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THE FATE OF HUMAN VIRUSES IN GROUNDWATER RECHARGE SYSTEMS

JAMES M. VAUGHN AND EDWARD F. LANDRY

March 1980

Prepared for the
UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
Under Contract No. 804776

LAND AND FRESHWATER ENVIRONMENTAL SCIENCES GROUP
DEPARTMENT OF ENERGY AND ENVIRONMENT

BROOKHAVEN NATIONAL LABORATORY
UPTON, NEW YORK 11973

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ABSTRACT

The overall objective of this research program was to determine the ability of a well-managed tertiary effluent-recharge system to return virologically acceptable water to the groundwater aquifer. The study assessed the quality of waters renovated by indigenous recharge operations and investigated a number of virus-soil interrelationships. The elucidation of the interactions led to the establishment of basin operating criteria for optimizing virus removal.

Raw influents, chlorinated tertiary effluents, and renovated wastewater from the aquifer directly beneath a uniquely designed recharge test basin were assayed on a weekly basis for the presence of human enteroviruses and coliform bacteria. High concentrations of viruses were routinely isolated from influents but were isolated only on four occasions from tertiary-treated sewage effluents. In spite of the high quality effluent being recharged, viruses were isolated from the groundwater observation well, indicating their ability to penetrate the unsaturated zone.

Results of poliovirus seeding experiments carried out in the test basin clearly indicated the need to operate recharge basins at low (e.g. 1 cm/hr) infiltration rates in areas having soil types similar to those found at the study site. The method selected for reducing the test basin infiltration rate involved clogging the basin surface with settled organic material from highly turbid effluent. Alternative methods for slowing infiltration rates are discussed in the text.

The influence of rainwater on the desorption and mobilization of soil-associated enteroviruses was found to be minimal in terms of its ability to significantly affect virus occurrence in the recharged aquifer. While viruses could migrate short distances following rainwater rinses, they were found to resorb at lower depths.

A series of laboratory column and soil core studies revealed the shortcomings inherent in the exclusive use of poliovirus type 1 (LSc 2ab) as a model system for predicting the occurrence and fate of other sewage associated virus types. A number of field and laboratory enteroviruses showed variable adsorption and elution characteristics.

An efficient method for the concentration of enteroviruses from large volume sewage effluent samples was developed. The method involved filter adsorption followed by elution with beef extract. The eluate was further concentrated by an organic flocculation method. The method combined the desirable characteristics of high efficiency with small final concentrate volumes.

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SECTION 1

INTRODUCTION

Ever increasing demands for new sources of clean, potable water have given rise to a variety of methods for supplementing groundwater reserves with treated domestic wastewater. Among methods proposed are: spray irrigation, overland flow, deep well injection, and basin recharge. The use of any of these schemes involving the augmentation of potential drinking water resources with wastewaters has often been opposed because of the health hazards posed by many of the ambient chemical and biological components of domestic wastewaters.

Among pollutants, some particularly perplexing problems have been posed by the presence of human viruses, particularly the enterovirus, in wastewaters used for recharging aquifers. While documented evidence for the waterborne transmission of disease by these viruses is lacking, their disease-producing potential is still a cause for concern. Numerous isolations have been made from chlorinated-treated wastewaters (Berg, 1973; Buras, 1976; Vaughn et al., 1978) and groundwaters receiving treated wastes (Schaub and Sorber, 1977; Wellings et al., 1975).

More recently, Vaughn et al. (1978) reported on numerous isolations of enterovirus from groundwaters beneath recharge basins on Long Island. The presence of these viruses in a groundwater system, which has been designated as a "sole source" aquifer, clearly demonstrated the need for more efficient recharge practices.

As part of that effort, a better understanding of the interactions of viruses in soils is needed. Significant informational gaps have historically existed with respect to several aspects of the incidence and movement of viruses through soil systems including such varied areas as: the mechanisms of viral adsorption to soils; the effects of infiltration on rates of virus removals; the role of rainwater on virus mobilization; the delineation of an appropriate virus model system; and system operational modes which will minimize entrance of virus particles to groundwater aquifers.

Using a unique experimental facility, the present study has focused on a number of the above aspects in an attempt to determine the overall ability of a basin recharge system, receiving tertiary effluent, to return virologically acceptable water to the aquifer.

SECTION 2

CONCLUSIONS

In general, the 12-Pines tertiary-treatment facility was effective in removing viruses from sewage. Occasionally, human enteroviruses were found in sewage effluents being used to recharge the aquifer. Viruses were subsequently isolated from the groundwaters beneath the test site recharge basin receiving effluent, indicating migration of the viruses through the soil profile.

The presence of coliform bacteria in the sewage effluent and the renovated waters could not always be correlated with the presence of viruses in the same samples. Viruses were generally isolated from samples with few coliforms, but not isolated from samples containing high concentrations of the bacteria.

The removal of viruses during basin seeding experiments was clearly dependent on the infiltration rate of the virus-containing effluent. At high infiltration rates (75-100 cm/hr), considerable concentrations of viruses were seen at all depths, including groundwater samples. Greatly reduced infiltration rates (0.5-1cm/hr) showed substantially better virus removal with few detected in the aquifer located 7.6 m below the floor of the recharge basin.

Reduced infiltration rates could be achieved by clogging the surface of the recharge basin. Clogging was effectively achieved by either prolonged recharge or dosing the basin with sewage effluent containing a high concentration of suspended solids.

Adsorption of human enteroviruses to Long Island soils was dependent upon the particular type of enterovirus tested. All strains of poliovirus, including field and laboratory strains, (except for guanidine-resistant mutants) showed a high degree of soil affinity. The adsorption efficiency of coxsackie and echoviruses varied with the strain and origin of the virus. Due to this varied soil affinity, the use of poliovirus as a model of virus interactions in soil was considered inappropriate.

In 4.2 x 12.5 cm soil cores taken from the test basin, the elution of adsorbed viruses by rainwater or sewage rinses was dependent upon the virus strain. Field and laboratory strains of poliovirus were found to be mobilized by sewage and/or rainwater rinses. Conductivity appeared to be more important than pH in the rainwater migration process.

In 10 x 75 cm cores, a majority of the virus removal occurred in the top 5 cm of soil. Few viruses were able to percolate the entire length of the core. Rainwater rinses, and to a lesser extent, sewage rinses caused some localized areas of virus migration, but all mobilized viruses were resorbed. The effectiveness of the cores to remove viruses during rinsing was equal, regardless of the type of treatment used.

Beef extract (3%) was superior to glycine in the recovery of viruses from concentrating filters used to process large volume samples. The efficiency of virus concentration from seeded wastewater was 85%. Beef extract was also efficient in recovering viruses from small volume groundwater samples.

Natural cores collected from operating recharge basins demonstrated better virus-adsorbing capacities than packed laboratory columns. Preconditioning of all laboratory columns was necessary for good virus removal.

SECTION 3

RECOMMENDATIONS

Results of the present study indicate the need for continued research in several areas. First, the precise mechanisms of virus removal during low-rate recharge should be investigated in order to establish the major factors of virus removal. For example, is primary removal a function of the low infiltration rate, or simply a result of the enhanced adsorption sites provided by the organic clogging layer? Secondly, the adsorption-desorption profiles extensively developed for poliovirus in this study should be tested for general applicability to other appropriate virus groups (e.g. rotavirus, adenovirus). Thirdly, other methods (viz. load-drying schemes) for establishing low infiltration rates in recharge basins should be studied for those basins having soil characteristics similar to those described and their virus-removing capacity compared to removal rates in clogged basins.

Several of the conclusions of this study may be directly applied to appropriate situations. In "sole-source" aquifer areas, such as Long Island, where soil characteristics permit extremely high infiltration rates during basin recharge, the authors recommend that careful consideration be given to the benefits of further wastewater treatment using recharge basins versus simple large scale disposal. The benefits of additional treatment (i.e. virus removal) by low-rate recharge clearly outweigh the expediency of rapid disposal of large volumes of wastewater. Measures should, therefore, be taken to introduce this element of treatment by allowing basins to partially clog and operate at reduced rates. (A necessary part of the implementation of this recommendation may involve the use of smaller basins.) The results of the present study also identify limits to the use of poliovirus LSc 2ab as a model system for virus behavior in soil systems. We strongly recommend that these limits be carefully considered before use of any polio-derived data to "typify" virus interactions with soil.

SECTION 4

MATERIALS and METHODS

EXPERIMENTAL SITE

The study site is located at the 12-Pines treatment facility in Medford, Suffolk County, New York (Figure 1). The treatment plant, which produces effluent for recharge, is designed to accommodate a population of 12,400 and has the capacity to treat 1.2 million gallons (4.7 million liters) of wastewater per day. Throughout the duration of the project (three years), the treatment plant operated at approximately one-half capacity. The sequence of treatment steps is shown in Figure 2. The plant combines conventional primary and secondary treatment processes with a denitrification step. Following final clarification, effluents are passed through filters composed of garnet, quartz, and anthracite and finally disinfected by chlorination. Final effluents are then pumped to large treatment plant recharge basins (seven basins - 2.5 total acres), or diverted to the test recharge basin. The typical physical and chemical characteristics of the tertiary effluent and the renovated wastewater can be seen in Table 1.

TABLE 1. CHEMICAL AND PHYSICAL CHARACTERISTICS OF 12-PINES
TERTIARY-TREATED SEWAGE EFFLUENT AND RENOVATED WATER

Determination*	Sewage effluent	Renovated water
Turbidity (NTU)†	4.58 (1.2-9.0) #	7.2 (2.5-15)
Conductivity (µmho)††	439.50 (393-500)	459.0 (389-500)
pH	6.62 (6.10-7.20)	6.11 (5.2-7.1)
Total alkalinity	95.38 (48-115)	67.12 (31-98)
Chloride	54.13 (47-64)	53.5 (50-56)
Sulfate	35.63 (28-54)	36.0 (29-56)
Nitrate-nitrogen	3.38 (0.07-16.0)	7.54 (.09-17)
Nitrite-nitrogen	0.11 (0.006-0.49)	0.026 (.003-.083)
Ammonia-nitrogen	5.34 (0.06-15)	0.38 (.16-1.1)
Total Kjeldahl nitrogen	6.63 (0.9-17)	2.27 (.6-10)
Ortho phosphate	6.13 (4.8-6.8)	6.07 (4.8-7.0)
Iron	<0.05 (<0.05)	0.05 (<0.05-0.1)
Manganese	0.02 (0.01-0.03)	0.078 (.02-.15)
Magnesium	4.53 (2.7-7.8)	4.26 (2.6-7.0)
Calcium	19.0 (15-24)	21.37 (17-33)
Sodium	58.25 (54-68)	59.12 (54-68)
Potassium	12.25 (11-13)	12.87 (12-14)
Total suspended solids	5.00 (1-14)	NT**
Total organic carbon	15.13 (10-29)	NT

*Except for pH and where noted, all determinations are in milligrams per liter (mg/l).

†NTU Nephelometric turbidity units.

††µmho, micro ohms per centimeters.

#Values in parentheses are ranges.

**NT, not tested.

Figure 1. Schematic diagram of advanced wastewater-treatment plant and U.S. Geological Survey testing facility, Medford, New York.

Figure 2. Major steps of the 12-Pines wastewater-treatment process.

Reprinted with permission of author (Prill et al., 1979).

Located in the northeast corner of the site is the main study facility built and operated by the U.S. Geological Survey (Figure 3). The structure is a miniaturized version of the adjacent recharge basins, consisting of a circular test basin 6.09 m in diameters (25 m^2) whose surface is approximately 7.62 m above the static water level. A manhole has been carefully constructed through the center of the basin to a depth of 6.4 m. Within the manhole, gravity samplers (38.7 cm^2 capture area) have been constructed at depths of 0.75, 2.25 and 5.34 m which extend 0.9 m into the surrounding basin. Platforms have been installed at depths of 2.5 and 4.9 m below the top of the manhole to provide working levels. At the bottom of the manhole, a well has been sunk into the water table to allow testing of waters which have percolated through the system. The test basin has been equipped with instrumentation for measuring water level, infiltration rate, temperature and conductivity. The soil in the basin consists primarily of coarse sand and fine gravel and contains an average of 1.12% silt and clay (Table 2). The study facility which receives treated effluent from the chlorine contact tanks, operates at a normal loading rate of 40,000 l per day and combines the advantage of a sufficiently large operating surface with the ability to control variations which might be experimentally applied to the system. As of this writing, approximately $62,000 \text{ m}^3$ of sewage effluent have been applied to the test basin.

TABLE 2. PARTICLE SIZE ANALYSIS OF TEST BASIN
SOIL FROM UNSATURATED ZONE

Particle diameter (mm)	Percent composition at depths below basin surface		
	0.75m	2.25m	5.34m
Silt and clay <0.0625	0.30	0.75	1.2
Sand			
0.0625-0.125	0.4	1.8	1.6
0.125-0.250	3.3	23.5	10.9
0.250-0.5	14.1	27.5	25.2
0.5-1	36.4	25.8	36.3
1-2	13.4	4.1	6.2
Gravel			
2-4	6.9	4.2	4.3
4-8	6.4	4.1	4.3
8-16	9.6	8.0	6.2
16-32	9.3	0.0	3.5
32-64	0.0	0.0	0.0

Figure 3. Schematic diagram of the U.S. Geological Survey test basin facility.

VIRUSES AND HOST CELLS

Poliovirus type 1 LSc 2ab was kindly provided by R. Reid (Carborundum Corp.). Poliovirus type 1 (V234-1), type 3 (V153-1 and V201-4) and coxsackievirus type B3 (V98-7) were previously isolated from secondary sewage effluents (Vaughn and Landry, 1977). Poliovirus type 3 (Leon), coxsackievirus type B3 (Nancy) and echovirus type 1 (Farouk) were obtained from the National Institute of Allergy and Infectious Disease (NIAID). Poliovirus type 3 (VR-62), coxsackievirus type B4 (VR-184) and echovirus type 6 (D'Amori - VR-36) were obtained from the American Type Culture Collection (ATCC). Poliovirus LScg^r-B, a spontaneous guanidine resistant mutant of strain LSc 2ab was selected by growth in guanidine-containing media (Melnick et al., 1961). The mutant was initially obtained by three serial passages in culture media containing increasing concentrations of guanidine (30, 50 and 100 µg/ml). Isolates were then plaqued in guanidine-containing medium (100 µg/ml) and finally plaque-purified three times. Poliovirus LScg^r-T was a gift from D.R. Tershak (Pennsylvania State University).

Viruses were propagated on monolayers of low-passage Buffalo Green Monkey kidney cells (BGM - Microbiological Associates) grown in Eagle's minimum essential medium (MEM) with Earle's balanced salt solution supplemented with 10% fetal calf serum, glutamine, and antibiotics (penicillin, streptomycin and gentamycin). Viruses were maintained in monodispersed stocks following the removal of aggregates by passage through serum-treated 50 nm filters (Jakubowski et al., 1975).

VIRUS ASSAY

Viral enumerations were carried out on monolayers of BGM cells grown as previously described. Sample volumes of 0.5 ml were placed on cell monolayers (25 cm² flasks) and incubated by rocking for one hour to facilitate virus attachment. After decanting excess inoculum, monolayers were overlaid with 4 ml of MEM plaquing medium supplemented with neutral red and agar (Vaughn et al., 1978). Flasks were incubated at 36°C under 5% CO₂ for three to ten day periods and analyzed daily for plaque-forming units (PFU). During the latter part of the project this single overlay (SO) technique was replaced by a double overlay (DO) method. Infected monolayers were first overlaid with a plaquing media without neutral red, incubated for 1-3 days, then stained with a neutral red agar overlay. While requiring additional handling of culture flasks, the DO method greatly enhanced plaque production. It has since been used exclusively for all viral enumerations conducted in this laboratory.

