Environmental Science & Technology

pubs.acs.org/est

Article

# Viral Community and Virus-Associated Antibiotic Resistance Genes in Soils Amended with Organic Fertilizers

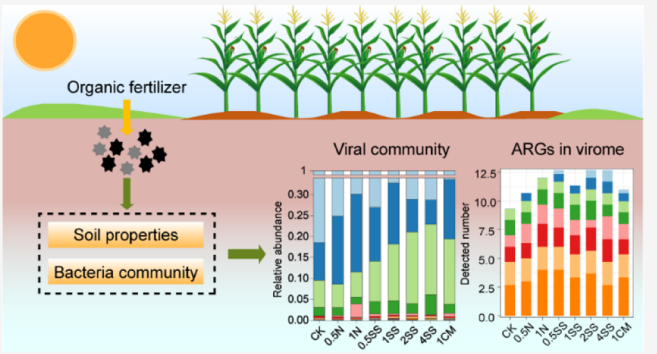
Mo-Lian Chen, Xin-Li An, Hu Liao, Kai Yang, Jian-Qiang Su, and Yong-Guan Zhu*

Cite This: *Environ. Sci. Technol.* 2021, 55, 13881−13890

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

**ABSTRACT:** Antibiotic resistance is a global health concern. Long-term organic fertilization can influence the antibiotic resistome of agricultural soils, posing potential risks to human health. However, little is known about the contribution of viruses to the dissemination of antibiotic resistance genes (ARGs) in this context. Here, we profiled the viral communities and virus-associated ARGs in a long-term (over 10 years) organic fertilized field by viral metagenomic analysis. A total of 61,520 viral populations (viral operational taxonomic units, vOTUs) were retrieved, of which 21,308 were assigned at the family level. The viral community structures were significantly correlated with the bacterial community structures ($P < 0.001$) and the dosage of applied sewage sludge ($r^2 = 0.782$). A total of 16 unique ARGs were detected in soil viromes, and the number of virus-associated ARG subtypes was higher in sewage sludge treatments (except for 1 SS) than others. The network analysis showed that the application of the organic fertilizer increased the bacteria−virus interactions, suggesting that the chances of ARG exchange between viruses and their hosts may increase. Overall, our results provide a novel understanding about virus-associated ARGs and factors affecting the profile of viral community in fertilized soil.




**KEYWORDS:** *bacteriophage, virome, antibiotic resistance genes, horizontal gene transfer, sewage sludge*



## INTRODUCTION

Viruses are abundant and ubiquitous in soil and greatly affect ecosystem functions, biogeochemical cycles, and food web dynamics.[1] It has been estimated that vial abundance in soils can range from ~$10^7$ to $10^9$ viral particles per gram dry weight.[2] Similar to the roles of viruses in marine and freshwater environments,[3,4] recent soil viral metagenome (virome) analysis has documented that viruses may impact microbial ecology and carbon metabolism in terrestrial ecosystems.[5] However, soil viruses and their functions remain poorly understood compared with marine viruses that have been studied for over 2 decades.[6,7]

Bacteriophages (phages) are believed to be the majority of identified soil viruses.[2,6] They could affect microbial community composition and nutrient cycling by lysing bacteria[7] or impact the genetic landscape and bacterial metabolism by transferring genes to their microbial hosts through specialized (lysogenic phages) or generalized transduction (lysogenic or lytic phages).[8,9] In particular, phages are capable of carrying part of bacterial DNA, including antibiotic resistance genes (ARGs), which may play an important role in ARG transmission.[10] Recent studies have found that virus-associated ARGs are prevalent in river and marine viromes,[11−13] indicating that viruses may act as significant reservoirs of ARGs in the environment.[14] Due to the relatively difficult recovery of enough viral DNA from the complex soil matrix and the limitation of publicly available viral databases,[1,15] soil virome is largely unexplored, and thus, soil viral diversity and virus-associated ARGs are poorly known.

In agricultural soil, the application of organic fertilizers can alter the bacterial diversity and structure by shifting soil nutrients, pH and organic matter, and so on.[16,17] Because bacteria play key roles in determining viral survival and production,[18,19] organic fertilizers may further influence soil viral communities by changes in the structure of bacterial communities. In addition, soil pH, organic matter, and other soil characteristics may affect the viral community distribution through influencing virus transport in soil.[20,21] Moreover, organic fertilizers (e.g., sewage sludge and manure) have been shown to contain many ARGs carried by viruses,[22,23] which may be transmitted to soil via fertilization, resulting in a potential threat to soil health. However, to the best of our knowledge, there are few studies that report the viral

Received: June 11, 2021
Revised: September 20, 2021
Accepted: September 21, 2021
Published: October 1, 2021

abundance and diversity in agricultural soil, and virus-associated ARG analysis has not been investigated in soil with long-term organic fertilization.

In this study, we recovered 24 soil metagenomes and 24 viromes from agricultural soils amended with organic/chemical fertilizers by illumina sequencing. Our aims were (1) to investigate the viral community and their main drivers, (2) to evaluate the ARG diversity in soil viromes, and (3) to explore the interactions between viruses and bacteria. This work may provide novel insights into the viral communities and their roles in the dissemination of ARGs in agricultural soil.

## MATERIALS AND METHODS

**Soil Sample Collection.** The sampling sites and details have been described in our previous study.[16] Soil samples were collected in August 2019 from a long-term field experiment site at the Key Experimental Station on Agricultural Resources and Ecological Environment in Dezhou, Ministry of Agriculture, China (37°20′ N, 116°38′ E). Briefly, organic fertilizers (sewage sludge and chicken manure) and chemical fertilizer (urea) have been applied in 24 plots annually since 2006, creating 8 different treatments with 3 replicate plots (Figure S1): 0.5 N (treated with 65.25 kg $hm^{-2}$ urea), 1 N (treated with 130.5 kg $hm^{-2}$ urea), 0.5 SS (treated with 65.25 kg $hm^{-2}$ urea and 4.5 t $hm^{-2}$ sewage sludge), 1 SS (treated with 65.25 kg $hm^{-2}$ urea and 9 t $hm^{-2}$ sewage sludge), 2 SS (treated with 65.25 kg $hm^{-2}$ urea and 18 t $hm^{-2}$ sewage sludge), 4 SS (treated with 65.25 kg $hm^{-2}$ urea and 36 t $hm^{-2}$ sewage sludge), 1 CM (65.25 kg $hm^{-2}$ urea and 10 t $hm^{-2}$ chicken manure), and CK (nonfertilized control plots). The surface soil (0−15 cm) was collected from each plot using the five-point sampling method, kept on dry ice, and transported to the laboratory immediately. Each soil sample was divided into two subsamples: one was stored at −80 °C for soil DNA extraction and the remaining was stored at 4 °C for viral DNA extraction.

**Soil Physicochemical Analysis.** Total nitrogen (TN) and total carbon (TC) contents of soil were measured using a CNS elemental analyzer (Elementar Analysensysteme GmbH, Germany), and dissolved organic carbon (DOC) was analyzed through a total organic carbon analyzer (Shimadzu, Japan) according to the report by Bolan et al.[24] The contents of soil organic matter (SOM) were also quantified by measuring absorbance at 590 nm wavelength using the colorimetric approach,[25] and heavy metals were determined using an X-ray fluorescence spectrometer (Axios-MAX, Panalytical). Additionally, soil pH measurement was performed in solution with a 1:2.5 ratio of soil to deionized water by a pH meter (Thermo Scientific, USA) at room temperature.

**DNA Extraction and Illumina Sequencing.** *Viral DNA Extraction.* Virus-like particles were acquired and purified as previously described with specific modifications.[26] Briefly, all soil samples were homogenized in sterilized bags and the large soil particles were broken down manually; 100 g soil of each sample was homogenized with 250 mL of phosphate buffered saline solution (PBS, 0.01 M, pH = 7.0) and suspended at room temperature by magnetic stirring for 45 min, and mitomycin C (final concentration, 1 μg $mL^{-1}$; Genview, American) was added into the suspension to induce temperate phages from host genome.[22,27,28] The mixture was shaken at 50 rpm overnight at 28 °C in the dark. The supernatant was then obtained by centrifugation at 3000*g* for 15 min at 4 °C. Bacteria cells were removed from the resulting supernatant by sequential filtration steps through 5.0, 0.45, and 0.22 μm low protein-binding poly ether sulfones membranes (Jin Teng, China). The filtrate was concentrated into 250 μL using 100 kDa Amicon Ultra centrifugal filter units (Millipore, USA) and subsequently refiltered by a sterile 0.22 μm Millex-GP filter (Millipore, USA).

