THE SURVIVAL OF ENTERIC VIRUSES IN SEPTIC TANKS AND SEPTIC TANK DRAIN FIELDS

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ABSTRACT

The persistence of enteric viruses in a functioning septic tank system was followed using poliovirus type 1 as a model. Poliovirus was incubated in dialysis membrane chambers suspended both in the chambers of a septic tank and in groundwater observation wells strategically placed in the adjacent drainfield. Poliovirus was also incubated, *in situ*, in the sandy loam drainfield soils to monitor virus movement and inactivation. Attempts were made to isolate indigenous enteric viruses from the septic tank and drainfield groundwater.

Indigenous enteric viruses were regularly recovered from the septic tank. Seeded poliovirus type 1 survived in the septic tank environment long enough to enter the drainfield. Viruses introduced into the drainfield remained infective for extended periods and moved with groundwater flows. Poliovirus incubated in shallow groundwater was rapidly inactivated, but contact with soil seemed to have a protective effect. A consistent disagreement between the results from laboratory experiments and field studies was noted.

The septic tank system used lay in the flood plain of the Rio Grande, within the Elephant Butte Irrigation District. Movement of indigenous enteric viruses through drainfield soils and into shallow groundwater during the initiation of irrigation-season river flows was reported.

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INTRODUCTION

An estimated 50,000,000 Americans depend on individual onsite wastewater treatment systems for disposal of human and household wastes (43,45,47), particularly on the septic tank with leach or drain field (48). A septic tank is an underground vault which collects wastewater and disposes of it through subsurface soil percolation and evapotranspiration while retaining settleable solids. These solids are reduced in volume through anaerobic decomposition and are manually removed from the vault at 3-5 year intervals (31). During the years of rapid suburban development following World War II, many tract homes were constructed with septic tank disposal systems. Although suburban homes have been increasingly "hooked-up" to expanding municipal sewage collection and treatment systems, the septic tank still serves 66 percent of the rural year-round housing units (50), highway rest areas, recreation facilities, vacation homes, and 10 percent of urban households in the U.S. (50).

Much concern and study has been directed toward the survival of enteric virus during municipal sewage treatment. This emphasis is understandable since more than 100 different enteric viruses are excreted in human feces (4,10), and these viruses can cause serious disease. Enteric viruses of the picornavirus group, include poliovirus, the causative agent in paralytic poliomyelitis; echovirus, responsible for meningitis and respiratory disease as well as diarrhea; and the coxsackieviruses, capable of causing herpangina, myocarditis, pleurodynia, meningitis, respiratory disease and diarrhea (18).

Virus concentrations of 463,500 infectious particles per liter have been isolated from raw sewage entering municipal sewage facilities (18). It has been shown that one viral plague-forming unit is capable of producing a human infection (20). Therefore, the presence of small numbers of viruses in a water supply poses a potential health hazard. Enteric viruses survive standard secondary sewage treatment processes and chlorination in sufficient numbers to be isolated regularly from municipal sewage effluents and sludges (10,23,30,36).

The actual public health hazard posed by enteric viruses in wastewater effluent and sewage sludge has not been determined. This is due in part to the inapparent nature of most viral infections (4,10) and the difficulties encountered in isolating small numbers of viruses from natural water supplies. A person may contract a viral infection by drinking contaminated water, and the virus can initiate an active respiratory or gastrointestinal infection without any overt symptoms of disease. This carrier can subsequently transmit the virus by coughing, sneezing, or poor personal hygiene to a large number of people, a few of whom may develop acute symptoms of disease (19). As population pressures and pollution-abatement legislation encourage increased recycling of water and the use of sewage sludges in agriculture, improving techniques of enteric virus detection, concentration, and enumeration will be used to quantify the possible health hazard posed by enteric virus in municipal sewage (5).

Less attention has been given to the potential pollution problems of septic tanks than to municipal sewage treatment facilities.

There are several legal and economic reasons for the lack of research in this area. Municipal sewage systems are large-volume point sources of environmental pollution which must meet increasingly rigorous federal standards and are monitored at the state level under provisions of Public Law 92-500. An individual septic tank is a point source of pollution, but for practical reasons, the tanks in a particular area represent a non-point source as defined by Section 208 of Public Law 92-500. In many states the regulation of septic tank construction and monitoring of performance is handled at the county level (3,29,32). Municipal sewage treatment systems are designed for observation and sampling while septic tanks often lie under carefully manicured lawns and are difficult to sample without costly disturbance of the landscape. The efficiency of municipal systems is a matter of civic responsibility because malfunctions can lead to civil suits and fines. Researchers are encouraged to identify and solve problems encountered in the operation of these systems and may even be employed by the municipalities. Since septic tanks are usually on private property, problems in operation are solely the concern of the owner; consequently little in the way of research has been directed towards improvement in the efficiency of individual, on-site septic tank systems. Homeowners, in fact, may discourage sampling of operating systems. They complain that such meddling will lead to increasing regulation and cost.

This study was undertaken to answer some of the basic questions concerning the ability of enteric viruses to survive septic tank

wastewater treatment. The current literature on septic tank performance is primarily concerned with efficiency, using chemical or bacterial parameters (7,28,42,43,44,48). Most data available on virus survival in comparable systems are from studies in anaerobic municipal treatment systems and these data may not be applicable to anaerobic septic tank systems. Further, the movement of virus in sewage effluent through soil has been studied in the deep, especially prepared percolation beds of municipal overland disposal sites (37) or in packed columns under laboratory conditions (25). Septic tank drain fields are often poorly designed and constructed, shallow with respect to bedrock and groundwater levels and subject to constant natural and human manipulation.

The following questions are addressed by this study:

1) Can viable enteric viruses be isolated from properly functioning septic tanks?

2) Will poliovirus type 1, used as a model for enteroviruses, remain viable in contact with septic tank liquor long enough to pass through the tank and into the drain field?

3) Once introduced into the drain field, will polioviruses adhere to or be rapidly inactivated by soil or will they remain viable and mobile?

4) If polioviruses move through soil and enter the groundwater, how long will they survive in contact with groundwater?

Enteroviruses were isolated in a recent study from septic tanks which had been opened for sludge removal (55). A sludge-filled tank may not be representative of properly functioning tanks because

viruses are associated with solid wastes (49) and are probably held in the accumulating sludge and may become concentrated in full tanks. Clogged tanks have a shorter retention time than those with more fluid capacity and there are changes in system chemistry as the ratio of fresh fluid to compacting sludge decreases. Only repeated sampling of a properly functioning system serving an average household could determine if viruses were routinely present in the tank fluid.

Septic tanks serving single-family homes have an average retention time of 24 hours (31). Viruses associated with solid wastes should settle to the bottom of the tank during this time as do an estimated 50 percent of the viruses entering a municipal treatment system with a 2-hour primary sedimentation period (16). While a large number of viruses may be immobilized in sludge and present a hazard only when the tank is cleaned, viruses shed by an individual with diarrhea are likely to be free in the fluids being passed. Several infectious hepatitis outbreaks have been traced to septic tank contamination of groundwater used for domestic supply (46,40). The virus responsible for infectious hepatitis, now believed to be a picornavirus (38,39), is excreted in the urine as well as the feces of infected individuals (54). Liquids from food preparation and the laundry enter septic tanks and can carry viruses into the system. Enteric viruses have been shown to persist on the surface of and within vegetables irrigated with virus-contaminated wastewater (41). These viruses could be transferred to the septic system when vegetables were washed. Any viruses that are not solids-associated

and can survive the septic tank environment for 24 hours will enter the drain field.

Pathogenic bacteria not killed by septic tank conditions are filtered from septic tank effluents as the fluids percolate through the soil (44). Immobilized bacteria gradually die off over a period of months (18,44). In a new adsorption bed, bacteria can move rapidly and for relatively long distances. Escherichia coli has been reported to move as much as 46 meters vertically and 70 meters horizontally in a newly constructed field (44). As soil pore spaces become clogged with organic solids over a period of use, the movement of bacteria is restricted (11). Movement of bacteria in soil is a function of soil porosity and the rate of groundwater flow (44). In dry soils there is less movement than in moist soils.

Studies of municipal sewage disposal by land spreading, involving a relatively small number of soil types, have shown that viruses are removed from percolating wastewater by adsorption rather than filtration (9,11). This process has not been adequately described. Monitoring at the Flushing Meadows Wastewater Renovation Project near Phoenix, Arizona, has not detected enteric viruses in receiving groundwater (20,21) but monitoring at a site near Fort Devens, Massachusetts, has detected enteroviruses sporadically in groundwater 183 meters horizontally from the wastewater application area (21). Viruses are amphoterically charged colloidal particles which are negatively charged at most soil pH values (11). Viruses adsorb to clays, glass, oxides of iron, silica and aluminum (11). This adsorption can be prevented or reversed by proteins such as egg

albumin and fetal-calf serum (11) suggesting a protein-soil interaction. In general, the virus adsorption capacity of a soil increases with increasing clay content, cation exchange capacity, and surface area and with decreasing organic carbon content (27). Thus, a clogged drain field which retains bacteria may have a decreased number of sites for viral adsorption, and may not retain these pathogens.

A recent study of five soils showed a soil of loamy texture with a pH of 7.2 and a low cation exchange capacity (8.9 meq/100g) did not adsorb viruses (11). This soil is similar to those in the area where this study was undertaken. Survival of viruses adsorbed to soil may also pose a health problem. Polioviruses have remained infective for 90 days in moist sand and for 77 days in dry sand (53,26). Desorption of viable viruses can occur as a result of heavy rains or septic tank use (25).

If viruses survive long enough and move freely with percolating water they will enter groundwater reservoirs. Infectious hepatitis virus has been shown to remain infective for 10 weeks in contaminated domestic well water (12). Recent studies in this laboratory showed poliovirus can survive extended periods in innoculated shallow groundwater samples (55). Groundwater is the principal source of drinking water in New Mexico. The Safe Drinking Water Act, P.L. 93-523, requires that public water supplies which serve 15 or more connections or 25 or more people must meet minimum standards with regard to chemical content and bacterial population, but virus contamination is not considered in these regulations and coliforms are not a reliable indicator of viral contamination (4). While most New Mexican towns draw their water from deep aquifers unlikely

to be affected by possible septic tank pollution, fissures in the bedrock can channel shallow groundwater into older, deeper supplies and cause contamination. Rural areas are most likely to have many septic tank disposal systems and are also most likely to depend on old shallow wells or improperly cased deep wells for domestic water supplies. These individual household wells represent the most probable source of water-borne disease in the future since the majority (71 percent) of reported water-borne disease between 1946 and 1970 resulted from contamination of private individual water systems (14,15). These systems are not monitored under the provisions of the Safe Drinking Water Act.

In the Lower Rio Grande Valley of New Mexico the shallow groundwater is an integral part of the river hydrologic system. The groundwater fluctuates with river flows, recharging directly from the river and returning to it through irrigation drains. Experiences in the late 1950's indicate that the deeper aquifers of the valley are also part of this system. Water levels in deep irrigation wells dropped 10 feet or more during an extended drought, only to return to previous levels when river flows returned to normal.* This pattern of interconnecting aquifers indicates the possibility of contaminating municipal water supplies with viruses from septic tank effluents if viruses remain viable in soil and groundwater for extended periods.

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*Personal Communication, 1976, John W. Clark, Former Director of the New Mexico Water Resources Research Institute.

More emphasis has been placed on the possible health hazard posed by enteric viruses in municipal sewage and sewage treatment products than on the possible health hazard posed by enteric viruses in septic tank effluents. Following naturally occurring enteric viruses and introduced, model poliovirus from introduction into a septic tank, through the system, into the soils of the drain field, through the percolation bed, and into the shallow groundwater below the drain field is a first step toward quantifying the possible health hazard in the Mesilla Valley.

MATERIALS AND METHODS

The Septic Tank

The septic tank system used in this study serves the farm manager's residence at the New Mexico State University Plant Science Farm (Figure 1). The system was installed in December 1976 and consists of a 2-chamber tank with a perforated clay tile discharge pipe set in a gravel-filled trench (Fig. 2) (42). Sampling access to both septic tank chambers was provided by replacing the existing concrete access lid with a pair of capped polyvinyl chloride (PVC) pipes set in a plywood frame. The access arrangement is shown in Figure 3.

Septic Tank Drain Field Observation Wells

The groundwater of the septic tank drain field was monitored through a system of shallow wells planned and placed by Chaur-Jyi Tzeng, a graduate student in the Civil Engineering Department of the New Mexico State University, College of Engineering, for his study of the chemical effects of septic tank effluent on groundwater quality (42). Five clusters of observation wells were dug at the locations shown in Figure 4. Each cluster consisted of three wells at different depths. Two single wells, numbers 6 and 7 in Figure 4, were added to the monitoring system after the study had started. Well clusters 1, 2, and 3 allowed sampling of groundwater near the septic tank outfall, along the discharge pipe in the middle of the drain field, and near the end of the discharge pipe, respectively. Well cluster 4 and well 6 were placed to