ISOLATE IDENTIFICATION

Where appropriate, isolates were "picked" from plaques with a sterile Pasteur pipette and grown on monolayers of BGM cells. Isolate identifications were carried out in plastic microtiter plates using polyvalent enterovirus typing pools obtained from NIAID.

TEMPERATURE MARKER ANALYSIS

The virulence of all poliovirus field isolates was determined by a temperature marker analysis (T-marker, Hsuing, 1964). Plaque assays at high temperatures were performed by submersing wrapped overlay flasks in a water bath (Magni-Whirl, Blue M Electric Co.) at 39.5°C. Temperature within the bags was constantly monitored with a Digitec 5810 thermometer. Temperature fluctuations never exceeded 0.3°C. Relative virulence was determined by calculating the efficiency of plating (EOP; PFU/ml^{39.5} divided by PFU/ml³⁷). Virus cultures exhibiting a log EOP of >-2.0 were considered to be virulent; those with a log EOP of -2.1 to -4.0 were considered to be attenuated viruses (Yoshioka et al., 1959).

COLIFORM STUDIES

Total and fecal enumerations from field samples were analyzed by the 5-tube most probable number method (Standard Methods, 1976).

OTHER PHYSICAL AND CHEMICAL TESTS

Routine testing of tertiary sewage effluent and renovated wastewater was carried out by the Suffolk County Department of Environmental Control (DEC). Physical and chemical characteristics assessed were those previously identified in Table 1. All determinations were made using appropriate standard techniques (Standard Methods, 1976).

FIELD SAMPLING

Sample Collection

Samples of raw sewage (plant influent) tertiary effluent and renovated water from the aquifer directly beneath the test basin floor were collected at weekly intervals for the first 1.5 years of the project. Influent grab samples were collected in sterile 4 l containers. Tertiary effluent (100 l) and renovated wastewater (400 l) samples were collected in sterile 220 l tanks (Plast-i-cube, Greif Brothers Corp.). Between sample collections, tanks were thoroughly rinsed with tap water, sanitized with 0.12 N hydrochloric acid (thirty minutes), and rinsed once again with tap water. Immediately before collection at each site, tanks and pumping equipment were rinsed with 40 to 80 l of the various waters which were to be sampled. These precautions were taken in order to obviate the chance of cross-contamination between different samples.

Virus Concentration Procedures

Viruses in raw sewage samples were concentrated by an inorganic flocculation procedure (Farrah et al., 1976). The resulting concentrates (20-50 ml) were supplemented with 10% fetal calf serum and stored at -72°C.

Viruses in large volume water samples (effluent and renovated wastewater) were initially concentrated by means of an Aquella Virus Concentrator (Carborundum Corporation) using a series of prefilters to remove debris. Viruses were adsorbed to fiberglass depth cartridge filters (K27), and epoxy-filter, glass-asbestos filters (1.0 and 0.45 µ Cox) at a pH of 3.5 and 0.5 mM AlCl₃ (Farrah et al., 1976). Elution of adsorbed virus was carried out with

two-1 & volumes 0.1 M glycine at pH 11.5. Eluates were then neutralized to pH 7.5 in an equal volume of pH 2.0 glycine. The concentration procedures routinely yielded a 4 & volume which was reconcentrated in the laboratory by means of an inorganic flocculation procedure (Farrah et al., 1976) to a final volume of 50-100 ml. After the addition of 10% fetal calf serum, samples were stored at -72°C to await assay.

The above large volume method was abandoned in favor of a new method soon after the beginning of the second project year (reasons for the change are explained in the Results and Discussion section). The new method also used an "Aquella-type" unit for initial virus concentration. The new unit employed no clarifying filters, with virus adsorption still carried out on the K27 and Cox filters (a few months later, use of the Cox filter was discontinued in favor of a pleated cartridge filter - Duo-Fine, Filterite, Timonium, Md.). The major difference between the two methods lay in the latter's use of two 660-ml volumes of 3% beef extract (pH 9.0) for elution of virus from concentrating filters. Because of difficulties in maintaining the alkaline pH during elution, a 0.5 M Tris buffered-beef extract (pH 9.5) eluant was used in later experiments. Reconcentrations were carried out according to Katzenelson et al., (1976). The pH of the eluate (~1200 ml) was lowered to 3.5 with 1.0 N HCl which caused the formation of a virus-adsorbing protein precipitate, or floc. The floc was then collected by centrifugation (5000 x g for 10 min) and the resulting pellets dissolved in 20-25 ml volumes of 0.15 M dibasic sodium phosphate (Na_2HPO_4 - pH 9.0). Samples were neutralized to pH 7.2 and stored as previously described. The final concentrate volumes which usually resulted from use of this method were 25-30 ml.

SMALL COLUMN AND CORE EXPERIMENTS

Initial studies of virus adsorption to 12-Pines soils, and their subsequent elution/mobilization by various media were carried out in small columns and soil cores. In column experiments, glass (Pyrex) laboratory columns of varying widths and lengths were packed with sand excavated from the test basin. Each column was fitted with a fine mesh screen to prevent the loss of sand during experiments. Sewage effluent (preconditioned or virus-seeded) was delivered to each column by means of a peristaltic pump (Buchler). The typical physical and chemical characteristics of tertiary effluent used in all column (and core) experiments may be seen in Table 3. Specific experiments in which soil columns were used are described in the Results and Discussion section.

Cores (4.2 x 12.5 cm) were collected from the surface of the test basin using 4.2 x 25.0 cm Lexan plastic tubing. During core extraction, overlying water was removed by vacuum and the bottom of each core carefully "capped" with fine mesh screening. For each experiment, the appropriate number of cores was matched according to similarities of infiltration rate (measured by comparing the percolation rate of 100 ml tertiary effluent in each core). Infiltration rates tended to be quite rapid, averaging ~83 cm/hr. Virus challenge to cores usually consisted of 200 ml volumes of seeded tertiary effluent. Effluents used in all experiments were allowed to sit at room temperature for a one to three-day period to dissipate any residual chlorine. Core filtrates were collected and assayed to determine total adsorbed virus. Core experiments involving rainwater employed an artificial rainwater mixture which was consistent with the typical rainfall in the northeastern United States (Evan et al., 1977). The rainwater, the chemical characteristics of which are shown in Table 3, was kindly provided by N. Gmur, Brookhaven

National Laboratory Acid Rain Program. Most of the experiments performed with small cores investigated varied aspects of viral adsorption-desorption to soil. Specific experiments and their rationale are covered in the Results and Discussion section.

TABLE 3. CHEMICAL AND PHYSICAL CHARACTERISTICS OF
TERTIARY-TREATED EFFLUENT AND ARTIFICIAL
RAINWATER USED IN COLUMN ELUTION EXPERIMENTS*

Determination*	Sewage effluent	Artificial rainwater
pH	7.47 (7.05-8.34)	4.4 (4.3-4.6)
Turbidity (NTU)	4.10 (1.0-12.5)	1.0
Conductivity (µmho)	606.00 (444-680)	40.0
Total alkalinity	95.38 (48-115)	NT
Total suspended solids	5.00 (1-14)	NT
Total organic carbon	15.13 (10-29)	NT
Chloride	54.13 (47-64)	2.70
Sulfate	35.63 (28-54)	5.00
Nitrate	3.38 (0.07-16.0)	0.80
Nitrite	0.11 (0.006-0.49)	NT
Ammonia	5.34 (0.06-15)	0.40
Total Kjeldahl-N	6.63 (0.90-17)	NT
Ortho-phosphate	6.13 (4.8-6.8)	NT
Iron	<0.05	0.010
Manganese	0.02 (0.01-0.03)	0.020
Magnesium	4.53 (2.7-7.8)	0.17
Calcium	19.00 (15-24)	0.506
Sodium	58.25 (54-68)	1.20
Potassium	12.25 (11-13)	0.20
Lead	NT	0.05
Zinc	NT	0.10
Nickel	NT	0.10
Cooper	NT	0.02
Cadmium	NT	0.02
Fluoride	NT	0.10

*See legend Table 1.
Evans et al., 1977.

TEST BASIN SEEDING EXPERIMENTS

A series of experiments was conducted to assess the virus-removing capacity of the test basin when operated at different infiltration rates. All experiments used a tertiary effluent which had been seeded with known numbers of poliovirus type 1 LSc 2ab or LSc g^r. Seeding was carried out because of the low virus numbers found in the tertiary effluent.

High Rate Recharge (75 cm/hr)

For several weeks preceding the high infiltration rate experiment, the test basin was allowed to dry out. One week before the experiment, the top

2.5 cm of the basin was removed and replaced with clean sand previously excavated during construction of the basin. These manipulations produced an infiltration rate of 75-100 cm/hr (as measured by neutron samplers). Mono-dispersed poliovirus LSc 2ab was added to 4000 l of unchlorinated tertiary effluent stored in two 2000 l Nalgene tanks to a final concentration of 2.3×10^6 PFU/l. Seeded sewage was then rapidly pumped into the test basin and allowed to drain through the basin surface. Normal recharge operation was then resumed with chlorinated tertiary effluent. During passage of the seeded "front" of water through the basin, 1 l samples were collected at timed intervals from the intermediate level gravity samplers. After passage of the "front", interval sampling was replaced by large volume composite (2-3 hr) sample collection. Large volume (380 l) renovated water samples were collected from the groundwater observation well located directly beneath the basin.

Small volume samples (1 l) were processed using the organic flocculation method of Katzenelson et al. (1976), while large volume composite and groundwater samples were processed through an "Aquella-type" concentrator using the 3% bccf extract elution method. Concentrates were analyzed for virus for total PFU per liter as previously described.

Low-Rate Recharge (6 cm/hr)

The initial low infiltration rate experiment was conducted following a prolonged period of recharge (six weeks) which produced sufficient basin clogging to result in a stable 6 cm/hr infiltration rate. Poliovirus type 1 LSc 2ab prepared as before was seeded into 8500 l of unchlorinated tertiary sewage effluent to a final concentration of $\sim 7 \times 10^4$ PFU/l. Seeded waters were then applied to the basin and allowed to drain through its surface as in the previous experiment. Normal recharge was then resumed. Composite samples of one to two liters were collected from gravity samplers over a period of several days. The reduced infiltration rate of this experiment produced significantly smaller sample volumes per unit time than the previous high rate experiment. Large volume samples (400 l) were collected over the same interval from the groundwater observation well. Samples were processed and analyzed for virus using the previously described methods.

Very Low-Rate Recharge (0.5 - 1.0 cm/hr)

In order to obtain infiltration rates of a lower order than the preceding experiment, it was necessary to clog the surface of the test basin. This was accomplished by resuspending settled solids in the treatment plant chlorine-contact chamber by manual mixing. The particulate-laden effluent was then pumped into the test basin and allowed to clog its surface. By using this method, infiltration rates of 0.5 - 2.0 cm/hr could be produced and maintained.

Two separate seeding experiments were conducted at low infiltration rates. The first of these was carried out at an infiltration rate of 1 cm/hr. The test basin was challenged with 4000 l tertiary effluent containing approximately 7.8×10^5 PFU/l poliovirus LSc 2ab. Following completion of infiltration, which required some 36 hours due to the low rate, normal recharge was resumed. Composite samples were collected from each level over an eight day period. Sample volumes varied from 0.17 to 809 l depending upon location of the sampler and the experimental stage at which samples were collected. Virus concentration and enumerations employed the previously described methods.

A second very low-rate experiment was conducted at 0.5 cm/hr with a guanidine-resistant strain of poliovirus type 1 (LSc 2ab g^r-T - the behavior of this strain in soil had been shown to be more consistent with those of other enterovirus types - see Results and Discussion). The experimental procedure was as described in the previous study with the exception that samples were collected over a sixteen-day period. The initial virus concentration in the seeded effluent was 1.92×10^6 PFU/l.

Large Core Experiments

In order to better delineate the role of rainwater in the desorption and mobilization of viruses through significant depths of soil, a series of experiments was conducted in the basin using large soil cores (10.1 x 100 cm). Before initiation of the actual experiment, the basin was operated at a high infiltration rate (>75 cm/hr) to insure complete flushing of its upper levels. The infiltration rate was then reduced by the previously described clogging method to <5 cm/hr. Cores (10.1 x 121 cm) of PVC builder's pipe (schedule 40) were then cut and tapered to facilitate placement in the basin. Cores were driven into the basin surface with a sledgehammer to a point within 21 cm of the core top. Basin recharge with suspended chlorine contact chamber solids was resumed until the infiltration rates of the cores returned to <5 cm/hr. Five cores were usually driven per experiment, with those matched by a similar infiltration rate (average rate in all experiments was 0.5-2 cm/hr) being used for each experimental run.

The general procedures for each experiment were similar. Experimental cores were allowed to drain and then inoculated with 2 l (~21 cm) volumes of unchlorinated tertiary effluent which had been seeded with a guanidine-resistant strain of poliovirus type 1 (LSc 2ab g^r-T) to a final concentration of approximately 10^3 PFU/ml. Seeded sewage was then allowed to drain through the soil surface. Control cores were then capped and carefully excavated from the basin and returned to the laboratory for analysis. Remaining cores were given a 2 l rinse with either tertiary effluent or the artificial rainwater described earlier. Following drainage of the appropriate rinse through the soil surface, these remaining cores were carefully excavated and returned to the laboratory.

Soil from each core was collected in 5 cm fractions (approximately 500 g/sample) and stored in plastic bags at 4°C to await extraction. In the extraction procedure, soil samples were supplemented with 500 ml of a 3% beef extract - 0.5 M Tris buffer solution (pH 9.5) and vigorously mixed for 5 minutes. Mixtures were then sonicated (Heat Systems - Ultrasonics Inc., Model W-220F) at 100 W/10 min and centrifuged (7500 x g/10 min). Supernatant fluids were then collected and acidified to pH 3.5 with 1.2 N HCl. The resulting floc was collected by centrifugation (7500 x g/10 min), and the pellets dissolved in 10-15 ml volumes of dibasic sodium phosphate (pH 9.5). Following neutralization to pH 7.0, samples were assayed for virus activity using the previously described method.

The overall efficiency of the extraction method was relatively low (30%). However, while this would likely influence the total number of viruses isolated, we assumed that a constant source of error would have no effect on the relative proportions of virus which were isolated between fractions or cores.

SECTION 5

RESULTS AND DISCUSSION

VIRUS ISOLATIONS DURING FIELD SAMPLING

Systems designed to use treated domestic wastewater effluents for recharging groundwater aquifers are often challenged due to a lack of information on the movement of potential disease-causing organisms within these systems. Of the waterborne pathogens, major concern has been directed toward the role of human enteric viruses commonly occurring in domestic wastewaters.

Sewage Influent

Prior to determining the ability of Long Island soils to remove indigenous human enteroviruses during normal recharge operation, it was necessary to obtain baseline information on the presence and concentration of these viruses in the sewage treatment plant influent and effluent. Table 4 presents the results of routine monitoring of plant influents for enteroviruses and coliform organisms. As expected, all samples (19) were positive for viruses and coliforms. An average of 784 PFU per liter were recovered over a one-year sampling period, with concentrations ranging from 20 to 5368 PFU/l. The large fluctuation in virus concentration likely resulted from the varied sewage input experienced by the treatment plant in daily operations. Similar fluctuations have been reported previously for other Long Island sewage treatment plants (Vaughn and Landry, 1977).