To remove external-free DNA fragments, viral concentrates were treated with DNase I (1000 U $mL^{-1}$; Beyotime Biotechnology, China) for 45 min at 37 °C according to the manufacturer's protocol. After heat inactivation of DNase I, PCR assays of bacterial 16S rRNA were performed using universal primers 27F/1492R to check the presence of free and contaminating bacterial DNA.[29] Encapsulated viral DNA was extracted by using a TIANamp Virus DNA/RNA Kit (Tiangen, China).[30] To obtain adequate viral DNA yields for sequencing virome, the REPLI-g Midi Kit [for multiple displacement amplification (MDA)] (Qiagen, German) was used to amplify total viral DNA according to the manufacturer's instructions.[31] The resulting products were purified using KAPA Pure Beads (Roche, Switzerland) and quantified using a Qubit double-stranded DNA (dsDNA) Assay Kit (Invitrogen, USA).

*Total DNA Extraction and Illumina Sequencing.* Total DNA was extracted using 0.25 g of frozen soil and a PowerSoil DNA Isolation Kit (Mo Bio Laboratories, USA) following the manufacturer's instructions. DNA concentrations were determined by using a NanoDrop spectrophotometer (ND-2000, Thermo Scientific, USA), and then, DNA was stored at −20 °C for further analysis.

The extracted total and viral DNA were sequenced on the Illumina HiSeq X-ten platform and the Illumina NovaSeq 6000 platform (MagiGene, Guangzhou, China), respectively. To acquire the clean data for subsequent analysis, the raw data were first screened with FastQC (v0.11.5) to assess quality, followed by trimming and filtering of low-quality reads using Trimmomatic (v0.36, parameters: TruSeq3-PE.fa 2:3:10, LEADING 5, TRAILING 5, SLIDINGWINDOW 4:20, and MINLEN 60).[32] Sequencing data were deposited at the NCBI (National Center for Biotechnology Information) Sequence Read Archive database with accession numbers PRJNA697905 (metagenome) and PRJNA705403 (virome).

**Viral Contig Assembly, Identification, and Phage Host Prediction.** Host sequences (≥80% reads coverage) were removed by mapping the clean reads against genomes from ribosome database (Silva 132) and bacteria RefSeq database using Bowtie2.[33] The remaining clean reads of each virome were then assembled into contigs using Megahit,[34] and lysogenic and lytic viral contigs were identified using a machine learning method implemented by VIBRANT.[35] Viral-like contigs (≥10 kb in size) and/or circular (assumed to be completed) were maintained, and redundancy was removed using CD-HIT (v4.7, parameter: -c 0.95 -n 5 -g 1 -aS 0.8).[36] Subsequently, the generation of "viral population (viral operational taxonomic unit, vOTU)" was performed by using a Perl script for clustering at >95% identity and >80% coverage.[37] The longest contig of each viral cluster (VC) was chosen as the representative sequences for further taxonomic assignment. Coverage of viruses in the viral fraction samples was determined by mapping clean reads to assembled viral contigs and calculated as the number of reads normalized to the length of the viral contig (number of total base pairs aligned to contig/base pair of contig) with Bowtie2 and perl script (jgi_summarize_bam_contig_depths),[33] and then, the relative abundance of individual vOTU was normalized to the


https://doi.org/10.1021/acs.est.1c03847
Environ. Sci. Technol. 2021, 55, 13881−13890

total coverage of all identified vOTUs in each sample.[38,39] The metagenome reads were also mapped to the assembled viral contigs (as described above for virome reads) to estimate their coverage and analyze the interactions between viruses and bacteria.

Using PILER-CR (v1.06) to identify CRISPR spacers of bacterial sequences by aligning with bacterial genome of RefSeq database, ambiguous sequences with continuous N were removed and then CRISPR spacers database was constructed. CRISPR spacers in the vOTU ("blastn-short" mode preset, *e*-value < $10^{-5}$ and bitscore ≥ 45) were identified by BLASTn against CRISPR spacers database,[5] and the best hit was selected as the possible host information (genus level) of the phage with ≥95% sequence identity and ≥80% sequence coverage. For each vOTU, redundant CRISPR spacer hits corresponding to the same bacterial genus were removed.

**Taxonomic Assignment and ARG Annotation of Viromes.** Open reading frames (ORFs) were predicted from vOTUs and reference genomes (virus RefSeq97) by using Prodigal (v2.6.3),[40] and then, the extracted protein sequences were clustered by using vConTACT [v2.0, parameters: pc-inflation 2.0, vc-inflation 1.5, MCL pcs-mode and ClustONE vcs-mode].[41] Briefly, viral proteins are grouped into homologous PCs by applying the Markov cluster algorithm (MCL), followed by the formation of VCs using ClusterONE. Those vOTUs clustered with reference genomes could be assigned to a known viral taxonomic genus. In order to assign the family level taxonomic annotations to vOTUs, the "Demovir" script was used to search for homologies between proteins encoded by vOTUs in question to a viral subset of TrEMBL database, and then, taxonomic assignment was determined by the voting approach.[42] ARGs of vOTUs were annotated by aligning with the deposited ones in SARG (v2.2) database, using BLASTp with a threshold of *e* value ≤ $10^{-5}$, query coverage ≥80, and ≥40% amino acid identity.[11]

**Detection of ARGs and Taxonomy in Microbial Metagenome.** Clean reads of metagenome were assembled by Megahit.[34] Assembled contigs of metagenome were taxonomically classified using a contig annotation tool pipeline with default parameters. Microbial taxonomic compositions were profiled quantitatively by Metaphlan2 with the default parameters.[43] ORF detection from microbial contigs was done in the same way as that from viral contigs. To quantify ARG abundances, predicted protein sequences after ORF detection were searched with diamond against NCBI nonredundant (nr) protein database, and the resulting ARG-like-sequences were annotated by using the ARG-OAP pipeline and the SARG (v2.2) database.[44] ARG abundances were normalized to the corresponding ARG reference sequence length and the number of 16S rRNA genes.

**Statistical Analysis.** All statistics analyses were performed using software-R (3.5.3 version). $\alpha$-diversity, nonmetric multidimensional scaling (NMDS) analysis, and multivariate analysis of similarity (ANOSIM) were performed using the "vegan" package. The differentially abundant vOTUs among treatments were identified using the linear discriminant analysis (LDA) effect size (LEfSe) method ($P$ < 0.01 and an LDA score >2.5).[45,46] According to the results of LEfSe, we further show the variation of the centered log ratio (CLR) transformed data of top 6 vOTUs enriched by the high dosage of sewage sludge treatment (4 SS). The CLR transformation of vOTU data was performed with "zComposition" and "rgl" packages. The proportions of dominant vOTUs (relative abundance ≥ 0.01%) shared between viromes were clustered using the complete method and visualized by the package "pheatmap".

To reveal the correlation between the dosage of the fertilizer and viral community composition, regression analysis based on the first coordinate of the principal coordinate analysis (PCoA1) was conducted by the "lm" function and Spearman's rank correlation test [false discovery rate (FDR)-adjusted $P$ value]. To investigate the relationship of viral and bacterial community patterns, mantel tests with the Spearman's rank correlation ($n_{perm}$ = 9999) and Procrustes test (Bray−Curtis dissimilarity based) were conducted in R with the package "vegan". To determine the relative importance of environmental factors (bacteria communities and soil properties) in structuring viral communities, multiple regression analysis was conducted using a multiple regression on the matrix (MRM) approach.[47] The R package "Hmisc" was used to assess the redundancy of the environmental variables by the "VARCLUS" function before applying MRM. The variables with Spearman' $\rho^2$ > 0.7 were removed from MRM analysis, and nonsignificant variables ($P$ < 0.05) were removed from model results, and then, the MRM test was rerun in R with "ecodist" package until the absence of nonsignificant variables.

Network analysis approaches are widely used to explore the interactions or associations among microbial taxa in ecological studies.[48] Because bacteria was the vital determinant of viral survival and production,[49] the co-occurrence patterns between virus and bacteria could be used to explore the association of the virus and bacteria. The co-occurrence patterns between virus and bacteria (species-level) were assessed using the network analysis approach. Correlation matrixes by calculating all possible pair-wise Spearman's rank correlations were constructed by the package "psych" in R with the threshold (strong coefficient $|\rho|$ > 0.8 and [FDR]-adjusted $P$ < 0.01),[48] and the correlations were visualized in a network created by Gephi (v. 0.9.2) using the Fruchterman Reingold algorithm. All scripts and intermediate files are available at: https://github.com/superahura/Virome_of_agricultural_soils.

## RESULTS

**Soil Properties in Different Fertilization Treatments.** The soil physicochemical properties under different fertilization applications are shown in Table S1. The content of SOM and arsenic in sewage sludge treatments (except for 0.5 SS) was the highest compared with the other treatments. Under the application of sewage sludge and chicken manure, concentrations of TC, TN, P, Cu, Zn, and DOC were higher than other treatments, while pH and C/N were relatively low in contrast to other treatments. In addition, soil physicochemical properties (e.g., TC, TN, Cu, and arsenic) did not change very much under the application of the chemical fertilizer, except for DOC, SOM, and Pb, which were higher than the control.

