of host lysis are high enough to offset the increased activity of non-infected populations. These alternative respiratory responses, in turn, reflect different experimental/environmental contexts, *e.g.*, constant environmental conditions (our initial incubation) vs. resource pulse scenarios. This range of possible responses is also evident in prior studies, where respiration has been observed to both increase (Albright et al., 2022; Tong et al., 2023) or decrease (Wei et al., 2021; Liang et al., 2024) with higher virus abundance.

It should also be noted that the differences in responses we observed among the different soils are probably related to the large differences in the concentrations of virus particles in the inocula across the soils. For example, the two soils that exhibited respiratory responses in the initial incubation were also the two with the highest VLP concentration in the inocula (prairie and conifer forest). In contrast, the deciduous forest soil had the lowest VLP abundance in the inoculum and never exhibited respiratory responses to the virus treatment. This reflects limitations to our virus extraction procedure, as the salt buffer we used is likely not effective for extracting viruses from soils with high organic matter, *e.g.*, our deciduous forest soil (Trubl et al., 2016; Göller et al., 2020). In preliminary trials, we did attempt to address this by using an organic (protein-supplemented) buffer (Göller et al., 2020), but the extracts proved to be unfilterable with our TFF system. On the other hand, it could be argued that the variation in the extractability of viruses among our soils could reflect meaningful differences in the mobility of viruses in the differing soil matrices. For example, VLPs might be more abundant in the deciduous forest soils than we were able to detect but might also be tightly bound to organic matter or mineral surfaces and thus less likely to infect hosts. In contrast, while VLP

concentration was also relatively low in the agricultural soil inoculum, that soil was low in organic matter and coarser-textured (sandy loam), and thus mobility of the added viruses might have been high due to less immobilization of the particles on mineral surfaces or on organic matter. Regardless, poor and varying extractability of viruses across different soil types represents a challenging limitation of this type of experimental approach. Yet another consideration is the potential for variation in the viability of the phages in the different extracts. While it is probably not feasible to directly assess the viability of highly diverse phage communities with mostly unknown and potentially unculturable microbial hosts, it is conceivable that phage particles were differentially viable among the extracts from the four soils, which could have contributed to the variable responses to virus addition.

In addition to variable virus abundance/extractability/viability, another factor that might underlie the diversity of responses among the soils is variation in the background rates and types of viral infection. For example, prior work has demonstrated that in some soils, most prokaryotic cells are already infected with lytic phages and that phage lysis is the primary cause of prokaryotic mortality in those soils (Bowatte et al., 2010). Therefore, if phages are already maximally influencing host populations in the control soils, there may be no effect of adding more virus particles. High background effects of viruses might also explain the generally small effect sizes of the virus treatments that we observed. If background rates of infection are high, the soils may be nearly or completely "saturated" with viruses. Because of the technical challenge of directly observing phage infections, we do not have information for our soils on background rates of infection, though it would be valuable for future studies to include this when conducting virus manipulations experiments like ours. Ideally, future work would also attempt to identify and quantify the biogeochemical implications of background rates of viral infection, *e.g.*,

by virus removal from soil, similar to what has been done in marine studies (Bonilla-Findji et al., 2008; Motegi et al., 2009). However, this represents a substantial methodological challenge since there is no simple way of removing viruses from soil that does not also remove cellular microorganisms and alter soil physiochemical properties (*e.g.*, gamma irradiation, autoclaving).

We also note that while we observed clear statistical effects of the virus treatments on several aspects of microbial C cycling, it remains difficult to directly attribute these effects to specific viral mechanisms. One mechanism that could underlie the effects we observed is viral lysis of hosts. We attempted to identify that mechanism by enumeration of VLP abundance in the soils at the end of the experiment, since lytic replication should release additional virus particles into the soil matrix. While we observed an overall increase in VLP abundance in the virus-treated soils, the differences were within the amount initially added, so we do not know whether those particles were remnants of the inoculation, were generated through viral replication, or some combination of the two. Future studies could resolve this issue by combining virus addition experiments like the one used here with DNA-stable isotope probing to trace isotopically labelled C substrates into specific viral taxa (Starr et al., 2021; Barnett and Buckley, 2023). This would allow for the pairing of quantitative estimates of the effects of viruses on soil C fluxes with identification of specific viral taxa involved. Those studies could be conducted with isotope tracing at higher temporal resolution and under different resource scenarios (*e.g.*, with and without C additions). Studies such as this could potentially shed light on specific viral mechanisms at play, *e.g.*, stress due to infection, lysis of microbial cells, etc.

Considering the range of possible mechanisms that underlie the statistical effects is particularly relevant for experimental approaches such as ours where controls are imperfect. For example, both our live virion inocula and the autoclaved control inocula contain organic matter

549 extracted from the soils, but in the autoclaved control inocula the organic matter is heat-treated
550 and the destroyed viruses in those inocula could represent additional C or nutrient sources for
551 microorganisms. That said, in preliminary trials, soils that received the autoclaved inocula were
552 indistinguishable from soils that received sterile buffer (Supplementary Fig. S1). Therefore, we
553 do not think that extracted organic matter in the inocula is likely to have played a significant role
554 in our experiment. Another possible experimental artifact is the potential for bacterial
555 contamination in the virus inocula, though we observed no evidence of contamination in our
556 inocula. A prior experiment with a similar TFF procedure also observed minimal bacterial
557 contamination in the virus inocula (Braga et al., 2020). Thus, while we cannot completely rule
558 out the potential role of bacterial contaminants or other experimental artifacts, we did not see
559 evidence of this in our experiment. Overall, we attribute treatment effects in this study to viral
560 influences, but the mechanisms remain elusive. To elucidate those mechanisms, it is critical to
561 continue using and improving these and other experimental approaches so that the key roles of
562 viruses in the functioning of soil ecosystems can be identified and quantified.