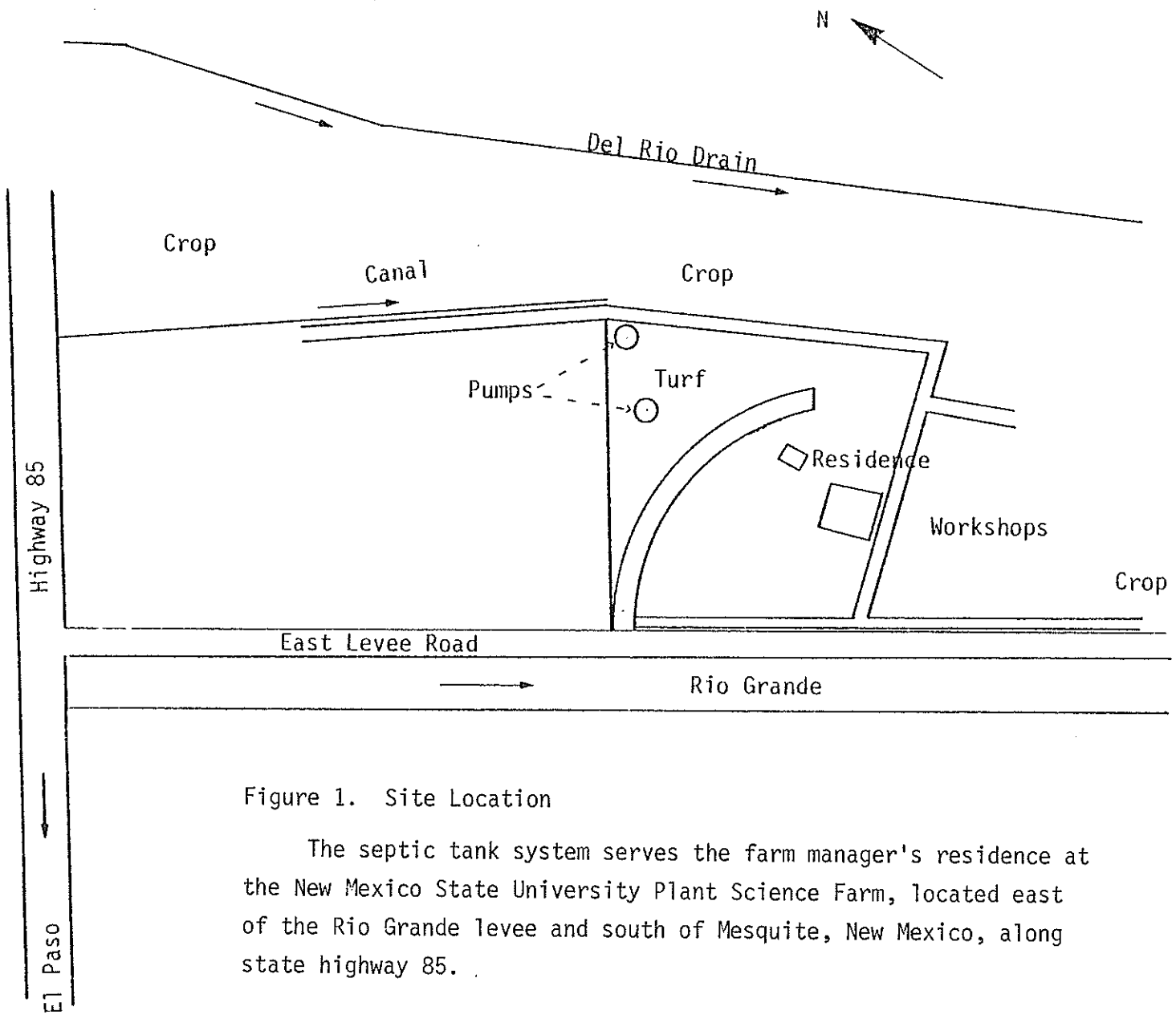

Figure 1. Site Location

The septic tank system serves the farm manager's residence at the New Mexico State University Plant Science Farm, located east of the Rio Grande levee and south of Mesquite, New Mexico, along state highway 85.

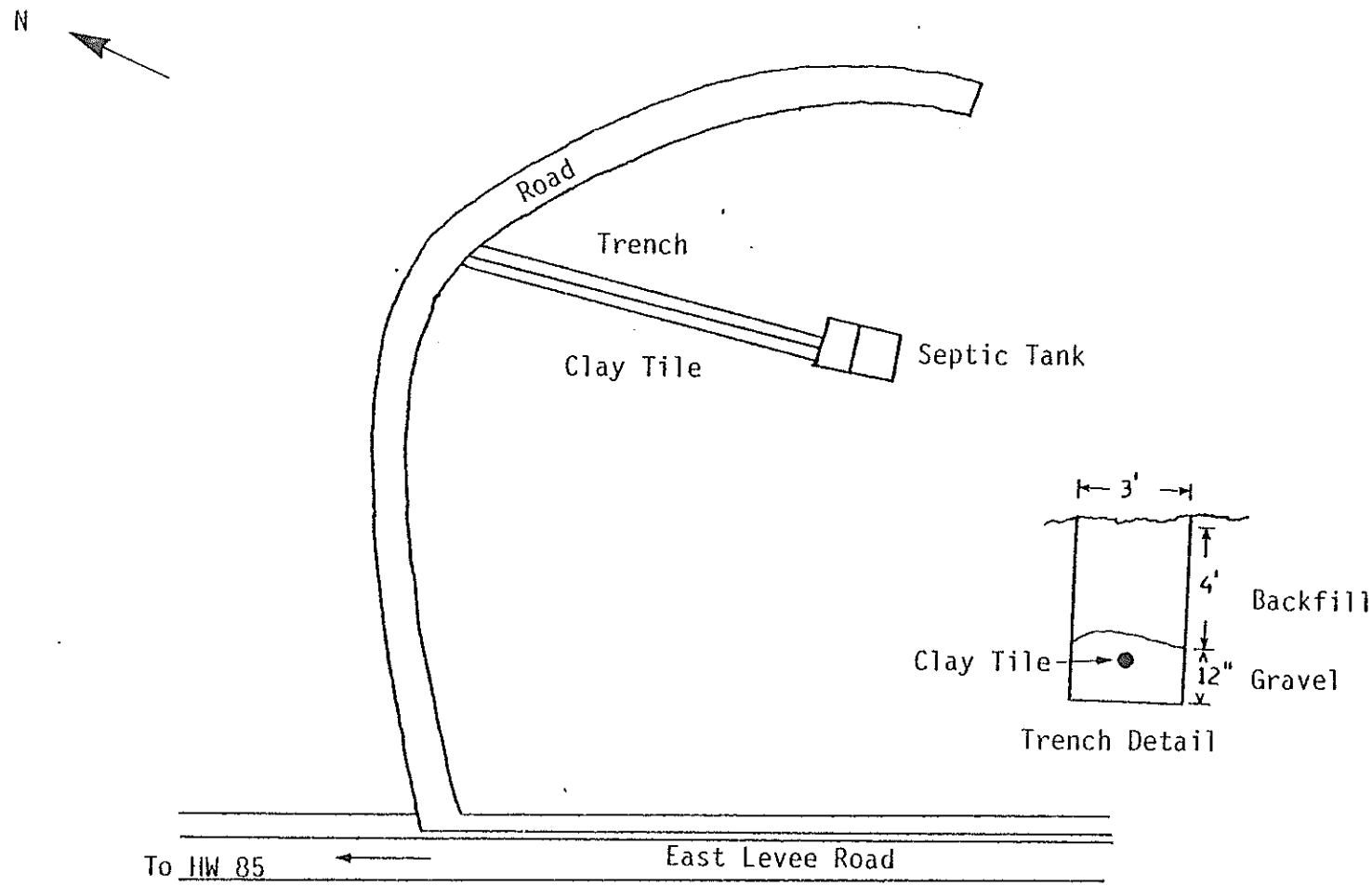

Figure 2. Septic Tank and Drain Field

The septic tank serving the farm manager's residence at the New Mexico State University Plant Science Farm has two compartments: a settling chamber, approximately 5.5 feet x 5 feet x 5 feet (1.7 m x 1.5 m x 1.5 m); and a sludge digestion chamber, 2.5 feet x 5 feet x 5 feet (.8 m x 1.5 m x 1.5 m), with a capacity of 62.5 cubic feet (111 m^3) (42). Effluent from the tank flows through a perforated 4 inch wide (10 cm), 95 foot (29 m) long clay tile pipe and discharges into a 3 foot (.9 m) wide trench filled with 12 inches (30 cm) of coarse gravel. The distribution system slopes at a one percent angle away from the outflow (42).

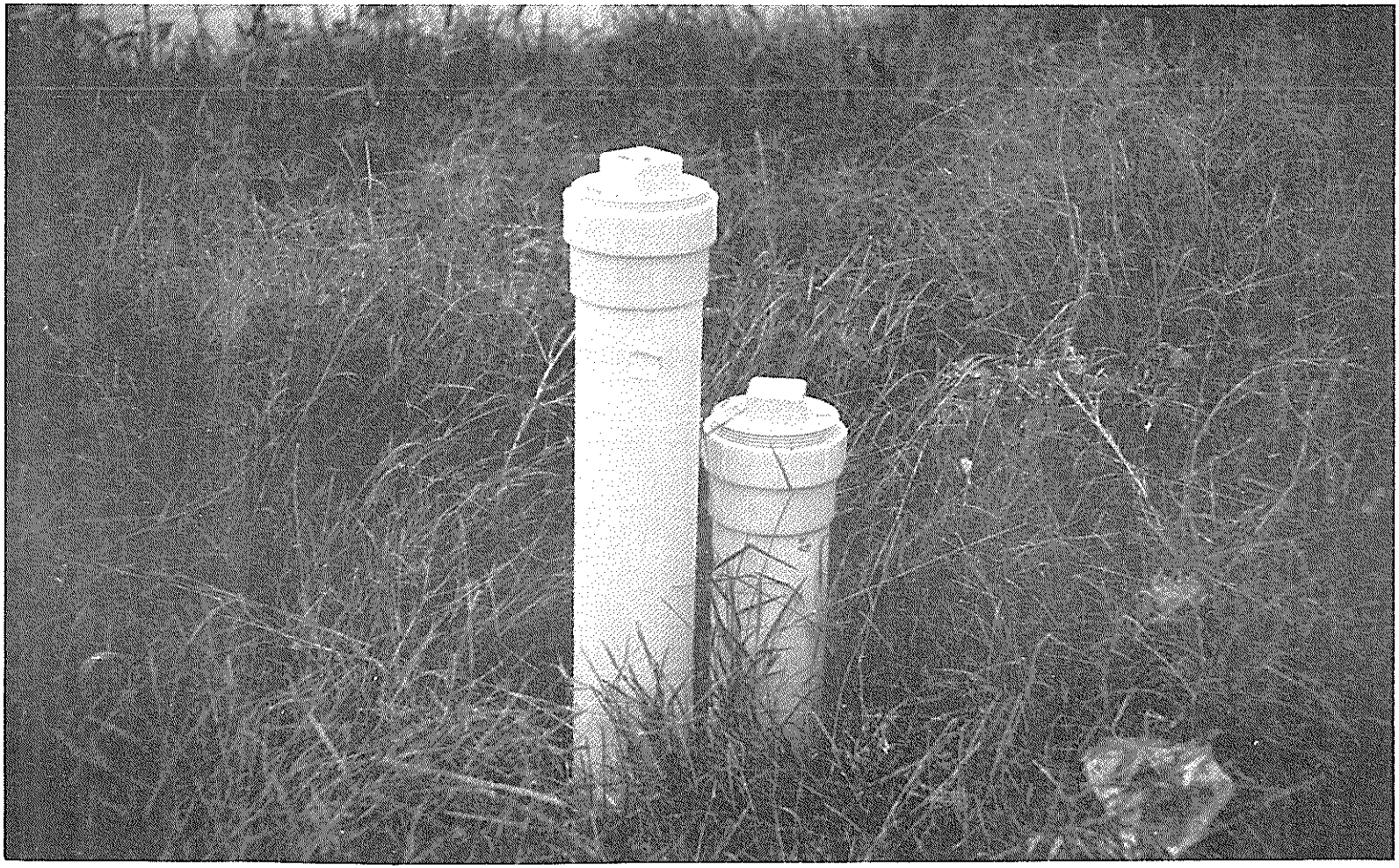

Figure 3. Access Pipes to Septic Tank

A pair of PVC pipes extended from the septic tank port and provided constant access to both chambers. The pipes were 4-1/2 inches (11 cm) in diameter and 60 inches (1.5 m) long. The right-hand pipe extended below the normal fluid level in the sludge digestion chamber while the left-hand pipe rested on concrete projections from the sides of the settling chamber, approximately 10 cm above the normal fluid level. Pipes extended 2 and 2.5 feet above ground.

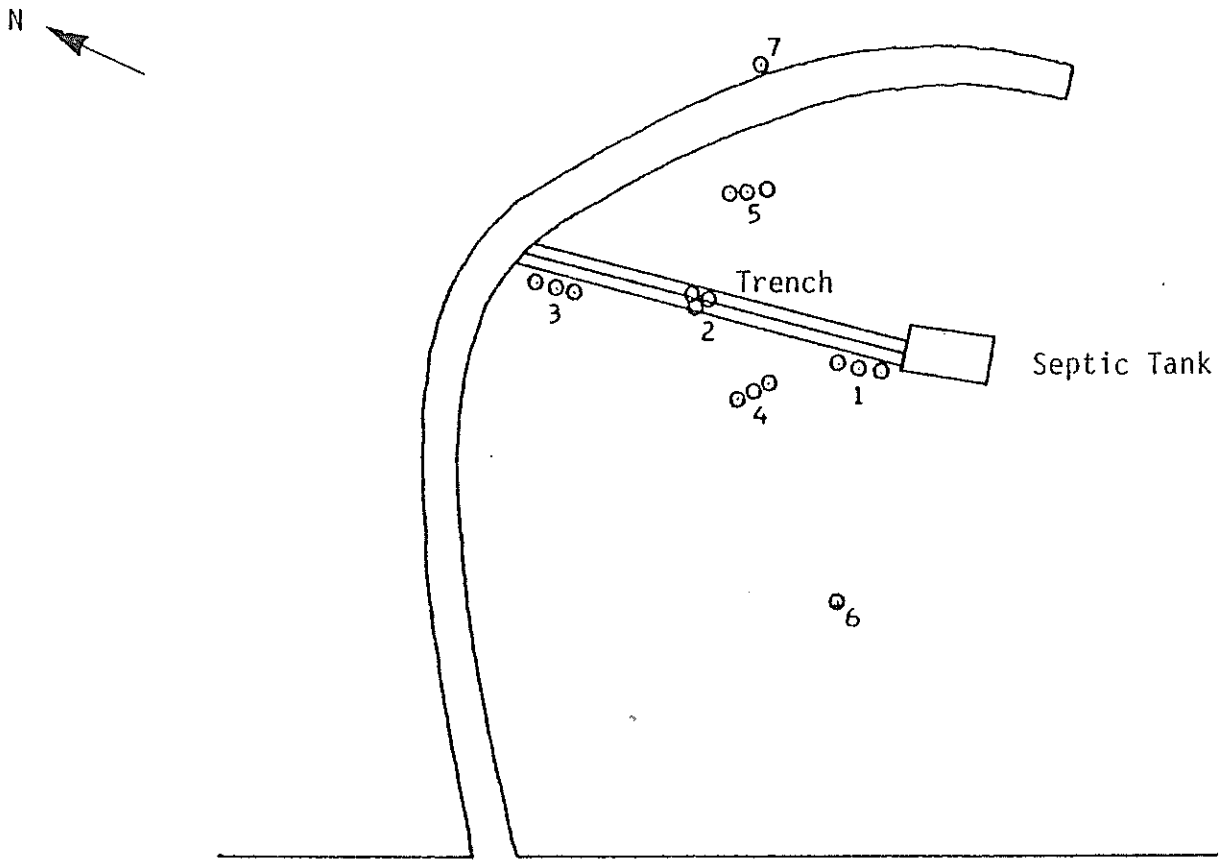

Figure 4. Location of Groundwater Observation Wells

Each well is represented by "O." Well clusters and the single outlying wells are assigned numbers. Well cluster 1 was located at the septic tank outfall; well cluster 2, above the discharge tile; well cluster 3, near the end of the discharge tile; well cluster 4, 11 feet (3.5 m) west of the trench containing the discharge pipe; and well cluster 5, 45 feet (14 m) east of the trench. The shallowest well in each cluster (10.5 foot) is located furthest south, the 12.5 foot well is in the middle and the deepest (14.5 foot) well is the most northern. The single wells located at 6 and 7 are 10.5 feet (3.2 m) deep. Well 6 is 60 feet (18 m) east of the trench and well 7 is an equal distance to the west.

determine background water quality, while well cluster 5 and well 7 were placed to determine the relationship between groundwater quality and groundwater movement in the downstream area.