**Characterization of Viral Community.** The detailed information of soil virome (Tables S2 and S3, Figure S2) is summarized in Text S1. As listed in Table S4, compared with control, the richness and Simpson diversity indexes were higher in fertilizer treatments. The Shannon diversity index in organic fertilizer treatments, especially 4 SS and 1 CM, was higher than that in others. NMDS analysis based on Bray−Curtis distance revealed the dissimilarity of viral communities between different treatments (ANOSIM, $R$ = 0.57, $P$ = 0.001, Figure 1a). As depicted in Figure 1b, despite the majority of



https://doi.org/10.1021/acs.est.1c03847
Environ. Sci. Technol. 2021, 55, 13881−13890

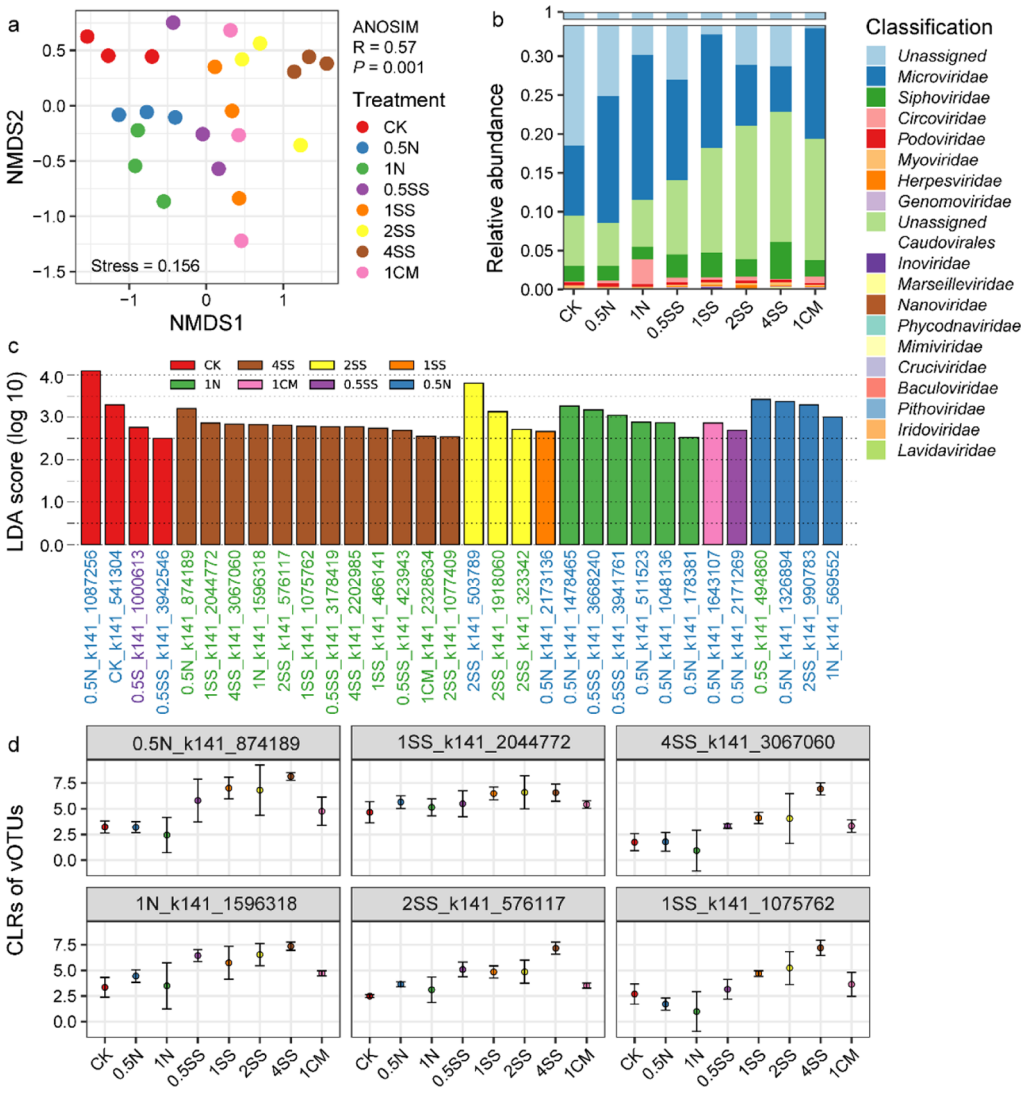



**Figure 1.** Composition and distribution of viral community in soil. (a) NMDS analysis of the viral community compositions based on Bray−Curtis similarities. The significance is assessed by ANOSIM. (b) Taxonomic distribution of vOTUs identified from eight treatments. The viral classification is described at the family level. (c) LEfSe analysis showing differentially abundant viruses (from the family level to population level) among treatments, based on $P < 0.01$ and a LDA score >2.5. Each column is labeled with the vOTU name, which was highlighted in blue, purple, and green referring to *Microviridae*, *Inoviridae*, and *Siphoviridae*, respectively. (d) CLRs of top 6 differentially abundant vOTUs identified in the high dosage of sewage sludge treatment (4 SS).

vOTUs was unassigned, the dominant families (relative abundance > 0.1%) were *Microviridae* and *Siphoviridae* among treatments (10.15−20.26%), and the relative abundance of *Siphoviridae* was elevated following the sewage sludge application. LEfSe analysis identified 3 families and 32 vOTUs ($P < 0.01$, LDA score > 2.5) that showed significantly different relative abundances among treatments (Figure 1c). *Siphoviridae* was a sole differentially abundant family in high dosage of sewage sludge treatment (4 SS), while *Microviridae* was the differentially abundant family in other treatments. At the viral population level, we observed that the top 6 vOTUs enriched in 4 SS were more abundant in sewage sludge treatments relative to the geometric mean (Figure 1d). The shared dominant vOTUs (relative abundance > 0.1% in any sample) among viromes are presented in Figure S3. It can be observed that two domains were generated. Control and chemical fertilizer treatments are grouped into a cluster because they shared more vOTUs, while organic fertilizer treatments are grouped into others.

**Drivers of Viral Community Composition.** The results of bacterial community analysis (Tables S5 and S6 and Figures S4 and S5) are described in Text S2. Regression analysis was used to investigate the impact of fertilizer concentration on viral communities (Figure 2a). For chemical fertilizer treatments, there was no significant linear regression between viral $\beta$-diversity and the dosages of the chemical fertilizer ($P = 0.197$). For sewage sludge treatments, there existed a positive correlation between viral $\beta$-diversity and the dosages of sewage sludge ($r^2 = 0.782$, Spearman $r = 0.982$, $P < 0.001$). A Mantel test showed that viral community was significantly correlated to bacterial communities ($r = 0.38$, $P < 0.001$). Procrustes analysis indicated that the viral and bacterial communities from each treatment clustered together and were separated from the other treatments, exhibiting a goodness-of-fit test (sum of squares $M^2 = 0.3832$, $P < 0.001$, $n_{perm} = 9999$, Figure 2b). The MRM was used to determine the relative contributions of bacterial communities versus soil properties to soil viral community similarity. The first model result was calculated


https://doi.org/10.1021/acs.est.1c03847
Environ. Sci. Technol. 2021, 55, 13881−13890