563 5. Conclusions

564 Our study provides experimental evidence that viruses can influence soil carbon cycling
565 across varying ecosystem types by reducing the growth yield and efficiency of microbial
566 communities. These effects may be attributed to increased microbial stress upon infection or
567 increased rates of host lysis and can manifest in altered respiratory loss of C from soil.
568 Respiratory responses to increased viral loads, however, appear to vary among different soil
569 types and are sometimes small in magnitude. In addition, this study examined microbial
570 functioning under elevated virus abundance scenarios, which might not be entirely representative
571 of the effects of viruses under baseline conditions. Additional research is required to elucidate

those influences, along with the specific viral mechanisms concerned. In particular, future studies should compare biogeochemical effects of viruses across soils with different degrees of phage "saturation" and/or different degrees of microbial adaptation to phages rather than simply considering influences of soil physicochemical properties and ecosystem type. Regardless, our results demonstrate that the effects of viruses on soil microorganisms resemble the phenomenon of viral shunting observed in better-characterized marine environments and shed light on the potential for viruses to alter the cycling and fate of carbon in terrestrial ecosystems.

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764

765 Figure Legends

766 Figure 1: Respiration during the initial 14-day incubation and following the addition of the ^{13}C-

767 glucose tracer. Note the different y-axis scales among soils. Symbols are treatment means while

error bars represent one standard error of the mean. The dotted vertical lines indicate the day of glucose tracer addition. For the initial 14-day incubation, respiration data were analyzed using a mixed model with 'virus treatment', 'soil type', and 'day' as fixed effects and 'jar' as a random effect. For respiration following tracer addition, data were analyzed using a linear model with 'virus treatment' and 'soil type' as factors. We detected significant virus treatment × soil type interactions for both the 14-day incubation ($\chi^2 = 7.9$, $p = 0.048$) and for the tracer incubation ($F = 4.4$, $p < 0.001$). Pairwise comparisons were performed within soil types (Tukey method) and symbols indicate significant differences between virus treatments at the following levels: ** $p < 0.01$, *** $p < 0.001$.

Figure 2: Effects of the virus treatments on respiration of soil organic matter (SOM) derived C during the ^{13}C-glucose incubation. We partitioned respired CO_2 between SOM and the added glucose tracer using a mass balance equation (see Supplementary Methods). Data were analyzed using a linear model with 'virus treatment' and 'soil type' as factors. There was a significant virus treatment × soil type interaction for SOM respiration ($F = 4.47$, $p = 0.009$). Pairwise comparisons were performed within soil types (Tukey method) and symbols indicate significant differences between virus treatments at the following levels: *** $p < 0.001$. Respiration of the glucose-C was much lower but exhibited similar patterns and is shown on Supplementary Fig. S4. On all panels, blue boxes indicate soils that received autoclaved control inocula while yellow boxes indicate soils that received the live virus inocula.

Figure 3: Effects of the virus treatments on ^{13}C incorporation into microbial biomass, *i.e.*, microbial growth yield. All ^{13}C equations are provided in the Supplementary Methods section. Data were analyzed using a generalized linear model with 'virus treatment' and 'soil type' as factors. There was a significant main effect of virus treatment on microbial growth ($\chi^2 = 11.7$, $p <$

0.001). Pairwise comparisons were performed within soil types (Tukey method) and symbols indicate significant differences between virus treatments at the following levels: *** $p < 0.001$. On all panels, blue boxes indicate soils that received autoclaved control inocula while yellow boxes indicate soils that received the live virus inocula.

Figure 4: Effects of the virus treatments on carbon use efficiency (CUE) of the glucose substrate. CUE calculations are provided in the Supplementary Information. Data were analyzed with a linear model with 'virus treatment' and 'soil type' as factors. There was a significant main effect of virus treatment on CUE ($F = 17.4$, $p < 0.001$). Pairwise comparisons were performed within soil types (Tukey method) and symbols indicate significant differences between virus treatments at the following levels: * $p < 0.05$, *** $p < 0.001$. On all panels, blue boxes indicate soils that received autoclaved control inocula while yellow boxes indicate soils that received the live virus inocula.

Figure 5: Principal coordinates analysis (Bray-Curtis dissimilarities) showing responses of 16S rRNA gene ASV composition. Presented p values are from PERMANOVA with Bray-Curtis dissimilarities and with 'soil type' and 'virus treatment' as factors. Analysis of the four soils separately is shown on Supplementary Fig. S8.

Figure 6: Virus-like particle (VLP) abundance in the microcosm soils at the end of the experiment. Data were analyzed using a generalized linear model with 'virus treatment' and 'soil type' as fixed effects. We detected a significant main effect of virus treatment ($\chi^2 = 5.5$, $p = 0.019$). Pairwise comparisons were performed within soil types (Tukey method), none of which were statistically significant (all $p > 0.05$). Results for VLP:16S ratios are provided on Supplementary Fig. S9. On all panels, blue boxes indicate soils that received autoclaved control inocula while yellow boxes indicate soils that received the live virus inocula.