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Sampling Natural Viral Communities from Soil for Culture-Independent Analyses

Kurt E. Williamson,[1] K. Eric Wommack,[1]* and Mark Radosevich[2]

Department of Plant and Soil Sciences, University of Delaware, Newark, Delaware 19716,[1] and Department of Biosystems Engineering and Environmental Science, University of Tennessee, Knoxville, Tennessee 37996[2]

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An essential first step in investigations of viruses in soil is the evaluation of viral recovery methods suitable for subsequent culture-independent analyses. In this study, four elution buffers (10% beef extract, 250 mM glycine buffer, 10 mM sodium pyrophosphate, and 1% potassium citrate) and three enumeration techniques (plaque assay, epifluorescence microscopy [EFM], and transmission electron microscopy [TEM]) were compared to determine the best method of extracting autochthonous bacteriophages from two Delaware agricultural soils. Beef extract and glycine buffer were the most effective in eluting viable phages inoculated into soils (up to 29% recovery); however, extraction efficiency varied significantly with phage strain. Potassium citrate eluted the highest numbers of virus-like particles from both soils based on enumerations by EFM (mean, 5.3 $\times$ 10^8 g of dry soil^{-1}), but specific soil-eluant combinations posed significant problems to enumeration by EFM. Observations of virus-like particles under TEM gave confidence that the particles were, in fact, phages, but TEM enumerations yielded measurements of phage abundance (mean, 1.5$\times$$10^8$ g of dry soil^{-1}) that were about five times lower. Clearly, the measurement of phage abundance in soils varies with both the extraction and enumeration methodology; thus, it is important to assess multiple extraction and enumeration approaches prior to undertaking ecological studies of phages in a particular soil.

The relatively recent discovery of high viral abundance in marine sediments (9, 17, 36) and the water column (11, 23, 42) has led to increasing interest in the possible effects of viral activity on ecosystem processes. As a result, viruses are now recognized as important participating members of aquatic microbial communities (13, 14; for reviews, see references 21 and 47). Through infection and lysis, viruses exert a direct effect on bacterial mortality (32, 47), although the regulation of the diversity of bacterial species within a community may be a more significant ecological effect (15, 32).

A growing body of evidence indicates that viruses have a significant impact on biogeochemical cycles in marine environments (7, 14, 20, 32). Furthermore, phage-mediated processes, such as transduction and lysogenic conversion, influence the genetic composition and in some cases the expressed phenotype of the host (12, 26, 45). Clearly, viruses possess an enormous potential for having a global impact on microbial ecology.

In contrast to the growing knowledge of the role of viruses in marine microbial communities, the ecological role of viruses in the microbial communities in soil remains relatively unexplored. Ashelford and coworkers (3, 5, 6) have performed the most extensive field studies, in which the population dynamics of six naturally occurring bacteriophages were monitored as they competed for an introduced *Serratia liquifaciens* host in the rhizosphere of sugar beets. The most striking of the researchers' findings was that the temporal dynamics of phage and host populations were almost identical over three consecutive years. In a microcosm study of the interactions between two phage strains and their *Bacillus subtilis* host, it was found that *B. subtilis* chemically ameliorated the soil environment for the phages (35). Furthermore, the dynamics of the phage-host population tended toward equilibrium, with bacteria outnumbering phages. More recently, Keel et al. (30) pointed out the overlooked potential of phage activity in soil when they found that the efficacy of the widely used biocontrol bacterium *Pseudomonas fluorescens* CHA0 was completely eradicated by an epidemic of autochthonous phage in the rhizosphere of cucumbers.

These studies unearthed vital information regarding the ecological role of viruses in soils; however, each study relied upon cultivable hosts for the detection and quantification of viruses. It is estimated that only 1 to 5% of autochthonous soil bacteria are culturable (43). Considering that most bacterial hosts can be infected by multiple phage strains, it is highly likely that less than 1% of extant soil phages have been isolated. Thus, culture-independent detection and analyses will be critical to obtaining a more complete understanding of the ecological impacts of viruses in soils.