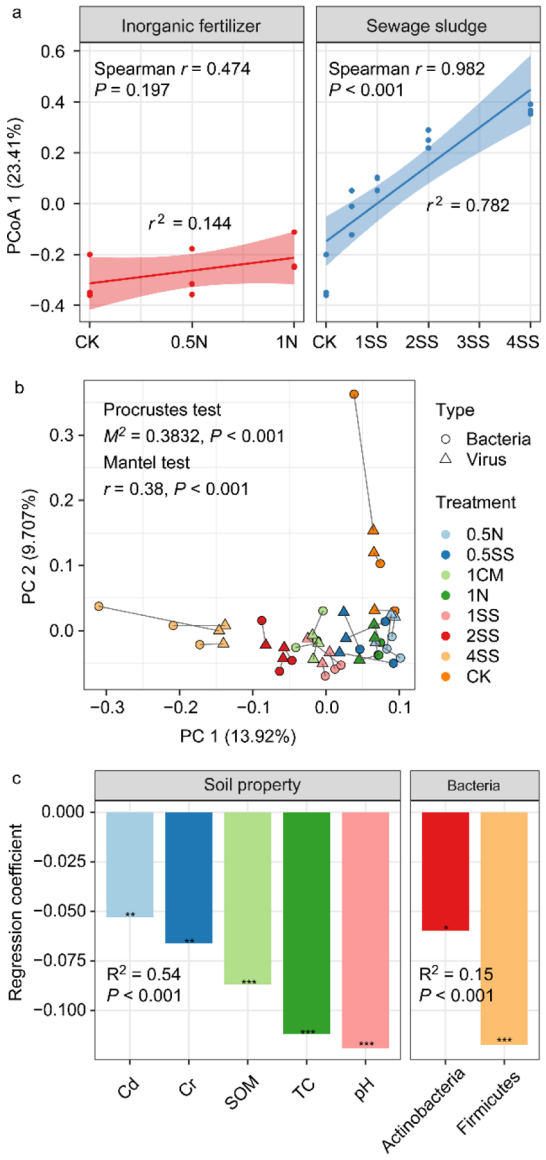



**Figure 2.** Potential factors that contribute to the variation of viral community $\beta$-diversity. (a) Regression analysis between the first dimension (PCoA 1) of viral community and the dosages of the applied fertilizer. The Spearman correlation test ([FDR]-adjusted $P$ value) was performed to evaluate the correlation between viral community and the dosages of the applied fertilizer. (b) Procrustes analysis and mantel test depicting the significant correlation between viral composition and bacterial community on the basis of Bray–Curtis dissimilarity metrics. (c) Results of MRM analysis. The first model results were calculated using soil properties as predictors, while the second model results were calculated using bacteria communities as predictors. The partial regression coefficients (b) and associated $P$-values of the remaining variables in the final MRM model are reported from the permutation test ($n_{perm}$ = 9999) if their significance levels are < 0.05. The variation ($R^2$) of ln community similarity (1 minus Bray–Curtis distance) is explained by the remaining variables. *$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.001.

from a multivariate multiple regression analysis using soil properties as predictors, and the second model result was calculated from a multivariate multiple regression analysis using bacterial communities as predictors. In this study, we only reported the variables contained in the final MRM model (Figure 2c). MRM analysis results indicated that the overall changes in viral community structures were best explained by soil properties ($R^2$ = 0.54, $P$ < 0.001), while bacterial communities only explained about 15% of the variations in viral community ($P$ < 0.001). In the first MRM model, soil pH, SOM, and TC showed the larger partial regression coefficient ($|b|$ = 0.119, 0.087 and 0.112, $P$ < 0.001, respectively), with Cr and Cd showing a smaller but significant partial regression coefficient ($|b|$ = 0.066 and 0.053, $P$ < 0.01, respectively). In the second MRM model, Firmicutes showed a larger effect on viral community similarity ($|b|$ = 0.117, $P$ < 0.001) than Actinobacteria ($|b|$ = 0.06, $P$ < 0.05).

**Diversity of ARGs in Virome and Metagenome.** The analysis results of ARGs detected in metagenome (Figures S6 and S7) are described in Text S3. After filtering with a threshold ($e$ value ≤ $10^{-5}$, query coverage ≥80 and ≥40% amino acid identity), 67 ARG-carrying vOTUs and 16 unique ARGs were retrieved. Four vOTUs were affiliated to unassigned *Caudovirales*, while the others could not be assigned to any known viral taxa (Tables S7 and S8). Most of the vOTUs (50 vOTUs, 80.65%) carried ARG-encoding resistance to chloramphenicol and macrolide–lincosamide–streptogramin (MLS). As shown in Figure 3a, the detected number of the virus-associated ARG subtype in each virome ranged from 9 to 13, with 0.5 SS and CK harboring the highest (13) and lowest (9) number of ARGs, respectively. These detected ARGs conferred resistance to aminoglycoside, chloramphenicol, MLS, multidrug, tetracyclines, trimethoprim, vancomycin, and others. Genes for trimethoprim resistance were the dominant ARG subtypes in all samples, including dfrA1, afrA12, afrA20, dfrB2, and dfrB6. The number of the ARG subtype detected in sewage sludge treatments was higher than other treatment, except 1 SS (Figure 3a). Moreover, NMDS analysis showed that the profiles of the ARG-carrying vOTU structure were different among treatments (ANOSIM, $R$ = 0.369, $P$ = 0.002, Figure 3b).

**Virus–Host Linkage Analyses.** By using in silico strategies, we next assigned putative hosts of vOTUs from bacterial RefSeq database. A total of 1357 unique CRISPR spacer matches (including 2 archaea spacers) were identified, consisting of 1019 vOTUs and 302 host genera (Figure S8). Among them, 184 vOTUs have been annotated, 168 vOTUs have multiple hosts, and 29 bacterial host genera are identified in soil metagenome (Figure S8b,c). The top 50 host genera with high frequency of matching vOTUs (occur in ≥50% of samples) are shown in Figure S9. The putative hosts could be clustered into five modules (marked with different capital letters in the heat map), while no obvious changes in taxa were observed across treatments. Analysis of CRISPR spacer sequence matches suggests that the majority of viruses infect *Pseudomonas*, *Klebsiella*, *Streptomyces*, and *Salmonella*, while most of these putative hosts could be identified as rare taxa or even unidentifiable in soil metagenome. For vOTU-carrying ARGs, 43.28% (29 vs 67 vOTUs) of them were linked to five putative bacterial phyla (Table 1). None of them infect more than one putative host. In addition, it was found 13 vOTUs sharing the same host genera (i.e., *Clostridium*, *Chryseobacterium*, *Defluviimonas*, *Bacteroides*, and *Olivibacter*).

**Co-occurrence Patterns between Virus and Bacteria.** In the present study, the co-occurrence patterns between virus and bacteria (species-level) were assessed using the network analysis approach. A total virus–bacteria coexistence network was constructed containing all samples (Figure S10a). The total network consists of 528 nodes linked by 1378 edges with


https://doi.org/10.1021/acs.est.1c03847
Environ. Sci. Technol. 2021, 55, 13881–13890

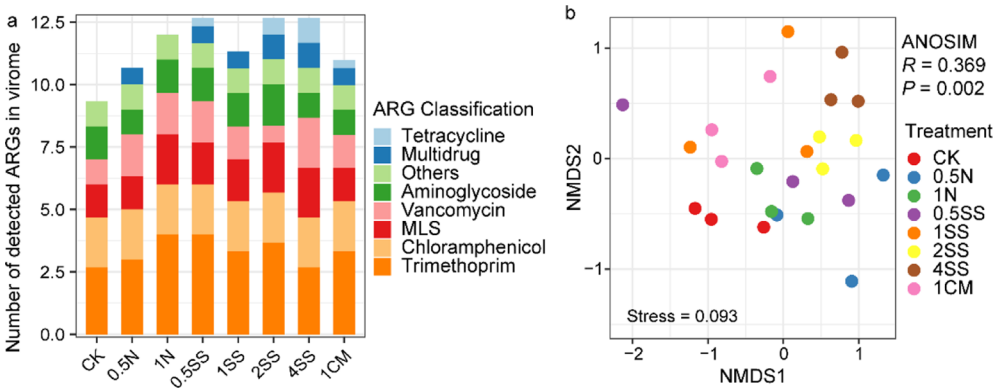



**Figure 3.** Virus-associated ARG composition and distribution in soil. (a) Numbers of ARG subtypes detected in soil vOTUs. (b) NMDS analysis of ARG-carrying viral population composition based on Bray−Curtis similarities. The significance is assessed by ANOSIM.

