ENTEROVIRUS AND BACTERIOPHAGE INACTIVATION IN SUBSURFACE WATERS AND TRANSLOCATION IN SOIL

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ABSTRACT

Inactivation of poliovirus 1, coxsackievirus B-1 and bacteriophages was studied in surface and subsurface waters and in septic tank liquor. In situ experiments were conducted in Rio Grande River water and in shallow ground water using dialysis membrane chambers with sidewalls constructed of dialysis tubing. Laboratory inactivation studies were performed using deep groundwater and septic tank liquor. Additionally, soil adsorption-elution characteristics of the viruses and bacteriophages studied using the surface and subsurface waters as eluents.

Results showed that the viruses and bacteriophages were inactivated at different rates in the waters tested. The rate of inactivation varied with virus and bacteriophage type and with water type. Extended survival of poliovirus 1 and coxsackievirus B-1 was demonstrated in shallow groundwater and septic tank liquor when compared to survival in deep groundwater. Inactivation of bacteriophages was generally faster than the viruses, and statistical comparison of inactivation rates showed that none of the bacteriophages studied would serve as suitable general indicators of enteric virus inactivation in the waters tested.

Soil adsorption-elution studies showed that virus adsorption characteristics varied with virus, water, and soil type. The elution of the enteric viruses from soils was more efficient with septic tank liquor and deep groundwater. The results suggest that a health hazard could exist if pathogenic, enteric viruses were introduced into surface

and groundwaters which are destined for domestic or other uses. The results also show that it is impossible to make valid generalizations regarding virus survival or virus soil adsorption characteristics based on studies using only one virus, soil, or water type.

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INTRODUCTION

The finite nature of the world's water resources coupled with increased demands resulting from population pressures and industrialization, makes greater reuse of wastewaters an eventual necessity (20). At present wastewater is being recycled directly into potable water supplies in some arid regions (35) and is being used for irrigation of crops in several areas (40, 44). Other waters receiving wastewater discharge are used for domestic, industrial, agricultural, or recreational purposes after continually shortening recharge times in groundwater aquifers or shortened residence times in surface water supplies. Increased water reuse and the projected impact of the Federal Water Pollution Control Amendments of 1972 (Public Law 92-500) which require zero pollutant discharge into the nation's waters by 1985, have made the study of water pollutants and their removal a critical priority (20).

Among water pollutants of major concern for a number of years are the human enteric viruses including the causative agent of infectious hepatitis. These viruses proliferate in the human respiratory or gastrointestinal tract. Over 100 types are excreted in the feces of diseased and asymptomatic infected individuals (6, 9). The viruses are excreted into domestic wastewater disposal systems where they are routinely found in effluents which have undergone varying degrees of treatment (9, 22). These virus-contaminated effluents are subsequently discharged into receiving waters which will ultimately be recycled for various consumer uses.

Although the primary mode of transmission of most enteric viruses is not aquatic (34), waterborne transmission may serve to seed a community with low levels of viral infections which can then be spread by more conventional routes (6). Viruses can survive for periods in excess of 188 days in receiving waters (20), and one plaque forming unit (PFU) may be capable of causing infection (36). This indicates that the presence of any viruses in water poses a potential health hazard.

There are several enteric viruses of major concern in the problem of waterborne transmission of viral diseases. The picornaviruses (small RNA viruses) including poliovirus, echovirus, and coxsackievirus, which have all been isolated from wastewater effluents, surface waters, and groundwaters (9, 22, 30), have been implicated in waterborne outbreaks of disease (34). Other virus types including reovirus and adenovirus have also been implicated in waterborne disease outbreaks (34). Perhaps of greatest concern in the transmission of disease by water is the viral agent(s) responsible for infectious hepatitis. Infectious hepatitis represents the best documented cases of waterborne viral disease outbreaks, the most notable of which occurred in New Delhi, India in late 1955. An estimated 30,000 cases of the disease were traced to a contaminated water supply which was shown to be bacteriologically safe for drinking (16). Other well-documented cases of waterborne infectious hepatitis have occurred and continue to occur, indicating the real nature of this health hazard (15, 43).

The entry of enteric viruses into recyclable waters can occur in several ways. The most common method is the discharge of wastewater which has undergone various degrees of treatment into surface receiving waters where they may be transported for considerable distances (22). The viruses survive for varying periods of time depending upon the water temperature, concentration of suspended solids and degree of sewage contamination of the receiving waters (11, 20). Viruses may enter groundwater aquifers by direct discharge into wells (29), after percolation of irrigation wastewater through soil (44), and by discharge of effluent from septic tanks which serve the needs of up to one-third of the nation's population (4, 41). Very little information is available on the survival of enteric viruses in septic tanks or the contribution of septic tanks to the viral pollution of groundwaters (41), but several infectious hepatitis outbreaks have been traced to septic tank pollution of groundwaters used for domestic drinking supplies (43). The agent of infectious hepatitis has been shown to remain infective for up to 10 weeks in contaminated well water (12).

Pollution of waters by enteric viruses of animal origin is a neglected area of study which is worthy of consideration. Viruses have been detected in runoff from feedlots and in the effluents from abbatoirs (31) which are often discharged into domestic wastewater treatment systems or directly into receiving waters. Since certain animal viruses have been shown to be oncogenic in unnatural hosts, their presence in water could pose a significant health hazard (5).

The movement of viruses through soils to groundwater aquifers is a complex process (8). and viruses have been shown in recent work by Wellings, et al. to percolate through soil as much as 6.1 m vertically and up to 18 m laterally from the site of application to monitoring wells (44, 45).

Extended survival of soil associated viruses was shown by Lefler and Kott (28) in studies in which poliovirus remained infective in excess of 90 days on moist sand and greater than 77 days on dry sand. This study amply demonstrates the survival potential of soil associated viruses which can percolate to underlying groundwaters. Upon reaching subsurface waters, bacteriophage T-4 tracers have been detected in an underground stream one mile from the point of phage injection (18). This study indicates a potential for considerable translocation of viruses that are introduced into aquifers. Virus contaiminated water in aquifers may then be reused after varying residence times in the aquifer and could pose a health hazard to subsequent consumers. Enteric viruses have also been shown to persist on the surface and within vegetables which have been irrigated with virus-contaminated wastewater (3, 27). These observations demonstrate that viruses need not be consumed in drinking water in order to pose a potential hazard. The potential hazard is amplified by the enhanced survival of viruses associated with solids in soils and waters (19).

The potential for movement of a virus through a soil system is dependent upon a complex set of parameters including virus type,

soil type, soil cation exchange capacity (CEC), soil mineral content, and the pH and cation content of the aqueous medium in which the virus may be applied to or eluted from the soil (21). Viruses, by virtue of their protein capsid, behave as colloids when placed in an aqueous medium, and the factors affecting their removal are thought to be the same factors that affect other colloidal systems (38). The rate of virus application to a soil has also been shown to be important in a soil's ability to remove viruses (21).