The first step in any such analysis is the effective extraction of viruses from soil. Several studies have detailed the mechanics and kinetics of virus adsorption to soil particles (16, 22, 29, 31), as well as the physical and chemical factors influencing adsorption and desorption, including solution pH, the isoelectric point of phage capsids (18), ionic strength and composition of the eluant (37, 49), and hydrophobic interactions (22; for a review, see reference 19). Obviously, several concerted factors dictate the phage-soil interactions. Therefore, rather than attempting to develop an ideal eluant, methods were selected based upon empirical values obtained in extracting viruses from soils and other porous media.

In soils, 10% beef extract has been commonly used for ex-

* Corresponding author. Mailing address: Delaware Biotechnology Institute, 15 Innovation Way, Newark, DE 19711. Phone: (302) 831-4362. Fax: (302) 831-3447. E-mail: wommack@dbi.udel.edu.

TABLE 1. Physical and chemical properties of soils[a]

Soil	pH	OM (%)	% Sand	% Silt	% Clay	ECEC (cmol/kg)
Matapeake silt loam	5.9	1.7	9	75	16	4.8
Evesboro loamy sand	6.1	0.1	85	12	3	1.4

[a] Values are based on duplicate samples; all associated standard errors are within 5%. OM, organic matter content (% dry weight); ECEC, effective cation exchange capacity.

tracting viable, primarily enteric phages (27, 28); however, beef extract has not been evaluated for use with direct counting procedures. Analyses of viruses in other porous media, namely, sewage sludge and marine sediments, have relied on a variety of extraction techniques, many aimed at the direct counting of viruses. Glycine buffer (250 mM) (2) and 7% beef extract (33) worked well for eluting viable coliphages from sewage sludge. For marine sediments, several methods of eluting virus-like particles (VLPs) have been used, including 6% beef extract (36), 1% potassium citrate, Trypticase soy broth, and 10 mM sodium pyrophosphate (10, 17).

Clearly, a wide range of published methods have been successful in extracting phages from porous media; however, no reports have evaluated the applicability of multiple extraction-elution methods to direct counting or culture-independent studies of soil phages. In this experiment, four elution buffers (10% beef extract, 250 mM glycine buffer, 10 mM sodium pyrophosphate, and 1% potassium citrate) were compared to determine the most effective method of extracting indigenous viruses from two Delaware agricultural soils. Extraction efficiencies were determined by viable counts (plaque assay of inoculated phages), direct counts of VLPs by epifluorescence microscopy (EFM), and direct counts by transmission electron microscopy (TEM).

MATERIALS AND METHODS

Determination of extraction efficiency by viable counts. (i) Soils. Two agricultural soils were used in this study: Matapeake silt loam collected from the University of Delaware Agricultural Experimental Station, Newark, and Evesboro loamy sand collected from the University of Delaware Research and Extension Center, Georgetown (Table 1). Both soils were collected in June 2002, homogenized, air dried, and passed through a 2-mm sieve prior to use.

(ii) Bacteriophages. Phage inocula (Table 2) were selected on the basis of morphology, size, and host range. Morphologies representative of environmental isolates were chosen to simulate as closely as possible a cross-section of a natural phage community (1). As an additional criterion, we selected phage unlikely to encounter susceptible hosts in soil environments to eliminate the need for the sterilization of soils.

(iii) Eluant solutions. The beef extract (10%) solution contained, per liter, 100 g of beef extract V (BBL), 13.4 g of $Na_2HPO_4 \cdot 7H_2O$ (Fisher), and 1.2 g of citric acid monohydrate (Fisher), pH 9, as described by Hurst et al. (27). The potassium citrate (1%) solution contained, per liter, 10 g of potassium citrate (Fisher), 1.44 g of $Na_2HPO_4 \cdot 7H_2O$ (Fisher), and 0.24 g of KH_2PO_4 (Fisher), pH 7, as described by Paul et al. (36). The sodium pyrophosphate (10 mM) solution (pH 7) used was that described by Danovaro et al. (17). The glycine (250 mM) solution (pH 8) used was that described by Araujo et al. (2).