**Table 1. List of the Putative Bacterial Host for ARG-Carrying Virus**

| vOTU[a] | category | ARG classification[b] | ARG[c] | host phylum | host genus[d] |
|---|---|---|---|---|---|
| 4SS-k141-1849582 | lytic | chloramphenicol | catB | Crenarchaeota | *Sulfolobus* |
| 1SS-k141-1656925 | lytic | chloramphenicol | catB | Bacteroidetes | *Seonamhaeicola* |
| 4SS-k141-2191122 | lysogenic | chloramphenicol | CA | Bacteroidetes | *Chryseobacterium* |
| 1CM-k141-2385221 | lytic | chloramphenicol | CA | Firmicutes | *Peptostreptococcus* |
| 0.5N-k141-76089 | lysogenic | chloramphenicol | CA | Firmicutes | *Ruminiclostridium* |
| 1CM-k141-383223 | Lytic | chloramphenicol | CA | Firmicutes | *Clostridium* |
| 1CM-k141-1805741 | lysogenic | MLS | macB | Proteobacteria | *Gynuella* |
| 0.5N-k141-2031168 | Lytic | MLS | vatB | Bacteroidetes | *Prevotella* |
| 0.5N-k141-1496985 | Lytic | MLS | vatB | Bacteroidetes | *Bacteroides* |
| 2SS-k141-1803235 | Lytic | MLS | vatB | Bacteroidetes | *Bacteroides* |
| 2SS-k141-156123 | Lytic | MLS | vatB | Bacteroidetes | NA |
| 1SS-k141-328097 | Lytic | MLS | vatB | Bacteroidetes | *Olivibacter* |
| 4SS-k141-1504792 | Lytic | MLS | vatB | Bacteroidetes | *Olivibacter* |
| 1CM-k141-2188665 | Lytic | MLS | vatB | Bacteroidetes | *Chryseobacterium* |
| 1N-k141-146903 | Lytic | MLS | vatB | Firmicutes | *Tissierella* |
| 1CM-k141-768224 | Lytic | MLS | vatB | Facteroidetes | *Bacteroides* |
| 1CM-k141-2325400 | lysogenic | MLS | vatB | Firmicutes | *Clostridium* |
| 1CM-k141-139652 | Lytic | MLS | vatB | Bacteroidetes | *Bacteroides* |
| 4SS-k141-1304121 | Lytic | MLS | vatB | Firmicutes | *Vallitalea* |
| 4SS-k141-2740504 | lysogenic | MLS | vatB | Bacteroidetes | *Dyadobacter* |
| 1SS-k141-799572 | lysogenic | MLS | vatB | Proteobacteria | *Xenorhabdus* |
| 2SS-k141-175109 | Lytic | multidrug | ABC transporter | Firmicutes | *Lactobacillus* |
| 1CM-k141-647016 | Lytic | tetracycline | tetT | Firmicutes | *Streptococcus* |
| 1N-k141-636475 | lysogenic | trimethoprim | dfrB2 | Proteobacteria | *Defluviimonas* |
| 1CM-k141-1511081 | Lytic | trimethoprim | dfrB2 | Proteobacteria | *Defluviimonas* |
| 2SS-k141-1865148 | Lytic | trimethoprim | dfrB6 | Proteobacteria | *Agrobacterium* |
| 4SS-k141-461945 | Lytic | trimethoprim | dfrB6 | Firmicutes | *Aneurinibacillus* |
| CK-k141-252371 | lytic | trimethoprim | dfrB6 | Planctomycetes | NA |
| 2SS-k141-111207 | lytic | others | ArlR | Firmicutes | *Clostridium* |

[a]vOTU number is formed from the sample name and VC number. [b]MLS—macrolide−lincosamide−streptogramin. [c]CA—chloramphenicol acetyltransferase. [d]NA—missing genus classification.

an average degree of 5.22 (Table S9). Compared with the chemical fertilizer treatment network, there were more nodes and edges in sewage sludge treatment networks (Table S9). Meanwhile, the average degree and network density were higher in sewage sludge networks than those in chemical fertilizer treatment (Table S9). These networks could be divided into several main modules based on the clustering of nodes. The most densely connected nodes in each module were known as hubs, which were mainly from the phyla Proteobacteria, Actinobacteria, and Firmicutes (Figure S10). In addition, we observed that most of the virus−bacteria correlations were positive, and the sewage sludge treatment network (2.33 and 7.6%) had a higher proportion of negative correlations than the chemical fertilizer treatment network (1.41%).

## DISCUSSION

**Effects of Long-Term Organic Fertilization on the Viral Communities.** Fertilization plays a pivotal role in soil biogeochemical processes and affects the soil microbial community. In the study, we observed that the $\alpha$-diversity of viral community was higher after the application of chicken



https://doi.org/10.1021/acs.est.1c03847
Environ. Sci. Technol. 2021, 55, 13881−13890

manure and high dosages of sewage sludge (Table S4). The results were similar to recent observations that the viral diversity in soil treated with pig manure was higher than control.[49] One plausible explanation was that sewage sludge/chicken manure can introduce rich microorganisms to agriculture soils, including bacteria and viruses.[50] Additionally, *Microviridae*, *Siphoviridae,* and *Circoviridae* were the dominant classified viral families in all samples (Figure 1b), which were reported to be widespread and abundant in various agricultural soils, such as tomato field, paddy, and maize soils.[31,51] *Microviridae* are typical single-stranded DNA (ssDNA) phages that naturally infect Proteobacteria, *Spiroplasma*, and *Chlamydia*.[52] In general, the majority of soil viruses are dsDNA (tailed phages), and the number of ssDNA viruses is very low.[53] In the present study, however, the abundance of the ssDNA viruses, represented by *Microviridae* and *Circoviridae* (naturally infecting birds and mammals), is likely to be overestimated through the use of MDA amplification, making taxonomic results biased.[54] This might also be one reason why the proportion of *Microviridae* in soil is much higher than other families in some samples (Figure 1b). Furthermore, *Siphoviridae* (naturally infecting enterobacteria and vibrios) were observed in higher relative abundance in sewage sludge treatments (except for 2 SS) than other treatments (Figure 1b), and several of its vOTUs were elevated following sewage sludge treatment when compared with the geometric mean (Figure 1d). We speculate that this is probably a consequence of applying sewage sludge, as *Siphoviridae* were reported to be the most abundant dsDNA phages in wastewater treatment plant habitat (i.e., influent, activated sludge, and effluent).[55,56]

A previous study suggested that the application of different fertilizers could change soil characteristics as well as the soil viral abundance and diversity.[49] Consistently, we found that the structure of viral communities differed significantly among the eight treatments (Figure 1a, $P = 0.001$). Importantly, the variations of viral communities were significantly and positively correlated with the dosages of applied sewage sludge (Figure 2a, $P < 0.001$), suggesting that long-term sewage sludge application may have a greater effect on viral communities than the chemical fertilizer. Previous studies have shown that environmental factors play important roles in changing viral community structure and composition.[2,21,57] In this study, soil properties (e.g., pH, TC, and SOM) and bacteria (Actinobacteria and Firmicutes) significantly correlated with the viral community structure (Figure 2c), implying that these factors were the important drivers for viral distribution in agricultural soil. Especially, our results showed that soil properties could predict the variation of the viral community structure better than bacterial communities. According to previous reports, soil pH and SOM may affect the soil viral community distribution through influencing virus transport or bacterial communities,[20,49] and bacteria could affect viral community through directly influencing viral survival and production.[18] It should be noted that the understanding of soil viral community composition and its drivers through this study may be limited by a small sample size and high soil heterogeneity, even though the stringent standards were used during statistical analysis. Additionally, the induction procedure by mitomycin C could potentially change the original viral communities. In future studies, the extraction protocol should be optimized to avoid this limitation.

**Potential Risk of ARG-Carrying Virus in Agricultural Soil.** Currently, several studies have reported that viruses carried diverse ARGs in different environments, including soil, freshwater, marine, animal and human feces, and so on.[14,23,58−61] However, little is known about their potential contribution to the spread of ARGs in agricultural soil. The present study provided a comprehensive landscape of virus-associated ARGs in agricultural soil. A total of 67 ARG-carrying vOTUs and 16 unique ARGs were retrieved with a relative low sequence similarity (40.57−61.22%) when a lenient threshold was employed. The number of ARG subtype detected in metagenomes (171) was higher than that detected in viromes (16). The genes for trimethoprim resistance were the dominant ARG type in viromes, whose subtype number and normalized abundance were lower in metagenomes compared with other ARG types, indicating that the contribution of viruses to ARG dissemination maybe very limited in the present study. However, our results suggested that the application of sewage sludge increased the diversity of detected ARGs within viromes (except for 1 SS, Figure 3a). It was documented that sludge contains rich ARGs in virus fraction,[22] and the application of sewage sludge might facilitate the enrichment of ARGs in soil virus fraction. A virus is more persistent in ambient environments than other mobile genetic elements due to the protection of its capsids, making virus-associated ARGs a significant threat.[62] Moreover, further functional studies are required to fully determine their capacity of disseminating antibiotic resistance and evaluate the potential threat to public health.

Microbial network analysis has been demonstrated to be an important approach for investigating potential interactions among microbial taxa.[63] In this study, the association changes were explored among bacteria and virus in agricultural soil using network analysis based on spearman correlations ($\rho > 0.8$, $P > 0.01$; Figure S10). The application of high dosages of sewage sludge increased the interactions between bacteria and viruses, leading to a more intricate bacteria−virus ecological network (more nodes and potential edges). Considering that phage transduction involves the infection of the bacterial donor and recipient,[64] the complex and closer links between bacteria and virus may play an active role in promoting ARG exchange between them. For instance, *Chryseobacterium* is one of the hubs in the total network, which was identified as the host of virus-carrying vatB. This suggests the potential risk of transmitting vatB among *Chryseobacterium*, related co-occurring viruses, and their hosts. Because phage-mediated spread of resistance is more likely in eutrophic environments than in oligotrophic environments,[65] our findings indicate the potential risks of ARG transduction in organic fertilizer-amended soils and highlight the importance of good agricultural stewardship to support one health (human, animal, and environmental health).