TABLE 4. ENTEROVIRUS AND COLIFORM ISOLATIONS
FROM 12-PINES RAW SEWAGE INFLUENT

Sample no.	Date collected	Virus (PFU/l)*	Coliforms/100 ml	
			Total	Fecal
A1	11/10/76	473.7	4.6x10 ⁶	NT
A8	12/2/76	452.6	1.1x10 ⁷	4.6x10 ⁶
A11	12/6/76	2368.4	1.1x10 ⁷	4.6x10 ⁶
A14	1/21/77	5368.4	1.2x10 ⁷	5.0x10 ⁶
A16	5/6/77	47.4	NT	NT
A20	5/11/77	505.3	4.3x10 ⁷	1.5x10 ⁷
A23	5/18/77	155.3	NT	NT
A24	5/24/77	113.7	9.3x10 ⁷	9.3x10 ⁷
A29	5/26/77	118.4	4.3x10 ⁸	4.3x10 ⁸
A32	6/2/77	231.6	NT	NT
A35	6/7/76	707.4	NT	NT
A36	6/20/77	20.0	1.1x10 ⁸	4.3x10 ⁵
A41	6/27/77	2682.1	2.4x10 ⁸	2.3x10 ⁷
A42	7/13/77	137.4	2.3x10 ⁷	2.3x10 ⁷
A45	7/18/77	394.7	4.3x10 ⁷	2.3x10 ⁷
A48	7/27/77	490.3	7.5x10 ⁷	4.0x10 ⁵
A53	8/2/77	454.7	1.1x10 ⁹	1.5x10 ⁷
A56	8/8/77	10.7	1.5x10 ⁸	9.3x10 ⁷
A59	10/11/77	179.5	1.1x10 ⁹	1.1x10 ⁹

*PFU, plaque-formings units.

No demonstrable seasonal trend, as reported by other investigators (Buras, 1976), was observed with influent samples. High concentrations of viruses were observed during both winter (A11, A14) and summer samplings (A41). Reduced virus levels were also observed during both seasons. As expected, total and fecal coliform counts from raw influent were extremely high.

Tertiary Effluent

A significant reduction in virus and coliform concentration was noted in treated plant effluents (Table 5). Enteroviruses were detected on four occasions, with concentrations ranging from 0.37 to 81.1 PFU per liter. The paucity of isolations from effluents suggested that either the treatment plant was producing extremely high quality effluent, or that the virus concentration procedure was not effective. At that time, concentration

TABLE 5. ENTEROVIRUS AND COLIFORM ISOLATIONS
FROM 12-PINES TERTIARY SEWAGE EFFLUENT

Sample no.	Date collected	Virus (PFU/l)	Coliforms/100 ml	
			Total	Fecal
A2	11/10/76	81.1	3.8×10^5	NT
A3	11/10/76	0	2.3×10^5	NT
A7	11/29/76	0	<3	<3
A9	12/2/76	15.5	2.3×10^1	2.3×10^1
A12	12/6/76	0	1.5×10^1	1.5×10^1
A17	5/6/77	1.1	NT	NT
A19	5/11/77	0	1.7×10^1	8
A22	5/18/77	0	NT	NT
A25	5/24/77	0	4.6×10^2	2.3×10^1
A28	5/26/77	0	2.4×10^3	<3
A31	6/2/77	0	NT	NT
A34	6/7/77	0	NT	NT
A38	6/20/77	0	4.6×10^4	4.6×10^4
A40	6/27/77	0	2.4×10^4	4.3×10^3
A44	7/13/77	0	<3	<3
A46	7/18/77	0	<3	<3
A49	7/27/77	0	4	<3
A52	8/2/77	0	<3	<3
A55	8/8/77	0	4	<3
A58	10/11/77	0	<3	<3
A61	10/18/77	0	2.4×10^3	2.4×10^3
A65	10/26/77	0	2.3×10^1	1.1×10^1
A68	10/31/77	0	2.3×10^1	4
A69	1/26/78	0	NT	NT
A73	1/30/78	0	<3	<3
A76	2/14/78	0	4	<3
A79	2/21/78	0	<3	<3
A82	2/28/78	0.37	4	<3

procedures employed fiberglass depth cartridges (K27) and epoxy-fiber glass-asbestos filters (Cox) as virus adsorbants. Virus recovery was accomplished by glycine elution (pH 11.5) followed by an inorganic flocculation at pH 3.5. To determine whether the failure to isolate virus was due to poor concentrating procedures, a number of modifications were introduced and compared. Modifications included: the processing of larger sample volumes (200 l); the use of pleated filters (Gerba et al., 1978); and elution of virus-concentrating filters with beef extract (pH 9.0). Experiments on the development of a more efficient concentration system will be detailed in a later section. While more efficient in laboratory tests, these methods failed to increase the frequency of virus isolation, attesting to the good treatment capability of this plant during the sampling period.

Total coliform concentrations exceeded 100/100 ml on only seven occasions, while fecal coliform counts exceeded this level on three occasions (out of 23 and 21 sampling events, respectively). It was of interest to note that on one particular occasion of high virus incidence (A9 - 15.5 PFU/l), few coliform organisms were observed (23 total and 23 fecal coliforms per 100 ml). Conversely, large concentrations of both total and fecal coliforms had been found in other samples which yielded no enteroviral isolates. These data lend support to the theory that coliform standards do not reflect the occurrence of enterovirus in the environment (Gerba et al., 1979; Vaughn et al., 1979).

Renovated Waters

Since few enteroviruses were apparently being applied to the recharge basin during normal operations, sampling of recharged waters at intermediate soil levels (0.75, 2.25 and 5.34 m) was considered inappropriate because of the small volumes of water which could be collected from these samplers. Extensive sampling of the renovated water 7.6 m below the basin floor was carried out (Table 6). Ordinarily, 380 to 760 l of water was processed for each sampling using the best available concentration procedure. Enteroviruses were detected in six of 28 samples, with concentrations ranging from 0.14 to 0.66 PFU per liter. On two occasions when viruses were isolated from treated sewage effluents (A2, A17), they were also observed in renovated well water (A4, A15). However, on several other occasions, viruses were either detected in the effluent and not in the renovated water (A9, A82), or in renovated water (A30, A51, A57, A62) but not in the effluent. These results were not unexpected as groundwater samples represented earlier effluent applications than that being sampled on the same day. The weekly sample regime could easily have missed an effluent batch containing significant numbers of virus whose presence might later be detected in the groundwater observation well. The most important information provided by these data was the demonstration of viable virus particles in the recharged groundwater.

The coliform data varied quite extensively ranging from <3 to 9.3×10^3 per 100 ml for total, and <3 to 4.3×10^3 for fecal coliforms. As seen previously, no correlation could be made between the occurrence of viruses and coliform organisms.

A number of isolates from influent, effluent and renovated water samples were plaque-purified, passaged and identified. Among isolates identified were: polio types 1 and 2; coxsackie A16; coxsackie B2; coxsackie B3; and echovirus types E18, E21 and E25. Several of the isolates could not be identified with the typing pools which are specific for approximately 35-40% of the known enterovirus types.

TABLE 6. ENTEROVIRUS AND COLIFORM ISOLATIONS
FROM 12-PINES RENOVATED WATER

Sample no.	Date collected	Virus (PFU/l)	Coliforms/100 ml	
			Total	Fecal
A4	11/10/76	0.66	9.3×10^2	NT
A5	11/18/76	0	4.3×10^2	9.6×10^1
A6	11/29/76	0	9.3×10^3	4.3×10^3
A10	12/2/76	0	4.3×10^2	4.3×10^2
A13	12/6/76	0	1.2×10^2	4
A15	5/6/77	0.53	NT	NT
A18	5/11/77	0	4.9×10^1	2
A21	5/18/77	0	NT	NT
A26	5/24/77	0	7.5×10^1	9
A27	5/26/77	0	4.6×10^2	2.3×10^1
A30	6/2/77	0.21	NT	NT
A33	6/7/77	0	NT	NT
A37	6/20/77	0	7.5×10^2	3.9×10^2
A39	6/27/77	0	2.4×10^3	4.3×10^2
A43	7/13/77	0	4.3×10^1	4
A47	7/18/77	0	1.1×10^4	4.3×10^2
A50	7/27/77	0	2.4×10^3	2.3×10^1
A51	8/2/77	0.61	2.5×10^1	<3
A54	8/8/77	0.66	2.1×10^3	9
A57	10/11/77	0	2.8×10^3	3.0×10^2
A62	10/18/77	0.14	4.3×10^2	7.0×10^1
A64	10/26/77	0	<3	<3
A67	10/31/77	0	<3	<3
A70	1/26/78	0	NT	NT
A74	1/31/78	0	4	<3
A77	2/14/78	0	<3	<3
A80	2/21/78	0	<3	<3
A81	2/28/78	0	4	<3

Several problems arose during the field sampling study which greatly hampered the conduct of this phase of the program. The severe winters of 1977-78 and 1978-79 caused extensive damage to both treatment plant and recharge facilities. In 1978, frozen pumps, valves, and distribution lines caused a complete shutdown of all recharge operations. Following repair, the systems were winterized by burying pipelines below the frostline and by providing heat for pumping and monitoring equipment. These improvements allowed for more normal operations during later cold periods.

TECHNIQUES FOR THE RECOVERY OF VIRUS FROM WASTEWATER EFFLUENTS

The filter-adsorption-elution technique has been employed by a number of investigators for the recovery of enterovirus from drinking water (Hill et al., 1974), estuarine water (Payment et al., 1976), treated sewage (Vaughn and Landry, 1977), and seawater (Gerba et al., 1978). Although this method is one of the most reliable and widely used techniques, recovery efficiencies, as judged by seeding experiments using known concentrations of virus,

exhibited great variability, with recoveries ranging from 48 to 95% (Sobsey et al., 1973). While glycine has been recommended as the standard eluant for the recovery of enteric viruses from water and wastewater (Standard Methods, 1976), a number of alkaline proteinaceous substances have been successfully used for this purpose including: bovine serum-supplemented media (Wallis and Melnick, 1967); beef extract (Berg et al., 1971; Wellings et al., 1976); and nutrient broth (Hill et al., 1972). Recently, Katzenelson et al., (1976) reported the elution of virus from an epoxy-fiber, glass-asbestos filter using 3% beef extract (pH 9.0). In a series of comparative experiments using tap water seeded with poliovirus type 1, beef extract elution was found to be superior to glycine elution, yielding an average efficiency of 74.4%, with recoveries ranging from 60 to 91%. Elution with 0.05 M glycine (pH 11.5) showed a 35% mean recovery efficiency, with a range of 30 to 47%.

Initial experiments in our laboratory using a filter adsorption-elution system with a 0.10 M glycine (pH 11.5) eluant resulted in erratic virus recoveries in seeded wastewater experiments. In an effort to increase the recovery efficiency of the system, a study was conducted to determine the efficiency of the beef extract elution method. A major portion of this work has been recently published (Landry et al., 1978).

Beef Extract vs. Glycine Elution

A comparison of the efficiency of virus recovery using 3% beef extract (pH 9.0) or 0.10 M glycine (pH 11.5) was assessed in poliovirus-seeded 100-liter volumes of treated sewage effluent. The viruses were concentrated via filter adsorption-elution using a fiberglass cartridge depth filter (K27) and epoxy-fiber glass-asbestos filters (1.0 and 0.45 μ m porosity). Clarifying filters were not used in any of the experiments as the turbidity of the tertiary-treated sewage was quite low. The results of duplicate experiments (Table 7) indicated beef extract elution, followed by organic flocculation, to be superior to a glycine elution-inorganic flocculation method in the recovery of seeded poliovirus from sewage effluent. Beef extract elution yielded a recovery efficiency of >100% and 96% respectively in two trials, while the recovery efficiency using glycine was 31 and 41% respectively. In addition, the beef extract technique appeared to be more practical since the final sample volumes following reconcentration were considerably smaller than those resulting from the glycine method (e.g. 15 ml vs. 160 ml). This is

TABLE 7. COMPARISON OF POLIOVIRUS LSc 2ab ELUTION FROM VIRUS-CONCENTRATING FILTERS WITH 3% BEEF EXTRACT (pH 9.0) and 0.1M GLYCINE (pH 11.5)

Expt. no.	Total PFU in:			
	Beef extract		Glycine	
	Input	Eluate	Input	Eluate
1	3.03x10 ⁵	3.16x10 ⁵ (>100)*	5.68x10 ⁵	1.80x10 ⁵ (31.69)
2	3.31x10 ⁶	3.18x10 ⁶ (96.07)	2.27x10 ⁶	9.30x10 ⁵ (40.96)

*Values in parentheses represent percent recoveries of input values.

particularly important when dealing with large volume water samples having very low concentrations of virus which necessitates assay of most or all of the sample.

Comparisons of Beef Extract Type

Beef extract may be obtained in one of two forms, a paste (Difco), or a powder (Lab Lemco). The previous work had made use of the paste, and we wished to insure that similar results could be obtained by using the more convenient powdered form. A series of experiments was conducted with seeded 12-Pines effluent as described in the previous section using these types of beef extract as eluting fluid. The composite results of three experiments are shown in Table 8. In both cases, beef extract elution resulted in the recovery of a large percentage of the input viruses. The powdered form (Lab Lemco) yielded a recovery efficiency of 84%, and the paste form (Difco) allowed an overall recovery of 78%. Since the powdered form was more convenient, it was used in all subsequent experiments.

TABLE 8. ELUTION AND RECONCENTRATION OF POLIOVIRUS LSc 2ab FROM VIRUS-CONCENTRATING FILTERS WITH 3% BEEF EXTRACT (pH 9.0): LAB LEMCO POWDER VERSUS DIFCO PASTE

Type of beef extract	Total PFU recovered from:		
	Input	Eluate	Reconcentrate
Lab Lemco	1.45×10^7	1.19×10^7 (82.06)*	1.22×10^7 * (84.13)
Difco	2.33×10^7	1.41×10^7 (60.51)	1.82×10^7 (78.11)

*Numbers in parentheses represent percent recovery of input values.

Optimizing Beef Extract Elution

Additional studies were conducted to determine whether an extension of the contact time between the virus concentrating filters and the eluant would increase the efficiency of virus recovery. Comparative experiments were performed by seeding two 100 liter wastewater samples with identical concentrations of poliovirus (1.54×10^7 PFU). One sample was eluted in the manner previously described, while the other sample was eluted after allowing the beef extract to remain in contact with the virus-laden filters for a total of 30 minutes. Both samples were reconcentrated by flocculation. The results are shown in Table 9. Immediate elution resulted in the recovery of 1.34×10^7 PFU (87%) before reconcentration, and 1.05×10^7 PFU (68%) following

TABLE 9. POLIOVIRUS LSc 2ab RECOVERY FROM VIRUS-CONCENTRATING FILTERS BY IMMEDIATE OR DELAYED BEEF EXTRACT ELUTION

Type of elution	Total PFU recovered from:		
	Input	Eluate	Reconcentrate
Immediate	1.54×10^7	1.34×10^7 (87.01)*	1.05×10^7 (68.18)
30 min.	1.54×10^7	1.24×10^7 (80.51)	1.21×10^7 (78.57)

*Numbers in parentheses represent percent recovery of input values.

organic flocculation. Extended elution yielded similar results with a recovery of 1.24×10^7 total PFU (80.5%), and 1.21×10^7 (78%) before and after organic flocculation, respectively. These results suggested that the elution was likely instantaneous, and that poliovirus was not inactivated by prolonged contact (30 min.) with beef extract at pH 9.0.

In an effort to determine whether lower concentrations of beef extract could be effective in the recovery of poliovirus from seeded wastewater effluents, experiments were performed as previously described, with the exception that 1% or 3% beef extract solutions were used as eluants. Table 10 illustrates that both concentrations of beef extract were similar in their virus-eluting capacity with 76% of the virus being recovered with 1% beef extract, and 69% recovered with 3% beef extract.