All well holes were dug with a hand auger. One-inch galvanized pipes were coupled to 6-inch well points and set with an 8-pound hammer to the appropriate depth. Pipes were fitted with threaded pipe caps (Figures 5 and 6).

Methods of Sampling Septic Tanks and Groundwater

Samples of septic tank liquor were collected by fitting a sterile plastic bottle into a weighted wire frame and dropping the bottle at least 2 feet below the surface of the tank liquor. The filled bottle was sealed, disinfected with 5 percent chlorox, and stored at 25°C during transport.

Small samples of groundwater were taken from the observation wells by dropping sterile screw-cap test tubes down the pipes. Larger samples were collected using a hand-operated vacuum pump. For sampling, a length of 1/4-inch (.64 cm) tygon tubing was forced down an observation well pipe as far as possible. The tubing was connected to the manual pumping apparatus shown in Figure 7. A small amount of groundwater was pumped from a well and used to rinse the flask and tubing. To ensure that fresh groundwater was sampled, the well was then pumped until the water was clear and the turbid water was discarded. Approximately 700 ml of clear water was pumped into the filtering flask and transferred to a sterile plastic bottle. The apparatus was cleaned in the

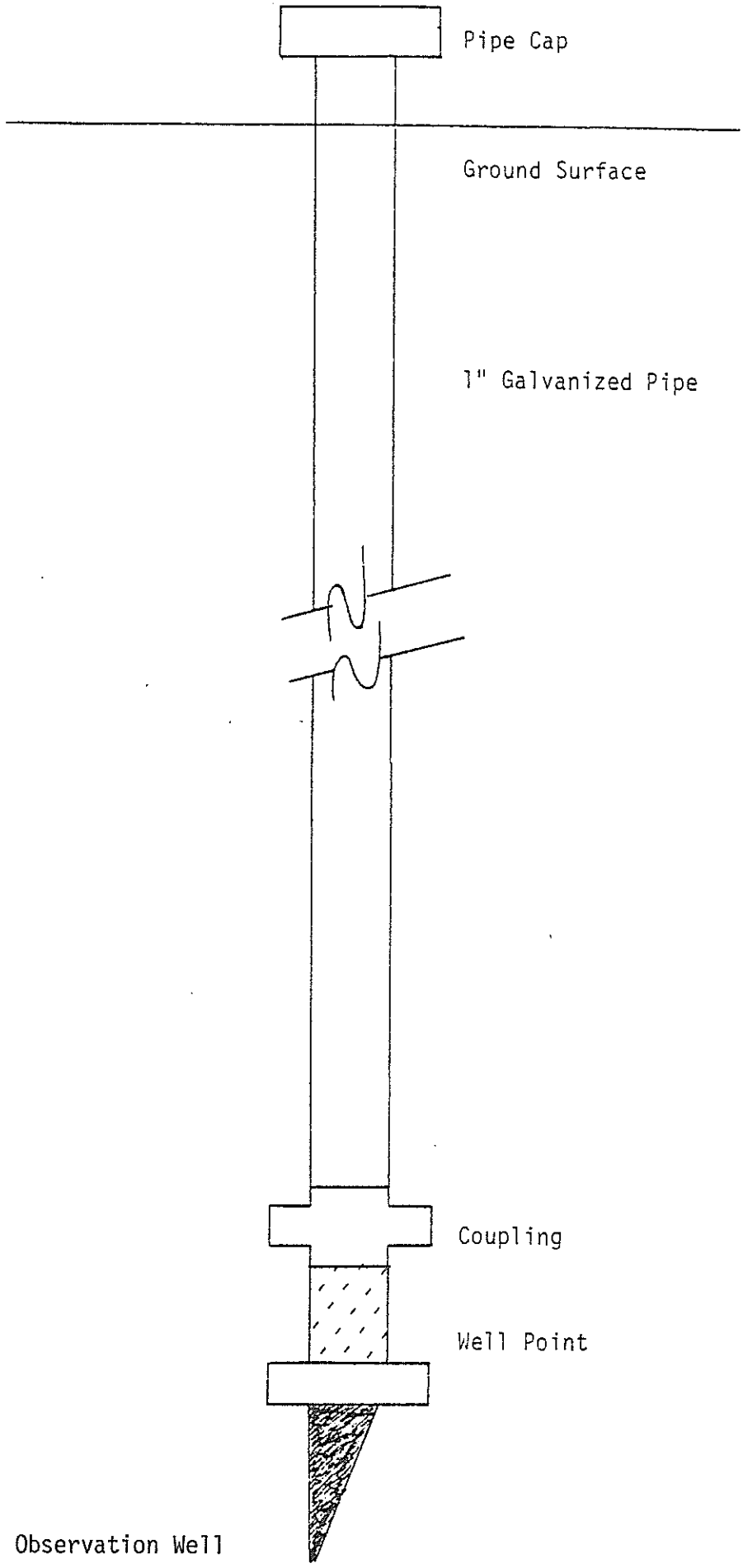

Figure 5. Observation Well

Diagram of observation well showing pipe, well point, and cap.

Figure 6. Observation Wells of Well Cluster 1

Well cluster 1, located near the septic tank outfall consisted of three observation wells at different depths (10.5, 12.5, and 14.5 feet).

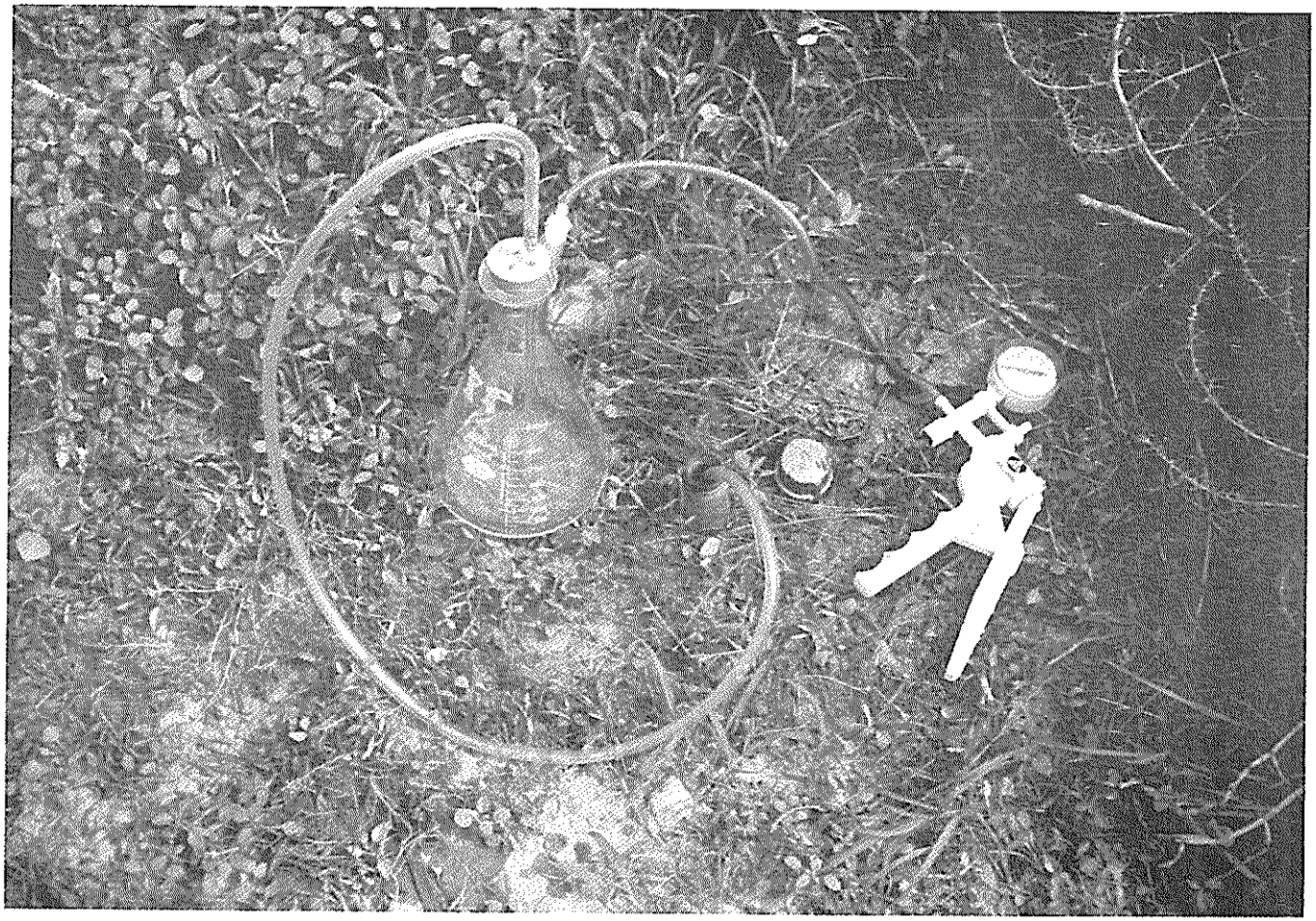

Figure 7. Manual Pumping Apparatus

Apparatus was used to sample groundwater in the septic tank drain field observation wells. A length of 1/4-inch diameter tygon tubing was forced down the well pipe. The free end of the tubing was connected to a glass tube running through the stoppered top of a 1-liter filtering flask. A Nalgene^{R} hand vacuum pump was connected to the side opening of the flask. Groundwater could be pumped with this apparatus when the water level was at least 3 inches (8 cm) from the bottom of a well.

field with 5 percent chlorox solution and rinsed with sterile distilled water if more than one groundwater sample was collected. All samples were kept at 25°C while at the site and during transport to the laboratory.

Dialysis Membrane Chambers

Plexiglas chambers, as described by Glass (22), were made by the New Mexico State University Physical Plant shop. These chambers were used for virus survival experiments in the Rio Grande and in the Plant Science Farm septic tank. The chambers consisted of dialysis membrane sheets (Bolab, Inc., membrane pore size of 4.8 nm and molecular exclusion of 12,000 daltons) secured between Plexiglas plates (Figure 8). The center chamber, bounded on either side by dialysis membrane, had a capacity of 20 ml. Access to the chamber was provided by two 18-gauge needles mounted in the middle plate and extending into the chamber interior. The needles were sealed with plastic syringe tips when the chamber was immersed.

For experiments in groundwater observation wells, modified dialysis membrane chambers were made from bottomless, perforated plastic test tubes (10 mm x 120 mm). Dialysis tubing, 6 mm in diameter (Fisher Scientific, membrane pore size of 4.8 nm and molecular exclusion of 12,000 daltons) was knotted at the bottom and tied at the top around an 18-gauge needle to form a bag with a 5 ml capacity. This bag was suspended within the protective plastic tube as shown in Figure 9.

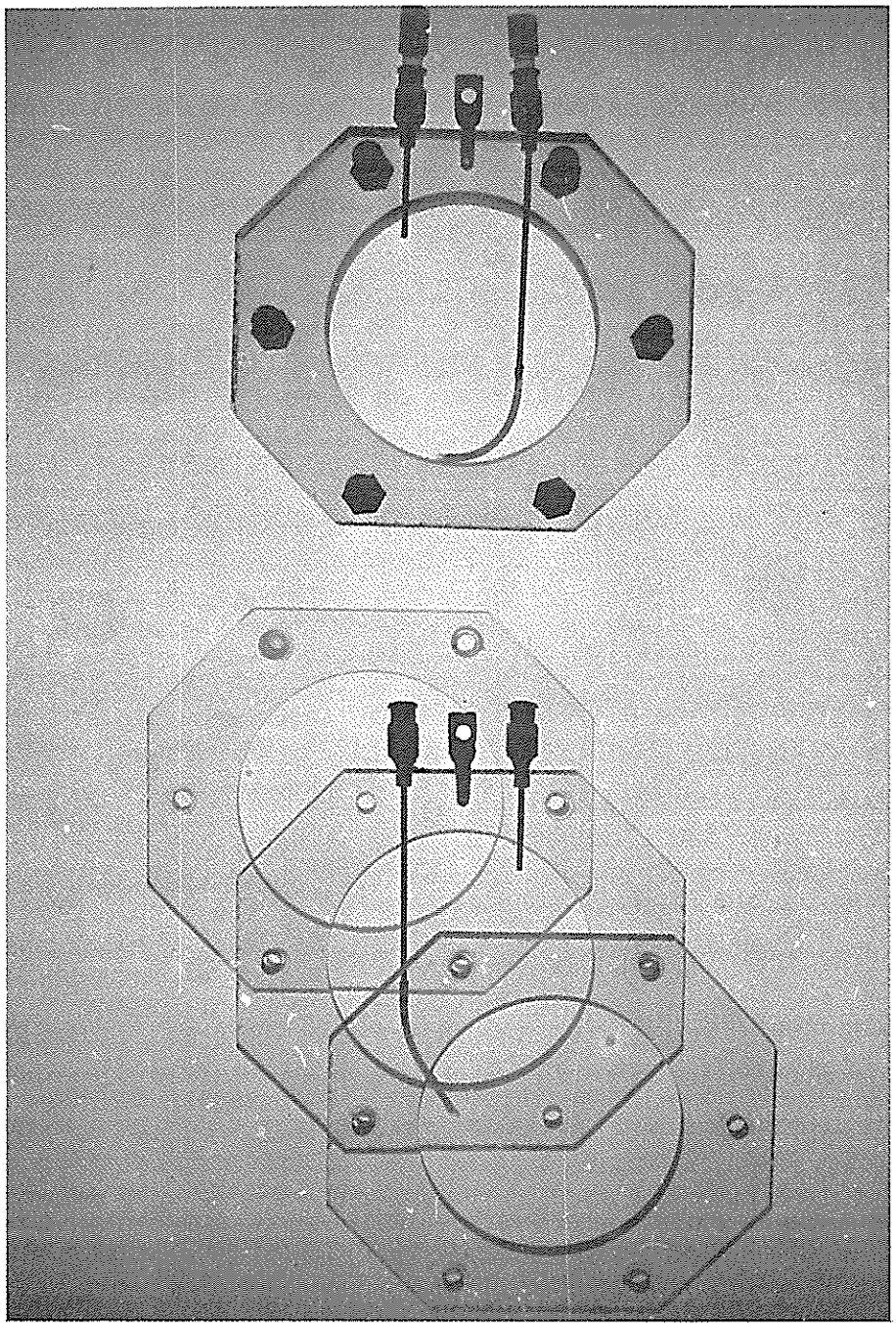

Figure 8. Dialysis Membrane Chamber

Three octagonal plates, with rectangular dimensions of 10 cm x 8.7 cm x 0.6 cm, and center openings 6 cm in diameter were used to secure dialysis membrane sheets (7 cm circles). The assembly was secured with stainless steel bolts. The center chamber had a capacity of 20 ml and was accessible through the 18-gauge needles mounted in the middle plate. Plastic syringe tips were used to seal the needles. The screw-eye was used to suspend the chamber from nylon cordage.