(iv) Extraction conditions. Duplicate samples of each soil were treated as follows, and the entire experiment was replicated twice. Subsamples (4.6 g of Evesboro or 4.0 g of Matapeake) were weighed in 50-ml Teflon-coated polyethylene centrifuge tubes, and sterile SM buffer (100 mM NaCl, 10 mM $MgSO_4 \cdot 6H_2O$, 50 mM Tris-Cl [pH 7.5]) or one-half-strength Trypticase soy broth (7.5 g/liter) containing a known concentration of phage was added to bring the dry sample to approximate field moisture capacity. Final sample weight was 5.0 g (0.6 ml of phage suspension was added to Evesboro samples, 12% moisture content; 1.0 ml of phage suspension was added to Matapeake samples, 20% moisture content). Soils were thoroughly mixed after the addition of phage suspensions and incubated at 4°C for 24 h prior to extraction.

A specific eluant (15 ml) was added to each tube. All samples were vortexed (Fisher Vortex Genie 2) at maximum speed for 10 s and incubated at 4°C for 15 min. Each sample was then sonicated with a Branson 185 Sonifier (Danbury, Conn.) at 100 W, 47 kHz, in an ice bath for a total of 3 min, with each minute being interrupted by 30 s of manual shaking. All tubes were centrifuged at 2,500 $\times g$ for 20 min at 4°C to sediment soil particles. Aliquots of the supernatant were used in conducting plaque assays to quantify extraction efficiency. Extraction efficiency was calculated according to the following formula: % recovery = [(initial phage titer − extract phage titer)/initial phage titer] × 100, where the initial phage titer is the concentration of the phage suspension (PFU ml^{-1}) used to inoculate the soil samples. Soil-free controls consisted of known titers of phages in SM buffer, which were carried through the extraction conditions listed above. The average recovery of phages was calculated based on the grand mean (2 × 2 replicates).

Determination of extraction efficiency of indigenous viruses by direct counts. (i) Extraction conditions. Duplicate samples of fresh, field-moist soils were collected and processed within 1 week; each extraction experiment was duplicated. Moisture content was determined gravimetrically for each soil, with triplicate samples. Subsamples (5.0 g) were weighed in 50-ml Teflon-coated polyethylene centrifuge tubes, and 15 ml of a specific eluant was added to each tube. All samples were vortexed, sonicated, and centrifuged as described above. Supernatants were passed through 0.20-μm Acrodisc filters (Pall Corporation, Ann Arbor, Mich.) to remove bacteria and small soil particles prior to further treatment. To determine the effects of nonencapsulated DNA on the enumeration of phage, aliquots of soil extracts were divided among the following treatments: heating to 98°C for 10 min, slow cooling to 20°C, followed by the addition of DNase I (Roche Diagnostics GmbH, Mannheim, Germany) to a final concentration of 1 U/μl; addition of DNase I (1 U/μl); or a control (no heat, no DNase).

TABLE 2. Properties of bacteriophages and their recovery from soil

Phage	Family	Avg capsid diam (nm)	Avg tail length (nm)	Genome	Source	Host	Mean % recovery from soil[c] (SE)
ϕX174	*Microviridae*	26	Tailless	ssDNA[d]	ATCC 13706-B1	*Escherichia coli* strain B	42 (7.1)
T4	*Myoviridae*	95	290	dsDNA	ATCC 11303-B4	*Escherichia coli* strain B	15 (3.9)
ϕCB38	*Podoviridae*	56	18	dsDNA	K. E. Wommack[a]	*Aeromonas* spp.	17 (6.2)
ϕCB45	*Podovioridae*	49	8	dsDNA	K. E. Wommack	*Aeromonas* spp.	41 (5.9)
ϕCB908	*Siphoviridae*	Unknown[b]	Unknown	dsDNA	K. E. Wommack	Unknown	3.1 (1.0)

[a] Samples were isolated from Chesapeake Bay water by using cultivable indigenous hosts and a plaque assay.
[b] Unknown (measurements not available).
[c] Values are from four replicates; mean recoveries were averaged across soil and eluant types.
[d] ssDNA, single-stranded DNA.

TABLE 3. Effect of eluant on recovery of viable phages and VLPs from soil

Eluant	Mean % recovery[b] of viable phage (SE)	Mean VLP[c] g of dry soil^{-1} (10^8) (SE)
Soil-free control[a]	53.1 (7.7)	NA
10% beef extract	26.0 (5.4)	ND
1% potassium citrate	16.9 (5.2)	5.3 (0.94)
10 mM sodium pyrophosphate	15.0 (5.8)	3.1 (0.88)
250 mM glycine	28.8 (6.9)	4.5 (0.91)
Sterile deionized water	2.3 (1.0)	NA

[a] Soil-free controls were aliquots of phage in storage buffer that were carried through the same procedures as the viruses inoculated into the soils.
[b] Values are based on four replicates; recoveries were averaged across soil types and phage strains.
[c] Values are based on four replicates; VLP counts were averaged across soils. VLPs could not be enumerated in beef extract due to high background fluorescence. NA, not applicable; ND, not determined.