TABLE 10. EFFICIENCY OF POLIOVIRUS LSc 2ab RECOVERY FROM VIRUS-CONCENTRATING FILTERS USING 1 or 3% BEEF EXTRACT ELUTION

Beef extract (%)	Total PFU recovered:	
	Input	Reconcentrate
1	2.22×10^6	1.67×10^6 (75.90)*
3	2.22×10^6	1.53×10^6 (68.91)

*Percent recovery of input value.

Virus Recovery from Pleated Filters

Recently, a number of investigators have adopted pleated cartridge filters for use in the recovery of enteroviruses from large volume water samples (Farrah et al., 1976; Gerba et al., 1978). The increased surface area of these filters eliminates clogging problems, allowing higher sample volumes to be processed. Experiments were, therefore, performed to insure that 3% beef extract (pH 9.0) was equally effective in systems using these filters. Seeded sewage effluent samples were processed through a filter series consisting of a fiberglass K27 filter and a $0.45 \mu\text{m}$ pleated filter. The results, presented in Table 11, indicated that an average of 65% of the input polioviruses were recovered following concentration and reconcentration. This efficiency appears to be slightly lower than that previously observed for elution-recovery using a fiberglass depth cartridge-membrane filter series. However, in spite of recovery values, the pleated filter series would appear to be most practical for the processing of large volume water samples because of their ability to resist clogging and maintain relatively high flow rates.

TABLE 11. EFFICIENCY OF POLIOVIRUS LSc 2ab RECOVERY FROM PLEATED CARTRIDGE FILTERS USING 3% BEEF EXTRACT (pH 9.0).

Expt. no.	Total PFU recovered from:	
	Input	Reconcentrate
1	3.28×10^6	2.04×10^6 (62.19)*
2	2.22×10^6	1.53×10^6 (68.91)

*Values in parentheses are percent recoveries based on input.

To illustrate the overall reproducibility and efficiency of the beef extract elution procedure for the recovery of virus from treated effluents, the data from a number of experiments are presented in Table 12. In seven trials the mean recovery efficiency of poliovirus was 85%, with a range of 68% to 100%.

TABLE 12. EFFICIENCY OF POLIOVIRUS LSc 2ab RECOVERY
FROM LARGE VOLUME SAMPLES USING THE 3% BEEF
EXTRACT (pH 9.0) ELUTION RECONCENTRATION METHOD

Expt. no.	Total PFU recovered from:		Percent recovery
	Input	Reconcentrate	
1	3.03×10^5	3.16×10^5	100.0
2	3.31×10^6	3.18×10^6	96.07
3	1.45×10^7	1.22×10^7	84.13
4	2.33×10^7	1.82×10^7	78.11
5	1.54×10^7	1.05×10^7	68.18
6	1.54×10^7	1.21×10^7	78.57
7	5.30×10^6	4.95×10^6	93.39
			Mean 85.49
			Range 68 - 100

Efficiency of Beef Extract in Second-Stage Reconcentration

In addition to the recovery of virus from large volume waste and renovated water samples, it was necessary to have an efficient technique for the recovery of virus from small volume renovated water samples collected from the intermediate gravity samplers during basin recharge experiments. Since Katzenelson et al., (1976) recently reported that an organic flocculation procedure, employing 3% beef extract was superior to an inorganic floc method using glycine for the recovery of seeded poliovirus from tapwater during second-stage reconcentration, it was necessary to determine whether a similar occurrence could be observed in poliovirus-seeded water collected from the basin gravity samplers. A 1 liter water sample was divided into 250 ml aliquots and the viruses concentrated using either organic or inorganic flocculation. The results (Table 13) showed that 85% of the input viruses were recovered with the organic method, while inorganic flocculation yielded an average recovery of 64%.

The previous concentration experiments utilized 3% beef extract. Since earlier experiments had indicated that lower concentrations of beef extract could be successfully employed in the recovery of virus from large volume samples (Table 10), reduced levels of the substance should be effective in second-stage reconcentration. Initial experiments were conducted using small volumes of renovated wastewater collected from observation wells located beneath wastewater recharge basins. Six liter volumes were seeded with poliovirus and dispensed into 1 l aliquots. Samples were concentrated by organic flocculation using beef extract at final concentrations of: 0.5; 1.0; 1.5; 2.0; 2.5; and 3.0% respectively. The results of this experiment (Table 14) indicated that lower concentrations of beef extract also yielded satisfactory virus recovery.

TABLE 13. COMPARISON OF THE EFFICIENCY OF RECONCENTRATING
SEEDED POLIOVIRUS LSc 2ab FROM SMALL VOLUME SAMPLES
BY INORGANIC OR ORGANIC FLOCCULATION METHODS

Expt. no.	Total PFU recovered after:	
	Inorganic flocculation	Organic flocculation
1	2.35×10^5 (68.11)*	3.04×10^5 (88.11)
2	2.07×10^5 (60.00)	2.87×10^5 (83.18)
Average	64.05	85.64

*Values in parentheses are percent recoveries based on an input value of 3.45×10^5 total PFU.

TABLE 14. RECOVERY OF POLIOVIRUS LSc 2ab FROM SMALL VOLUME SAMPLES
USING VARIOUS CONCENTRATIONS OF BEEF EXTRACT

% Beef extract	Total PFU recovered from*:		Mean recovery (%)
	Expt 1	Expt 2	
0.5	1.14×10^5 (74.03)	1.99×10^5 (80.56)	77.29
1.0	1.10×10^5 (71.42)	2.28×10^5 (92.30)	81.86
1.5	8.84×10^4 (57.40)	2.47×10^5 (100)	78.70
2.0	1.60×10^5 (100)	2.48×10^5 (100)	100.0
2.5	1.36×10^5 (88.31)	1.67×10^5 (67.61)	77.96
3.0	1.12×10^5 (72.72)	2.67×10^5 (100)	86.36

*Values in parentheses are percent recoveries based on input values of 1.54×10^5 for the first experiment and 2.47×10^5 for experiment 2.

Overall, beef extract has been shown to be superior to glycine in the recovery of poliovirus from both large and small volume seeded samples. It is not known whether the decreased efficiency of glycine elution in our system was the result of viral inactivation caused by the high pH of the glycine (pH 11.5). This pH appears to be necessary for efficient virus elution (Wallis et al., 1972), and has not been reported to be viricidal for poliovirus during the time required for filter elution and subsequent neutralization (Metcalf et al., 1974). However, Jakubowski et al. (1975) have suggested that lowering the pH may reduce the risk of viral inactivation, particularly since pH inactivation data on other members of the enterovirus group are unavailable. Glycine solutions having a pH of 9.8 and 10.5 have been shown to be effective in the elution of seeded poliovirus from Balston and nitrocellulose membrane filters (Jakubowski et al., 1975), and from pleated filters when using tapwater (Gerba et al., 1978). Fields and Metcalf (1975) reported that pH 10.5-11.5 glycine could not be used for the elution

of adenovirus type 5 from adsorbing filters because of the pH sensitivity of the virus. They found that 3% beef extract at pH 9.0 yielded a recovery efficiency of 90% during adenovirus seeding experiments in artificial or natural seawater.

ADSORPTION OF ENTEROVIRUS TO LONG ISLAND SOILS

Important to any overall analysis of the removal of enteroviruses during wastewater recharge operations is a thorough understanding of virus interactions with soil particles. The chief mechanism of virus removal during percolation through sand or soils was found to be adsorption, rather than filtration or sieving (Drewry and Eliassen, 1968). The process was found to be strongly influenced by a number of factors including the nature of the virus, the chemical composition of the soil and the recharged wastewater, the moisture content of the soil, and the rate and schedule of wastewater application (Gerba et al., 1975). During the course of this study, a number of these factors were analyzed to determine the fate of enteroviruses in Long Island soils by employing both laboratory soil columns and field cores.

Adsorption of Virus to Laboratory Soil Columns

Initial experiments involved the use of glass columns packed with clean, unused soil excavated from the 12-Pines recharge basin. The objective of these experiments was to determine if unconditioned, sandy soils, characteristic of most Long Island recharge basins (see Table 2), had any virus-retaining capabilities. A series of 4 laboratory columns (1.4 cm x 7.62 cm) were pretreated with 0, 3.8, 15 and 20 l of treated sewage effluent. Columns were inoculated with 160 ml volumes of treated sewage effluent seeded with poliovirus LSc 2ab. The wastewater was allowed to percolate through the column, and the filtrate collected for analysis of unadsorbed viruses. The results of the experiment are shown in Table 15. Virus retention in the columns was found to be dramatically influenced by the degree of preconditioning. Columns receiving larger volumes (15 to 20 l) of treated sewage effluent showed the removal of 87 and 91% of the virus, while columns receiving little or no conditioning failed to adsorb any viruses. This appears to support the premise that during conditioning or recharge, the filtration and adsorption of particulates, ions, and organics present in the sewage enhance the binding of viruses to the top layers of soil (Berg, 1973). However, this

TABLE 15. EFFECT OF COLUMN PRECONDITIONING ON VIRUS ADSORPTION
TO 1.4 cm x 7.62 cm SAND COLUMNS

Precondition volume (l)	Total PFU recovered from:		Percent retention
	Input	Filtrate	
0	1.70×10^5	1.67×10^5	1.77
3.8	1.70×10^5	1.82×10^5	0
15	3.10×10^5	4.00×10^4	87.10
20	3.10×10^5	2.74×10^4	91.17

may not hold true for all soils, especially those of differing clay and silt composition. This precise situation was recently reported by Lance and Gerba (1979) who demonstrated that unconditioned soils with higher clay and silt concentrations than seen in Long Island soils were quite efficient in virus removal.

The next phase of column experimentation attempted to compare the efficiency of poliovirus adsorption to soils using columns of varying length and diameter. Columns 1.4 or 4.2 cm in diameter, and 7.6, 12.7, 17.8 and 22.8 cm in length were preconditioned with 20 l of treated sewage effluent to enhance virus adsorption. A virus-containing sewage effluent (100 ml) was added to each column and the filtrates collected, measured, and analyzed for unadsorbed viruses. The amount of retained virus was calculated by subtracting the filtrate value from the measured virus input. The results are presented in Tables 16 and 17. Erratic results were obtained from columns 1.4 cm in diameter. Little or no virus retention (0 and 35%) was observed in 1.4 cm x 12.7 cm columns, and reduced adsorption (62%) was seen in 1.4 cm x 22.8 cm columns. Columns 4.2 cm in diameter yielded less erratic results, with the mean virus retention equalling 83.9, 78.4, 77.1 and 78.5%, respectively, for 7.62, 12.7, 17.7 and 22.8 cm column lengths. These results suggested that 1.4 cm columns may not be useful in studying the mechanics of virus adsorption to soils. The data gathered from 4.2 cm columns suggested that good

TABLE 16. RETENTION OF POLIOVIRUS LSc 2ab
IN 1.40 cm DIAMETER SAND COLUMNS

Column size (cm)	Percent virus retained on column*	
	Expt. 1	Expt. 2
1.40 x 7.62	91.1	87.1
1.40 x 12.70	0	35.5
1.40 x 17.78	73.2	94.6
1.40 x 22.86	63.5	86.1

*Values based on total input PFU of 3.10×10^5 .

TABLE 17. RETENTION OF POLIOVIRUS LSc 2ab IN 4.20 cm
DIAMETER SAND COLUMNS OF VARYING LENGTH

Column size (cm)	Percent virus retained on column	
	Expt. 1	Expt. 2
4.20 x 7.62	91.92	75.99
4.20 x 12.70	87.13	69.61
4.20 x 17.78	83.34	70.94
4.20 x 22.86	72.89	84.04

virus adsorption was occurring within the top 7.6 cm of soil. Since these columns presented less aberrant results, they were chosen for future laboratory analysis.

Elution of Adsorbed Virus from Soil Columns

In order to determine the depth of virus penetration in soil and their survival ability, an efficient method of virus elution from soil columns was needed. Eluents used previously to remove virus from soils and sediments e.g. distilled water (Duboise et al., 1976), glycine (Gerba et al., 1977), and beef extract were analyzed for their ability to recover virus from 12-Pines soil. Poliovirus LSc was adsorbed to preconditioned sand columns as previously described. Columns were then challenged with rinses of either 100 ml of distilled water (pH 7.3), 0.1 M glycine (pH 9.5), or 3% beef extract (pH 9.0). Column effluents were analyzed for total virus eluted, and the resulting efficiencies of elution were calculated. As seen in Table 18, neither distilled water nor glycine rinses released appreciable numbers of adsorbed virus. Rinses with 3% beef extract, however, yielded significant but somewhat erratic results, with over 100% of the virus eluted in the first trial, but only 55% released in the second trial. Since beef extract elution was clearly more efficient in these preliminary experiments, this technique was investigated further.

TABLE 18. ELUTION OF POLIOVIRUS LSc 2ab FROM 4.2 cm x 12.70 cm SAND COLUMNS

Total PFU representing:				
Expt. no.	Type of elution	Adsorbed virus*	Eluted virus	Percent eluted
1	Distilled H ₂ O (pH 7.3)	9.72×10^5	5.25×10^4	5.39
	0.1 M Glycine (pH 9.5)	9.87×10^5	1.05×10^5	10.63
	3% Beef Extract (pH 9.0)	9.77×10^5	2.20×10^6	100+
2	Distilled H ₂ O (pH 7.3)	4.65×10^5	6.50×10^3	1.39
	0.1 M Glycine (pH 9.5)	4.67×10^5	4.35×10^4	9.31
	3% Beef Extract (pH 9.0)	4.30×10^5	2.40×10^5	55.81

*These values represent the theoretical amount of adsorbed virus calculated by subtracting column filtrate values from Input virus titers of 1×10^6 and 5×10^5 PFU for experiments 1 and 2, respectively.

Using 4.2 cm x 12.7 cm packed and conditioned columns, varying concentrations of poliovirus LSc 2ab were adsorbed to basin soils. After collection of column filtrates, 100 ml of 3% beef extract (pH 9.0) was passed through each column and eluates collected, measured and analyzed for total PFU. The results (Table 19) again show the variability of virus recovery using this technique. Recoveries ranged from 35 to 100% in four trials. Recovery appeared to be more efficient when lower concentrations of virus were applied to columns.

To determine the efficiency of beef extract in the elution of viruses other than poliovirus LSc 2ab from basin soils, a series of laboratory

columns were challenged with a variety of enteroviruses including: a polio-virus type 3 field isolate; laboratory and field strains of coxsackie B3; and two laboratory strains of echovirus (E1 and E6). Experiments were performed as previously described, and the results are shown in Table 20. The elution efficiency of beef extract was quite variable, apparently depending upon the particular type of virus being used. Elution of polio type 3 resulted in the recovery of 62% of adsorbed viruses. An obvious strain-dependent elution characteristic was observed with the coxsackie viruses. While 100% of the CB3 field isolate could be eluted, only 0 and 8% of the laboratory strain was recovered. Echovirus elution also showed great variability, with none of the E1 being eluted, while over 50% of the E6 were recovered. In addition to strain-dependent elution characteristics, these viruses also demonstrated strain-dependent adsorption profiles to preconditioned laboratory columns. Both poliovirus type 3 and the field strain of coxsackie B3 (98-7) showed poor virus adsorption (46% and 25%, respectively). The laboratory strain of CB3 demonstrated an intermediate adsorptive ability (59 and 69%), while both echoviruses (E1 and E6) adsorbed well to soils (100 and 83%, respectively). These results were recently corroborated by Gerba and Goyal (1979) who reported the strain-dependent adsorption of 27 different enteroviruses to nine soil types.