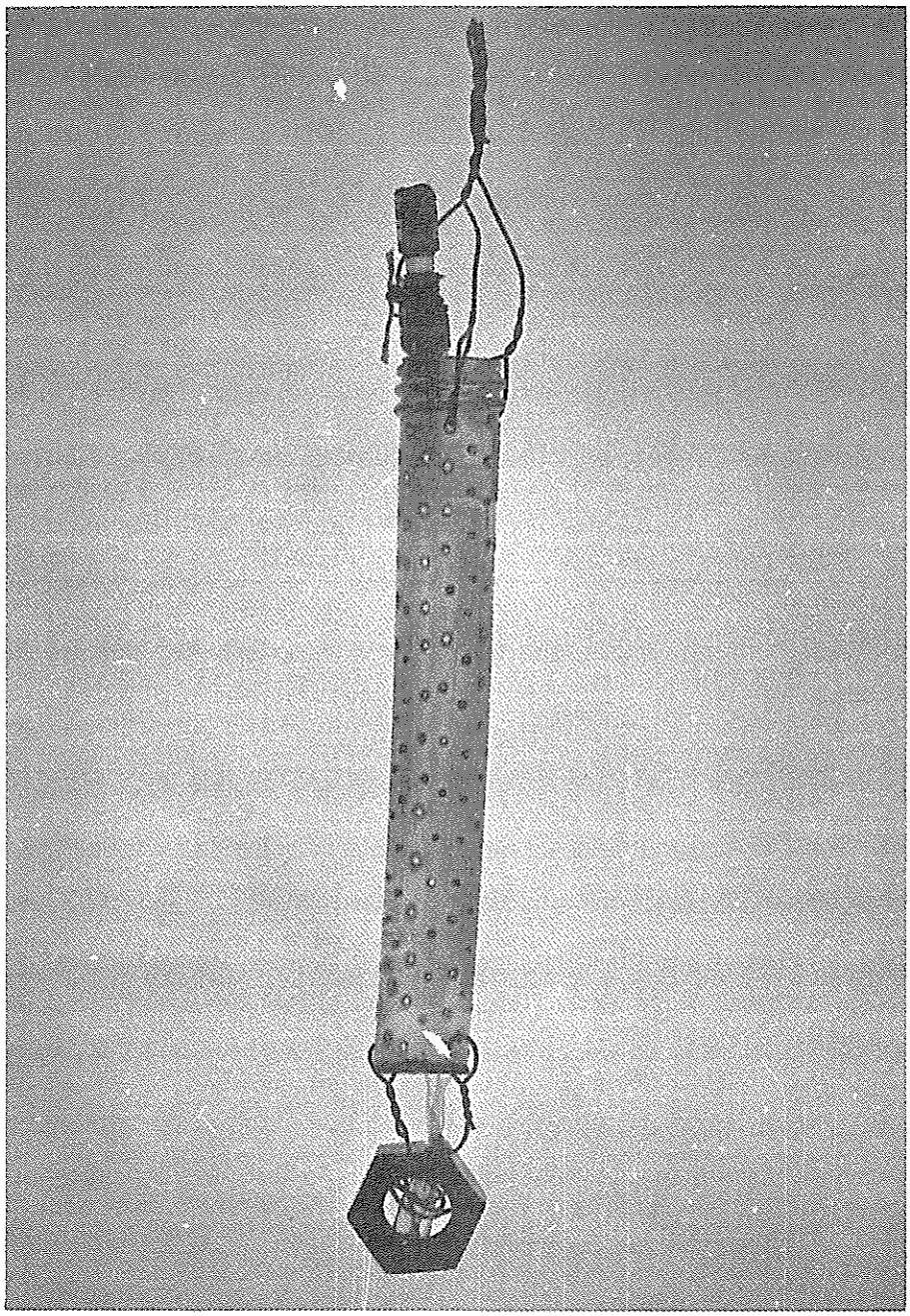

Figure 9. Modified Dialysis Chambers

A dialysis bag with a capacity of 5 ml was formed by knotting one end of a 15 cm length of 6 mm diameter dialysis tubing and tying the other end with sewing thread around an 18-gauge needle. This bag was suspended within a perforated 10 mm x 120 mm plastic test tube. The needle was sealed with a plastic syringe tip when the chamber was immersed. The chamber was weighted at the bottom with a stainless steel nut and stainless steel wire was used to form a three-point attachment to the tube rim. Nylon cordage was used to suspend the chamber in groundwater observation wells.

The dialysis membranes used for both chambers were boiled in three changes of distilled water before use. Chambers were assembled in the laboratory and transported to the study site in a container of distilled water to prevent drying of the membranes. Water which diffused into the dialysis chambers during transport was removed by aspiration before the chambers were filled with poliovirus innoculated septic tank liquor or groundwater samples. Fluids were introduced into the chambers through the access needles using standard 10-ml pipettes.

Both types of dialysis chambers were sampled by unplugging the access needles and screwing a 1-ml tuberculin syringe into a needle head. The fluid in the chambers was mixed by drawing a portion into the syringe and expelling it 5 times. After mixing, a sample was drawn into the syringe and transferred to a vial containing an amount of Eagle's minimal essential medium (MEM) equal to the sample.

Preparation of Poliovirus Suspensions

Poliovirus type 1 (Mahoney) was used throughout the study. Virus pools were prepared by infecting phosphate buffered saline (PBS) rinsed HeLa cell monolayers with .2 ml of stock poliovirus suspension. After a 1-hour infection period, the monolayers were covered with MEM and incubated for 24 hours at 37°C. The cultures were frozen and thawed five times to disrupt the cells, and cell debris was removed by low speed centrifugation. Poliovirus was pelleted from the resulting supernatant by centrifugation at 149,000 xg for two hours in a Spinco S-50 rotor. The virus pellet was resuspended in PBS.

Virus suspensions were assayed for infectivity using the methods described by O'Brien and Newman (35). Confluent HeLa cell monolayers grown in French square glass bottles (13 cm^2 surface area) were drained of MEM growth medium and infected with 0.2 ml of virus suspension diluted appropriately in PBS. The bottles were rotated immediately to distribute virus evenly over the monolayer and were rotated every 5 minutes during a 30-minute infection period at room temperature. Monolayers were then covered with 5 ml of overlay medium. Infected monolayers were incubated at 37°C for 30 to 40 hours, until plaques 1-2 mm in diameter were visible.

The overlay medium was removed and the monolayer stained with ethanolic crystal violet and rinsed with warm water. Plaques were counted immediately and again after the monolayers had dried.

Radioactively labeled virus preparations were made as previously described except that either [^{3}H]-uridine or [^{14}C]-protein hydrolysate was added 2 hours after infection. Crude virus preparations were further purified by centrifugation in 15 to 30 percent glycerol gradients. Radioactivity in gradient fractions was assayed by liquid scintillation spectroscopy and the most radioactive fractions pooled. After dilution of pooled gradient fractions with PBS, viruses were pelleted by centrifugation. Capsid labeled preparations were further purified by equilibrium density centrifugation in cesium chloride. The cesium chloride was removed by dialysis.

Bacterial Analysis

Groundwater and septic tank liquor samples were analyzed for total aerobic plate count and coliform content using techniques described in Standard Methods (1). Appropriate dilutions of each sample were plated on plate count agar and incubated for 48 hours at 37°C. Visible colonies were counted at 24 and 48 hours using a Quebec colony counter. Coliforms were determined by the 3-tube, most probable number technique using lauryl sulfate tryptose (LST) broth. Positive tubes were streaked on eosin methylene blue (EMB) agar plates for coliform verification.

Isolation of Enterovirus From Septic Tank Liquors

Septic tank liquor samples were collected from either the Plant Science Farm septic tank or from properly functioning septic tanks in the Las Cruces area opened specifically for sampling. Samples were taken as previously described. In the laboratory, samples were allowed to settle for 2 to 4 hours before 10 ml of liquid was pipetted from the top of the collection container and used for virus assay. The remainder of the sample was frozen. Confluent HeLa cell monolayers growing in milk dilution bottles (48 cm^2 surface area) were inoculated with 0.8 ml of untreated, fresh septic tank liquor. The monolayer was rinsed several times with PBS after a 30-minute virus attachment period and covered with overlay medium. Monolayers were incubated 72 to 84 hours; the incubation period was terminated if the overlay agar showed a significant change in pH or if HeLa cells began to slough off the glass.

Attempts were made to control bacterial or chemical destruction of the monolayer by septic tank liquor. These included: treating raw liquor with chloroform (2 parts liquor to 1 part chloroform) for 1 hour at 37°C prior to inoculation (22); adding 12.5 mg/ml aureomycin to the overlay medium; making samples of liquor isotonic with HeLa cells by adding 0.1 ml of 10X PBS to 0.9 ml of septic tank liquor and adjusting the pH of the resulting solution to 9.4; and using liquor samples cleared of particulate matter by low-speed centrifugation. None of these methods, alone or in combination, decreased sloughing significantly. When the inoculation with a particular, fresh liquor sample resulted in large areas of sloughing, the frozen portion of the sample was thawed, and the test was rerun.

French square glass bottles (13 cm^2 surface area) were used in later attempts to isolate enterovirus from the Plant Science Farm septic tank. These were handled in the same way as milk dilution bottles, except the inoculum was 0.2 ml of septic tank liquor. The French square bottles (plaque bottles) were more convenient to use and showed less monolayer sloughing than the larger milk dilution bottles.

In Situ Poliovirus Survival in Septic Tank Liquors

The survival of poliovirus type 1 was studied in the septic tank at the Plant Science Farm. A fresh sample of septic tank liquor was collected from the appropriate tank chamber and thoroughly mixed by shaking 50 times through a 2 foot arc. The liquor was allowed to settle for 15 minutes and 20 ml were added to a test tube

of virus suspension to give a final titer of 10^6 - 10^7 plaque forming units per milliliter of innoculated liquor. The virus-liquor mixture was shaken as described above and between 10 and 15 ml of inoculated liquor was pipetted into a dialysis membrane chamber. The filled chamber was plugged and introduced into the septic tank chamber from which the liquor had been taken. After 30 minutes, the chamber was drawn from the tank and an initial sample was taken. The sample was transferred to a vial containing an equal amount of MEM. Samples, ranging in size from 0.5 - 1 ml, were taken periodically throughout each experiment. Samples were kept at 25°C during transport to the laboratory and until 2 hours had passed from time of collection to allow antibiotics in MEM to control bacteria in the septic tank liquor. Sample vials were then frozen until an experiment was completed. All samples from a given experiment were assayed at the same time. Periodically, portions of samples were assayed immediately after collection to determine approximate titer and suitable termination times for each experiment.

Each time a sample was taken for viral assay, a sample was taken for bacterial analysis. These samples were not diluted with MEM and were processed within 2 hours of collection.

The length of a particular virus survival experiment was often limited by destruction of the dialysis membranes and corrosion of metal chamber parts by septic tank liquors. Chambers were occasionally lost to the tank when nylon suspending cords deteriorated or attachments broke.

Laboratory Study of Poliovirus Survival in Septic Tank Liquors

Poliovirus type 1 survival in septic tank liquor was also studied in the laboratory. Fresh septic tank liquor samples were collected from the Plant Science Farm tank or septic tanks used in enterovirus isolation studies. The liquor was mixed with appropriate amounts of virus in 10 x 20 cm sterile glass screw-cap test tubes. Small sterile magnets were added to each tube, and the test tubes were incubated in a circulating water bath maintained at 22°C. Initial samples were taken 30 minutes after mixing. Samples were treated and assayed in the same way as those from field studies.

Poliovirus Survival in Groundwater

The survival of poliovirus type 1 in groundwater was studied in the 14.5 foot deep observation wells of well clusters 1 and 4 as described in Figure 2 and in the laboratory using groundwater samples collected from these wells. For field studies, poliovirus suspension was diluted at the site with freshly collected groundwater. The mixture was shaken as previously described and 3-5 ml was pipetted into the dialysis bag of a modified dialysis membrane chamber. The chamber was plugged and introduced into the well from which the groundwater sample was taken. Groundwater levels in the area of the septic tank drain field varied 4 feet (1 m) or more during the study period. Chambers were suspended 12 feet (3.7 m) below ground level when possible, but were dropped as low as 14 feet (4.3 m) when necessary to maintain immersion in the groundwater.

The initial sample was taken 30 minutes after groundwater was added to the virus. Other samples were taken at intervals of 24 hours ± 2 hours. Samples were collected, treated and assayed as previously described for septic tank studies.

Poliovirus Survival in Soil

Septic tank liquor was collected from the digestion chamber of the Plant Science Farm septic tank. A small frozen juice can (5.2 cm x 9.8 cm), with both ends removed, was pressed about 2 cm into undisturbed sandy loam soil similar to that in the septic tank drain field. The soil was moistened by slowly adding 200 ml of fresh liquor to the can, which maintained a fairly constant pressure head while containing the liquor to a relatively small area. After 1 hour, 24 ml of fresh septic tank liquor inoculated with 1 ml of [^{3}H]-uridine labelled poliovirus type 1 (468,000 cpm/ml) was added to the soil surface enclosed by the can. After 1 hour, a 20 cm long core was taken with a standard coring device. The core was removed from the corer in 1-cm slices starting from the surface. Each slice (1 cm x 2 cm) was taken aseptically and placed in a sterile, capped 10 cm x 120 cm test tube. Two milliliters of glycine buffer (pH 11.5) were added to each sample, and the tubes were tightly capped.