Although the percentage of ARG-carrying vOTUs was less than 0.1% of the total vOTUs, nearly half of their hosts were putatively assigned to the pathogenic bacterial species (Table 1). For instance, some species affiliated with *Bacteroides* (e.g., *Bacteroides fragilis*) are frequently observed in clinical infectious diseases such as bacteriuria, appendicitis, and peritonitis. *Clostridium* spp., commonly found in the river, soil, animal, and human intestines, play important roles in human diseases.[66] Some of these connections were previously reported in NCBI, including the connections between *Bacteroides* and *Clostridium* mentioned above and their corresponding viruses. We also found the connections between some ARG-carrying vOTUs and pathogenic species that have


https://doi.org/10.1021/acs.est.1c03847
Environ. Sci. Technol. 2021, 55, 13881−13890

not been identified as hosts of viruses previously, including *Peptostreptococcus* and *Streptococcus* that cause pleuropulmonary, oral, and skin infections in human.[67] In addition, some vOTUs carrying different ARGs were linked to the same host (Table 1), which may promote the emergence of multidrug resistance pathogenic bacteria. These findings indicated that soil ARG-carrying viruses could serve as hidden reservoirs of ARGs available for exchange with pathogens, acting as vehicles for ARG transfer among pathogens.[68] Previous studies have shown that phages were capable of transferring ARGs to their hosts through generalized and specialized transduction.[8,9] For example, recent research by Gabashvili et al. has documented the transduction of the macrolide resistance gene (mel) between lysogenic phages (*Streptococcus* phage and *Erysipelothrix* phage) and bacterial pathogens (*Streptococcus pneumoniae* and *Bacillus coagulans*).[69] Liu et al. isolated T7-like lytic phages from the pig farm sewage treatment system and found that the ARG fragments could be acquired by phages through potential packaging during phage−host interaction, suggesting that these lytic phages have potential to participate in ARG transfer.[70] In another study, lytic coliphages randomly isolated from chicken meat were found to be capable of transferring antibiotic resistance into *Escherichia coli* through generalized transduction.[71] Considering that lytic phages were the major ARG-carrying viruses in this study, further studies are necessary to test and determine the transduction ability and the contribution of these phages to antibiotic resistance transfer, although generalized transduction is a rare event that relies on erroneous packaging.[72]

In summary, this study provides a comprehensive characterization of viral communities and virus-associated ARGs in agriculture soils. Our results demonstrate that the long-term application of organic fertilizers can alter the structure of viral communities and restructure the bacteria−virus ecological networks. Additionally, the application of sewage sludge may promote the diversity of virus-associated ARGs. These findings could provide guidelines for fertilization management to limit the spread of ARGs. In order to evaluate the potential risks of ARG dissemination via transduction, more studies are needed to verify the function of ARGs harbored by viruses. Our results underline the necessity of monitoring the virus introduced by organic fertilization prior to field application in order to mitigate the virus-mediated horizontal transfer of ARGs in microbiomes of agricultural soils, especially to pathogen hosts.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.1c03847.

Overviews of soil virome; bacterial community and ARG analysis; soil physiochemical properties; general characteristics of sequencing data; sketch map of sampling plot distribution; details on the results of soil bacteria and ARG analysis; additional information about virome, and network analysis results (PDF)

## ■ AUTHOR INFORMATION


### Corresponding Author

**Yong-Guan Zhu** − *Key Lab of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China; State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China;* orcid.org/0000-0003-3861-8482; Email: ygzhu@rcees.ac.cn

### Authors

**Mo-Lian Chen** − *Key Lab of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China; University of Chinese Academy of Sciences, Beijing 100049, China;* orcid.org/0000-0001-8057-9910

**Xin-Li An** − *Key Lab of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China*

**Hu Liao** − *Key Lab of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China; University of Chinese Academy of Sciences, Beijing 100049, China*

**Kai Yang** − *Key Lab of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China;* orcid.org/0000-0002-3554-3334

**Jian-Qiang Su** − *Key Lab of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China;* orcid.org/0000-0003-1875-249X

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.est.1c03847


### Author Contributions

M.-L.C.: experiment and data analyses, visualization, and writing the manuscript. X.-L.A.: project administration, analysis of data with constructive discussions, and review and revision of the manuscript. H.L.: analysis of DNA sequencing results (produce metadata) and analysis of data. K.Y.: visualization and review and revision of the manuscript. J.-Q.S.: review and revision of the manuscript. Y.-G.Z.: review and revision of the manuscript, supervision, and funding acquisition.

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

This work was supported by the Natural Science Foundation of China (42021005, 21936006, 32061143015, and 81991535) and the National Key Research and Development Plan (2020YFC1806902). We want to express our appreciation to Qian Xiang and Xin-Yuan Zhou (from the Y.-G.Z. laboratory) for helping with sample collection, especially to the editor and anonymous referees for their valuable suggestions and great efforts on our manuscript.

## ■ REFERENCES

(1) Emerson, J. B. Soil viruses: a new hope. *mSystems* **2019**, *4*, No. e00120-19.

(2) Williamson, K. E.; Fuhrmann, J. J.; Wommack, K. E.; Radosevich, M. Viruses in soil ecosystems: an unknown quantity within an unexplored territory. *Annu. Rev. Virol.* **2017**, *4*, 201−219.

(3) Brum, J. R.; Sullivan, M. B. Rising to the challenge: accelerated pace of discovery transforms marine virology. *Nat. Rev. Microbiol.* **2015**, *13*, 147−159.

(4) Kimura, M.; Jia, Z.-J.; Nakayama, N.; Asakawa, S. Ecology of viruses in soils: past, present and future perspectives. *Soil Sci. Plant Nutr.* **2008**, *54*, 1−32.

(5) Emerson, J. B.; Roux, S.; Brum, J. R.; Bolduc, B.; Woodcroft, B. J.; Jang, H. B.; Singleton, C. M.; Solden, L. M.; Naas, A. E.; Boyd, J.