The Mesilla Valley in southern New Mexico offers a unique opportunity to study several viral water pollution possibilities. The waters of the Rio Grande River are held in reservoirs north of the valley and are released periodically for irrigation use. The city of Las Cruces and several small upriver communities discharge variously treated municipal wastewaters into the river which is then used for irrigation in the Mesilla Valley and downstream areas. Potentially virus laden irrigation water may be returned to the river by a system of return drains, or the water may recharge the groundwater aquifer where it could serve as a source of supply for shallow domestic or irrigation wells. More concentrated wastewater, potentially contaminated with viruses, is discharged to the soil from septic tanks and privies that serve large portions of the valley's rural population. These effluents may enter shallow groundwater systems which in some cases are only a few feet below the ground surface (25). Contaminated water from this aquifer may by returned to the river by direct flow or the drainage canal system, or it may serve as a source of supply for shallow wells.

In order to adequately assess the potential for virus pollution of waters in the area, it is necessary to characterize the inactivation of viruses in various aquatic environments in the valley that could be subjected to viral contamination. It is also necessary to study virus adsorption and elution characteristics in some indigenous soils in order to provide a measure of the potential for virus movement through these soils. The determination of these characteristics could enable their extrapolation to other viral pathogenic agents.

The detection of low numbers of enteric viruses in water is a formidable task even with improved detection and concentration technology. The use of bacteriophages as enteric virus pollution indicators has been widely suggested and has even shown promise in some studies (26). Since assay procedures for bacteriophages are far less time consuming and technically less demanding than the procedures used with animal viruses, the potential for the use of phages as a virus indicator system in local aquatic environments deserves consideration.

An objective of the work reported here is to compare the inactivation of two enteric viruses and three bacteriophages in Rio Grande River water, shallow groundwater, deep groundwater, and septic tank liquor. A second objective is the comparison of the soil adsorption and elution characteristics of the same viruses and phages in two indigenous soil types that might be encountered in the local water recycling scheme. The water samples used in the inactivation studies will be used in the soil adsorption-elution

studies. Finally, the inactivation and adsorption-elution characteristics of the viruses and phages used will be compared in order to determine the suitability of the phages as viral pollution indicators in the aquatic systems studied.

MATERIALS AND METHODS

Viruses and bacteriophages. All animal viruses used in inactivation and soil studies and HeLa cell cultures used for infectivity assays were obtained from Dr. L. C. McLaren, Department of Microbiology, School of Medicine, University of New Mexico. Poliovirus 1 (Mahoney) and coxsackievirus B-1 were used throughout the studies. Virus pools were prepared by infecting phosphate buffered saline (PBS) rinsed HeLa cell monolayers. Following a one hour infection period, Earle's minimal essential medium (MEM) (Grand Island Biological Co.) supplemented with 10% newborn calf serum, 10% tryptose phosphate broth, 200 U/ml penicillin, and 10 ug/ml streptomycin was added, and the infected monolayers were incubated 18 to 20 h at 37 C. Following incubation, the cultures were freeze-thawed five times, and cell debris was removed by centrifugation at 3,000 rpm for 10 min. The crude virus pools were washed three times by centrifugation at 37,000 rpm for two hours in a Spinco S-50 rotor and resuspended in PBS. The resultant virus pools with infective titers of approximately 1×10^8 PFU/ml were stored in PBS at 4 C.

Bacteriophages used in the studies were f2, and RNA phage specific for F^+ or Hfr strains of Escherichia coli, N-1 a double stranded DNA phage specific for Micrococcus lysodeikticus ML-1, and an RNA coliphage designated CP-1 which was isolated from septic tank liquor using E. coli A-19 Hfr as the host. Bacteriophage f2 was obtained from Miles Laboratories, and phage N-1 was obtained

from Dr. C. E. Cords, Department of Microbiology, School of Medicine, University of New Mexico. Lysates of f2 and CP-1 were prepared by inoculating log phase cultures of E. coli A-19 grown in R-medium (33). The infected cultures were incubated overnight at 37 C with slow rotary shaking. Cultures were then treated with chloroform (5% v/v) and incubated at 37 C for 30 min to lyse remaining viable bacteria. The crude lysates were centrifuged at 5,000 rpm for 10 min to remove cell debris. The cleared lysates were washed by the procedure used for enteric viruses to remove residual R-medium. The lysates with an infective titer of approximately 1×10^{11} PFU/ml were stored in PBS at 4 C. Lysates of phage N-1 were prepared and stored as described above with the exception that the M. lysodeikticus ML-1 host was grown in a tryptone (0.8%), yeast extract (0.5%), NaCl (0.5%) medium with 0.002 M $CaCl_2$. Final infective titers of the N-1 lysates were approximately 1×10^{8} PFU/ml.

Assay of virus and bacteriophage infectivity. Enteric virus infectivity assays were performed using the method of Holland and McLaren (23) as modified by Cords, et al. (14). HeLa cell monolayers were grown to confluency in 2 oz French square glass bottles (13 cm^2 surface area) and infected with 0.2 ml of virus suspension diluted appropriately in PBS. Depending upon the virus being assayed, the infected monolayers were incubated for 40 to 60 h at 37 C prior to staining with ethanolic crystal violet for plaque enumeration. Assay for viruses indigenous to septic

tank liquor were performed using a modification of the direct inoculation technique described by Buras (9). In order to approximate isotonicity, 0.1 ml of 10X PBS was added to 0.9 ml of septic tank liquor. HeLa cell monolayers grown to confluency in 200 ml milk dilution bottles were infected with 1 ml of the PBS-septic liquor suspension for 20 min followed by two PBS rinses and agar overlay. Plaques were enumerated as previously described after 48 h of incubation at 37 C.

Bacteriophage infectivity assays were done using the soft agar overlay technique of Adams (1). The host for f2 and CP-1 was E. coli A-19, and the host for N-1 was M. lysodeikticus ML-1. One ml assay samples were treated with chloroform (20% v/v) for 30 min at 37 C to reduce contaminating microflora. Samples were then diluted in dilution broth (33), and 0.1 ml of appropriate dilutions was combined with 0.2 to 0.3 ml of log phase host culture. After an adsorption period of 10 min, the phage-host mixture was added to molten soft agar and poured over a base of hard agar in a petri dish. Hard and soft agars were prepared by adding 1.5% or 0.5% agar to the appropriate growth medium. Plates containing f2 or CP-1 were incubated at 37 C for 24 h prior to plaque enumeration. Due to the slow growth of M. lysodeikticus ML-1, plates containing N-1 were incubated at 37 C for 48 h before plaque enumeration. Indigenous microfloral contamination of N-1 samples in soil experiments led to consistent overgrowth of host lawns by soil microflora. This problem was circumvented by

filtering the assay samples through a 0.22 u Millipore filter prior to assay as described above. Control experiments showed no significant adsorption of N-1 to the membrane filter.