TABLE 19. EFFICIENCY OF BEEF EXTRACT FOR ELUTION
OF POLIOVIRUS LSc 2ab FROM SOIL COLUMNS

Total PFU recovered as:				
Input virus (A)	Adsorbed virus* (B)	% Adsorbed (B÷A)	Eluted virus (C)	% eluted (C÷B)
3.15×10^5	2.50×10^5	79.36	1.18×10^5	47.20
3.15×10^5	2.84×10^5	90.16	1.00×10^5	35.21
4.52×10^4	4.32×10^4	95.57	2.60×10^4	60.18
1.92×10^3	1.92×10^3	100	2.20×10^3	>100

*Adsorbed virus was calculated by subtracting the total PFU recovered in column effluents from the input value.

TABLE 20. EFFICIENCY OF BEEF EXTRACT FOR THE ELUTION OF A
VARIETY OF ENTEROVIRUS TYPES FROM SOIL COLUMNS

Virus Type		Total PFU recovered as:		
		Input	Adsorbed	Percent recovered Eluted
P3	(V98-1 field isolate)	6.33×10^4	2.94×10^4	1.84×10^4 62.59
CB3	(98-7 field isolate)	8.4×10^4	2.15×10^4	3.06×10^4 >100
CB3	(Nancy)	8.65×10^4	6.02×10^4	5.00×10^3 8.30
CB3	(Nancy)	8.65×10^4	5.16×10^4	0 0
E1	(Farouk)	1.53×10^6	1.53×10^6	0 0
E6	(D'Amori)	5.31×10^4	4.43×10^4	2.37×10^4 53.50

Virus Adsorption to Natural Cores

Preliminary evidence has demonstrated variable adsorption and elution of enteroviruses using small packed laboratory columns. While strain differences are probably the largest contributing factor, some of the variability may be due to unequal conditioning of the columns. To avoid this problem and more closely duplicate the natural recharge soil, a series of soil cores, or plugs, were collected from operating recharge basins, and analyzed for their ability to adsorb viruses relative to adsorption profiles in packed soil columns. Experimentally, each of two cores was challenged with 200 ml of nonsterile virus-containing tertiary sewage effluent seeded with laboratory strains of polio 1 or coxsackie B3. Sewage effluents were monitored to insure that no residual chlorine was present. Filtrates were collected and assayed for total virus. At the same time, 43 x 125 mm columns were packed with clean, used soils from the banks of the recharge basin. Since preliminary experiments had indicated poor viral adsorption to clean, unused Long Island soils, columns were conditioned by passage of 15 l of unchlorinated secondarily-treated sewage through each column. Column effluents were collected and analyzed for total PFU. The results of both experiments are listed in Table 21, representing the average of at least two experimental runs. Reference strains of polio and coxsackie viruses adsorbed efficiently to natural cores (94% and 98%, respectively), while adsorption to packed, conditioned columns in both cases was somewhat decreased (76% and 64%, respectively). These data indicated that the continual exposure of soils to sewage-effluent, rather than short-term conditioning, better enhanced their ability to adsorb viruses. Based on these results, natural cores were used in all subsequent experiments for optimal enterovirus adsorption.

Adsorption-Elution of Viruses Using Natural Cores

As previously mentioned, Gerba and Goyal (1978) reported the strain-dependent adsorption characteristics of 27 different enteroviruses to nine different soil types, using a 30-minute batch adsorption procedure. The authors observed that all three types of poliovirus, including reference and field isolates, adsorbed extremely well to a majority of the test soils, while other enteroviruses (coxsackie and echoviruses) demonstrated varied adsorption patterns depending on the origin of the virus. Reference strains of coxsackievirus (B1 through B6) were found to readily adsorb to soils, while field isolates of coxsackie B4 adsorbed poorly. Adsorption of field-isolated

TABLE 21. COMPARISON OF THE ADSORPTIVE CAPACITY OF POLIOVIRUS 1Sc AND COXSACKIE B3 TO PACKED, CONDITIONED COLUMNS AND NATURAL CORES

Total PFU recovered as:				
Virus Type	Column type	Input (A)	Unadsorbed (B)	Theoretical adsorbed (A-B)
Polio 1	packed	3.78×10^5	8.89×10^4 (23.54)*	2.89×10^5 (76.46)
"	natural core	2.85×10^6	1.73×10^5 (6.97)	2.68×10^5 (94.03)
CB3	packed	1.04×10^5	3.65×10^4 (35.10)	6.75×10^5 (64.90)
"	natural core	6.97×10^5	6.46×10^3 (1.01)	6.90×10^5 (98.99)

*Numbers in parentheses represent percentages of the input value.

coxsackie viruses to Flushing Meadow soil was greatly enhanced by the presence of 10 mM Ca^{++} . The adsorption of both reference and field-isolated echoviruses was found to be greatly dependent on the particular virus strain. Reference strains of echo 1 and 29 were poor adsorbers, while the remaining echovirus types tested were efficiently adsorbed. Field isolates of echo 1 failed to adsorb to most of the soils tested. Because these experiments were performed with soil slurries rather than soil cores or columns and involved soil types which were quite different from Long Island soils, it was necessary for us to examine strain-dependent adsorption of a variety of field and laboratory strains of enteroviruses using natural cores. At the same time, we wished to examine other factors governing the fate of virus in soil, particularly those that might affect their movement through the unsaturated zone of a recharge basin. Since adsorbed viruses are not irreversibly bound to soil particles, they may be desorbed and move through soil under certain conditions. The conductivity of the percolating solution appears to play an important role in desorption and mobilization of virus. This was first observed by Wellings et al. (1975) following the isolation of enterovirus from groundwater beneath a recharge basin after a period of heavy rainfall. Mobilization of adsorbed viruses by low ionic strength fluids was also reported by Lance et al. (1976) and Gerba and Lance (1978) using 10 x 250 cm packed and conditioned columns. These authors observed the movement of poliovirus type 1 (LSc 2ab) to greater column depths following the application of deionized water which simulated a burst of rainfall. Desorbed viruses were found to resorb at lower levels in the column, with few being detected in the column outflow. In addition, viral desorption and movement was greatly reduced or eliminated by the addition of salts, the immediate application of sewage effluent, or by drying the column for three to five days before application of deionized water. Duboise et al. (1976), studying the removal efficiency of poliovirus type 1 (CHAT) in nonsterile natural forest soils, also observed virus mobilization using low ionic strength fluids. The authors demonstrated that peak viral elution from 19.5 x 5.6 cm soil cores coincided with the minimum column effluent conductivity brought about by distilled water rinses.

Clearly, conductivity is important in viral movement through soils. However, all studies to date have employed distilled water at neutral pH to mimic the effects of rainwater. Rather than repeat this likely source of error, experiments were designed to use an artificial rainwater mix. Results obtained from these experiments would be more consistent with the adsorption and elution of enteroviruses in natural recharge systems. A major portion of these studies has recently been published (Landry et al., 1979).

Effects of Rainwater Rinse on Poliovirus Types 1 and 3--

In the first series of experiments, soil cores were collected as described, and challenged with 200 ml of tertiary sewage effluent containing either polio 1 LSc 2ab or V234-1, a polio type 1 isolated from secondary sewage effluent during a previous study (Vaughn et al., 1978). After filtrates were analyzed for unadsorbed virus, the cores were rinsed with 200 ml volumes of tertiary sewage effluent, or artificial rainwater. The second filtrates were analyzed for total virus released during each rinse. The results are shown in Table 22. Both the reference strain and the field isolate adsorbed well to cores, ranging from 93-96% and 82-89%, respectively. While the adsorption characteristics of both virus types were the same, the elution or

TABLE 22. ADSORPTION - ELUTION PROFILES OF FIELD AND LABORATORY STRAINS OF POLIOVIRUS TYPE 1 IN NATURAL SOIL CORES

Total PFU recovered from:					
Virus type	Input (A)	Unadsorbed (B)	Theoretical adsorbed (A-B)	Sewage filtrate	Rainwater filtrate
LSc 2ab	2.85×10^6	1.73×10^5 (6.07)*	2.68×10^6 (93.93)*	3.68×10^4 (1.37)	-
"	2.85×10^6	1.06×10^5 (3.72)	2.74×10^6 (96.28)	-	7.76×10^4 (2.83)†
V234-1	7.26×10^7	1.28×10^7 (17.63)	5.98×10^7 (82.37)	8.34×10^6 (13.94)	-
"	7.26×10^7	7.64×10^6 (10.51)	6.49×10^7 (89.49)	-	2.15×10^7 (33.13)

*Values in parentheses are percent recoveries based on the input.

†Values in parentheses are percent recoveries based on the theoretical adsorbed virus.

mobilization patterns with sewage effluent or artificial rainwater rinses were quite different. Few LSc viruses were released with either a sewage (1.37% average), or a rainwater rinse (2.83%). Substantially more field isolates were released from cores by rinses with either substance, with an average of 14% being eluted by sewage effluent and 33% eluted by simulated rainwater.

As strains of poliovirus type 1 were seen to exhibit similar adsorption, but differing elution profiles, we investigated the likelihood of similar patterns occurring with field and laboratory strains of polio type 3. The strains chosen for this study were type 3 Leon (ATCC VR-62), and the field strains V153-1 and V201-4, isolated and identified during a previous study (Vaughn et al., 1978). Temperature sensitivity (T-marker tests) performed on these latter two strains revealed a high efficiency of plating (EOP) at an elevated temperature (39.5°C - Table 23). As a result of this finding, these isolates were tentatively classified as nonattenuated (i.e. virulent) strains of polio 3.

TABLE 23. VIRULENCE OF POLIOVIRUS TYPE 3 FIELD ISOLATES

Virus type	PFU recovered at:		EOP*	Log EOP
	39.5C (A)	37C (B)		
LSc 2ab (control)	1.00×10^2	1.30×10^7	7.69×10^{-6}	-6.8859
V153-1	1.20×10^6	7.80×10^6	1.5×10^{-1}	-1.1761
V201-4	3.40×10^5	7.40×10^5	4.5×10^{-1}	-1.6532

*EOP = efficiency of plating (A+B).

As seen in Table 24, adsorption patterns of both reference and field strains were similar to those of poliovirus type 1, with over 97% of the viruses adsorbing to the cores. While sewage effluent rinses did not appreciably mobilize either reference or field strains, the rainwater rinse resulted in significant movement of all types through the core.

As previously illustrated (Table 1), the artificial rainwater used in all experiments possessed an ambient pH of 4.3 and a conductivity of 40 μ MHOS, while the treated sewage effluent rinses had an average pH of 7.47 and conductivity of 606 μ MHOS. Since both pH and conductivity are known to affect the mobilization of virus in soil (Gerba et al., 1975), it was necessary to determine which factor played the major role in the movement of poliovirus. Polio type 3 field strain V153-1 was chosen for these experiments. Matching virus-seeded cores were treated with either the described sewage effluent rinse, a rainwater rinse, or a rainwater rinse adjusted to pH 6.7. pH adjustments resulted in an increase in the conductivity to 300 μ MHOS. Core filtrates were collected and analyzed for total PFU. The results, shown in Table 25, indicated the inability of the sewage rinse to mobilize any

TABLE 24. ADSORPTION - ELUTION PROFILES OF LABORATORY AND
FIELD STRAINS OF POLIOVIRUS TYPE 3 IN NATURAL CORES

Total PFU recovered from:						
Poliovirus strain	Input (A)	Unadsorbed (B)	Theoretical adsorbed (A-B)	Sewage filtrate	Rainwater filtrate	
VR-62	5.85×10^6	9.35×10^4	(1.60)*	5.76×10^6	(98.40)*	1.11×10^4 (0.19)
(Leon)	5.85×10^6	9.50×10^4	(1.62)	5.75×10^6	(98.38)	-
V201-4	7.57×10^6	1.06×10^5	(1.40)	7.46×10^6	(98.60)	7.92×10^5 (1.06)
"	6.60×10^6	7.99×10^4	(1.21)	6.52×10^6	(98.79)	-
V153-1	2.28×10^7	4.35×10^5	(1.90)	2.23×10^7	(98.10)	8.92×10^4 (0.40)
"	2.28×10^7	6.64×10^5	(2.91)	2.21×10^7	(97.09)	-
						1.47×10^7 (66.51)

*Values in parentheses are percent recoveries of the input.

†Values in parentheses are percent recoveries of theoretical adsorbed.

viruses. Rinsing with normal rainwater, however, resulted in the removal of 27% of the adsorbed virus. Elution with higher pH rainwater of intermediate conductivity resulted in mobilization of about 20% of the viruses. These data appeared to indicate that conductivity was a more important factor than pH in the release of soil-adsorbed V153-1.

TABLE 25. ELUTION OF POLIOVIRUS TYPE 3 FIELD ISOLATE
(V153-1) FROM CORES WITH pH 6.7 ARTIFICIAL RAINWATER

Elution media	pH	Conductivity (µmho)	Total PFU recovered as:	
			Adsorbed virus	Eluted virus
Sewage-effluent	7.34	650	1.36×10^6	6.62×10^3 (0.48)*
"	7.23	800	2.14×10^6	1.43×10^4 (0.06)
Rainwater	4.30	50	1.43×10^6	3.93×10^5 (27.48)
"	6.70	300	2.12×10^7	4.17×10^6 (19.67)

*Values in parentheses are percent recoveries of adsorbed viruses.

Rainwater Mobilization of Echovirus Types 1 and 6--

In order to determine whether the varied patterns of adsorption and desorption observed with poliovirus also occurred in other enterovirus types, a series of experiments using reference strain echoviruses was carried out using the described experimental procedure. The results are presented in Table 26. Echovirus type 1 exhibited the greatest soil affinity of all enteroviruses tested with over 99% of the virus adsorbing and no detectable levels being eluted with either a sewage effluent or a rainwater rinse. Echovirus type 6 showed somewhat less soil affinity with 78% of the virus adsorbing. These viruses were mobilized to some extent by both sewage effluent and rainwater rinses.

Rainwater Mobilization with Mutant Strains of Poliovirus Type LSc 2ab--

Since the adsorption and elution pattern of poliovirus LSc 2ab was different from all other enteroviruses tested, it was of interest to determine whether mutant forms of this virus also possessed altered soil affinities. Two independently isolated guanidine-resistant mutants of poliovirus type 1 LSc 2ab were propagated, tested for guanidine resistance, and analyzed for their ability to adsorb and be eluted from cores by rainwater. Table 27 illustrates the effect of guanidine, an inhibitor of viral protein synthesis (Tereshak, 1978) on the replication of various strains of poliovirus LSc 2ab. The efficiency of plating of the sensitive reference strain was extremely

TABLE 26. ADSORPTION AND RAINWATER ELUTION OF ECHOVIRUS TYPES 1 and 6 in SOIL CORES

Total PFU recovered from:					
Echovirus type	Input (A)	Unadsorbed (B)	Theoretical adsorbed (A-B)	Sewage filtrate	Rainwater filtrate
1 (Farouk)	1.53×10^6	0 (0)*	1.53×10^6 (100)*	-	0 (0)†
"	1.53×10^6	1.16×10^3 (0.08)	1.52×10^6 (99.92)	0 (0)	-
6 (D'Amori)	5.31×10^4	8.75×10^3 (16.48)	4.43×10^4 (83.52)	-	6.71×10^3 (15.14)
"	5.31×10^4	1.46×10^4 (27.50)	3.85×10^4 (72.50)	5.25×10^3 (13.63)	-

*Values in parentheses are percent recoveries based on the input.