A.; Hodgkins, S. B.; Wilson, R. M.; Trubl, G.; Li, C.; Frolking, S.; Pope, P. B.; Wrighton, K. C.; Crill, P. M.; Chanton, J. P.; Saleska, S. R.; Tyson, G. W.; Rich, V. I.; Sullivan, M. B. Host-linked soil viral ecology along a permafrost thaw gradient. *Nat. Microbiol.* **2018**, *3*, 870−880.
(6) Pratama, A. A.; van Elsas, J. D. The "neglected" soil virome − potential role and impact. *Trends Microbiol.* **2018**, *26*, 649−662.
(7) Breitbart, M. Marine viruses: truth or dare. *Annu. Rev. Mar. Sci.* **2012**, *4*, 425−448.
(8) Touchon, M.; Moura de Sousa, J. A.; Rocha, E. P. Embracing the enemy: the diversification of microbial gene repertoires by phage-mediated horizontal gene transfer. *Curr. Opin. Microbiol.* **2017**, *38*, 66−73.
(9) von Wintersdorff, C. J. H.; Penders, J.; van Niekerk, J. M.; Mills, N. D.; Majumder, S.; van Alphen, L. B.; Savelkoul, P. H. M.; Wolffs, P. F. G. Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. *Front. Microbiol.* **2016**, *7*, 173.
(10) Gómez-Gómez, C.; Blanco-Picazo, P.; Brown-Jaque, M.; Quirós, P.; Rodríguez-Rubio, L.; Cerdà-Cuellar, M.; Muniesa, M. Infectious phage particles packaging antibiotic resistance genes found in meat products and chicken feces. *Sci. Rep.* **2019**, *9*, 13281.
(11) Moon, K.; Jeon, J. H.; Kang, I.; Park, K. S.; Lee, K.; Cha, C.-J.; Lee, S. H.; Cho, J.-C. Freshwater viral metagenome reveals novel and functional phage-borne antibiotic resistance genes. *Microbiome* **2020**, *8*, 75.
(12) Calero-Cáceres, W.; Balcázar, J. L. Antibiotic resistance genes in bacteriophages from diverse marine habitats. *Sci. Total Environ.* **2019**, *654*, 452−455.
(13) Colombo, S.; Arioli, S.; Neri, E.; Della Scala, G.; Gargari, G.; Mora, D. Viromes as genetic reservoir for the microbial communities in aquatic environments: a focus on antimicrobial-resistance genes. *Front. Microbiol.* **2017**, *8*, 1095.
(14) Debroas, D.; Siguret, C. Viruses as key reservoirs of antibiotic resistance genes in the environment. *ISME J.* **2019**, *13*, 2856−2867.
(15) Göller, P. C.; Haro-Moreno, J. M.; Rodriguez-Valera, F.; Loessner, M. J.; Gómez-Sanz, E. Uncovering a hidden diversity: optimized protocols for the extraction of dsDNA bacteriophages from soil. *Microbiome* **2020**, *8*, 17.
(16) Chen, Q.; An, X.; Li, H.; Su, J.; Ma, Y.; Zhu, Y.-G. Long-term field application of sewage sludge increases the abundance of antibiotic resistance genes in soil. *Environ. Int.* **2016**, *92*−*93*, 1−10.
(17) Trandel, M. A.; Vigardt, A.; Walters, S. A.; Lefticariu, M.; Kinsel, M. Nitrogen isotope composition, nitrogen amount, and fruit yield of tomato plants affected by the soil−fertilizer types. *ACS Omega* **2018**, *3*, 6419−6426.
(18) Gómez, P.; Buckling, A. Bacteria-phage antagonistic coevolution in soil. *Science* **2011**, *332*, 106.
(19) Lawson, K. A.; Barnet, Y. M.; McGilchrist, C. A. Environmental factors influencing numbers of rhizobium leguminosarum biovar trifolii and its bacteriophages in two field soils. *Appl. Environ. Microbiol.* **1987**, *53*, 1125−1131.
(20) Dowd, S. E.; Pillai, S. D.; Wang, S.; Corapcioglu, M. Y. Delineating the specific influence of virus isoelectric point and size on virus adsorption and transport through sandy soils. *Appl. Environ. Microbiol.* **1998**, *64*, 405−410.
(21) Zhao, B.; Zhang, H.; Zhang, J.; Jin, Y. Virus adsorption and inactivation in soil as influenced by autochthonous microorganisms and water content. *Soil Biol. Biochem.* **2008**, *40*, 649−659.
(22) Calero-Cáceres, W.; Melgarejo, A.; Colomer-Lluch, M.; Stoll, C.; Lucena, F.; Jofre, J.; Muniesa, M. Sludge as a potential important source of antibiotic resistance genes in both the bacterial and bacteriophage fractions. *Environ. Sci. Technol.* **2014**, *48*, 7602−7611.
(23) Yang, Y.; Xie, X.; Tang, M.; Liu, J.; Tuo, H.; Gu, J.; Tang, Y.; Lei, C.; Wang, H.; Zhang, A. Exploring the profile of antimicrobial resistance genes harboring by bacteriophage in chicken feces. *Sci. Total Environ.* **2020**, *700*, 134446.
(24) Bolan, N. S.; Baskaran, S.; Thiagarajan, S. An evaluation of the methods of measurement of dissolved organic carbon in soils, manures, sludges, and stream water. *Commun. Soil Sci. Plant Anal.* **2008**, *27*, 2723−2737.
(25) Lu, R. *Methods of Soil Agricultural Chemical Analysis*; China Agricultural Science and Technology Press: Beijing, 1999; pp 108−109.
(26) Jin, M.; Guo, X.; Zhang, R.; Qu, W.; Gao, B.; Zeng, R. Diversities and potential biogeochemical impacts of mangrove soil viruses. *Microbiome* **2019**, *7*, 58.
(27) Oliveira, J.; Mahony, J.; Hanemaaijer, L.; Kouwen, T. R. H. M.; Neve, H.; MacSharry, J.; van Sinderen, D. Detecting lactococcus lactis prophages by mitomycin C-mediated induction coupled to flow cytometry analysis. *Front. Microbiol.* **2017**, *8*, 1343.
(28) Quirós, P.; Colomer-Lluch, M.; Martínez-Castillo, A.; Miró, E.; Argente, M.; Jofre, J.; Navarro, F.; Muniesa, M. Antibiotic resistance genes in the bacteriophage DNA fraction of human fecal samples. *Antimicrob. Agents Chemother.* **2014**, *58*, 606−609.
(29) Suzuki, M. T.; Taylor, L. T.; DeLong, E. F. Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5′-nuclease assays. *Appl. Environ. Microbiol.* **2000**, *66*, 4605.
(30) Ye, M.; Sun, M.; Zhao, Y.; Jiao, W.; Xia, B.; Liu, M.; Feng, Y.; Zhang, Z.; Huang, D.; Huang, R.; Wan, J.; Du, R.; Jiang, X.; Hu, F. Targeted inactivation of antibiotic-resistant Escherichia coli and Pseudomonas aeruginosa in a soil-lettuce system by combined polyvalent bacteriophage and biochar treatment. *Environ. Pollut.* **2018**, *241*, 978−987.
(31) Han, L.-L.; Yu, D.-T.; Zhang, L.-M.; Shen, J.-P.; He, J.-Z. Genetic and functional diversity of ubiquitous DNA viruses in selected chinese agricultural soils. *Sci. Rep.* **2017**, *7*, 45142.
(32) Bolger, A. M.; Lohse, M.; Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **2014**, *30*, 2114−2120.
(33) Langmead, B.; Salzberg, S. L. Fast gapped-read alignment with Bowtie 2. *Nat. Methods* **2012**, *9*, 357−359.
(34) Li, D.; Liu, C.-M.; Luo, R.; Sadakane, K.; Lam, T.-W. MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics* **2015**, *31*, 1674−1676.
(35) Kieft, K.; Zhou, Z.; Anantharaman, K. VIBRANT: automated recovery, annotation and curation of microbial viruses, and evaluation of viral community function from genomic sequences. *Microbiome* **2020**, *8*, 90.
(36) Fu, L.; Niu, B.; Zhu, Z.; Wu, S.; Li, W. accelerated for clustering the next-generation sequencing data. *Bioinformatics* **2012**, *28*, 3150−3152.
(37) Gregory, A. C.; Zayed, A. A.; Conceição-Neto, N.; Temperton, B.; Bolduc, B.; Alberti, A.; Ardyna, M.; Arkhipova, K.; Carmichael, M.; Cruaud, C.; Dimier, C.; Domínguez-Huerta, G.; Ferland, J.; Kandels, S.; Liu, Y.; Marec, C.; Pesant, S.; Picheral, M.; Pisarev, S.; Poulain, J.; Tremblay, J.-É.; Vik, D.; Babin, M.; Bowler, C.; Culley, A. I.; de Vargas, C.; Dutilh, B. E.; Iudicone, D.; Karp-Boss, L.; Roux, S.; Sunagawa, S.; Wincker, P.; Sullivan, M. B.; Acinas, S. G.; Babin, M.; Bork, P.; Boss, E.; Bowler, C.; Cochrane, G.; de Vargas, C.; Follows, M.; Gorsky, G.; Grimsley, N.; Guidi, L.; Hingamp, P.; Iudicone, D.; Jaillon, O.; Kandels-Lewis, S.; Karp-Boss, L.; Karsenti, E.; Not, F.; Ogata, H.; Pesant, S.; Poulton, N.; Raes, J.; Sardet, C.; Speich, S.; Stemmann, L.; Sullivan, M. B.; Sunagawa, S.; Wincker, P. Marine DNA viral macro-and microdiversity from pole to pole. *Cell* **2019**, *177*, 1109−1123.
(38) Trubl, G.; Jang, H. B.; Roux, S.; Emerson, J. B.; Solonenko, N.; Vik, D. R.; Solden, L.; Ellenbogen, J.; Runyon, A. T.; Bolduc, B.; Woodcroft, B. J.; Saleska, S. R.; Tyson, G. W.; Wrighton, K. C.; Sullivan, M. B.; Rich, V. I. Soil viruses are underexplored players in ecosystem carbon processing. *mSystems* **2018**, *3*, No. e00076-18.
(39) Manrique, P.; Bolduc, B.; Walk, S. T.; van der Oost, J.; de Vos, W. M.; Young, M. J. Healthy human gut phageome. *Proc. Natl. Acad. Sci. U.S.A.* **2016**, *113*, 10400.
(40) Hyatt, D.; Chen, G.-L.; LoCascio, P. F.; Land, M. L.; Larimer, F. W.; Hauser, L. J. Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinf.* **2010**, *11*, 119.