Aqueous environments used in inactivation studies. Virus and phage inactivation was studied in Rio Grande river water (f2 and N-1 only), shallow ground water from a 2.7 m United States Geological Survey test well, deep ground water obtained from a 24.4m well located on the New Mexico State University Agronomy Farm, and in pooled septic tank liquor samples obtained in the local area.

Bacteriological analyses of all aqueous media were performed according to Standard Methods (2). Total aerobic plate count (Standard Methods Agar), total coliform (LST broth at 37 C), and fecal coliform (EC broth at 44.5 C) determinations were done. Total and fecal coliform densities were determined using the five tube most probable number (MPN) technique. Anaerobic plate counts were performed on septic tank liquor using a Torbal anaerobic chamber and BBL Gas Pak disposable H_2-CO_2 generators.

All aqueous media were also analyzed to determine pH, conductance, anionic (NO_3^-, SO_4^{-2}, Cl^-, HCO_3^-, CO_3^{-2}), and cationic (Ca^{+2}, mg^{+2}, Na^+, K^+) content by Bruce Franzen, Department of Biology, New Mexico State University. Temperature was recorded at the time of sample collection, and dissolved oxygen (DO) was measured with a Delta Scientific Corporation Model 75 Oxygen Meter.

Inactivation studies. Depending upon the accessibility of the test waters, either in situ or in vitro laboratory inactivation studies were done. Experiments in Rio Grande River water were conducted in a location not accessible to the public. Test phages suspended in 20 ml of raw river water were introduced into dialysis membrane chambers as described by McFeters, et al. (32), equipped with sidewalls cut from three inch dialysis tubing (12,000 dalton exclusion limit). Chambers suspended from styrofoam floats were immersed in the river current. Samples were withdrawn at prescribed intervals, after thorough mixing of chamber contents, and assayed immediately.

Shallow groundwater studies were performed by introducing 15 to 20 ml of phage or virus suspended in well water into a dialysis bag constructed form 1.5 inch dialysis tubing (12,000 dalton exclusion limit) fitted with a sealed hypodermic needle which provided sampling access. The dialysis bags were suspended in the previously bailed two inch well casing which was sealed to prevent public access. Samples were withdrawn at prescribed intervals after thorough mixing of the bag contents. Phages were assayed immediately after sampling, and the viruses were diluted 1:1 in MEM, allowed to stand at room temperature for 4 h, and frozen at -70 C for later assay.

Field studies with septic tank liquor and deep ground water were precluded by the inaccessibility of the sampling sites. Water samples were obtained from field sites and inactivation studies

were done in the laboratory. Phages or viruses were diluted into septic tank liquor or deep ground water, and 20 ml samples were placed in sterile 20×150 mm screw cap tubes equipped with magnetic stirrers. The tubes were incubated with continuous stirring in a water bath maintained at appropriate field condition temperature. Septic liquor samples were maintained at 25 C, and deep groundwater samples were kept at 18 C. Samples were withdrawn and assayed as described above with the addition of an assay for indigenous E. coli A-19 specific bacteriophage from uninoculated tubes of septic liquor.

All inactivation studies were conducted using two to four replicates, and most were conducted for a period of time which ensured a one log (90%) reduction in infective titer of virus and phage. Virus assays were done by adsorbing 0.2 ml aliquots of appropriately diluted virus samples onto HeLa cell monolayers grown to confluency in prescription bottles.

Soil studies. Soil adsorption and elution experiments using viruses and phages, with the exception of CP-1, were performed using two soil types. A sandy soil was obtained from the bed of the Rio Grande River, and sandy loam was obtained from an adjacent agricultural field. Physical and chemical parameters of the soils were determined as described for the aqueous samples, and additional cation exchange capacity (CEC), organic carbon, and hydrometer soil typing determinations were done by the Soil and Water Testing Laboratory at New Mexico State University.

Two elution methods were used to determine phage and virus adsorption-elution properties under vigorous and mild elution conditions. Soil samples were screened through a 1.4 mm U.S.A. Standard Testing Sieve, and 5 g was placed in sterile 20×150 mm screw cap tubes and in 20×150 mm glass columns fitted with nylon mesh bottoms to prevent soil loss. Viruses or phages were diluted in the waters used in the inactivation studies, and one void volume of the suspension (2 ml for sandy loam and 1.2 ml for sandy soil) was placed on the soil samples in the tubes and columns. Total PFU applied were less than the previously determined virus adsorption capacity of the soil in order to minimize the elution of unadsorbed viruses and phages. After allowing 1 h for virus adsorption, the samples were eluted by two methods. The samples in the screw cap tubes were eluted four times using 12.5 ml aliquots of the same waters used for suspending the virions. Each elution consisted of a 1 min vortexing of the soil-eluent mixture followed by centrifugation for 15 min at 5,000 rpm to remove suspended soil. The supernatants for the four elutions were pooled in sterile bottles, and aliquots were removed for assay. Assay samples were treated as described in the aqueous inactivation studies. Elution of the viruses and phages from the soil columns was accomplished by gently pouring four 12.5 ml aliquots of the appropriate eluent through the soil columns and collecting the eluates in sterile bottles. After mixing, aliquots of eluent were removed for assay. Three replicate experiments were done in all elution studies.

Analysis of data. Data from the inactivation studies were converted to surviving fraction of PFU, and lines were fit to the data using least squares and regression techniques. Slopes of the best fit lines for each aqueous regime were analyzed using one way analysis of variance (ANOVA) and least significant differences (LSD) techniques to test for differences in the slopes of the best fit lines (42). The time required for a one log (90%) reduction in infective titer (LRT) of phages or viruses in a specific medium were calculated from the slopes of the best fit lines. The percent of PFU eluted in the soil experiments was calculated by dividing the total PFU eluted by the total PFU applied to the soil.

RESULTS

Inactivation studies. Analysis of data dervied from the inactivation studies showed that the data best fit lines which indicated exponential (first order) inactivation of phages and viruses with time. This relationship yielded a straight line on semi-log plots as indicated by Figure 1. This observation is consistent with the results of other inactivation studies in which viral aggregation was minimal (7). This type of plot allowed the calculation of the time required for a 90% reduction in infective titer (LRT) from the slopes of the best fit semi-log plot inactivation curves.

Shallow groundwater and river water virus inactivation studies were conducted in the field. The septic tank liquor and deep groundwater sampling sites were inaccessible, and laboratory inactivation studies were done. Samples obtained from the field were placed in sterile screw cap tubes equipped with magnetic stirrers and seeded with virus or phage. Samples were continuously stirred and were incubated at appropriate in situ temperatures. Results of these experiments are summarized in Table 1 and Figure 2. The range of LRT values for individual experiments shows little variation between replicates.