After the addition of DNase, all samples, including the control, were incubated at 20°C for 20 min.

(ii) EFM. Aliquots of the soil extract (100 μl) were suspended in 900 μl of sterile deionized water and vacuum filtered (~50 mm Hg) through a stack of 25-mm filters consisting of a 0.02-μm Anodisc (Whatman International, Ltd., Maidstone, England), a 0.22-μm Supor (Pall Corporation), and a glass fiber filter (Pall Corporation). Anodisc filters containing captured virus particles were stained by adding 400 μl of 1× SYBR Gold (Molecular Probes, Eugene, Oreg.). Filters were incubated for 15 min in the dark and analyzed by EFM with an Axioskop[2] microscope (Carl Zeiss Microimaging, Inc., Thornwood, N.Y.) with a fluorescein isothiocyanate excitation filter. Ten fields per sample were digitally photographed at a magnification of ×1,000 with an ORCA-ER camera (Hamamatsu Corporation, Bridgewood, N.J.). VLPs were counted with the Fovea Pro plug-in for Adobe Photoshop. VLPs were discriminated from bacteria or detritus based on pixel dimensions. The relative extraction efficiency was determined by comparing the total number of phage eluted by each eluant. Average VLP counts were calculated based on the grand mean (2 × 2 replicates).

Analysis of soil extracts with TEM. Sodium pyrophosphate extracts of both soils (50 g each) were pooled and purified via CsCl density gradient centrifugation (38). One milliliter of purified extract was suspended in 9 ml of deionized water and spun down onto Formvar-coated 650 copper mesh grids by the method of Wommack et al. (48). Grids were stained with 1% uranyl acetate for 1 min and examined at ×85,000 magnification in a CEM 902 transmission electron microscope (Carl Zeiss Microimaging, Inc.). Viruses were enumerated by counting particles in 10 fields of triplicate grids per sample, or until about 200 virus particles had been counted per grid. Virus abundance per gram of dry soil was calculated as follows: VLP gram of dry soil^{-1} = {[average VLP count × (πr_{grid}^2/A_{field}) × 10]/volume sampled} × (milliliter of eluant/gram of most soil) × (gram of most soil/gram of dry soil), where the volume sampled is equal to $\pi\ r_{grid}^2 \times h$, and h is the height of the column of solution above the grid in the centrifuge tube.

Statistical analyses. Analysis of variance was used to test for significant differences between experimental treatments and virus or VLP counts by using Super ANOVA software (Abacus Concepts, Berkeley, Calif.). Fisher's least significant difference test was used post hoc for specific means comparisons. Significant differences are quoted at a P value of 0.05 unless stated otherwise.

RESULTS

Enumeration of viable inoculated phages. There was no significant difference in phage recovery between the two soils, averaged across eluant type and phage strain. Averaged across soil types and phage strains, the comparison of eluant extraction efficiency revealed that all treatments were significantly better than deionized water in extracting introduced phages from soils. Glycine buffer was ca. 10% more efficient than potassium citrate or sodium pyrophosphate in recovering viable phages. Beef extract was 1% less efficient than glycine in extracting phages but was not found to be significantly better than the remaining buffers (Table 3).

TABLE 4. Effects of soil and eluant combinations as measured by EFM direct counts of VLPs

Soil	Eluant	Mean VLP[a] g of dry soil^{-1} (10^8) (SE)
Matapeake	Potassium citrate	5.9 (1.9)
Evesboro	Potassium citrate	4.7 (0.47)
Matapeake	Glycine	7.5 (0.8)
Evesboro	Glycine	1.5 (0.6)*
Matapeake	Sodium pyrophosphate	NA*
Evesboro	Sodium pyrophosphate	6.2 (0.78)

[a] VLPs could not be enumerated in Matapeake soil and sodium pyrophosphate due to high background fluorescence. NA, not available; *, significant difference.