†Values in parentheses are based on the theoretical adsorbed value.

low, while both resistant strains (T and B) replicated well in the presence of 100 µg/ml of guanidine. The behavior of the guanidine-sensitive and resistant strains differed markedly in soil cores (Table 28). Poliovirus LSc 2ab adsorbed well to soil cores (93%) and was not affected by either a sewage or a rainwater rinse. Although the adsorption and sewage elution patterns of the guanidine-resistant B strain were similar to the sensitive strain, the rainwater rinse resulted in an increased amount of virus in the core filtrate. The guanidine-resistant T strain of LSc not only demonstrated a reduced adsorptive ability to cores, averaging 72%, but showed a significantly greater amount of virus in the sewage filtrate. About 37% of the T strain viruses were eluted from the soil by the rainwater rinse.

TABLE 27. EFFECT OF GUANIDINE ON THE REPLICATION OF SENSITIVE AND RESISTANT STRAINS OF POLIOVIRUS TYPE 1 LSc 2ab

Poliovirus strain	PFU/ml		EOP (A+B)
	No guanidine (A)	Guanidine at 100 g/ml (B)	
LSc 2ab	9.9×10^7	$>1.0 \times 10^2$	$>1.01 \times 10^{-6}$
LSc 2ab g ^r -T	4.3×10^5	4.0×10^5	0.93
LSc 2ab g ^r -B	6.6×10^5	4.20×10^5	0.63

TABLE 28. ADSORPTION - ELUTION PROFILES OF GUANIDINE - SENSITIVE AND RESISTANT STRAINS OF POLIOVIRUS LSc 2ab

Poliovirus strain	Total virus	recovery	as (%)
	Theoretical adsorbed (A)	Sewage filtrate† (B)	Rainwater filtrate (C)
LSc 2ab	91.82	-	0.46
"	95.67	<0.44	-
LSc 2ab g ^r -B	71.33	0.00	38.63
"	96.51	0.87	6.62
LSc 2ab g ^r -T	68.55	4.07	59.73
"	76.50	9.49	15.54

*Calculated by subtracting unadsorbed viruses from the input.

†Based on the theoretical adsorbed.

Overview of Adsorption-Elution Processes--

The results obtained in the above studies, coupled with the recent findings of Gerba and Goyal (1978), indicate that the behavior of enteroviruses in soil is not uniform, and caution should be exercised in extrapolating from data based on a limited number of virus and soil types. The data has shown the adsorption of a number of enteroviruses to a highly permeable, coarse sandy soil, which is not considered to be a good virus-adsorbing soil

(Bitton, 1975). The elution of the viruses by sewage effluent or rainwater rinses appeared to be strain-dependent, indicating a strain or type variation in the overall charge characteristics of each virus. Poliovirus strain LSc failed to be mobilized by either a sewage or rainwater rinse, while other polioviruses were mobilized by either, or both. In addition, two echovirus types demonstrated strain-dependent adsorption-elution behavior. Since adsorption is basically an electrostatic attraction (Bitton, 1975), electro-negative charges in virus and soil particles, influenced by changes in the pH or ionic strength of the percolating fluids, could result in desorption and mobilization of the virus. The results also showed conductivity to be a more important mobilizing factor than pH in this system. Both acid (pH 4.3) and near-neutral (pH 6.7) rainwater rinses of low ionic strength were found to elute viruses effectively. While movement of soil-bound enteroviruses has been demonstrated in these studies, no statement can be made concerning their resorption because of the size constraints of the test cores. At least one virus type, polio type 1 LSc 2ab, has been shown to resorb at greater column depths following mobilization by distilled water rinses (Lance et al., 1976). Later experiments presented in this report will verify the above findings using mutant strains of LSc 2ab. Neither study can comment on the possible resorption of other enterovirus types (coxsackie or echoviruses), which have previously been shown to occur more frequently in glacial aquifers beneath wastewater recharge basins (Vaughn and Landry, 1978; Vaughn et al. 1976). These latter data tend to support the concept of variable resorption ability suggested by the experimental results.

Since researchers are limited to studying avirulent enterovirus in most field and laboratory experiments, we sought to derive a mutant LSc which retained its avirulent nature but had altered capsid properties, resulting in altered adsorption-elution characteristics. Such a virus may more closely simulate other enterovirus-soil interactions. The results presented here appear to verify such an option. Two independently isolated guanidine-resistant mutants were analyzed and found to have adsorption-elution behaviors different from the parent strains. Strain B resembled the parental LSc strain in that it was not significantly mobilized by sewage. However, it demonstrated a significant elution with rainwater. Strain T also differed from the parent strain in demonstrating a significantly different sewage and rainwater elution profile. Some variability in the adsorption and elution pattern within each experimental run was observed and may have been due to a mixed subspecies population within the strain. While the strains were plaque-purified three times, the efficiency of plating in 100 µg/ml guanidine was less than that in the absence of guanidine, possibly indicating the presence of a residual guanidine-sensitive population. Extensive purification may further increase the efficiency of plating. These avirulent mutant strains were used in other laboratory and field studies for predicting the fates of certain enteroviruses.

RECHARGE BASIN SEEDING EXPERIMENTS

Currently practiced sewage treatment methods generally cannot insure the removal of all human viruses from treated wastewater (Buras, 1976; Vaughn et al., 1978; Clarke et al., 1951). The presence of these organisms has been viewed as a potential health hazard for wastewater reuse operations, especially those involving aquifer supplementation by recharge (Berg, 1973; Bernard, 1973; Hori et al., 1970). Recently, a number of field-monitoring studies have demonstrated the presence of naturally-occurring viruses in

wastewater recharge systems. Wellings, et al., (1975) reported the isolation of polio and coxsackie viruses from groundwater beneath a cypress dome receiving secondarily-treated effluent. Schaub and Sorber (1977) observed the sporadic occurrence of enteroviruses in recharged groundwater following rapid infiltration of treated wastewater through silty sand and gravel. Vaughn, et al., (1978) demonstrated the presence of a variety of enteroviruses in groundwater aquifers adjacent to wastewater recharge basins at three separate sites on Long Island.

Although the health significance of the possible contamination of groundwater by these agents cannot be accurately assessed due to the lack of epidemiological evidence, optimizing land treatment systems for removal of enterovirus appears to be a reasonable and prudent goal. To date, no attempts have been made to control virus movement in operating recharge basins. The 12-Pines recharge facility offered a unique opportunity to initiate such a study. We felt that by altering certain operating modes, an effective basin management program could be developed for virus removal.

A number of factors appear to influence the movement of virus through soils during recharge including: the chemical composition of the soil and wastewater; pH; conductivity; and the rate and schedule of wastewater application (Gerba et al., 1975). Of these possibilities, control of the infiltration rate appeared to be the most practical and attainable at the 12-Pines site. The objective of experiments outlined in this section was to determine whether virus migration in soil could be minimized by controlling the application of virus-containing wastewaters. Portions of this work have already been published (Vaughn and Landry, 1978).

Virus Migration During High Rate Recharge (75-100 cm/hr)

The initial experiments were designed to determine the extent of virus migration in soils during the "normal" recharge operation. Since a majority of the recharge basins on Long Island are composed of coarse, sandy soils, the usual infiltration rates experienced during recharge are quite high (50-75 cm/hr), with little or no ponding of wastewater in the basins. Such high infiltration rates do not appear to be necessary for disposal of all the wastewater, since most basins are designed to handle recharge at a much slower rate (5 gallons/day/ft² - 0.8 cm/hr). However, since local recharge was conducted at high rate, this condition was chosen as a starting point for our experiment using the test basin.

Four-thousand-liter volumes of unchlorinated sewage effluent were seeded with poliovirus LSc 2ab, and allowed to infiltrate at a rate of 75 to 100 cm per hour. The final concentration of virus in the seeded effluent was 2.32×10^6 PFU/l. The results of the high rate experiment, shown in Table 29, clearly indicate extremely poor virus retention during this rate of recharge. Large numbers of virus were detected at all sampling levels. The seeded effluent apparently moved through the first level (0.75 m) in a sharply defined band (samples 1 through 3), with decreasing numbers of viruses observed following the passage of the sewage front (samples 4 - 9) through this area. By the time the band had reached level 2 (2.25 m), it had been rendered less compact by diffusion within the soil column. Fewer viruses were isolated at this level, indicating that some virus adsorption had occurred between the first and second level. The high virus titer encountered in the first sample from level 3 (5.34 m) may have resulted from channeling through portions of the basin. This early arrival of virus was unexpected based upon estimates of the travel time of the lead water. The band of water containing

TABLE 29. THE MOVEMENT OF POLIOVIRUS LSc 2ab IN A RECHARGE BASIN
DURING HIGH RATE RECHARGE (75-100 cm/hr)

Depth below surface of basin floor (m)	Sample no.	Time after seeding (hr)	Virus recovered* (PFU/ℓ x 10 ⁴)
0.75 (Level 1)	1	0.60-0.81	78.00
"	2	0.86-0.91	97.50
"	3	1.03-1.06	26.50
"	4	1.20-1.23	1.46
"	5	1.41-1.45	1.94
"	6	1.58-1.61	1.22
"	7	1.75-1.78	0.79
"	8	2.00-2.03	0.90
"	9	2.41-2.45	0.03
2.25 (Level 2)	1	1.20-1.30	2.44
"	2	1.41-1.50	8.58
"	3	1.58-1.63	6.70
"	4	1.75-1.81	5.40
"	5	2.00-2.08	1.81
"	6	2.25-2.31	1.38
"	7	2.50-2.56	0.31
"	8	2.58-3.86	0.05
5.34 (Level 3)	1	1.78-2.15	8.82
"	2	2.16-2.28	0.54
"	3	2.33-2.41	0.27
"	4	2.50-2.55	1.81
"	5	2.81-2.85	10.10
"	6	3.00-3.03	9.80
"	7	3.28-3.31	3.32
"	8	3.50-3.53	1.96
"	9	3.53-4.03	0.12
7.62 (Level 4)	1	2.50-2.66	0.11
"	2	4.66-4.83	0.001

*Virus input = 2.3×10^6 PFU/ℓ

a high concentration of viruses appeared to pass this third sampler between 2.8 - 3.0 hours. Later sampling showed a decline in the number of viruses. However, these samples still contained a substantial virus population. Reduced numbers of virus were also recovered from the aquifer, 7.6 m below the floor of the recharge basin. The reduction was likely the result of the dilution of the virus-containing water following entrance into the groundwater table.

These data clearly indicate that significant numbers of virus can move appreciably vertical distances during high rate recharge. It is important to note that the virus type used in this study, polio LSc 2ab, had previously been shown to adsorb to basin soils more efficiently than other enterovirus types (Landry et al., 1979). This suggests that viruses which adsorb poorly to Long Island soil (e.g. coxsackie B3) may move in greater concentrations and to greater depths than the experimental strain. This selective adsorption might explain the isolation of certain virus types (e.g. echovirus) and not others (e.g. poliovirus) from aquifers beneath a recharge basin noted during a previous study (Vaughn et al., 1978).

Virus Migration During Low Rate Recharge (0.5 - 6 cm/hr)

Prior to determining whether lower infiltration rates would promote virus adsorption during basin recharge, a number of methods for decreasing the infiltration rate had to be analyzed including: compacting the soil; clogging the surface by prolonged recharge, or a burst of high suspended solids material; addition of a gravel layer over the basin floor; and alternate modes of wastewater loading or pulsing. Compacting the sand to increase its bulk density was considered impractical for large scale basin experiments, as was the addition of gravel to the basins. Studies on alterations of loading and drying schedules were postponed to hopefully dovetail with proposed U.S.G.S. studies on low-rate recharge which will investigate chemical and bacterial removal. However, no virus studies have been funded. The only practical method of choice for attaining low infiltration rates appeared to be by clogging the basin surface.

The first low rate experiment was conducted using a surface which had been naturally clogged by prolonged recharge (~6 weeks) of the test basin. During the experiment, an infiltration rate of 6 cm/hr was maintained. Approximately 8500 l of unchlorinated tertiary-treated effluent were seeded with poliovirus LSc 2ab to a final concentration of 7×10^4 PFU/l. The seeded wastewater was allowed to infiltrate the basin surface before normal recharge was resumed. Sampling was conducted as previously indicated.

Data from the experiment, presented in Table 30, indicated that a significant reduction in viral numbers occurred as a result of passage of the wastewater through the recharge basin. The peak virus concentrations in the 0.75 m sampler were noted between 4 and 10 hours after inoculation. These concentrations (16 - 20 PFU/l) were greatly reduced compared to input values (7×10^4 PFU/l). Samples collected the following day yielded even fewer isolates, suggesting that significant adsorption was occurring within the first 0.75 m of soil. Low concentrations of virus were also recovered in most of the samples collected from level 2. A peak in virus concentration was noted at 11 hours in this level. At the 5.3 m sampler, no viruses were recovered during passage of the leading band of water. A spot check of this sampler on the following day indicated that some viruses were still moving through this sampling area at about 24 hr. Extensive sampling of the observation well over a three-day period indicated that few viruses were able to travel through the entire 7.6 m of soil to reach the aquifer. The peak concentration of viruses in this level (0.35 PFU/l) was noted at 24 hr. These results represent a significant improvement in virus removal over that obtained at higher rates.

Although a substantial number of viruses were retained by the soil during recharge at 6 cm per hour, a few viruses were shown to have successfully moved through the entire unsaturated zone. In order to determine whether migration to these depths could be totally eliminated, experiments were conducted at even lower infiltration rates (e.g., 1 cm/hr.). These rates were achieved by clogging the surface of the recharge basin with sewage effluent containing a high concentration of suspended solids. About 4000 l of unchlorinated sewage effluent were seeded with poliovirus LSc 2ab to a final concentration of 7.8×10^5 PFU/l. Sampling was conducted over an 8-day period as previously described.

TABLE 30. THE MOVEMENT OF POLIOVIRUS LSc 2ab IN A RECHARGE BASIN DURING LOW RATE RECHARGE (6 cm/hr)

Depth below surface of basin floor (m)	Sample no.	Time after seeding (hr)	Virus recovered* (PFU/l)
0.75 (Level 1)	1	1.45-2.25	0
"	2	2.25-3.00	0
"	3	3.00-4.63	7.90
"	4	4.63-6.66	16.90
"	5	6.66-8.53	20.00
"	6	8.53-9.25	17.70
"	7	9.25-10.08	25.25
"	8	23.63-24.25	1.90
"	9	28.81-29.33	3.70
2.25 (Level 2)	1	2.26-2.91	5.45
"	2	2.91-3.61	0
"	3	3.61-5.26	3.18
"	4	5.26-7.66	3.60
"	5	7.66-10.08	11.60
"	6	10.08-11.16	2.20
"	7	11.16-11.91	23.30
"	8	23.68-24.33	1.59
"	9	28.78-29.41	19.50
5.34 (Level 3)	1	6.83-9.41	0
"	2	9.41-11.91	0
"	3	23.83-25.08	38.20
"	4	28.75-29.83	0
7.62 (Level 4)	1	0	0
"	2	12	0
"	3	24	0.35
"	4	30	0
"	5	48	0
"	6	54	0.08
"	7	72	0.07

*Virus input = 7.0×10^4 PFU/l

As seen in Table 31, significant virus removal was observed during recharge at 1 cm/hr. At this low rate, viruses were not detected at 0.75 m until 24 to 48 hours after basin inoculation, and then, only at greatly reduced concentrations. Subsequent samples at this level revealed a consistent decrease in virus concentration over time. Results from sampling at levels 2 and 3 similarly revealed low concentrations of viruses. However, it appeared that the initial band of virus-containing water (level 1, 24-48 hr) retained the same concentration of viruses as it moved past level 2 (48-97 hr). Large-volume samples (400-800 l) collected from the observation well (level 4) revealed that few viruses were able to completely penetrate the length of the basin.