In deep groundwater (24.4 m well) poliovirus 1 was more resistant to inactivation than coxsackievirus B-1, coliphage f2, and phage N-1. The LRT values for coxsackievirus B-1 and coliphage f2 cannot be said to differ due to the overlap of LRT values obtained from

Figure 1. Inactivation curves for poliovirus 1 (○), coxsackievirus B-1 (□), bacteriophage f2 (▷), and bacteriophage N-1 (×), in septic tank liquor at 25 C. Curves are plotted as log-percent surviving fraction of virus or phage PFU/ml versus time. Viruses and phages were suspended in 20 ml of septic tank liquor in 20×150 mm screw cap tubes and magnetically stirred in a constant temperature water bath. Initial virus and phage titers ranged from 1×10^4 to 1×10^5 PFU/ml.

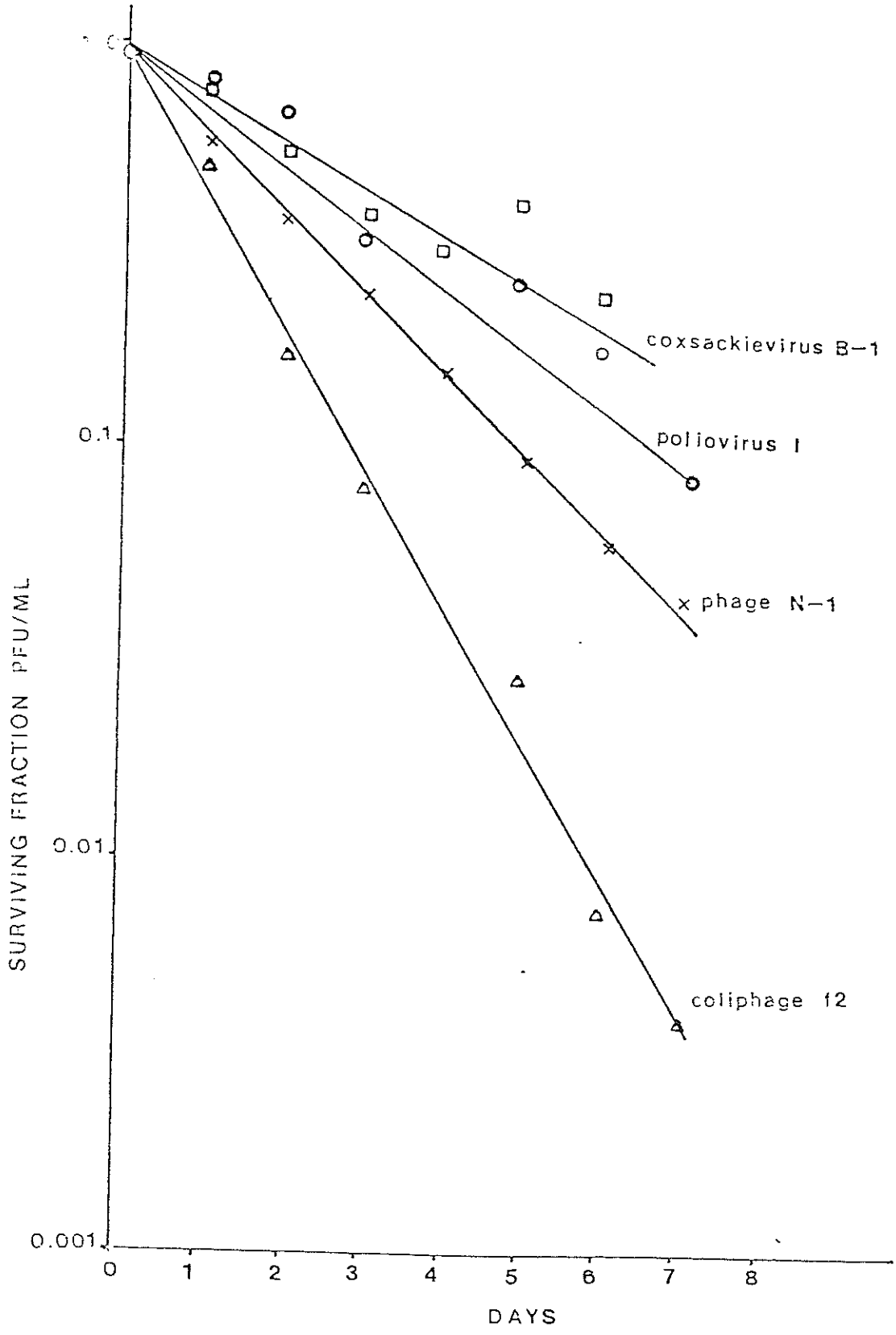

SURVIVING FRACTION PFU/ML
1
0.1
0.01
0.001
0
1
2
3
4
5
6
7
8
DAYS
coxsackievirus B-1
poliovirus I
x phage N-1
coliphage f2

Table 1. Inactivation of enteric viruses and bacteriophages in aquatic environments.[a]

Virus Phage	Suspending Medium	LRT (days)[b]	LRT Range[c] (days)	Temp C[d]
Poliovirus 1	Deep groundwater	4.8(.96)[e]	4.0- 5.9	18
Coxsackievirus B-1	Deep groundwater	3.0(.95)	2.9- 3.3	18
Coliphage f2	Deep groundwater	2.7(.88)	2.3- 4.3	18
Phage N-1	Deep groundwater	1.9(.97)	1.7- 2.1	18
Coliphage f2	River water	1.6(.99)	1.6	25
Phage N-1	River water	2.1(.99)	2.1	25
Poliovirus 1	Shallow groundwater	13.0(.96)	12.9-13.1	18
Coxsackievirus B-1	Shallow groundwater	10.5(.98)	9.8-11.5	18
Coliphage f2	Shallow groundwater	1.9(.98)	1.8- 2.0	18
Phage N-1	Shallow groundwater	3.1(.99)	3.0-3.2	18
Poliovirus 1	Septic tank liquor	5.9(.94)	5.4- 6.9	25
Coxsackievirus B-1	Septic tank liquor	8.1(.90)	6.7-10.6	25
Coliphage f2	Septic tank liquor	2.8(.98)	2.5- 3.2	25
Phage N-1	Septic tank liquor	5.9(.83)	4.9- 6.2	25
Coliphage CP-1	Septic tank liquor	5.9(.81)	4.3- 7.7	25

a. Each experiment included two to four replicates.