The tubes were transported to the laboratory at room temperature and each was vortexed for 3 minutes to elute the viruses from the soil. The tubes were then centrifuged at low speed (7710 xg) for 30 minutes to remove soil particles, and the supernatant was decanted

into a small capped tube. A 0.1-ml alliquot of the supernatant from each soil core slice was diluted with 2 ml of "aquasol" scintillation cocktail and assayed for radioactivity on a Packard Tri-carb liquid scintillation counter.

Forty-eight hours after the soil had been first wetted, 500 ml of distilled water were added to the juice can which had been left in position. After 1 hour a second core was taken. This core was sliced in the same way as the first, and each sample treated as described above.

The supernatant from selected soil slices was assayed for virus infectivity on confluent HeLa cell monolayer grown in plaque bottles.

The experiment described above was repeated using an unlabeled poliovirus suspension containing 5×10^9 plaque-forming units per milliliter. Seven days after the initial core was taken, the can was filled with 500 ml of distilled water and after 1 hour, 2 horizontal cores were cut through the wetted soil at depths of 1 cm and 3 cm below the surface. Then a second vertical core was taken in an undisturbed portion of the wetted soil. The supernatant from the soil slices in this experiment were assayed for virus infectivity on HeLa cell monolayers.

Isolation of Enterovirus from Groundwater

The Rio Grande does not flow below the Caballo Reservoir Dam during the winter. When water was released for the 1978 irrigation season, the front of the water was followed until it reached the

Plant Science Farm (Figure 1). The water level in the groundwater observation wells was monitored. When the water level rose, each well with sufficient water was sampled. In the laboratory an aliquot of water was removed from each sample and assayed for coliform bacteria. The remaining portion of each sample was frozen. Coliform positive samples were used in attempts to isolate enteric viruses.

Samples were thawed and thoroughly mixed. Approximately 150 ml was removed from each bottle and centrifuged (7710 xg) for 20 minutes to remove particulate matter. The supernatant was divided in 12-ml portions among polyallomer centrifuge tubes (Beckman, No. 326814). The tubes were capped and placed in a Beckman general purpose fixed-angle type 50 Ti rotor and centrifuged at 96,592 xg for 60 minutes to pellet any virus present. The supernatant was discarded and the pellet was suspended in 0.8 or 3 ml of PBS. Virus suspensions from all 12 tubes, when 0.8 ml was used, were combined and assayed for virus on HeLa cell monolayers in 1-quart medicine bottles (112 cm^2 surface area). When 3 ml of PBS was used to resuspend virus, each tube was vortexed several times during a 30-minute elution period and .2 ml aliquots were inoculated onto HeLa cell monolayers in plaque bottles. Inoculated monolayers were incubated for 72 hours before being stained and examined for plaques.

RESULTS

Isolation of Enteric Viruses from Septic Tank Liquors

Fifteen families living outside the city limits of Las Cruces, New Mexico, were contacted for permission to sample the septic tanks serving their residences. Five households were willing to have their tanks opened. Only 3 of the 5 septic tanks were operating normally and were sampled. Conditions in these tanks varied, as is shown in Table 1, but viruses capable of infecting HeLa cell monolayers were isolated from each tank. The number of viruses isolated from septic tank liquors ranged from 1600/l to 3700/l. As expected, tanks serving households with children had the higher virus concentrations. The numbers of viruses isolated represent minimum numbers since all enteric viruses are not detected on HeLa cell monolayers. No attempt was made to identify the viruses isolated.

Five attempts were made, over a 12-month period, to isolate enteric viruses from the septic tank at the New Mexico State University Plant Science Farm. The family served by this system included 2 adults and 3 children, with 2 other children being frequent guests. The temperature in the septic tank chambers varied from 27°C during the summer to a low of 20°C in the digestion chamber in the winter. Specific conductivity of the liquors was consistently 1 millimho/cm and the pH was regularly 7.4 in both chambers. The results of virus isolation attempts are shown in Table 2. Throughout the trials, 1/2 of all plaque bottles

Table 1. Isolation of Viable Enteric Viruses from Septic Tank Liquors.[a]

	HOUSEHOLD SERVED		
	#1	#2	#3
Condition of Septic Tank	Functioning Well	Beginning to Fill	Needed Cleaning
# Children in Household	2	3	0
# Adults in Household	8[b]	2	1
Temp. of Liquor in Tank (°C)	27	25	25
Conductivity of Liquor (mmhos/cm)	1.0	1.0	1.5
pH of Liquor	7.2-6.6	6.4	8.6
Nitrogen as NH_4 (mg/l)	.48	3	5.2
Viruses/l of Liquor	2500	3700	1600
Bacteria/ml of Liquor	52×10^8	36×10^8	24×10^5
Coliphage/l of Liquor	10^{12}	10^{14}	10^{11}

(a) Three functioning septic tanks were opened and sampled. Bacteriological analyses were done according to Standard Methods (1). Coliphage were assayed by the soft agar overlay technique (33) using Escherichia coli A-19 as a host. Viruses were assayed on HeLa cell monolayers. Nitrogen content was determined using the Nessler method. Specific conductivity was measured with a portable meter.

(b) This septic tank system serves both a home and an office which employs 6 adults.

Table 2. Isolation of Enteric Viruses from New Mexico State University Plant Science Farm Septic Tank[a]

Month of Sample	Sedimentation Chamber PFU/ml[b]	Digestion Chamber PFU/ml
July 1977	1.7	4.2
October 1977	5.8	4.2
January 1978	4.2	5.0
April 1978	0.8	3.3
June 1978	5.8	7.5
Mean	3.7	4.6

(a) Samples of raw septic tank liquor collected from the 2 chamber septic tank at the Plant Science Farm were assayed for viruses on HeLa cell monolayers.

(b) Plaque-forming units/ml of liquor.

inoculated with fresh liquor showed visible plaques. Those plaques which did form included several distinct types: small-sized plaques which did not enlarge beyond 2 mm in diameter during a 72-hour incubation period; opaque plaques similar in size to those formed by poliovirus type 1 on HeLa cell monolayers; larger plaques, up to 7 mm in diameter, with smooth edges; and plaques similar in morphology to those formed by poliovirus. While no attempts were made to identify the viruses, the different plaque morphologies suggested at least 4 virus types were present. There was no obvious seasonal fluctuation in the number of viruses isolated, nor was there any observable pattern in the types of plaques seen. There was no statistically significant difference in the number of viruses isolated from the two chambers of the septic tank nor was there a difference in plaque types.

The numbers of viruses isolated from the 4 septic tanks examined in this study were much lower than those isolated by Yeager (55) from a septic tank opened for cleaning and those reported by Buras in undiluted domestic sewage (10). HeLa cells are susceptible to enterovirus infection but may not be sufficiently sensitive in demonstrating the presence of other enteric viruses. The ages, immunization records, and general states of health of the occupants of the sample households were investigated. There was only 1 child under the age of 5 in the 4 families. None of the septic tank users had recently received immunization against any viral disease and no incidents of gastrointestinal or respiratory illness were reported during the sampling period. The average of

3.2×10^3 PFU/l of septic tank liquor reported here as opposed to an infective titer of 2.5×10^6 PFU/l reported by Yeager (55) may indicate the normal dilution factor of functioning systems that are not receiving infectious excrement.

Effect of pH on Survival of Poliovirus Type 1 in Septic Tank Liquor

Ward and Ashley (49) have shown that ammonia is virucidal in sewage sludge. Ammonia has no effect on virus as ammonium ion, but is active against enteroviruses in the non-ionic, aqueous form present at alkaline pH. Ammonia has shown significant virucidal activity only in sludges with a pH over 7.5. To determine if ammonia was responsible for virus inactivation in septic tank liquor, a sample of septic tank liquor was collected from the tank serving household #1 (Table 1). The sample was divided, with one portion adjusted to pH 7 and the other to pH 9.4, and virus inactivation was compared in the two systems. Table 3 shows 1/2 of all viruses inoculated into both systems survived a 24-hour period, which approximates the average holding time of a domestic septic tank. The different pH levels had no noticeable effect on virus survival, indicating that ammonia may not be a factor in virus inactivation in septic tank systems. The level of ammonia in the tank sampled may have been too low to be virucidal. Note that fewer virus were isolated from septic tank #3 in Table 1 and that this tank had the highest ammonia concentration.

Table 3. Effect of pH on the Survival of Poliovirus Type 1 in Septic Tank Liquor[a]

pH	Percent Infectivity Recovered	
	24 Hours	72 Hours
7	74	4
9.4	60	9

(a) Fresh septic tank liquor buffered with 1N Tris and adjusted to pH 7 and 9.4 with 1N NaOH was seeded with 1×10^6 poliovirus/ml. Viruses were assayed on HeLa cell monolayers.

Survival of Poliovirus Type 1 in a Septic Tank

Dialysis membrane chambers described in Figure 8 were filled with fresh septic tank liquor inoculated with poliovirus type 1. The chambers were suspended in the appropriate compartments of the Plant Science Farm septic tank for up to 20 days. Data from 6 separate experiments are presented in Table 4 and Figure 10. An initial rapid inactivation of virus was followed by a gradual decline in infective virus titers. Fifty percent of the viruses introduced into either compartment were inactivated after 24 hours and 95 percent were inactivated after 20 days.

Aerobic bacterial plate counts and most probable number tube tests were made on samples removed from the dialysis membrane chamber during 2 virus-survival experiments (Appendix Table 1). Total plate counts were consistently 10 percent higher than coliform counts estimated by the MPN technique. Both methods of determining bacterial numbers indicated an exponential decrease in bacteria during the first 5 days of each experiment. A 1 $\log_{10}$ reduction (90 percent) in viable bacteria was noted after 2 days, and an over 2 $\log_{10}$ reduction after 5 days. The decline in bacterial numbers slowed after 5 days, and the number of bacteria remained nearly constant for the remainder of the experiment (Figure 11). Samples of liquor from both septic tank chambers were periodically subjected to bacterial analysis. Numbers of bacteria in the septic tank chambers remained constant at 80-100 x 10^5 colony forming units per ml (CFU/ml) in the sedimentation chamber and 20-40 x 10^5 CFU/ml in the digestion chamber over the

TABLE 4. SURVIVAL OF POLIOVIRUS TYPE 1 IN A FUNCTIONING SEPTIC TANK[a]

	Percent Infectivity Recovered					
	October		November		December	
Sample Day	Chamber 1[b]	Chamber 2[b]	Chamber 1	Chamber 2	Chamber 1	Chamber 2
0	100	100	100	100	100	100
1	50	50	53	47	50	50
2	-[c]	-	-	-	33	47
3	60	60	-	-	32	47
4	-	-	-	-	20	40
5	18	50	-	-	22	35
7	-	-	20	12	-	-
10	20	20	-	-	17	30
15	-	-	19	3	-	17
20	4	5	-	-	-	9

(a) Fresh septic tank liquor from each chamber of the septic tank at the New Mexico Plant Science Farm was innoculated with 1 x 10^7 PFU/ml poliovirus type 1 and was incubated in dialysis membrane chambers suspended in the appropriate chamber. Samples were taken at intervals and assayed on HeLa cell monolayers.

(b) Chamber 1 is the sedimentation chamber and chamber 2 is the digestion chamber of the septic tank.

(c) Dash means no sample was taken from this chamber on this day.

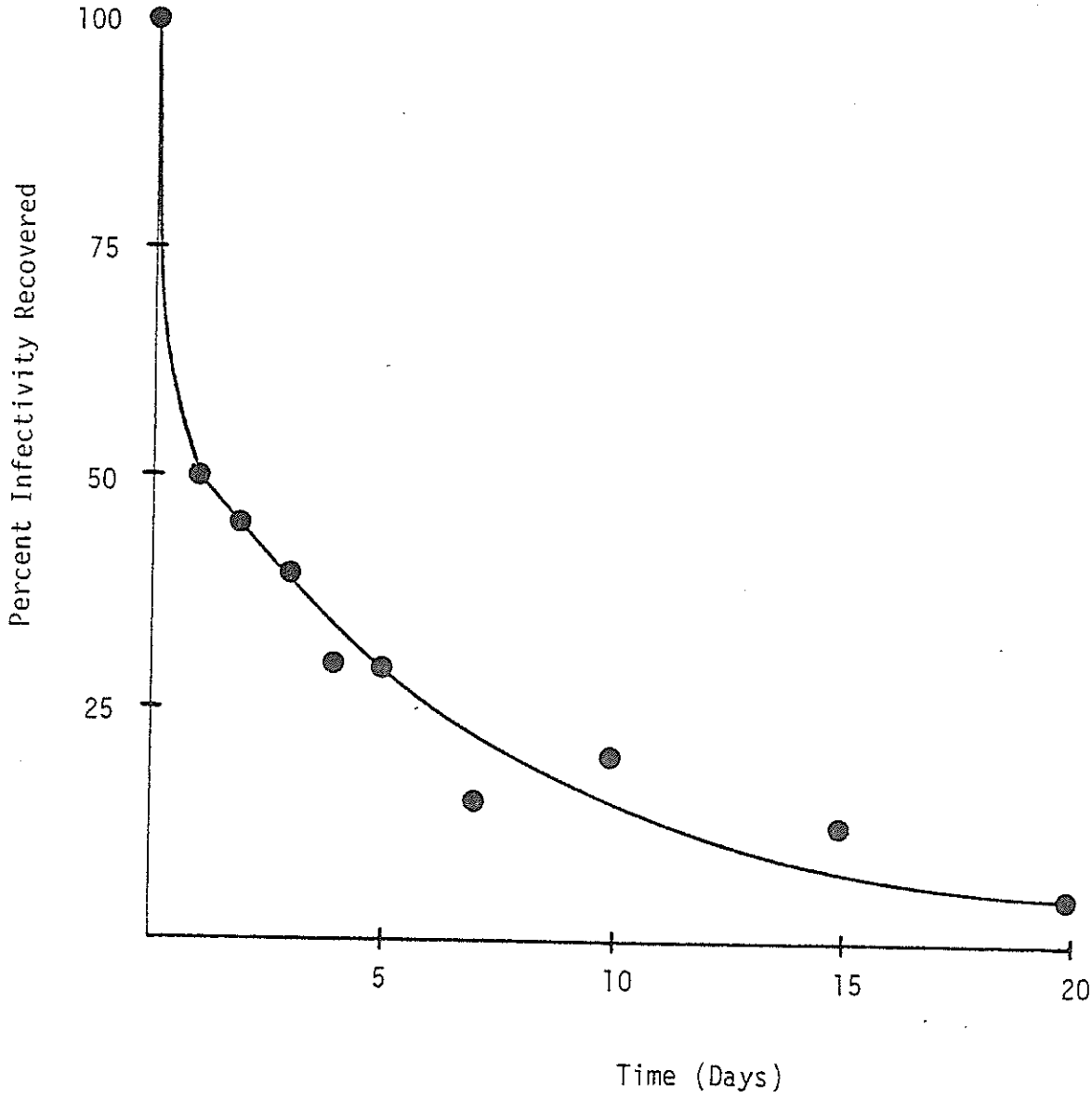

Figure 10. Poliovirus Recovered from Septic Tank Dialysis Membrane Chambers

Fresh septic tank liquor from both chambers of the Plant Science Farm septic tank was inoculated with 1×10^7 PFU/ml poliovirus type 1 and was incubated in dialysis membrane chambers suspended in the appropriate tank chamber. Samples were taken at intervals and assayed on HeLa cell monolayers. Virus infectivity recovered from 6 trials is averaged.