Previous data (Tables 22-26) had shown that virus interactions with soil varied with virus type. While poliovirus LSc 2ab was adsorbed extremely well, other virus types were less efficient in their adsorption to soil. Since all large-scale basin-seeding experiments required the use of an

avirulent virus strain (viz., poliovirus LSc 2ab), the possibility existed that data generated from the above experiments were inappropriate for extrapolation to other enterovirus types. Earlier in this report we indicated that guanidine-resistant strains of poliovirus LSc 2ab reacted differently in soil adsorption-elution studies from the parent strain (Table 28). These viruses showed a reduced adsorption capacity, along with a greater likelihood of mobilization by a rainwater rinse. Since these data were more consistent with the adsorption-desorption characteristics of other enteroviruses, this virus was chosen for further basin studies.

TABLE 31. THE MOVEMENT OF POLIOVIRUS LSc 2ab THROUGH A RECHARGE BASIN DURING VERY LOW RATE RECHARGE (1 cm/hr)

Depth below surface of basin floor (m)	Sample no.	Time after seeding (hr)	Virus recovered* (PFU/ℓ x 10 ²)
0.75 (Level 1)	1	8-24	0
"	2	24-48	4.07
"	3	48-72	2.29
"	4	72-98	0.20
"	5	98-118	0.20
2.25 (Level 2)	1	34-48	0
"	2	48-72	4.13
"	3	72-97	5.71
"	4	97-120	0
"	5	120-168	0.24
5.34 (Level 3)	1	74-99	0
"	2	99-122	0
"	3	122-144	0.81
"	4	144-170	1.94
7.62 (Level 4)	1	73	0
"	2	98	0
"	3	121	0
"	4	143	0.04
"	5	170	0.00013
"	6	194	0.00066
"	7	218	0.00124

*Virus input = 7.8×10^5 PFU/ℓ

To investigate whether strain differences would result in a different virus migration pattern during low-rate recharge, poliovirus LSc 2ab g^r-T was seeded into 4000 ℓ of tertiary sewage effluent to a final concentration of 1.92×10^6 PFU/ℓ. The seeded effluent was pumped into a basin previously clogged by suspended solids, and allowed to infiltrate into the soil at 0.5 cm/hr. After complete infiltration, recharge was resumed. All sampling was carried out as previously described. The results, shown on Table 32, show a dramatic decrease in virus migration at all levels. A tremendous reduction in the concentration of viruses was noted at the 0.75 m level, presumably indicating significant adsorption above this level. Few, if any, viruses were recovered from the groundwater observation well, with only two of the nine large volume samples being positive.

The extensive virus adsorption seen in this experiment could have been the result of three factors: 1) the strain difference; 2) the decreased

TABLE 32. THE MOVEMENT OF POLIOVIRUS LSc 2ab g^r (TER) IN A RECHARGE BASIN DURING LOW RATE RECHARGE (0.5 cm/hr)

Depth below surface of basin floor (m)	Sample no.	Time after seeding (hr)	Virus recovered (PFU/l)*
0.75 (Level 1)	1	8-23	0
"	2	23-47	0
"	3	47-71	0
"	4	71-95	14.0
"	5	95-119	15.3
"	6	119-168	0
"	7	168-213	13.6
2.25 (Level 2)	1	9-40	0
"	2	40-64	0
"	3	64-88	14.4
"	4	88-112	0
"	5	112-161	toxic
"	6	161-206	0
5.34 (Level 3)	1	73-143	0
"	2	143-194	0
"	3	194-242	175.2
"	4	242-290	0
"	5	290-338	0
"	6	338-386	159.2
7.62 (Level 4)	1	97	0
"	2	121	0.001
"	3	144	0.032
"	4	170	0
"	5	194	0
"	6	242	0
"	7	290	0
"	8	338	0
"	9	386	0

*Virus input = 1.92×10^6 PFU/l

infiltration rate; or 3) the increased amount of suspended solids on the surface of the basin. It is unlikely that the strain difference contributed to the increased retention. Based on laboratory experiments using small cores, (Table 28) this strain was found to adsorb less efficiently than LSc 2ab. If anything, therefore, it should have migrated to greater soil depths during this experiment. It is not known if the surface mat created by the settling of the added suspended solids significantly contributed to the increased adsorption of viruses. Given the available data, it would be difficult to independently analyze the effect of the mat on adsorption, and compare it directly to likely influence of reduced infiltration rate without further experimentation. While the addition of suspended solids might change the composition of the soil and make it more favorable to virus adsorption at the surface, it would have no affect on adsorption to the underlying soils. Therefore, an analysis of the relative rates of virus removal with depth (at

low infiltration rate) with high and low suspended solid content might provide the answer. Regardless of the exact removal mechanism, the above data indicate that proper basin control can virtually eliminate poliovirus migration to the aquifer during recharge.

Overview of Virus Migration During Recharge

The preceding experiments indicate the importance of basin control during recharge. Recharge can be used for both water disposal (assuming good quality wastewater is being recharged), and for further treatment of lower quality effluents. For sewage treatment plants which generally produce good quality effluent, e.g. 12-Pines (see Table 5), basin control may not be important. However, for plants producing lower quality effluents (e.g. secondary treatment plants), basin control becomes crucial as a method of further treatment. Since several such treatment plants have been identified on Long Island (Vaughn and Landry, 1977), basin control should be an important aspect of treatment plant operation. Generally, the pattern of recharge basin management on Long Island involves rotating the sewage effluent discharge into one of several basins. This basin is then used exclusively until sufficient clogging occurs to cause ponding. Effluent discharge is then shifted to an alternate basin and the ponded basin allowed to dry, whereupon it is scarified by tractor or hand-raking in order to break up the surface mat. This "renovated" basin is then considered ready to receive further discharge. The present study indicates that high infiltration rates which occur during the initial phases of sewage recharge into these renovated basins may facilitate the movement of viruses into the groundwater aquifer. The stage when virus removal could be optimized (e.g. by low infiltration rate) appears to be when ponding begins and recharge is shifted to an alternate basin. By allowing recharge to continue at slower rates through the partially clogged basin, the potential of viral contamination of groundwater would be minimized.

At this time, we can make no comments on the extent of virus migration through the aquifer. Experiments had originally been planned to trace the horizontal movement of aquifer-entrained virus. New wells were to be drilled along the plume-line of recharged groundwater flow. The depth to groundwater in the down-gradient area (>9.1m) necessitated the use of a large submersible pump (Teel) which required a well diameter of 10 cm. Since wells of this size can only be driven by a large commercial drilling rig, the Suffolk Dept. of Environmental Control, which owns such a rig, was engaged to perform the task. The DEC drilling crew was unable (in two attempts) to locate the rig in the desired area (within a grid to the southeast of test basin) because of the inability of the sandy soil to support the weight of the rig and its tow vehicle. On one occasion the rig had sunk so low in the sand that a bulldozer was required for its extraction. With no other options for locating the observation well, this phase of the study was abandoned.

VIRUS MIGRATION IN LARGE CORES (75 cm)

Previous data have indicated that substantial viral adsorption occurs within the top 75 cm of soil during low-rate basin recharge (Tables 30-32). Because sampling could only be performed at the 75 cm level, it was not possible to ascertain the exact pattern(s) of virus distribution within this section. Adsorption may either have been confined to a defined soil depth,

or have taken place over a greater distance. Previous evidence (Tables 22-28) suggested that most viral adsorption occurred in a narrow zone near the surface of the soil. However, these cores were not of sufficient length to observe adsorption below 12.5 cm. Lance et al., (1976) using packed 10 x 250 cm soil columns observed that 90% of seeded poliovirus LSc adsorbed to the top 2 cm of Flushing Meadow soils. Percolation through an additional 38 cm of soil was necessary for a further 90% reduction. In order to compare laboratory adsorption data to adsorption occurring during recharge in the field, several experiments were conducted. Since it was impractical to conduct large-scale basin experiments, large field cores (10.1 x 100 cm) were employed.

In addition to analyzing the adsorption profile of poliovirus, the large cores facilitated the investigation of what changes might occur in the pattern of virus distribution following the application of rainwater or sewage rinses. As shown previously with small cores, some viruses were mobilized by such rinses. Unfortunately, these cores were too small to provide information on virus resorption. It was felt that 10.1 x 100 cm field cores would be of sufficient size to allow resorption to occur, and would thus help delineate the role of rainwater in overall virus migration.

Experimentally, a series (4-5) of large field cores was driven 100 cm into a recharge basin which had been clogged on the surface by a mat of settled material from high-turbidity sewage effluent. Following a two to four day period of normal basin recharge to stabilize and slow the infiltration rates of the cores, each core was inoculated with 2 l of poliovirus-seeded (LSc 2ab g^r-T) tertiary-treated sewage. After the seeded effluent had completely infiltrated the basin surface (~24 h), one core was removed and returned to the laboratory where it was sectioned into 5 cm fractions. The remaining cores received a 2 l rinse (~21 cm) of either tertiary-treated effluent, or artificial rainwater. Following complete infiltration of the appropriate rinse (~24 hr), these cores were also removed and returned to the laboratory for sectioning and analysis.

Virus Distribution in Control Cores

Cores receiving the initial 2 l of seeded effluent (~10³ PFU/l) were designated as controls, designed to determine the initial virus distribution profile. The histogram presented in Figure 4 represents the mean values of duplicate core experiments. Recoveries in 5 cm sections are expressed as a percentage of the total PFU recovered per core. The results show that the majority (77%) of the viruses were removed within the first 5 cm of soil during the primary adsorption stage, with an additional 11% removed over the next 5 cm of soil. By 25 cm, over 96% of the total virus had been adsorbed. The remaining 4% was uniformly distributed over the last 50 cm of soil. All soil sections were found to contain a minimum 0.23% of the total viruses recovered.

Virus Distribution Following a Sewage Rinse

Figure 5 presents the mean distribution of viruses in three cores following a rinse with 2 l of tertiary-treated sewage effluent. As previously seen in the control profile, the maximum virus population was located in the top 5 cm of soil (82%), with another 7% present in the 5-10 cm zone. Ninety-four percent of the total viruses were found in the top 25 cm of soil. Below this depth, the mean virus population per core section never fell below 0.2%, but was never higher than 1% of the total virus population.

Figure 4. Distribution Profile of Poliovirus LSc 2ab g^T-T in 10.1 x 75 cm Control Cores. Values represent the means of duplicate experiments.

Figure 5. Distribution Profile of Poliovirus LSc 2ab g^T-T in 10.1 x 75 cm Cores Rinsed once with Sewage Effluent. Values represent the Means of Triplicate Experiments.

Virus Distribution Following Rainwater Rinse

The mean distribution profile of poliovirus LSc g^TT following a 2 l rinse with artificial rainwater is presented in Figure 6. As in the preceding experiments, the top 5 cm of soil retained the highest percentage of virus (68%). Appreciable numbers of viruses (16%) were present in the 5-10 cm core section. This latter value is somewhat higher than those resulting from the control core (11%) and the sewage-rinse (7%) data. A total of 93% of the viruses were found in the top 25 cm of rainwater-rinsed soil. Below this level, the mean virus concentration ranged from 0.4 to 1%.

From these data we can conclude that the first 5 cm of soil are extremely important to virus removal during recharge. This data agrees well with previous observations made during this study (Landry et al., 1979), and by others (Lance et al., 1976; Gerba and Lance, 1978). It is interesting to note the extent to which viruses migrated in all cores. Although the percentage of virus detected below the 25 cm depth was small, some viruses were able to penetrate the entire length of the core. A similar distribution pattern was recently reported by Lance and Gerba (1979) using 10 x 250 cm packed laboratory columns. The authors reported that the majority of seeded polio LSc viruses were adsorbed in the top 5 cm of soil, while others were found to move to greater depths. They also noted that the distribution of LSc in soil columns was essentially unchanged over a wide concentration of applied viruses, and that as the concentration was increased, the number of viruses at each soil depth was found to increase proportionally. The authors suggested that slight variations of charge strength within the virus population was responsible for the distribution pattern. They proposed that viruses having stronger negative charges adsorbed immediately, while a subpopulation, with a somewhat reduced negative charge, moved further down the column. It was not known, however, if the subpopulation of lower-charged viruses was affected by mobilizing rinses (e.g. rainwater or sewage effluent) to the same extent as more strongly-charged viruses. Duboise, et al., (1976) maintained that viruses adsorbed further from the surface of a soil column would be more easily mobilized as a function of a decreasing ionic gradient within the column. Ions from percolating fluids would be adsorbed at the surface until reaching a point of saturation. Fewer ions would then be available for adsorption at greater depths. Thus, viruses initially adsorbed at greater depths may have had a weaker charge, as well as a less optimal adsorbing environment. Such viruses might easily be influenced by drastic changes in pH and ionic strength. It appears that this question could be resolved in further experimentation by subjecting the lower-adsorbing virus population to extensive rainwater or sewage rinses, and determining the change in the adsorption profile with depth.

Statistical Comparison of Virus Migration in Cores

The histograms presented in the previous section (Figures 4-6) indicate that all three cores were effective in removing viruses, even following sewage or rainwater rinse. In order to determine whether significant differences occurred in the overall effectiveness of virus removal following each treatment, data sets for each core were individually subjected to regression-ANOVA analysis, and compared via the Simultaneous Test Procedure for determining differences among sets of regression coefficients (Sokal and Rohlf, 1969). Because the untransformed data approximated a power curve fit ($y =$

Figure 6. Distribution Profile of Poliovirus LSc 2ab g^r-T in 10.1 x 75 cm Cores Following a Rainwater Rinse. Values represent the Means of Triplicate Experiments.

ax^b, see Figures 4-6) it was necessary to perform a log-transformation of both the x and y coordinates in order to linearize the data sets for regression analysis.

Best fit regression lines for control (Figure 7), sewage (Figure 8) and rainwater rinsed cores (Figure 9) were significant ($p < 0.001$) for each data set. Coefficients of determination (r^2), representing the goodness of fit for these regression lines, ranged from 0.74 to 0.96 for the 8 cores analyzed. These values indicate that from 74 to 96% of the variation from the mean is explained by linear regression.

A comparison of slopes of these regression lines was made to determine whether differences in the overall virus distribution existed after various types of core treatment (i.e. no treatment; sewage rinse; rainwater rinse). The comparison of the slopes indicated that there were significant differences ($p < 0.01$) among the 8 cores (Table 33 -- Regression ANOVA for all regression lines are listed in Tables A-1 to A-3). However, this analysis was not sufficient to separate the slopes into homogenous subgroups. Further examination of the data was required to determine which cores were significantly different from each other in their overall virus distribution profiles. This analysis identified two homogenous subsets (Table 34). Bracketed groups represent homogenous subsets of cores whose regression slopes were not significantly different ($p > 0.05$), as determined by the Simultaneous Test Procedure. The largest of the subsets included seven of the eight cores tested. The only core not included in this subset was the initial rainwater-rinsed core run on 7/9/79. The slope of this rainwater-rinsed core was significantly different ($p < 0.05$) from the two rainwater-rinsed cores run on different days. On the other hand, the slope of the 7/9 rainwater-rinsed core was not significantly different ($p > 0.05$) from the control and sewage-rinsed cores carried out on the same day. It was also not significantly different from the two other sewage-rinsed cores from experiments carried out in subsequent experiments (9/12; 10/13).

TABLE 33. REGRESSION ANOVA TABLE TEST FOR THE EQUALITY OF SLOPES IN CONTROL SEWAGE-RINSED AND RAINWATER-RINSED CORES

Description	DF*	SS†	MS††	F _s #
Variation among regression slopes	7	8.5060	1.2151	3.7643**
Average variations within regressions	104	33.5712	0.3228	
Total variations	111	42.0772		

*DF, degrees of freedom

†SS, sum of squares

††MS, mean squares

#F_s, critical F value

**p < 0.01

Figure 7. Best fit regression lines for virus distribution in control cores. Data points represent means of at least three replicates.