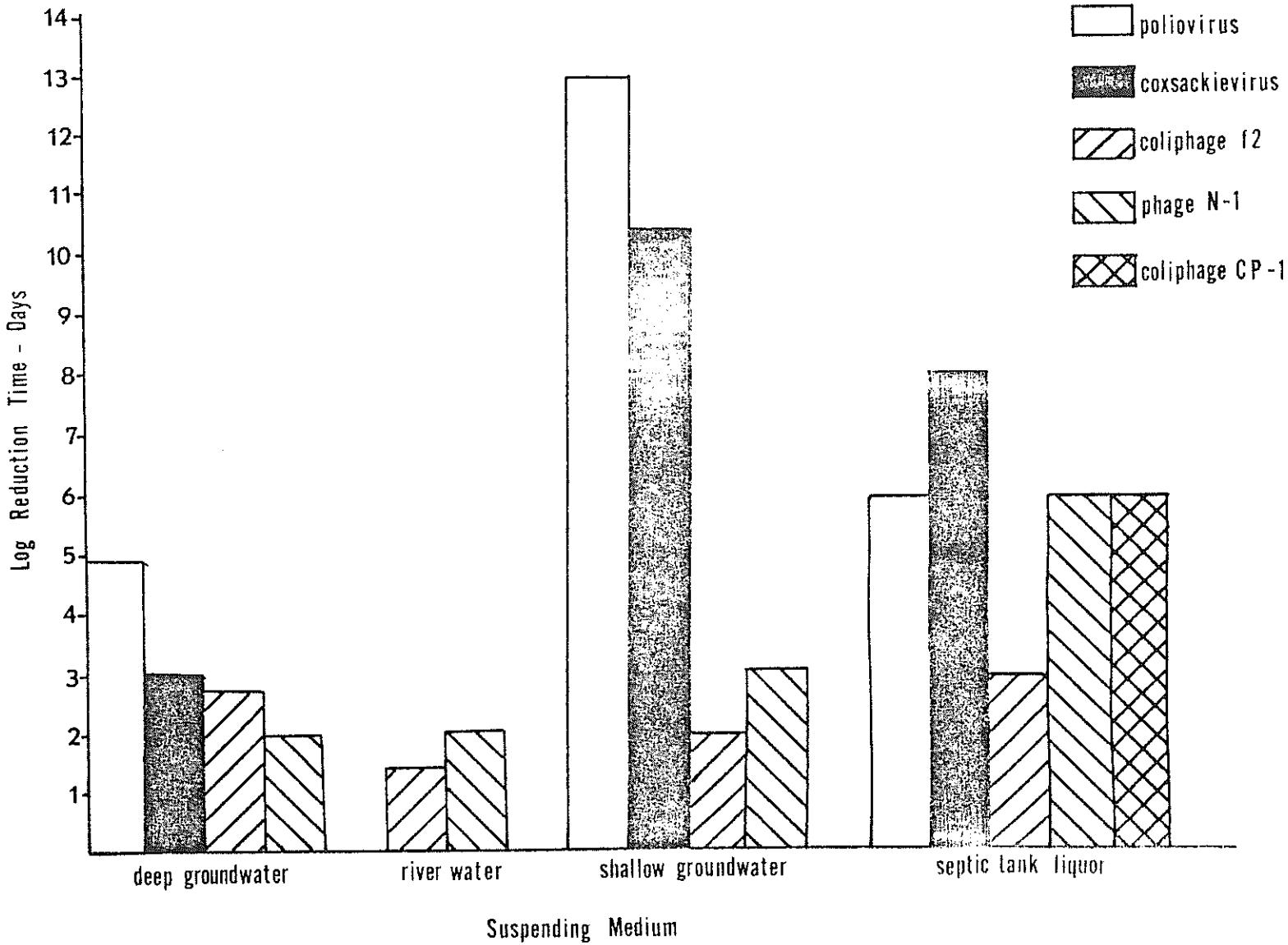
b. Log reduction time. The time required for a 90% loss in infective titer. Calculated from slopes of best fit regression line for all replicates.

c. Log reduction time calculated from individual replicate experiments for each virus or phage.

d. Temperatures are appropriate in situ values.

e. Number in parenthesis is the correlation coefficient of the best fit line for all replicate experiments.

Figure 2. Comparison of log reduction times (time required for 90% loss in infective titer) for poliovirus 1, coxsackievirus B-1, coliphage f2, Micrococcus lysodeikticus phage N-1, and coliphage CP-1 in each of the waters tested.


poliovirus
coxsackievirus
coliphage f2
phage N-1
coliphage CP-1
Log Reduction Time - Days
14
13
12
11
10
9
8
7
6
5
4
3
2
1
deep groundwater
river water
shallow groundwater
septic tank liquor
Suspending Medium

replicate experiments. The results obtained in shallow groundwater (2.7 m well) show that the enteric viruses were much more resistant to inactivation than were the bacteriophages. The results indicate that the enteroviruses could persist for extended periods of time in the shallow groundwater, and, depending upon soil types and other conditions in the aquifer, the viruses could be transported considerable distances within the aquifer. A similar conclusion can be drawn for the survival of the enteroviruses in septic tank liquor. The data in Table 1 and Figure 2 show that the LRT values for poliovirus 1 and coxsackievirus B-1 in septic liquor were 5.9 and 8.1 days, respectively. Previous studies of enteric virus inactivation in the waters of the Rio Grande conducted in this laboratory showed LRT values of 25 h and 28 h for poliovirus 1 and coxsackievirus B-1, respectively, at 25 C (R.T. O'Brien, personal communication). These values do not differ greatly from the LRT vlaues obtained in Rio Grande water for coliphage f2 and phage N-1 (Figure 2) and indicate that these phages might serve as suitable indicators of enterovirus inactivation in this environment. In view of the disparities between LRT values obtained in the various waters, it would appear that different inactivation processes were operating in the different waters, or that the concentration of some common virus inactivation factor varied from one water type to another.

The effect of temperature on the inactivation of viruses and phages in the various waters was not determined since studies

were carried out at appropriate in situ temperatures which showed little variation during the study period. In view of the differences in LRT values obtained in deep and shallow groundwater at 18 C, it would appear that the differences in inactivation rates are due to differences in the virus inactivating capacity of the waters rather than to temperature.

In order to evaluate the similarity of LRT values obtained for viruses and phages in a given aquatic environment, the slopes of the best fit inactivation curves for each virus and phage were compared at the 5% confidence level using the LSD multiple comparisons test (42). The results of these comparisons are shown in Table 2 where it can be seen that the LRT values for poliovirus 1 and coxsackievirus B-1 in deep groundwater were significantly different. In contrast to this result, the LRT values for poliovirus 1 and coxsackievirus B-1 in shallow groundwater and septic tank liquor were not significantly different. The comparisons shown in Table 2 show no consistent patterns of similarity between the LRT values for viruses and phages in the different waters used in the studies which would preclude the use of the phages tested as general indicators of enteric virus inactivation in these environments. The comparisons of LRT values in septic tank liquor indicate that coliphage CP-1 might serve as a suitable indicator for the inactivation of poliovirus 1 and coxsackievirus B-1 in septic tank systems.