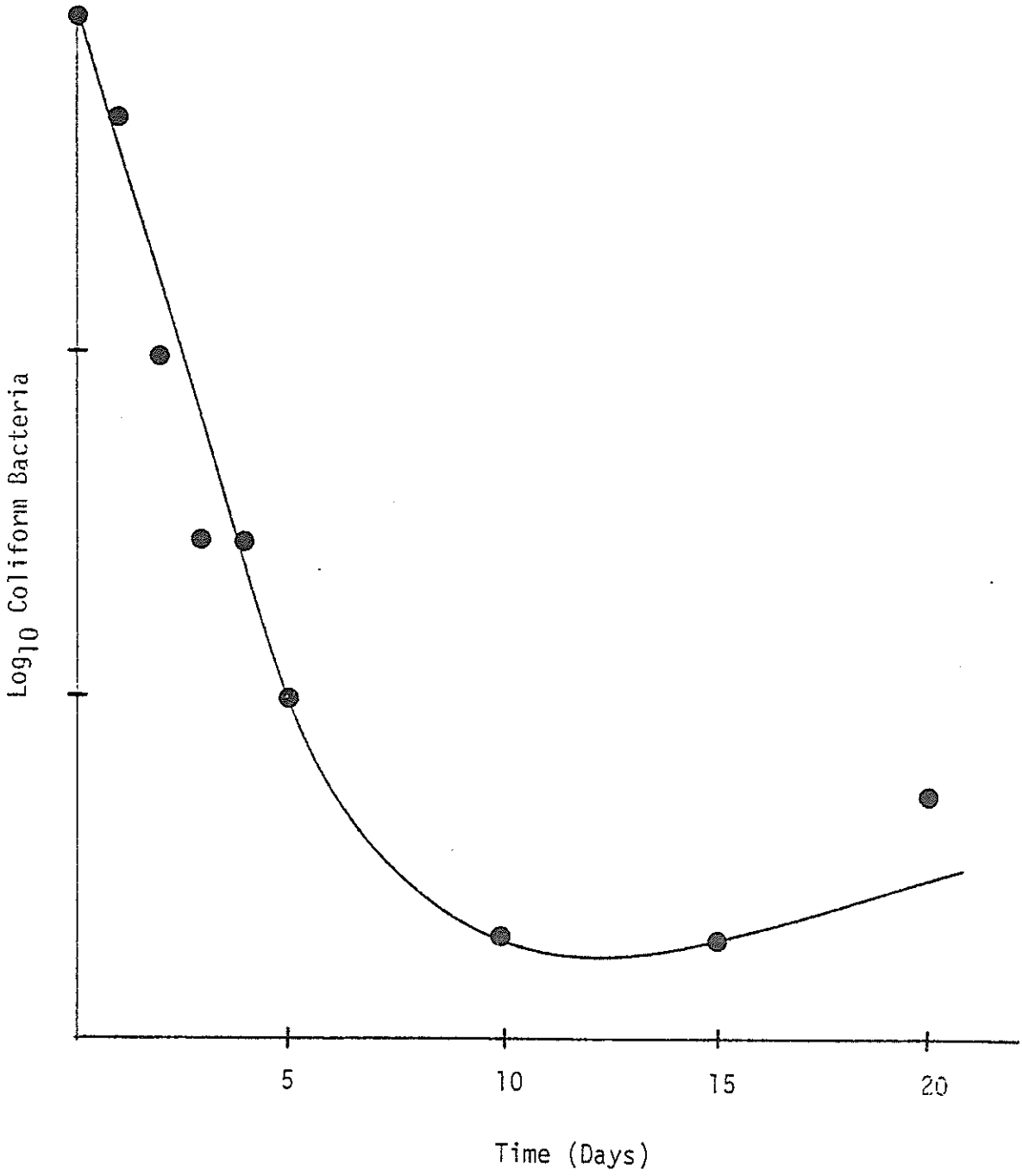

Figure 11. Coliform Bacteria in Septic Tank Liquor Incubated in a Dialysis Membrane Chamber

Septic tank liquor from the digestion chamber of the Plant Science Farm septic tank was inoculated with poliovirus and incubated in a dialysis membrane chamber suspended in the tank. Liquor samples were removed at intervals and assayed for coliform bacteria by the most probable number tube test technique according to Standard Methods (1).

12-month study period. Since bacterial counts were made aerobically, only aerobes and facultative anaerobes were counted. These data indicate that the bacterial population of a septic tank remains at some steady-state level.

Poliovirus Type 1 Survival in Groundwater Adjacent to a Septic Tank Drain Field

Groundwater samples seeded with poliovirus were suspended for up to 12 days in the groundwater observation wells using the chambers described in Figure 5. Positions of observation wells in the drain field are shown in Figure 4. The rate of inactivation of poliovirus, as shown in Table 5, was more rapid in the groundwater environment than in the septic tank chambers. Virus counts were reduced approximately 90 percent in 24 hours in each of the groundwater survival experiments compared with a 50 percent reduction in 24 hours and 90 percent reduction after 15 days in the septic tank. Groundwater studies could only be performed during June and July when river flow and irrigation schedules were such that groundwater levels in the septic tank drain field were at least as high as 10 feet below the surface.

The differences noted in the rates of poliovirus inactivation in septic tank liquor and groundwater led to a series of laboratory experiments to further characterize virus inactivation in these environments. Mixtures of septic tank liquor and groundwater were inoculated with poliovirus, incubated with stirring in a circulating water bath at 22°C and sampled at daily intervals.

Table 5. Survival of Poliovirus Type 1 in Groundwater Observation Wells[a]

	Percent Infectivity Recovered			
	Cluster 1[b]		Cluster 2	Cluster 4
Sample Day	12.5 ft	14.5 ft	14.5 ft	14.5 ft
0	100	100	100	100
1	10	9	4	-
2	-	7	-	-
3	13	6	3	11
6	6	.8	-	-
8	-	-	.2	6
12	-	-	.06	1

(a) Groundwater from observation wells in the drain field of the Plant Science Farm septic tank was inoculated with 1×10^7 PFU/ml poliovirus type 1 and was incubated in modified dialysis membrane chambers suspended in the appropriate well. Samples were taken at intervals and assayed on HeLa cell monolayers.

(b) Well cluster number and well depth as described in Figure 4.

The mixtures were designed to simulate the fluid gradient as tank effluent contacted groundwater. The data, presented in Table 6, show that although inconsistencies are apparent in the data, there are no differences between inactivation rates of poliovirus in septic tank liquor, groundwater, or in mixtures of the two fluids under the conditions of the experiment. In each system approximately 60 percent of the seeded viruses were recovered after 24 hours, and approximately 7 percent were recovered after 5 days. However, the data recorded in this experiment were not in agreement with either set of field data. In field studies, an average of 30 percent of viruses incubated in septic tank liquor were recoverable after 5 days while an average of only 4 percent of viruses incubated in groundwater were recoverable in the same period.

Studies in this laboratory by Yeager (55) have shown poliovirus to have a $\log_{10}$ reduction time of 5.9 days in septic tank liquor and 13.0 days in shallow groundwater. These data were obtained in a series of laboratory experiments. The data for septic tank liquors is very close to that recorded during the laboratory phases of this study, including the specific experiments reported in Tables 3 and 6. Apparently, small volumes of septic tank liquor held under laboratory conditions do not adequately reproduce the physical and chemical regime of a functioning septic tank. Virus survival in shallow groundwater may be a function of water history. All field studies were done when groundwater was flowing from irrigated plots toward the river. The

Table 6. Survival of Poliovirus Type 1 in Mixtures of Septic Tank Liquor and Groundwater[a]

Sample Day	Percent Infectivity Recovered				
	100% Septic Tank Liquor	25% Liquor 25% Groundwater	50% Liquor 50% Groundwater	25% Liquor 75% Groundwater	100% Groundwater
0	100	100	100	100	100
1	61	39	63	68	62
3	11	11	35	30	10
5	7	5	3	11	7

(a) Septic tank liquor from the sedimentation chamber of the Plant Science Farm tank was mixed with groundwater from the 12.5-foot observation well of cluster 2 (Figure 4) in the percentages given. These solutions were inoculated with 1×10^7 PFU/ml poliovirus type 1 and incubated at 22°C with constant stirring. Samples were taken at intervals and assayed for virus on HeLa cell monolayers.

sample used in the laboratory study was taken later in the year when the river was still flowing but irrigation at the site had ceased. Groundwater was flowing from the Rio Grande toward the drain field. Differences in survival have been noted previously between viruses incubated in shallow and in deep groundwater (55). Virus inactivation in Rio Grande water has been reported to be more rapid than in flood-plain groundwater (35).

In view of the inconsistencies between field and laboratory data and the variable data presented in Table 6, studies were undertaken to determine if adsorbtion of viruses to particulate matter in septic tank liquor was involved. In one experiment, septic tank liquor was inoculated with poliovirus and incubated at room temperature. Visible particulate material in the suspension was allowed to settle-out naturally. Every 15 minutes a sample of the suspension was removed for viral assay. After 1 hour the remaining suspension was centrifuged at 7710 xg for 10 minutes to pellet particulate material. A sample of the supernatant was taken. The pellet was resuspended in a volume of PBS equal to the supernatant and sampled. There was no consistant reduction in virus titer during the hour incubation period, and only 9 percent of the total virus inoculum was recovered from the pelleted material (Appendix Table 2). To quantitate virus association with solids more precisely over a longer period, a second experiment was done using [^{3}H]-uridine labelled poliovirus incubated in septic tank liquor for 70 hours. The virus-septic liquor suspension was centrifuged at 7710 xg for 10 minutes at 7-hour intervals. An aliquot of the supernatant was taken, and its radioactivity measured

by liquid scintillation spectrometry. The virus-septic tank sample was vortexed to resuspend the pellet after each centrifugation and sampling. There was a slow decline in radioactivity in the supernatant during the first 21 hours, followed by a steady rise to nearly the original count by the end of the experiment. No irreversible adsorption of virus to settlable particulates was noted in the studies (Figure 12). However, the results of the labelled virus experiment suggest that transient adsorption to particulates may have contributed to the variability of the data in other experiments.

Survival and Movement of Poliovirus Type 1 in Sandy Loam Soils

Septic tank liquor was seeded with [^{3}H]-uridine labeled poliovirus type 1 and the suspension was allowed to infiltrate soil in a restricted cone. A core of soil from beneath the area of infiltration was removed immediately and viruses were eluted from 1-cm slices of the soil core. A second core was removed 48 hours later. The distribution of radioactivity in both cores is shown in Figure 13. Eluates from 5 soil-core slices were used to inoculate HeLa cell monolayers. Eluates were chosen to check core slices which showed significant radioactivity. Viable viruses were recovered from all soil-core slices taken from soil depths moistened by the infiltrating liquid. The data in Table 7 suggest a large number of virus moved with the front of the infiltrating liquid.

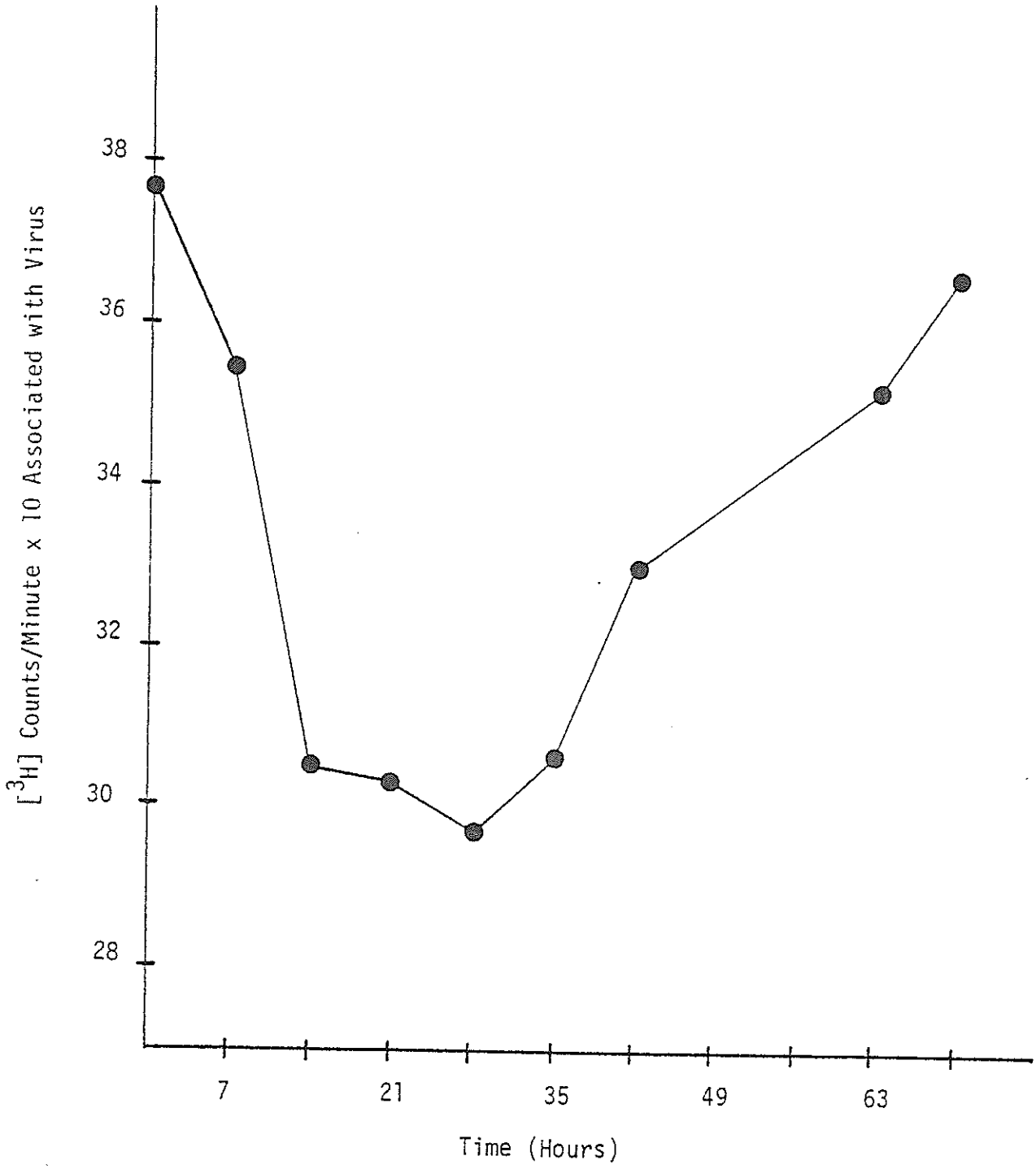

Figure 12. Adsorption of Poliovirus type 1 to Particulate Matter in Septic Tank Liquor

Polioviruses labeled with [^{3}H]-uridine were incubated in fresh septic tank liquor for 70 hours. The suspension was centrifuged at intervals to pellet particulate matter. The radioactivity in the supernatant was determined.