Averaged across soils and eluant types, the maximum possible efficiency of extraction depended largely on the characteristics of the individual phages used in this study. Phages ϕX174 and ϕCB45 were relatively easy to extract from soil, with 42 and 41% of the initial inocula recovered, respectively. Phages ϕCB38 and T4 were not as readily extracted, with 17 and 16% recovered, respectively. In the case of ϕCB908, an extremely low extraction efficiency (3% recovery) was observed relative to the other phages (Table 2).

EFM direct counts of natural viral communities. Potassium citrate was significantly better than sodium pyrophosphate at extracting autochthonous phages from both soils, independent of DNase treatment, extracting 2.15×10^8 more VLPs g of dry soil^{-1}. The relative extraction efficiency of potassium citrate was also higher than extraction with glycine by 4.0×10^7 VLPs g of dry soil^{-1}; however, this difference was not statistically significant (Table 3). Beef extract could not be utilized as an eluant in direct counts because it was extremely difficult to filter through Anodisc filters, and when the filters were stained, accurate enumeration of VLPs was prohibited by the opalescence of the optical field.

A definite effect was observed with specific soil-eluant combinations (Table 4). The apparent extraction efficiency was significantly lower than all other treatments in two particular cases: (i) Evesboro soil with glycine buffer as the eluant and (ii) Matapeake soil with sodium pyrophosphate as the eluant. In both cases, the VLPs could not be accurately enumerated due to the opalescence of the optical field. In spite of these differences, when the number of VLPs extracted from each soil was compared independently from eluant type, no significant difference was seen between the two soils.

Counts of VLPs were slightly lower in unheated, DNase-treated samples than in untreated samples (data not shown); however, this difference was not found to be statistically significant. By contrast, soil extracts that were heated and subsequently treated with DNase contained significantly fewer VLPs. Microscopic examination of these soil extracts revealed no VLPs.

TEM direct counts. Observations with TEM confirmed that viruses were being extracted from both soils, even if particles could not be visualized by EFM (Fig. 1). The average number of viruses extracted from Matapeake soil with sodium pyrophosphate was 1.2×10^8 VLP g of dry soil^{-1}, while the average

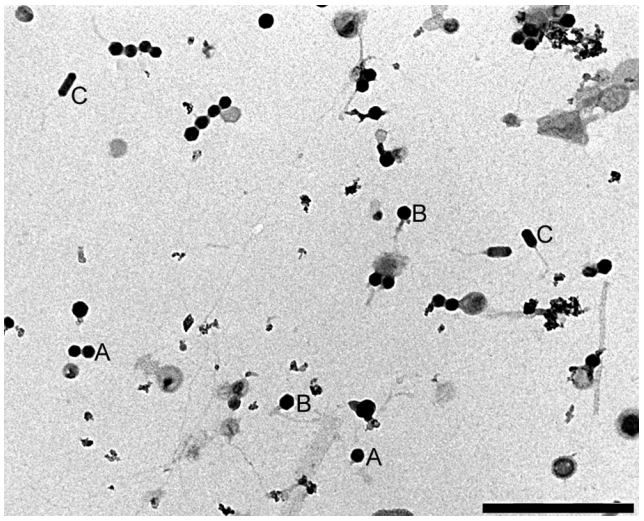

FIG. 1. Transmission electron micrograph of phage extracted with sodium pyrophosphate from Matapeake soil. This extract could not be enumerated by EFM; however, an abundance of diverse morphotypes (*Podoviridae* [A], *Myoviridae* [B], and elongate capsid [C]) was observed, indicating that phages were eluted. Bar, 500 nm.

extracted from Evesboro under the same conditions was 1.8×10^8 g of dry soil^{-1}. The grand mean averaged across the soils was 1.5×10^8 g of dry soil^{-1}, about five times lower than epifluorescence-based counts.

DISCUSSION

Viable counts of introduced phages. The Evesboro loamy sand has a much lower clay content, organic matter content, and effective cation exchange capacity than Matapeake silt loam (Table 1). Because of the lower specific surface area for adsorption, it was expected that phage extraction from Evesboro soil would be more efficient than from Matapeake soil; however, the two soils were not significantly different in their abilities to retain introduced phage particles. These results suggest that the physicochemical properties of soils may not be the most significant factors in determining the overall extraction efficiency of viable phages.