Table 2. Comparison of log reduction time (LRT) values for viruses and phages in each aqueous environment.[a]

Aqueous Environment	LRT Values Compared	Significant Difference
Deep groundwater	Poliovirus vs. Coxsackievirus[c]	+[b]
	Poliovirus vs. Coliphage f2	+
	Poliovirus vs. Phage N-1	+
	Coxsackievirus vs. Coliphage f2	-
	Coxsackievirus vs. Phage N-1	+
	Coliphage f2 vs. Phage N-1	+
River water	Coliphage f2 vs. Phage N-1	+
Shallow groundwater	Poliovirus vs. Coxsackievirus	-
	Poliovirus vs. Coliphage f2	+
	Poliovirus vs. Phage n-1	+
	Coxsackievirus vs. Coliphage f2	+
	Coxsackievirus vs. Phage N-1	+
	Coliphage f2 vs. Phage N-1	+
Septic tank liquor	Poliovirus vs. Coxsackievirus	-
	Poliovirus vs. Coliphage f2	+
	Poliovirus vs. Phage N-1	-
	Poliovirus vs. Coliphage CP-1	-
	Coxsackievirus vs. Coliphage f2	+
	Coxsackievirus vs. Phage N-1	+
	Coxsackievirus vs. Coliphage CP-1	-
	Coliphage f2 vs. Phage N-1	+
	Coliphage f2 vs. Coliphage CP-1	+
	Coliphage CP-1 vs. Phage N-1	-

a. LRT values were compared using the least significant difference (LSD) multiple comparisons test (42) at the 5% confidence level.

b. A + denotes significant difference, - denotes no significant difference ($p<0.05$).

c. Poliovirus 1, Coxsackievirus B-1.

Soil adsorption and elution. In view of the results of the inactivation studies which indicated the potential for prolonged persistence of the enteroviruses in the septic tank and shallow groundwater environments, soil adsorption-elution studies were performed to assess the potential for movement of viruses through soil systems. Soil of two types and two virus elution methods were used in the studies. The data from all of the experiments can be found in Appendix Table 5. Since, in the natural environment, the most probable process of elution of soil adsorbed viruses would be by percolation of an eluent through a virus-contaminated soil, only the data obtained for the elution of enteric viruses from the soils by percolation are presented in Table 3. The PFU eluted ranged from less than 0.2% for poliovirus 1 eluted from sandy loam with deep groundwater to 73.8% elution of coxsackievirus B-1 from sandy soil with septic tank liquor. The data also show that shallow groundwater and septic liquor were more efficient in virus elution than the other waters tested. This observation is pertinent in view of the fact that the greatest LRT values for the inactivation of enteroviruses were also obtained in these same two water types. The soil elution data also demonstrate that, regardless of the eluting medium used, poliovirus 1 was more tightly bound to sandy loam than to sandy soil. Conversely, coxsackievirus B-1 was more tightly bound to sandy soil except in the septic liquor elutions where the sandy loam bound the coxsackievirus more tightly. The tighter binding of coxsackievirus B-1 by sandy soil is contrary to what

Table 3. Elution of enteric viruses from two soil types by percolation.[a]

Virus	Suspending and Eluting Medium	Soil Type	% Total PFU Eluted[d]
Poliovirus 1	Deep groundwater	sand[b]	14.7 (6.7-20.8)[e]
	Deep groundwater	sandy loam[c]	<0.2 (<0.1-0.3)
Coxsackievirus B-1	Deep groundwater	sand	<0.3 (<0.3-0.4)
	Deep groundwater	sandy loam	6.5 (6.3-7.1)
Poliovirus 1	River water	sand	17.5 (13.1-21.4)
	River water	sandy loam	0.7 (0.5-0.9)
Coxsackievirus B-1	River water	sand	<0.2 (<0.2)
	River water	sandy loam	0.8 (0.8-0.9)
Poliovirus 1	Shallow groundwater	sand	41.6 (17.4-65.9)
	Shallow groundwater	sandy loam	1.5 (1.3-1.7)
Coxsackievirus B-1	Shallow groundwater	sand	4.7 (4.2-5.8)
	Shallow groundwater	sandy loam	31.7 (21.1-42.3)
Poliovirus 1	Septic tank liquor	sand	20.8 (14.6-25.0)
	Septic tank liquor	sandy loam	7.4 (3.3-15.6)
Coxsackievirus B-1	Septic tank liquor	sand	73.8 (67.8-83.3)
		sandy loam	61.5 (48.4-68.8)

a. Five g of soil in a 20×150 mm glass column were loaded with about 1×10^5 viruses or phages and eluted by pouring four 12.5 ml aliquots of eluent through the column.

b. Sandy soil was obtained from the dry bed of the Rio Grande.

c. Sandy loam was obtained from an agricultural field adjacent to the Rio Grande.

d. Average of three replicate experiments.

e. Range of % PFU eluted in replicate experiments.

might have been expected since soils with higher clay content are generally thought to be more efficient adsorbers of virus than soils with low clay content (21). This result shows that virus type and soil type are important in determining virus adsorption characteristics, and that generalizations based on data obtained from a single virus or soil type may not be valid.

Microbiological and physico-chemical analysis. Microbial antagonism (13) and other physical and chemical factors may play an important role in aquatic virus inactivation and virus removal by soil. A number of these parameters were measured in the soils and waters used in these studies. These determinations were done on the premise that the results might aid in explaining observed differences in inactivation or elution characteristics of viruses and phages. A summary of the results of these analyses is presented in Table 4 which shows that the standard plate counts from the natural water samples were not high enough to be a factor in enterovirus inactivation. The aerobic and anaerobic plate counts from the septic liquor were three to four orders of magnitude higher than the counts in the natural waters, but the rate of enteroviral inactivation was much lower than all waters except shallow groundwater. This result eliminates microbial antagonism as a major factor in the inactivation of enteroviruses in these studies. River water contained 43.1 meq/l of the ions tested for, deep groundwater contained 29.k meq/l, and shallow groundwater contained 24.1 meq/l. River water also had a greater content of SO_4^{-2}, Cl^{-1}, Ca^{+2}, Mg^{+2},

Table 4. Bacteriological and physio-chemical analysis of soils and aquatic media used in inactivation and soil adsorption-elution studies.[a]