(41) Bin Jang, H.; Bolduc, B.; Zablocki, O.; Kuhn, J. H.; Roux, S.; Adriaenssens, E. M.; Brister, J. R.; Kropinski, A. M.; Krupovic, M.; Lavigne, R.; Turner, D.; Sullivan, M. B. Taxonomic assignment of uncultivated prokaryotic virus genomes is enabled by gene-sharing networks. *Nat. Biotechnol.* **2019**, *37*, 632−639.
(42) Shkoporov, A. N.; Clooney, A. G.; Sutton, T. D. S.; Ryan, F. J.; Daly, K. M.; Nolan, J. A.; McDonnell, S. A.; Khokhlova, E. V.; Draper, L. A.; Forde, A.; Guerin, E.; Velayudhan, V.; Ross, R. P.; Hill, C. The human gut virome is highly diverse, stable, and individual specific. *Cell Host Microbe* **2019**, *26*, 527−541.e5.
(43) Truong, D. T.; Franzosa, E. A.; Tickle, T. L.; Scholz, M.; Weingart, G.; Pasolli, E.; Tett, A.; Huttenhower, C.; Segata, N. MetaPhlAn2 for enhanced metagenomic taxonomic profiling. *Nat. Methods* **2015**, *12*, 902−903.
(44) Yang, Y.; Jiang, X.; Chai, B.; Ma, L.; Li, B.; Zhang, A.; Cole, J. R.; Tiedje, J. M.; Zhang, T. ARGs-OAP: online analysis pipeline for antibiotic resistance genes detection from metagenomic data using an integrated structured ARG-database. *Bioinformatics* **2016**, *32*, 2346−2351.
(45) Segata, N.; Izard, J.; Waldron, L.; Gevers, D.; Miropolsky, L.; Garrett, W. S.; Huttenhower, C. Metagenomic biomarker discovery and explanation. *Genome Biol.* **2011**, *12*, R60.
(46) Bi, L.; Yu, D.-T.; Du, S.; Zhang, L.-M.; Zhang, L.-Y.; Wu, C.-F.; Xiong, C.; Han, L.-L.; He, J.-Z. Diversity and potential biogeochemical impacts of viruses in bulk and rhizosphere soils. *Environ. Microbiol.* **2021**, *23*, 588−599.
(47) Wang, X.-B.; Lü, X.-T.; Yao, J.; Wang, Z.-W.; Deng, Y.; Cheng, W.-X.; Zhou, J.-Z.; Han, X.-G. Habitat-specific patterns and drivers of bacterial $\beta$-diversity in China's drylands. *ISME J.* **2017**, *11*, 1345−1358.
(48) Li, B.; Yang, Y.; Ma, L.; Ju, F.; Guo, F.; Tiedje, J. M.; Zhang, T. Metagenomic and network analysis reveal wide distribution and co-occurrence of environmental antibiotic resistance genes. *ISME J.* **2015**, *9*, 2490−2502.
(49) Chen, L.; Xun, W.; Sun, L.; Zhang, N.; Shen, Q.; Zhang, R. Effect of different long-term fertilization regimes on the viral community in an agricultural soil of southern China. *Eur. J. Soil Biol.* **2014**, *62*, 121−126.
(50) Bibby, K.; Peccia, J. Identification of viral pathogen diversity in sewage sludge by metagenome analysis. *Environ. Sci. Technol.* **2013**, *47*, 1945−1951.
(51) Santos-Medellin, C.; Zinke, L. A.; ter Horst, A. M.; Gelardi, D. L.; Parikh, S. J.; Emerson, J. B. Viromes outperform total metagenomes in revealing the spatiotemporal patterns of agricultural soil viral communities. *ISME J.* **2021**, *15*, 1956−1970.
(52) Brentlinger, K. L.; Hafenstein, S.; Novak, C. R.; Fane, B. A.; Borgon, R.; McKenna, R.; Agbandje-McKenna, M. Microviridae, a family divided: isolation, characterization, and genome sequence of $\varphi$MH2K, a bacteriophage of the obligate intracellular parasitic bacterium bdellovibrio bacteriovorus. *J. Bacteriol.* **2002**, *184*, 1089−1094.
(53) Segobola, J.; Adriaenssens, E.; Tsekoa, T.; Rashamuse, K.; Cowan, D. Exploring viral diversity in a unique South African soil habitat. *Sci. Rep.* **2018**, *8*, 111.
(54) Kim, K.-H.; Bae, J.-W. Amplification methods bias metagenomic libraries of uncultured single-stranded and double-stranded DNA viruses. *Appl. Environ. Microbiol.* **2011**, *77*, 7663−7668.
(55) Wang, Y.; Jiang, X.; Liu, L.; Li, B.; Zhang, T. High-resolution temporal and spatial patterns of virome in wastewater treatment systems. *Environ. Sci. Technol.* **2018**, *52*, 10337−10346.
(56) Parmar, K.; Dafale, N.; Pal, R.; Tikariha, H.; Purohit, H. An Insight into phage diversity at environmental habitats using comparative metagenomics approach. *Curr. Microbiol.* **2018**, *75*, 132−141.
(57) Adriaenssens, E. M.; Kramer, R.; Van Goethem, M. W.; Makhalanyane, T. P.; Hogg, I.; Cowan, D. A. Environmental drivers of viral community composition in antarctic soils identified by viromics. *Microbiome* **2017**, *5*, 83.
(58) Lekunberri, I.; Subirats, J.; Borrego, C. M.; Balcázar, J. L. Exploring the contribution of bacteriophages to antibiotic resistance. *Environ. Pollut.* **2017**, *220*, 981−984.
(59) Colombo, S.; Arioli, S.; Guglielmetti, S.; Lunelli, F.; Mora, D. Virome-associated antibiotic-resistance genes in an experimental aquaculture facility. *FEMS Microbiol. Ecol.* **2016**, *92*, fiw003.
(60) Modi, S. R.; Lee, H. H.; Spina, C. S.; Collins, J. J. Antibiotic treatment expands the resistance reservoir and ecological network of the phage metagenome. *Nature* **2013**, *499*, 219−222.
(61) Fancello, L.; Desnues, C.; Raoult, D.; Rolain, J. M. Bacteriophages and diffusion of genes encoding antimicrobial resistance in cystic fibrosis sputum microbiota. *J. Antimicrob. Chemother.* **2011**, *66*, 2448−2454.
(62) Calero-Cáceres, W.; Muniesa, M. Persistence of naturally occurring antibiotic resistance genes in the bacteria and bacteriophage fractions of wastewater. *Water Res.* **2016**, *95*, 11−18.
(63) Albert, R.; Barabási, A.-L. Statistical mechanics of complex networks. *Rev. Mod. Phys.* **2002**, *74*, 47−97.
(64) Balcazar, J. L. Bacteriophages as vehicles for antibiotic resistance genes in the environment. *PLoS Pathog.* **2014**, *10*, No. e1004219.
(65) Arya, S.; Todman, H.; Baker, M.; Hooton, S.; Millard, A.; Kreft, J.-U.; Hobman, J. L.; Stekel, D. J. A generalised model for generalised transduction: the importance of co-evolution and stochasticity in phage mediated antimicrobial resistance transfer. *FEMS Microbiol. Ecol.* **2020**, *96*, fiaa100.
(66) Patrick, S. 91-Bacteroides. In *Molecular Medical Microbiology*; Sussman, M., Ed.; Academic Press: London, 2002; pp 1921−1948.
(67) Wexler, H. M.; Tang, Y.-W.; Sussman, M.; Liu, D.; Poxton, I. Chapter 48—Anaerobic infections: a clinical overview. In *Molecular Medical Microbiology*, 2nd ed.; Schwartzman, J., Ed.; Academic Press: Boston, 2015; pp 875−897.
(68) Dinsdale, E. A.; Edwards, R. A.; Hall, D.; Angly, F.; Breitbart, M.; Brulc, J. M.; Furlan, M.; Desnues, C.; Haynes, M.; Li, L.; McDaniel, L.; Moran, M. A.; Nelson, K. E.; Nilsson, C.; Olson, R.; Paul, J.; Brito, B. R.; Ruan, Y.; Swan, B. K.; Stevens, R.; Valentine, D. L.; Thurber, R. V.; Wegley, L.; White, B. A.; Rohwer, F. Functional metagenomic profiling of nine biomes. *Nature* **2008**, *452*, 629−632.
(69) Gabashvili, E.; Osepashvili, M.; Koulouris, S.; Ujmajuridze, L.; Tskhitishvili, Z.; Kotetishvili, M. Phage transduction is involved in the intergeneric spread of antibiotic resistance-associated blaCTX-M, mel, and tetM Loci in natural populations of some human and animal bacterial pathogens. *Curr. Microbiol.* **2020**, *77*, 185−193.
(70) Liu, J.; Liu, P.; Feng, F.; Zhang, J.; Li, F.; Wang, M.; Sun, Y. Evaluation of Potential ARG Packaging by Two Environmental T7-Like Phage during Phage-Host Interaction. *Viruses* **2020**, *12*, 1060.
(71) Shousha, A.; Awaiwanont, N.; Sofka, D.; Smulders, F. J. M.; Paulsen, P.; Szostak, M. P.; Humphrey, T.; Hilbert, F. Bacteriophages isolated from chicken meat and the horizontal transfer of antimicrobial resistance genes. *Appl. Environ. Microbiol.* **2015**, *81*, 4600−4606.
(72) Volkova, V. V.; Lu, Z.; Besser, T.; Gröhn, Y. T. Modeling the infection dynamics of bacteriophages in enteric Escherichia coli: estimating the contribution of transduction to antimicrobial gene spread. *Appl. Environ. Microbiol.* **2014**, *80*, 4350−4362.


https://doi.org/10.1021/acs.est.1c03847
*Environ. Sci. Technol.* 2021, 55, 13881−13890