*, trial 1 - 5/11/79; $Y = 6.819 - X \cdot 2.198$; $r^2 = 0.815$; $N = 86$

+, trial 2 - 7/11/79; $Y = 6.953 - X \cdot 1.791$; $r^2 = 0.752$; $N = 58$

Figure 8. Best fit regression lines for virus distribution in sewage-rinsed cores. Data points represent means of at least three replicates.

+, trial 1 - 7/9/79; $Y = 6.236 - X \cdot 1.93$; $r^2 = 0.767$; $N = 45$
 x, trial 2 - 9/12/79; $Y = 8.014 - X \cdot 2.039$; $r^2 = 0.891$; $N = 54$
 square, trial 3 - 10/13/79; $Y = 6.242 - X \cdot 1.695$; $r^2 = 0.754$; $N = 60$

Figure 9. Best fit regression lines for virus distribution in rainwater-rinsed cores. Data points represent means of at least three replicates.

+, trial 1 - 7/9/79; $Y = 5.891 - X.1.223$; $r^2 = 0.741$; $N = 59$

x, trial 2 - 9/12/79; $Y = 7.873 - X.2.565$; $r^2 = 0.849$; $N = 54$

$\square$, trial 3 - 10/13/79; $Y = 7.665 - X.2.492$; $r^2 = 0.959$; $N = 58$

TABLE 34. GROUPING OF HOMOGENOUS CORES BASED ON A STATISTICAL COMPARISON OF THEIR SLOPES

Date	Core type*	Slope	Homogenous subgroups
7/9	R	-1.224	
10/13	S	-1.695	
7/9	C	-1.791	
7/9	S	-1.933	
9/12	S	-2.040	
5/11	C	-2.198	
10/13	R	-2.493	
9/12	R	-2.566	

*R = rainwater-rinsed cores; S = sewage-rinsed cores; C = control cores

Taken together, these data indicate that certain experimental differences, present in at least the initial trial, may have influenced the overall distribution of viruses in cores. A careful analysis of each replicate experiment indicated that all experimental designs were essentially identical. One possible source of variability could have been in the amount of suspended solids (e.g. organic material) used to clog the basin or core surface. Although each experiment followed the same general clogging procedure, no absolute analysis of the amount of material used to clog the basins or cores was made. It is plausible that one core (e.g. 7/9 rainwater core) received a smaller dose of suspended solids material on a day when the suspended solids load was low. This would have resulted in a measurable decrease in the overall adsorption efficiency of this core, as seems to be indicated in Figure 9.

In spite of the differences, these data indicated that there were no significant differences in virus distribution profiles in control, sewage-rinsed and rainwater-rinsed cores run on the same day. We can conclude from these analyses that, over the entire length of a 75 cm core, the capacity for virus removal is independent of the type rinse used. Both sewage and rainwater rinses failed to alter the overall virus distribution profile.

In order to confirm this last conclusion, an experiment was conducted to compare the total amount of virus eluted from a 75 cm core following a sewage or rainwater rinse. Two 75 cm cores were set in the clogged test basin as previously described, removed immediately and returned to the laboratory.

TABLE 35. ELUTION OF POLIOVIRUS LSc g^{-1} -T FROM 75 cm CORES FOLLOWING A RAINWATER OR SEWAGE RINSE

Sample type	Total PFU recovered	Percent of input PFU
Sewage Core		
Input	2.11×10^6	100
Filtrate	0	0
Eluate	3.39×10^3	0.16
Rainwater Core		
Input	3.20×10^6	100
Filtrate	7.05×10^3	0.22
Eluate	5.11×10^3	0.16

The infiltration rate of each was slowed to 1 to 2 cm/hr by continuous application of tertiary-treated sewage containing suspended solids. Clogged cores were then inoculated with 2 l poliovirus (LSc 2ab g^r-T) seeded sewage effluent, and the filtrates collected for virus analysis. Thereafter, each core received 2 l of either a sewage, or an artificial rainwater rinse. Column effluents were collected and analyzed for total PFU eluted by each treatment. The results are shown in Table 35. In each case only 0.16% of total input viruses was eluted by either type rinse. This clearly confirmed the equal effectiveness of both cores for the removal of viruses following sewage or rainwater treatment.

Despite the high significance of linear regression in these data sets, significant deviations ($p < .001$) from regression were also present for at least some levels within the cores (see Appendix). This indicated that although the regression described the overall distribution of viruses over the entire length of the core to a statistically significant level, it may not have adequately described variations which occurred at increments within the cores. To more closely examine the possible variations occurring within internal structures during the three core treatment, a two-way ANOVA on data collected from the 7/9/79 experimental run was conducted. This two-way ANOVA analysis was followed by a comparison of means using the Least Significant Difference (LSD) technique (Sokal and Rohlf, 1969). The use of cores that were only run on the same day precluded any possible variation between replicate experiments not run on the same day (see Table 34). As seen in Table 36, significant differences ($p < 0.001$) were found to exist between core depths and the various core treatments. Further, the interaction term was also found to be significant ($p < 0.001$), indicating that although differences were present between cores at some levels, they may not have been present at all levels or for all cores. The use of the Least Significant Difference technique permitted the grouping of related sections in all cores. In Table 37, the core sections are arranged in decreasing orders of magnitude. From this table, it is clear that the top 5 cm of soil in each experimental core (i.e. control, rainwater-rinsed or sewage-rinsed) did not differ significantly ($p > 0.05$) from each other. A greater amount of virus was found in the rainwater-rinsed cores (18%) than in the sewage-rinsed (4%), or control cores (9%). It is of interest to note that in the 10-15 cm section, data from all three treatment modes were not significantly different ($p > 0.05$) from each other. While the subgroupings became more complicated at greater core depths, a few general trends could be identified. First, as mentioned previously, the top 5 cm of each core type (i.e. treatment mode) was not significantly different

TABLE 36. TWO-WAY ANOVA ANALYSIS OF THE EFFECT OF CORE TREATMENT AND SAMPLE DEPTH ON VIRUS DISTRIBUTION

Source of variation	DF	SS	MS	F _s
Among core types	14	43.3044	3.0931	109.30***
Among core depths	2	5.2207	2.6103	92.24***
Interaction of core types and depth	28	5.8910	0.2103	7.43***
Within Subgroups	90	2.5473	0.0283	
Total	134	56.9635		

*** $p < 0.001$

TABLE 37. GROUPS OF STATISTICALLY SIMILAR CORE SECTIONS FOR CONTROL,
RAINWATER AND SEWAGE CORES (7/9/79)

Sample type -- section no.*	Groupings [†]	Mean%	Mean log%
S-1		81.02	-0.0914
C-1	I	63.08	-0.2001
R-1		51.11	-0.2915
R-2		18.25	-0.7387
C-2		9.29	-1.0319
C-3		4.86	-1.3132
S-2		4.36	-1.3610
R-3		4.34	-1.3622
R-4		3.10	-1.5088
S-3		2.99	-1.5244
R-8		2.78	-1.5559
R-14		2.49	-1.6033
R-5		2.20	-1.6570
R-10		1.78	-1.7486
S-7		1.69	-1.7781
S-5		1.66	-1.7810
R-9		1.61	-1.7942
R-15		1.53	-1.8139
R-6		1.46	-1.8356
S-9		1.45	-1.8374
R-12		1.35	-1.8682
S-10		1.34	-1.8732
S-4		1.26	-1.8996
C-4		1.25	-1.9018
R-13		1.19	-1.9258
S-8		1.04	-1.9816
R-7		1.02	-1.9901
R-11		0.98	-2.0088
S-6		0.88	-2.0556
C-8		0.66	-2.1800
C-7		0.57	-2.1944
C-14		0.51	-2.2940
C-13		0.51	-2.2940
C-9		0.50	-2.3052
C-15		0.46	-2.3390
C-11		0.46	-2.3390
C-10		0.43	-2.3702
C-5		0.41	-2.3878
C-12		0.38	-2.4208
C-6		0.34	-2.4743
S-15		0.28	-2.5494
S-12		0.25	-2.5951
S-11		0.19	-2.7196
S-14		0.17	-2.7693
S-13		0.09	-3.0555

*S,C, and R refers to sewage, control and rainwater cores. The number following the dash is the section number. The section number times 5 equals the maximum section depth per core.

[†]Designated section groups are not significantly different based upon the Least Significant Difference technique. LSD .05 = 0.2757.

from the others in terms of virus content. This indicated that viruses adsorbed in this zone were not mobilized by either a sewage or a rainwater rinse. Secondly, the lower third of Table 37, includes only the lower core sections in sewage-rinsed (S11-S15) and control cores (C9-C15), while the intermediate portion of the table is dominated by the lower sections of the rainwater-rinsed cores (R11-R15). This indicated that the lower sections of the rainwater-treated cores had significantly more viruses than comparable sections in the sewage-rinsed or control cores.

Table 38 presents a less rigorous treatment of data. Subgroups that do not violate the continuity of the cores are defined by bars, and the mean percent virus for each group is given. The data indicates a redistribution of the virus population to greater depths as affected by rinses. A larger percent of virus was found in rainwater cores between 25 and 75 cm depths than in either the sewage-rinsed or control core. A higher percentage of virus was found at greater depths in sewage-rinsed cores than in the untreated control.

Overview of Field Core Experiments

An analysis of large core data leads to the conclusion that passage of seeded-effluent through 75 cm cores results in significant removal of virus. A majority of the viruses were removed by adsorption in the top 5 cm of soil, with substantially few viruses being found below depths of 25 cm. However, viruses were found in all soil sections analyzed. Less than 0.3% of the total virus recovered were found to migrate through the entire 75 cm core. These data agree with the results of large-scale basin-seeding experiments

TABLE 38. GROUPINGS* OF STATISTICALLY SIMILAR CORE SECTIONS BY PERCENT FOR CONTROL, SEWAGE- AND RAINWATER-RINSED CORES

Section no.	Maximum Core depth (cm)	Core type†		
		R	S	C
1	5	51.11	81.02	63.08
2	10	18.25	3.68	7.08
3	15	3.17		
4	20			1.25
5	25			
6	30		1.32	
7	35			
8	40			
9	45	1.67		
10	50			0.59
11	55			
12	60			
13	65		0.20	
14	70			
15	75			

*Designated section groupings are not significantly different based upon the Least Significant Difference technique. Note that other subgroups are possible, but only those that do not violate the continuity of the cores are presented.

†R,S,C refer to rainwater-, sewage-rinsed or control cores, respectively. Values represent the mean percent values over the distances defined by the groups or lines.

(Tables 30-32), where a majority of viruses were found to adsorb within the first 75 cm of soil during low rate recharge. However, as seen above, a small fraction of the virus population was able to penetrate to lower depths, leading to an occasional isolation in the groundwater aquifer.

Treatment of field cores with either a sewage or a rainwater rinse did not have a significant effect on the overall ability of the core to remove viruses. The distribution profile of the viruses was not significantly altered by rinses, and fewer than 0.2% of the total virus were eluted from the cores. A careful analysis of the internal structure of the core indicated that there were localized areas of virus migration and resorption resulting from treatment of cores with sewage or rainwater. There was more movement following rainwater rinses than with additional sewage applications. The migration of desorbed viruses was not extensive, as few viruses could be detected in core effluents.

The above findings clearly complement and extend the previous data (Tables 22-28) which indicated the occurrence of strain-dependent mobilization of viruses by rainwater. While previous data demonstrated mobilization and elution of certain virus types from 12.5 cm cores, it is apparent that these viruses would resorb to soil at lower depths. Similar results have been reported for laboratory columns (Gerba and Lance, 1978; Funderberg et al., 1979; Lance et al., 1976).

Still in question is the fate of viruses adsorbed at greater soil depths. While these viruses are hypothesized to have a lesser soil affinity than surface adsorbed viruses, (and thus be more influenced by pH and ionic changes) it is unlikely that rainwater would play a major role in any mobilization. Apparently, a burst of rainwater will not move through the soil of an operating recharge basin as an intact band of water, but rather will mix with the resident water. This mixing would likely dilute the unique virus-mobilizing characteristics of the rainwater. However, it is not known if viruses residing deep in soil profiles would be continually mobilized and resorbed several times during continual sewage recharge, and eventually migrate to the aquifer. This problem needs to be investigated further.

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APPENDIX A

TABLE A-1. Regression Anova for the Distribution of Viruses
in 75 cm Control Cores*

Trial no.	Date	Source of variation	DF	SS	MS	F _s
1	5/11/79	among groups	14	45.9384	3.2813	126.3159***
		regression	1	38.9508	38.9508	72.4652***
		deviation	13	6.9876	0.5375	20.6917***
		within groups	71	1.8443	0.0260	
		total	85	47.7827		
2	7/9/79	among groups	14	25.8718	1.8480	66.7552***
		regression	1	20.3597	20.3597	48.0168***
		deviations	13	5.5121	0.4240	15.3166***
		within groups	43	1.1904	0.0277	
		total	57	27.0622		

* The slopes and Y intercepts for the regression lines are: trial 1, -2.1983 and 6.8193; trial 2, -1.7912 and 6.9531.

*** p < 0.001

APPENDIX B

TABLE A-2. Regression Anova for the Distribution of Viruses
in 75 cm Sewage-Treated Cores*

Trial no.	Date	Source of variation	DF	SS	MS	F _s
1	7/9/79	among groups	14	21.9236	1.5659	27.8402***
		regression	1	18.1150	18.1150	61.8324***
		deviation	13	3.8086	0.2929	5.2084***
		within groups	30	1.6874	0.0562	
		total	44	23.6110		
2	9/12/79	among groups	14	24.5156	1.7511	237.1108***
		regression	1	22.1042	22.1042	119.1660***
		deviations	13	2.4114	0.1855	25.1165***
		within groups	39	0.2880	0.0074	
		total	53	24.8036		
3	10/13/79	among groups	14	23.3212	1.6658	57.5033***
		regression	1	18.5691	18.5690	50.7984***
		deviations	13	4.7512	0.3655	12.6186***
		within groups	45	1.3036	0.2897	
		total	59	24.6248		

* The slopes and Y intercepts for the regression lines are: trial 1, -1.9338 and 6.2360; trial 2, -2.0399 and 8.0143; trial 3, -1.6956 and 6.2424.

*** p < 0.001

APPENDIX C

TABLE A-3. Regression Anova for the Distribution of Viruses
in 75 cm Rainwater-Treated Cores*

Trial no.	Date	Source of variation	DF	SS	MS	F _s
1	7/9/79	among groups	14	12.3401	0.8814	59.7017***
		regression	1	9.6357	9.6357	46.3191***
		deviation	13	2.7043	0.2080	14.0903***
		within groups	44	0.6496	0.0147	
		total	58	12.9896		
2	9/12/79	among groups	14	45.3648	3.2403	192.1422***
		regression	1	39.1072	39.1072	81.2449***
		deviations	13	6.2575	0.4813	28.5425***
		within groups	39	0.6577	0.1686	
		total	53	46.0224		
3	10/13/79	among groups	14	39.6519	2.8322	248.9836***
		regression	1	38.5183	38.5183	441.7566***
		deviations	13	1.1335	0.0871	7.6635***
		within groups	43	0.4892	0.0113	
		total	57	40.1410		

* The slopes and Y intercepts for the regression lines are: trial 1, -1.2235 and 5.8917; trial 2, -2.5658 and 7.8731; and trial 3, -2.4925 and 7.6653

*** p < 0.001