Soil/Water	Standard Plate Count/ml	MPN Fecal Coliform/ 100 ml	Temp C	Dissolved Oxygen(mg/l)	pH	Specific Conductance (umho/cm)	meq/l									CEC (meq/100g)[d]	Organic Matter%
							NO_3^{-1}	SO_4^{-2}	Cl^{-1}	HCO_3^{-1}	CO_3^{-2}	Ca^{+2}	Mg^{+2}	Na^{+1}	K^{+1}		
Deep groundwater	$2.5x10^2$	0	18	3	7.6	750	0.03	3.8	2.6	4.1	0	4.1	1.9	12.4	0.2	ND	ND
River water	$2.5x10^3$	0	25	4	8.2	1200	0.06	5.8	5.6	4.8	0	6.8	2.4	17.3	0.3	ND	ND
Shallow groundwater	$2.0x10^3$	0	18	3	7.6	700	0.03	2.0	1.6	4.5	0	3.6	1.4	10.8	0.2	ND	ND
Septic tank liquor	$9.0x10^6$ $1.2x10^7$[b]	$2.3x10^6$	25	<.5	7.2	9000	0.04	2.5	2.1	5.0	0	0.5	0.5	12.0	0.6	ND	ND
Sand	ND[c]	ND	ND	ND	8.0	820	0.08	2.8	2.9	3.5	0	4.0	1.2	8.6	0.3	0.35	0
Sandy loam	ND	ND	ND	ND	7.7	1450	0.06	6.2	2.2	7.9	0	6.0	2.9	13.6	0.3	17.9	0.9

a. Bacteriological analyses were done according to Standard Methods (32). Plate counts were done with Standard Methods Agar. Fecal coliform analysis was done using a five tube MPN series incubated at 44.5 C.

b. Anaerobic plate count.

c. ND, not done.

d. CEC, cation exchange capacity.

and Na^{+1} than the other natural waters. Deep groundwater had a greater content of these ions than did shallow groundwater. Organic matter in soils is know to be a more effective virus adsorbant than clay (13), and the presence of organic material in a soil should enhance its virus adsorption capacity. The analyses of the two soils used showed 0.9% organic matter present in the sandy loam and no detectable organic matter in the sandy soil. The cation exchange capacity of a soil is generally thought to be an indication of virus adsorbing capacity of a soil. The soil CEC determinations showed that the sandy loam had a CEC of 17.9 meq/100 g, and the sandy soil showed a CEC of 0.35 meq/100 g. The CEC and organic matter results do not offer an explanation for the apparent anomaly of the tighter binding of coxsackievirus B-1 to the sandy soil.

Primary isolation of virus from septic tank liquor. A modification of the direct inoculation techniques of Buras (9) was used to isolate indigenous viruses from septic tank liquor samples using HeLa cell monolayers. One sample from a septic tank servicing a family of four with two small children yielded an infective titer of 2,500 PFU/ml, a figure in agreement with the observations of Buras (9) and others (11, 22) who have isolated viruses from domestic sewage. This result indicates that, even though most virus particles in domestic sewage are trapped in settleable solids (9), significant numbers of viruses may be recovered from the aqueous portion of domestic wastewater.

DISCUSSION

The enterovirus and phage inactivation studies were done in order to assess their survival potentials in locally occurring groundwater environments. The results of the studies clearly show that inactivation of enteroviruses and phages closely approximate first order kinetics, however, the inactivation rates of the individual viruses and phages were different. The three-fold difference between the LRT values for the enteroviruses in deep and shallow groundwater were obtained using the same water temperature (18 C). The differences in inactivation rates are assumed to be due to differences in the virus inactivating capacity of groundwater at different depths. The LRT values for poliovirus 1 and coxsackievirus B-1 in each of the groundwater environments also demonstrate the differences in the stabilities of each of the enterovirus types.

Virus inactivation in aquatic environments is not a well understood process. Numerous studies have been done on the effects of environmental parameters on viral inactivation. For example, it is well documented that there is an inverse relationship between water temperature and the rate of virus inactivation (3, 21, 34, 39). Since all of the experiments done in the groundwaters were conducted at the temperature actually measured in the wells (18 C), the differences in the inactivation rates in deep and shallow groundwater environments cannot be attributed to water temperature. Similarly, the pH of the water was not a factor in inactivation

since all of the samples from deep and shallow wells as well as the river water and septic tank liquor samples were in the pH range of 7.2 to 8.2. This range of pH is not known to affect virus stability. Another factor which has been suggested as affecting virus inactivation is microbial antagonism (13). No experiments to investigate this possibility were done here. However, standard plate count data were obtained, and the counts were probably too low to be a significant factor. Furthermore, a bacterial floc which appeared in the dialysis bags suspended in the shallow well had no effect on the rates of inactivation since the slopes of the inactivation curves did not change upon appearance of the floc.

The analyses of the cation and anion content (Table 4) of the natural groundwater samples show that virus inactivation rates varied inversely with the ionic strength of the water. River water in which the most rapid inactivation of enteroviruses occurred contained a total of 43.1 meq/l of the ions tested. Shallow groundwater in which the slowest enteroviral inactivation rates were obtained contained a total of only 24.3 meq/l of the ions. This apparent inverse relationship between total ionic content and rate of viral inactivation was also true individually for SO_4^{-2}, Cl^{-1}, Mg^{+2}, and Ca^{+2} content. The structural integrity of the proteins in the viral capsid is largely maintained by hydrogen bonding, hydrophobic interactions, and weak electrostatic forces. Differences in ionic concentrations could alter the stability of the viral capsid proteins in natural, aquatic environments.

A reason for the prolonged persistence of enteric viruses in septic tank liquor is not clear, however, it is known that suspended organic matter exerts a protective effect for viruses (14, 37), and this may be the case with the septic tank liquor. Further experiments conducted with raw and filtered septic liquor could clarify this possiblity. The results clearly show that enteroviruses introduced into septic tank or shallow groundwater environments can maintain infectivity for extended periods. This is especially true for shallow groundwater where poliovirus 1 introduced at an initial concentration of 1×10^4 PFU/ml could theoretically contain at least one infective unit after 52 days. In shallow groundwater aquifers this period of time could allow considerable movement of viruses from the point of introduction into the aquifer.

There is considerable interest in perfecting indicators of viral pollution in natural waters. The comparison of LRT values for viruses and phages shown in Table 2 show that the statistical similarities between the LRT values for poliovirus 1, coxsackievirus B-1, and coliphage CP-1 in septic liquor are such that coliphage CP-1 could serve as an indicator for the inactivation of enteroviruses in septic tank waste disposal systems. However, the lack of consistent patterns of similarity between phage and enterovirus inactivation in natural groundwaters indicate that none of the phages tested would be suitable general indicators of enterovirus inactivation in these environments.