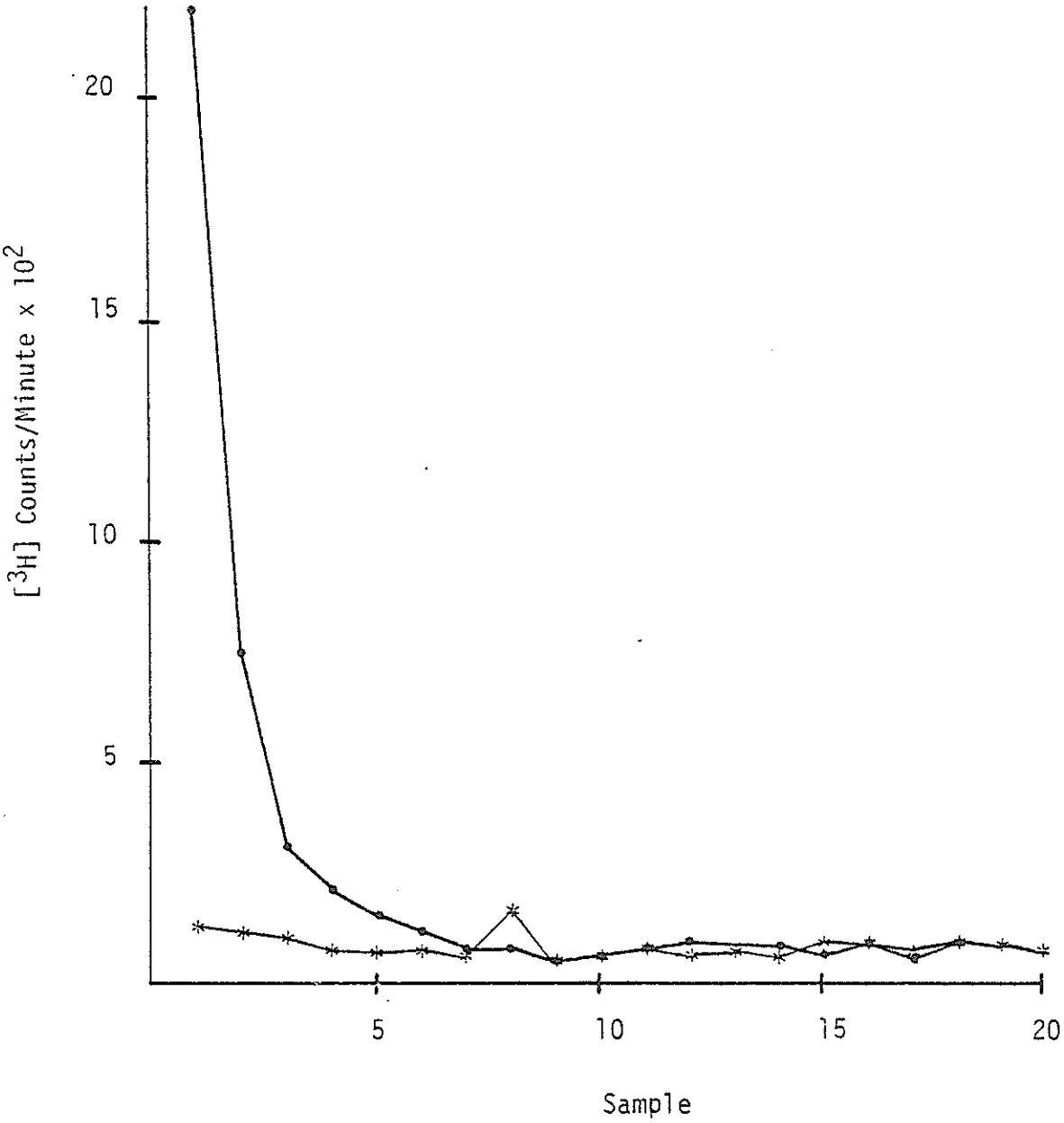

Figure 13. Distribution of [^{3}H]-Uridine Labeled Polioviruses in Soil Core

Septic tank liquor inoculated with [^{3}H]-uridine labeled poliovirus type 1 was allowed to infiltrate sandy loam soil. A 20 cm vertical soil core (●——●) was taken from the infiltration site, 1-cm-thick slices of the core were eluted with glycine buffer (pH 11.5) and the eluates assayed for radioactivity. A second core (*——*) was taken 48 hours later and treated in the same manner. Sample number indicated distance from the soil surface in centimeters.

Table 7. Virus Recovery From Soil Cores[a]

	Sample [b]	PFU/ml[c]
Initial Core	1	TNTC[d]
	6	TNTC
	7	TNTC
	13	230
	18	0
Core Taken 48 Hours Later	1	TNTC
	8	280
	10	250
	17	320
	20	TNTC

(a) [^{3}H]-uridine labeled poliovirus type 1 inoculated septic tank liquor was allowed to infiltrate undisturbed sandy loam soil. A 20-cm-long vertical soil core was taken from the infiltration site; 1-cm-thick slices of the core were eluted with glycine buffer (pH 11.5) and .2 ml of the eluates were assayed on HeLa cell monolayers. A second core was taken 48 hours later and treated in the same manner.

(b) Sample number indicates the depth from the soil surface of each 1-cm-thick slice of soil core. Samples which showed significant radioactivity were chosen (Figure 13).

(c) Plaque-forming units/ml of soil core eluate.

(d) Plaques too numerous to count.

Soil studies were repeated using unlabeled viruses. Cores were taken 7 days apart. Horizontal cores were taken before the second vertical core to determine whether the coring tool had carried virus to deeper soil locations. Infective viruses were recovered 17 cm below the surface in the initial core. Infective viruses were recovered 20 cm below the surface in the core taken 7 days after application of poliovirus seeded septic tank liquor. The number of plaque-forming units recovered from the cores is shown in Table 8. The original titer of virus in the septic tank liquor was approximately 1×10^7/ml. The number of viruses recovered cannot be used to estimate the fraction of virus surviving 7 days in soil because horizontal movement of viruses must be assumed and cannot be quantified. The soil slice eluants from the horizontal core were positive for virus only where the core crossed the zone of liquid inflitration. Infective viruses could be recovered for over 30 days from moist core slices kept in the laboratory at room temperature.

Viruses were not isolated from soil below the level of fluid infiltration. The core slices taken 17-20 cm below the soil surface in the initial core were dry and no virus were recovered from them. Similarly, core slices taken 21-23 cm below the surface in the day 7 vertical core were not positive for virus. Cores could only be taken when the soil was moist. These soil studies were conducted during the winter. Soil temperatures were low and there was a heavy rain between the time the initial core was taken and the second. The soil surface was moist to the

Table 8. Virus Distribution in Soil Cores[a]

	Plaque-Forming Units/.2ml Soil Eluate	
Vertical Core Sample[b]	Initial Core	Day 7 Cores
1	11 x 10^2	1 x 10
2	59 x 10^2	4 x 10
3	15 x 10^2	9 x 10
4	76 x 10	19 x 10
5	67 x 10^2	14 x 10
6	47 x 10	26 x 10
7	43 x 10	57 x 10
8	59 x 10	64 x 10
9	13 x 10	46 x 10
10	11 x 10	12 x 10
11	27 x 10	13 x 10
12	35 x 10	10 x 10
13	40 x 10	65
14	10 x 10	73
15	2 x 10	76
16	4	31
17	2	88
18	0	50
19	0	32
20	0	31
21	-[c]	0
22	-	0
23	-	0
Horizontal Core Sample[d]		
1-1		0
1-2		13
1-3		49 x 10
1-4		40 x 10
1-5		20
1-6		3
3-1		-
3-2		-
3-3		42 x 10
3-4		85
3-5		-
3-6		20

(a) Poliovirus type 1 inoculated septic tank liquor was allowed to infiltrate undisturbed sandy loam. A 20-cm-long vertical soil core was taken from the infiltrated soil; 1-cm-thick slices of the eluates were assayed for virus on HeLa cell monolayers. Seven days later 2 horizontal cores were taken across the infiltration site at depths of 1 and 3 cm below the soil surface and a second vertical core was taken. Core slices were made and treated as above.

(b) Sample number indicates the depth from the soil surface of each 1-cm-thick slice of soil core.

(c) Dash indicates sample could not be assayed.

(d) Horizontal core sample numbers indicate the depth from the soil surface of the core followed by the horizontal distance from the side cut made to allow sampling across the infiltration site

touch for the entire 7-day period.

Bacterial Counts of Groundwater

Bacterial counts on water samples from each of the 3 wells in the 5 original groundwater observation well clusters were made at 3-month intervals. Water was not pumpable from the shallower wells during the fall and none of the wells were pumpable during the winter. Total aerobic plate counts and most probable number tube tests for coliforms were run on all samples. Coliform bacteria were isolated from the wells only once during the 1977 irrigation season (March 1977 - October 1977). The 12.5 foot and 14.5 foot wells of cluster 2 were positive for coliforms and fecal coliforms in July when the area around the wells collapsed following heavy rains. Surface water channeled down along the well casings. Samples from both wells were negative for coliforms one week later. Bacterial counts in the 14.5 foot wells were consistently 20-30 x 10^2 CFU/ml; in the 12.5 foot wells, 100 x 10^3 CFU/ml; and in the 10.5 foot wells, 100 x 10^3 CFU/ml.

Wells were sampled in the spring of 1978 when water released from Caballo Reservoir reached the Plant Science Farm. There was a 4-foot rise in groundwater levels when the front of the approaching river was opposite the study area. Levels dropped 2 feet over the next 3 days. Water samples were taken from the 12.5 foot and 14.5 foot wells when the water level was highest. After a portion of each sample was removed for bacterial counts, the groundwater samples were frozen for later virus assay. Samples from the 14.5 foot well

of cluster 1, the 14.5 foot well of cluster 2, and the 12.5 foot well of cluster 4 were positive for coliform bacteria. In subsequent bacterial analyses during the summer of 1978, all well water samples were negative for coliform bacteria.

Isolation of Enteric Viruses from Groundwater Influenced by Septic Tank Effluent

Groundwater samples which were positive for coliform bacteria in the spring of 1978 were concentrated as previously described and assayed for virus on HeLa cell monolayers. Results of the isolation attempts are shown in Table 9. Viruses capable of infecting HeLa cells were isolated from each coliform positive sample. No viruses were isolated from coliform-negative groundwater samples run as a control. As in previous isolations, plaques formed on the HeLa cell monolayers had varying morphologies. No attempts were made to identify virus isolates.

Table 9. Isolation of Enteric Viruses from Groundwater[a]

Well[b]	PFU/240ml
Cluster 1, 14.5 ft	1
Cluster 2, 14.5 ft	3
Cluster 2, 14.5 ft	7
Cluster 4, 12.5 ft	6

(a) Groundwater samples found positive for coliform bacteria were concentrated by centrifugation and assayed for viruses on HeLa cell monolayers.

(b) Groundwater observation wells as identified in Figure 4.

DISCUSSION

The five questions posed in the introduction have been partially answered by this research effort. Viable enteric viruses were regularly isolated from properly functioning septic tanks. Poliovirus type 1, often used as a model for enterovirus, remained viable in contact with septic tank liquor for longer than the 24 hours necessary to pass through a tank and into a septic tank drain field. Once introduced into the drain field, poliovirus remained viable for extended periods, and moved with groundwater flows. In contact with the groundwater, poliovirus was rapidly inactivated but contact with soil seemed to have a protective influence. Enteric viruses were isolated from groundwater influenced by septic tank effluent.

Several questions have not been answered. The number of viruses normally found in septic tank liquor is obviously influenced by the size, age range, and health of the group using the system. How the operation of septic tank systems affects this number is not known. It is possible that newer, efficient systems may inactivate enterovirus more rapidly or may trap more virus in the sedimenting solids than older or malfunctioning systems. This would explain the differences between the findings of this study and the greater numbers reported elsewhere. Differences in numbers may be reflective of naturally occurring variation. A survey of functioning septic tanks for virus and bacteria concentrations would answer this question. It is also possible that the method of gaining

continuous access to the New Mexico State University Plant Science Farm septic tank affected the results. The caps used to seal the access pipes were not sufficiently tight to maintain anaerobic conditions in the settling chamber. The access pipe in this chamber rested on projections above the normal fluid level. Flies, apparently hatching in the air space between pipe and liquor surface, were found in the pipe on several occasions during the warmer months. Insecticide was sprayed on the sides of the pipe to control the problem. The temperature in the tank chambers was also affected by the access pipes. Air temperature in the pipes was within a few degrees of ambient air temperature, changing with the season and time of day.

Poliovirus type 1 was used as a model for enterovirus in this study. Data for poliovirus is often assumed to be representative of all viruses in this group. The validity of this assumption should be questioned. Coxsackievirus B-1 has been shown to survive longer than poliovirus type 1 in septic tank liquor (55). Echovirus 11 is less sensitive than poliovirus to inactivation during anaerobic sludge digestion (8). Of particular concern is whether poliovirus models hepatitis A virus.