When plaque assays were used as the method of detection, glycine buffer appeared to be most effective overall in recovering viable phages from both soils, followed closely by beef extract. Both of these buffers had been adjusted to alkaline pH, whereas potassium citrate and pyrophosphate were at neutral pH. Higher pH is likely to increase electrostatic repulsion between phages and soil surfaces by deprotonating the ionizable sites on both (22). In this case, hydrophobic interactions would be the main force to overcome in desorbing phages from soil surfaces. Beef extract consists of large organic molecules that most likely disrupt hydrophobic interactions between phages and soil surfaces (22, 37; Y. Jin, personal communication), whereas glycine is a low-molecular-weight amphipathic amino acid and may be able to solvate viruses and prevent hydrophobic interactions between phages and soil particles (8, 16, 22). For these reasons, glycine and beef extract may be more effective than potassium citrate or sodium pyrophosphate in displacing adsorbed phages from binding sites. Support for these conclusions is offered in a similar comparison study in which beef extract was found to be more effective than other buffers in eluting viable phages from sewage sludge (33). However, given these results, it is not possible to exclude the lower viability of phages in potassium citrate and sodium pyrophosphate as a possible cause of the lower apparent extraction efficiencies of these eluants.

The most significant factor in recovery of viable introduced phages from soil samples was the specific strain of phage used in each experiment. It has been suggested that capsid size (i.e., effective diameter) plays a direct role in sorption by increasing the number of surface charges available for interaction with soil particles (18). However, phages ϕCB38 and ϕCB45, both *Podoviridae* of similar capsid diameter (Table 2), were found to have significantly different extraction efficiencies. Furthermore, phages ϕX174 and ϕCB45, and likewise ϕCB38 and T4, were not found to have significantly different extraction efficiencies, despite the obvious differences in capsid diameters and morphologies (Table 2). Based on the recovery of phage from soil-free controls, the very low observed extraction efficiency for ϕCB908 is likely the result of inactivation due to interactions with soil particles (Table 2). These data suggest that capsid diameter and gross morphology play minor roles in extracting a particular phage strain.

It is more likely that the isoelectric point and hydrophobicity of individual phage capsids are the most important factors influencing phage-soil interactions and, thus, relative extraction efficiency by any one method (16, 18). The fact that some phages are easier to extract than others leads to issues concerning the representative sampling of phage communities: are the phages extracted from a soil sample representative of the extant phage community? With present methods, this question is intractable for the extraction of viable bacteriophages. It is possible that extraction inactivates viruses yet still extracts intact virus particles. If this is true, extraction bias may have only a limited impact on subsequent interpretations of viral diversity in soil from culture-independent studies.

Direct counts. Direct counts of introduced model phages were not attempted due to a general inability to distinguish them from naturally occurring phages, which would be coeluted from nonsterile soils. Such an approach would have required prior sterilization of soils (i.e., autoclaving), consequently altering the physical and chemical properties of the soils and thus confounding the environmental relevance of our results (34, 39, 40). For example, in a study of population dynamics of a host and phage inoculated into sterile potting soil, autoclaving the soil reduced the pH from 6.0 to 4.5 (35). This drop in pH caused a significant decrease in the infectivity of the inoculated phage, altering the dynamics of the model system under study.

The use of fluorescently labeled phages as inocula would enable differentiation between introduced and preexisting phage populations, but this approach has been attempted only in studies of aquatic marine phages (25, 41), and it remains unclear whether such methods would work in soil extractions. For the time being, we elected to compare total VLP counts from nonsterile soils. Since only naturally occurring viruses were extracted in the direct count experiments, the best extraction was considered the method giving the highest yield of VLPs relative to all other extractions for a given soil type.

An added complication to enumerating naturally occurring phage populations is the uncertainty of whether differences in the extraction efficiency among soils (or lack thereof) exist due to differences in extractability or to differences in the total abundance of phages in a particular soil. For example, it may be more difficult to extract phages from Matapeake soil than from Evesboro, but if Matapeake soil also harbors a higher number of total phage, similar numbers may be obtained for both soils. While the recovery of introduced phages was apparently unaffected by soil properties (e.g., clay content), this may not be the case with resident viral communities. At present, the distinction between measured phage abundance and phage extractability cannot be drawn.