The soil adsorption-elution studies were done in order to determine the potential for migration of enteroviruses in soil-groundwater environments in the local area. Table 3 shows that the viruses tested have unique adsorption-elution characteristics. These individual characteristics appear to be related to virus type, soil type, and eluting medium. For example, regardless of the eluting medium, poliovirus 1 was eluted more readily from sandy soil than from sandy loam. Elution ranged from less than 0.2% eluted from sandy loam by deep groundwater to 41.6% eluted from sandy soil by shallow groundwater. Conversely, coxsackievirus B-1 was eluted more readily from sandy loam by groundwaters than from sandy soils. Elution ranged from less than 0.2% eluted from sandy soil by river water to 31.7% eluted from sandy loam by shallow groundwater. As previously mentioned, the generally tighter binding of coxsackievirus B-1 to sandy soil is inconsistent with the generalization that soils with higher cation exchange capacities are more efficient in virus adsorption. Coxsackievirus B-1 differs in surface charge from poliovirus 1 even though the size and mass of the virions are virtually identical (37). It is not clear whether this difference in surface charge on the virions would be sufficient to account for the drastically different elution characteristics. Many virus adsorption experiments performed in the laboratory employed a single, well-characterized clay type such as bentonite and montmorillonite. It is likely that in the natural soil environment the relative proportions of

of different clay types along with other factors could result in less efficient virus adsorption to soils, including those with significant clay content. The apparent anomaly seen in the adsorption and elution of the coxsackievirus may be another exception to a laboratory generated generalization.

As in the case of the inactivation studies, it is probable that viral adsorption properites are determined by a complex variety of factors in the natural soil environment. The pH and ionic character of the eluent, virus type, soil type and other unidentified factors are all undoubtedly involved in determining the soil adsorption characteristics of viruses. Valid generalizations encompassing all of these variables are probably impossible. A virus percolating through a natural soil system would probably encounter continually changing soil and eluent conditions (17). This probability would further confound generalizations derived solely from laboratory data. The fact that even one infective virus particle may be eluted from the soil by the natural waters and septic liquor demonstrates a potential for virus movement through soil into aquatic systems.

The inactivation and elution studies done with septic liquor and shallow groundwater dramatically illustrate a potential for virus pollution of local waters. These two systems may be intimately linked in areas where a shallow water table leads to the probability that septic tank effluents, which we have shown to contain viruses, may be discharged almost directly into the

shallow aquifer. Tables 1 and 3 show that shallow groundwater and septic tank liquor are efficient eluents of viruses from soils and are relatively mild environments with respect to virus inactivation. Thus, after percolation through a septic tank drainfield, enteric viruses could be transported considerable distances in the aquifer. The public health significance of this hazard is well demonstrated by the numerous documented cases of viral pollution of wells by septic tank systems which often led to outbreaks of serious disease (30, 41, 43).

Some of the results obtained in these studies will require additional verification. Other fruitful avenues of inquiry which have emerged from the studies will require more study if the public health significance of the data is to be determined. The differences in the characteristics of the enteroviruses studied make it clear that consideration of other enteric viruses is vital if valid generalizations are ever to be made. These studies show that it is impossible to make meaningful generalizations based on studies of one virus or phage type or of one aquatic environment. The removal and/or inactivation of viruses by septic tank systems deserves considerable attention. Septic waste disposal systems are generally less efficient than aerobic systems in aspects of wastewater treatment such as biochemical oxygen demand (BOD) removal and total suspended solids (TSS) removal (24). Relatively inefficient inactivation of enteric viruses by septic systems should not be surprising. The movement of viruses through selected soils

and the potential for their entry into groundwater systems deserves more thorough field and laboratory study. There is a great need for studies of the viral inactivation process at the molecular level in aquatic systems. This understanding could lead to the formulation of a useful model for the process of aquatic viral inactivation which could have broad practical application in the design of wastewater treatment systems which would minimize the chances of viral pollution of receiving waters.

These limited studies show enhanced survival of enteric viruses in septic tank liquor and shallow groundwater. These waters were also shown to efficiently elute viruses from local soil types. These observations demonstrate a potential health hazard in certain areas of the Mesilla Valley if pathogenic viruses are introduced from septic tanks or privies into groundwater which could eventually be destined for domestic or other uses.

APPENDIX: VIRUS AND BACTERIOPHAGE SOIL ADSORPTION-ELUTION DATA

Table 5. Elution of adsorbed virus and phage from soils.[a]

Virus/Phage	Aqueous Medium	Elution Method	Soil Type	% Eluted
Coliphage f2	DGW[b]	V[c]	sand	6.2
	DGW	P	sand	2.9
	DGW	V	sandy loam	28.4
	DGW	P	sandy loam	30.6
Phage N-1	DGW	V	sand	3.8
	DGW	P	sand	4.7
	DGW	V	sandy loam	2.8
	DGW	P	sandy loam	3.8
Poliovirus 1	DGW	V	sand	6.9
	DGW	P	sand	14.7
	DGW	V	sandy loam	<0.2
	DGW	P	sandy loam	<0.2
Coxsackievirus B-1	DGW	V	sand	<0.5
	DGW	P	sand	<0.5
	DGW	V	sandy loam	12.5
	DGW	P	sandy loam	6.5
Coliphage f2	RW	V	sand	<0.1
	RW	P	sand	<0.1
	RW	V	sandy loam	<0.1
	RW	P	sandy loam	<0.1
Phage N-1	RW	V	sand	2.1
	RW	P	sand	6.9
	RW	V	sandy loam	1.4
	RW	P	sandy loam	4.5
Poliovirus 1	RW	V	sand	13.1
	RW	P	sand	17.5
	RW	V	sandy loam	0.9
	RW	P	sandy loam	0.7
Coxsackievirus B-1	RW	V	sand	<0.2
	RW	P	sand	<0.2
	RW	V	sandy loam	12.5
	RW	P	sandy loam	0.8
Coliphage f2	SGW	V	sand	12.9
	SGW	P	sand	6.5
	SGW	V	sandy loam	10.6
	SGW	P	sandy loam	11.7

Virus/Phage	Aqueous Medium	Elution Method	Soil Type	% Eluted
Phage N-1	SGW	V	sand	4.1
	SGW	P	sand	5.2
	SGW	V	sandy loam	4.4
	SGW	P	sandy loam	11.2
Poliovirus 1	SGW	V	sand	46.1
	SGW	P	sand	41.6
	SGW	V	sandy loam	1.5
	SGW	P	sandy loam	1.5
Coxsackievirus B-1	SGW	V	sand	6.0
	SGW	P	sand	4.7
	SGW	V	sandy loam	30.8
	SGW	P	sandy loam	31.7
Coliphage f2	SL	V	sand	29.8
	SL	P	sand	31.7
	SL	V	sandy loam	28.5
	SL	P	sandy loam	28.6
Phage N-1	SL	V	sand	16.2
	SL	P	sand	12.7
	SL	V	sandy loam	2.8
	SL	P	sandy loam	27.8
Poliovirus 1	SL	V	sand	34.9
	SL	P	sand	20.8
	SL	V	sandy loam	0.9
	SL	P	sandy loam	3.3
Coxsackievirus B-1	SL	V	sand	35.2
	SL	P	sand	73.8
	SL	V	sandy loam	57.1
	SL	P	sandy loam	61.5

a. Procedures as described in Materials and Methods.

b. DGW = Deep groundwater, RW = River water, SGW = Shallow groundwater, SL = Septic tank liquor.

c. V = vortexing, P = percolation.

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