Between 1946 and 1974, 31 waterborne outbreaks of hepatitis were reported (15). Each resulted from the use of untreated groundwater. It is strange that outbreaks due to other enteroviruses have not been reported. This could be explained by the premise that hepatitis A virus is more resistant to inactivation than the other enteroviruses. It is also possible that waterborne outbreaks of

other enterovirus diseases do occur but go unrecognized. Hepatitis is a reportable disease with a fairly discrete set of symptoms while other enteric viral infections may be subclinical in nature or give transitory distress for which professional help is not sought. A retrospective study done in Israel of a large number of kibbutzim offers evidence that even overt enteric disease may not be recognized (24). In 77 kibbutzim practicing spray irrigation with untreated wastewater, the summer incidence of infectious hepatitis (probably hepatitis A), confirmed shigellosis, salmonellosis, and typhoid fever was 2 to 4 times higher than in 130 kibbutzim which did not irrigate with wastewater. Individuals in the affected kibbutzim had no awareness of the higher incidence of illnesses (6).

The effect of soils and groundwater on virus survival is almost completely undefined. There are 102 recognized soil associations in New Mexico alone (2). Waters percolating through soils to the groundwater table dissolve and carry with them a variety of chemicals representative of the overlying soil. Each soil type and the groundwater associated with it may inactivate enterovirus at a different rate. Only a few generalizations have been made in the literature (51) and there were supported in this study. Poliovirus were detected in soils at the study site for longer periods of time than were expected from their survival when suspended in the groundwater observation wells. Adsorption of virus to solids has been reported to increase virus survival time (13). Viruses were easily eluted from soils in this study and were infective. Whether the viruses were adsorbed to soil particles or held in soil-associated fluids

is not known. Viruses have been reported to desorb from soil without loss of infectivity (17). Virus adsorbed to solids can still be infective (34). The apparent disagreement between laboratory and field studies in this effort emphasizes the need to understand the principles of virus inactivation mechanisms. Laboratory results give insights into inactivation processes but care should be taken in assigning too much importance to this data in predicting virus survival in the environment.

Previous studies have indicated that the organics in wastewater reduce the adsorption capacity of silicate mineral soils as well as the rate of adsorption. This would suggest that enteric virus could move with septic tank effluent infiltrating drain field soils and may not be adsorbed, particularly as drain fields become saturated with organic material. The peak of [^{3}H]-uridine radioactivity 13 cm below the soil surface, shown in Figure 13, has not been explained. It may represent the mean depth of percolation of the pulse of virus-seeded septic tank liquor. Other soil experiments did not show a peak. Table 8 shows a steady decline in detectable virus with depth. The soils of the study area were dry before the labeled virus experiment but fairly damp before the other runs. The soil moisture content should affect the rate of liquor percolation and virus movement.

The recovery of enterovirus from groundwater has been previously reported (52). What is of interest in this study is the pattern of recovery. Coliform bacteria were isolated from the groundwater observation well samples only during the "Spring Flood". In the

Mesilla Valley this is a controlled event. The flood gates of the Caballo Reservoir Dam are opened to release water for the Elephant Butte Irrigation District each spring after a winter period during which no water flows in the bed of the Rio Grande or in the irrigation canals. Groundwater in the study area was more than 14 feet below the surface during the winter. When the "flood" came in the spring, there was a sudden 4-foot rise in groundwater level. A wedge of water from the river moved not only south in the river bed but also east and west below the low-lying areas on either side. This wedge of water apparently moved bacteria and viruses from the soil directly under the septic tank drain downward to the wells. When the normal summer water table was reestablished under the influence of continuous river flows and irrigation of surrounding fields, there was probably no further downward washing through the drain field resulting in no isolation of bacteria or viruses during the summer. The effluent reaching the drain field is probably rapidly evaporated from the soil. This idea is supported by the lack of chemical pollution reaching the observation wells (42). It is not surprising that the 14.5 foot well of cluster 4 yielded coliform bacteria and virus. Winter groundwater flow is toward the river.

The phenomenon of persistence until conditions in soil permit movement described above merits further study. Pathogens in general survive longest in the environment during cool weather. Enterovirus in particular survive freezing. In many areas of the country, soils freeze in the winter. During spring snow-melts and general flooding, soils in residential areas become saturated including the soils of

septic tank drain fields. Viruses may enter the soil with septic tank effluents during the winter when the ground is partially frozen and move to the surface during spring flooding. This speculation is supported by observations in the Ohio Valley during the 1950's. Severe diarrhea was common among preschool children and young adults during the spring. The disease was blamed on the flooding Ohio River. It is possible that the disease was tied to septic tank overflows. Systems often backed up at this time. Since small children play in the mud more than older children and their mothers are the ones who remove muddy boots, one would expect a higher incidence of disease in these groups. No record of spring-flood-associated diarrhea has been located in the available literature, but there are still residents of Hamilton County, Ohio, who expect diarrhea when the Ohio floods. Epidemiologic studies to identify enteric disease of viral origin are needed. Folklore of the type mentioned above is a logical starting place. Testing the groundwater in the observation wells at the Plant Science Farm every spring for several years is another.

Throughout the study reported here, laboratory experiments did not correlate with field studies. While the controls expected of laboratory experiments are rarely possible in the field, there is a great need to study pathogens in the undisturbed natural environment. Disposal of human wastes through septic tank systems may pose a threat to public health. This threat can only be defined by looking at functioning systems.

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APPENDIX

MEDIA

A. Cell Culture

1) Eagle's Minimal Essential Medium (MEM)

Eagle's Basal Diploid (Gibco; Berkley, CA; Cat. No. G-13)

Penicillin	100 g/ml
Streptomycin sulfate	100 g/ml
Fungizone	25 g/ml
Newborn calf serum (Inactivated at 56°C for 30 minutes before use)	10%
$NaHCO_3$	2.5%(v/v)
Bacto tryptose phosphate broth	10%(v/v)

2) Trypsin stock (1%)

Bacto trypsin (1:250)	10 g
$NaHCO_3$	2 g
GKN medium (10x)	100 ml
Distilled water	900 ml

3) $NaHCO_3$ Solution (7.5%)

$NaHCO_3$	75 g
NaCl	9 g
Distilled water	1000 ml

4) GKN Medium (10x)

Glucose	10 g
NaCl	80 g
KCl	4 g
Phenol red (1% in 0.3 N NaOH)	1 ml

5) Working Trypsin (0.05%)

Stock trypsin (1%)	50 ml
$NaHCO_3$ solution	1.5 ml
EDTA solution	50 ml
Distilled water	900 ml

6) EDTA Solution

Disodium ethylenediaminetetraacetic acid	1.86 g
Tetrasodium ethylenediaminetetraacetic acid	1.90 g
Distilled water	1000 ml

B. Enterovirus Assays

1) Phosphate-buffered Saline (PBS)

KCl	2 g
NaCl	80 g
Na_2HPO_4	11.5 g
K_2HPO_4	4 g
Distilled water	1000 ml

2) Overlay Agar

MEM medium		440 ml
$MgCl_2$ (0.3 M in GKN medium)		60 ml
$MgCl_2$	17.9 gm	
GKN 10x	100 ml	
H_2O	900 ml	
Ion agar		100 ml

3) Ion Agar

Noble agar	2.4 g
GKN	100 ml

4) Alcoholic Crystal Violet

Crystal violet	5 g
Ethanol (95%)	40 ml
Distilled water	960 ml

C. Bacterial Assays

1) Lauryl Sulfate Tryptose Broth

Tryptose	20 g
Lactose	5 g
KH_2PO_4	2.75 g
K_2HPO_4	2.75 g
NaCl	5 g
Sodium lauryl sulfate	0.1 g
Distilled water	1000 ml

2) Nutrient Agar

Beef extract	3 g
Peptone	5 g
Agar	15 g
Distilled water	1000 ml

3) Plate Count Agar (Tryptone Glucose Yeast Agar)

Peptone-tryptone	5 g
Yeast extract	25 g
Glucose	1 g

Agar	15 g
Distilled water	1000 ml

4) Eosin Methylene Blue Agar (EMB Agar)

Peptone	10 g
Lactose	10 g
K_2HPO_4	2 g
Agar	15 g
Eosin Y	0.4 g
Methylene blue	0.065 g
Distilled water	1000 ml

D. Soil Elution

1) Calf Serum Eluent (pH 11.5)

Glycine	11.3 g
EDTA	6.5 g
Fetal calf serum	15 ml
Distilled water	1500 ml

Table 10. Bacteria in Septic Tank Liquor Held in Dialysis Membrane Chambers.[a]

	Number of Bacteria								
Sample Day	0	1	2	3	4	5	10	15	20
Sedimentation Chamber									
MPN[b]	(100)[d]	(20)	(3)	(2)	(5)	(2)	(.4)		
	23×10^4	46×10^3	72×10^2	43×10^2	12×10^3	46×10^2	10×10^2	-	-
TPC[c]	(100)	(5)	(1)	(1)	(2)	(1)	(1)		
	99×10^5	49×10^4	100×10^3	110×10^3	170×10^3	91×10^3	120×10^3	-	-
Digestion Chamber									
MPN	(100)	(52)	(10)	(2)	(2)	(.9)	(.2)	(.2)	(.5)
	46×10^4	24×10^4	46×10^3	11×10^3	11×10^3	40×10^2	10×10^2	10×10^2	24×10^2
TPC	(100)	(11)	(8)	(10)	(7)	(4)	(10)	(6)	(9)
	250×10^4	270×10^3	200×10^3	250×10^3	170×10^3	100×10^3	240×10^3	140×10^3	230×10^3

(a) Septic tank liquor from both chambers of the Plant Science Farm septic tank was inoculated with poliovirus and incubated in dialysis membrane chambers suspended in the appropriate tank. Liquor samples were removed at intervals and bacterial analysis was run.

(b) Most probable number tube test estimate of coliform bacteria.

(c) Total aerobic plate count.

(d) Percentage of initial bacterial count.

Table 11. Association of Viruses with Particulate Matter in Septic Tank Liquor - One-Hour Study.[a]

Sample		Percent Infectivity Recovered
Solution at Time	0 min	100
	15 min	73
	30 min	90
	45 min	125
	60 min	80
Fraction of Solution after Centrifugation		
	Supernatant	70
	Resuspended Pellet	9

(a) Fresh septic tank liquor from the Plant Science Farm tank was inoculated with poliovirus type 1 and incubated at room temperature with constant stirring. Samples were taken every 15 minutes and assayed for viruses on HeLa cell monolayers. After 1 hour the suspension was centrifuged to remove particulate matter. The supernatant and the resuspended pellet were assayed for virus.

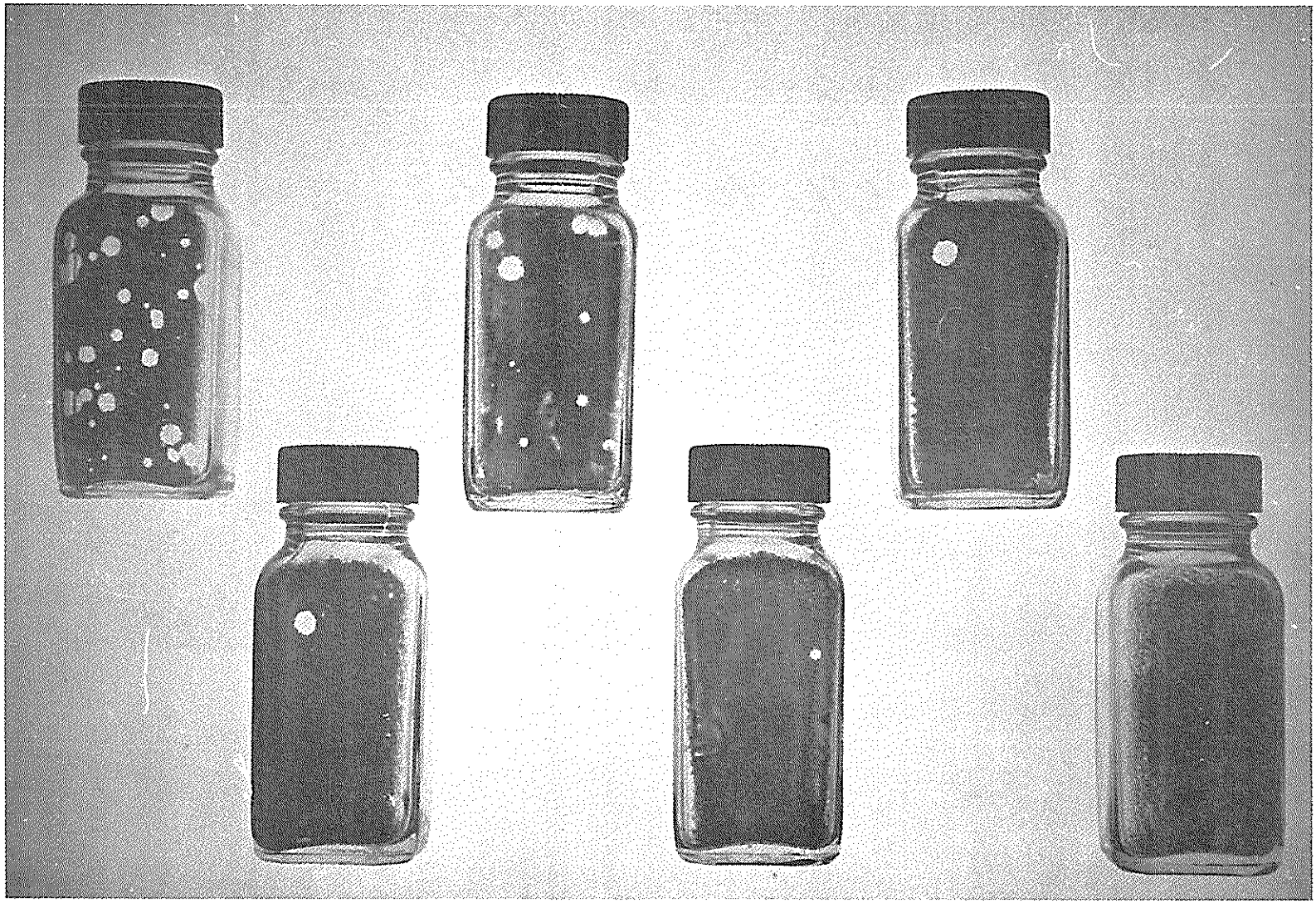

Figure 14.

Examples of plaques obtained on HeLa cell monolayers with poliovirus type 1 and indigenous viruses recovered from septic tanks.