Another concern with direct enumeration, particularly with EFM, is the overestimation of viral abundance due to the possible inclusion of stained nonviral particles (4). To ensure that EFM total counts included only VLPs, extracts were subjected to heating and/or treatment with DNase I. In the absence of prior heat treatment, DNase had little effect on VLP counts as protein capsids protected viral genomic DNA from enzymatic degradation. However, initial heating to 98°C irreversibly denatured viral capsids, releasing genomic DNA. The subsequent addition of DNase destroyed phage genomic double-stranded DNA (dsDNA), eliminating the fluorescence signal in these samples. The use of SYBR Gold stain should have allowed for visualization of both dsDNA and single-stranded DNA viruses, as well as RNA viruses (38, 44), but the absence of any fluorescence signal following DNase treatment suggests that the majority of extracted viruses contained dsDNA genomes.

While nonspecific staining posed little difficulty, background fluorescence or opalescence of the optical field was problematic under certain conditions. In particular, beef extract prohibited the accurate enumeration of VLPs with SYBR Gold due to high background fluorescence. As a result, beef extract could not be used with either soil for direct counting with EFM. A similar problem occurred in the case of Matapeake soil extracted with sodium pyrophosphate. When extracting viruses from marine sediments, Danovaro et al. (17) noted that raising the concentration of pyrophosphate above 10 mM resulted in opalescent optical fields under EFM. Based on the rich brown color of sodium pyrophosphate extracts and the organic matter content of Matapeake soil (Table 1), it is thought that pyrophosphate is more efficient in extracting from soil humic acids, which subsequently interfere with epifluorescence staining. In any case, the background fluorescence of Matapeake sodium pyrophosphate extracts was extremely high, making enumeration difficult. Enumeration of VLPs in glycine extracts of Evesboro soil was prohibited by a similar problem with background fluorescence; however, this observation was puzzling since Evesboro has a low organic matter content (Table 1). It may follow that humic acids are not the only extractable components of soils that interfere with EFM techniques.

Because sodium pyrophosphate extracts of Matapeake soil and glycine extracts of Evesboro soil could not be enumerated, potassium citrate, which could be used to successfully enumerate VLPs from both soils, emerged as the best overall eluant for the soils examined in this study (Table 4). A comparison of these results with those of the viable count extractions made it clear that the best eluants for extracting viable phages (i.e., beef extract and glycine buffer) were not compatible with EFM. Furthermore, specific combinations of soils and eluants severely impeded the accurate enumeration of VLPs by EFM. Thus, extraction approaches will likely have to be tailored to the demands of the enumeration methodology. It should be noted, however, that in most cases an appreciable number of phages were still being eluted from the soils, even if they could not be enumerated under EFM. When soil extracts which had been problematic under EFM were processed and scanned under TEM, many viruses were observed (Fig. 1).

Recently, Ashelford and coworkers (4) extracted both indigenous and introduced phages by bead beating rhizosphere soil with water and subsequently enumerating phages by TEM. Our results of viable phage extractions indicated that water was a poor eluant; thus, water was not used in subsequent direct count enumerations. However, the phage abundance extracted with sodium pyrophosphate and enumerated by TEM was 1.5×10^8 VLP g of dry soil^{-1}, ca. 10 times higher than the figure reported by Ashelford et al. (4). TEM enumerations of VLPs from marine water and sediment samples have been about seven times lower than EFM enumerations of comparable samples (17, 24, 46). In this study, the mean VLP abundance as measured by TEM was about five times lower than the mean abundance as measured by EFM, which varied from 3.1×10^8 to 5.3×10^8 VLP g of dry soil^{-1}, depending on the extraction matrix. Clearly, the perceived abundance of VLPs in soils varies with both the extraction and enumeration methodology.

These elution techniques will be assessed for additional soils and applied toward more extensive culture-independent analyses. Future goals include the comparison of the total abundance of viruses among soils, tracking seasonal fluctuations in viral abundance, and the use of molecular techniques such as pulsed-field gel electrophoresis and metagenomic sequencing to evaluate the genetic diversity of viral communities in soil.

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