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MASTER

DATA REPORT

THE FATE OF HUMAN ENTERIC VIRUSES IN A NATURAL SEWAGE RECYCLING SYSTEM

JAMES M. VAUGHN AND EDWARD F. LANDRY

September 1980

DEPARTMENT OF ENERGY AND ENVIRONMENT

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ABSTRACT

A two-year study was conducted to determine the virus-removing capacity of two man-made ecosystems designed for the treatment of raw domestic wastewater. The first treatment system consisted of two meadows followed by a marsh-pond unit (M/M/P). The second system contained individual marsh and pond units (M/P). All systems demonstrated moderate virus removal, with the marsh/pond system yielding the most consistent removal rates. Within this system, the greater potential for virus removal appeared to occur in the marsh unit.

In addition to the production of system-oriented data, improved techniques for the concentration and enumeration of human viruses from sewage-polluted aquatic systems were developed.

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I. INTRODUCTION

The increasing demands, with respect to efficient disposal of domestic wastewater and the establishment of new sources of potable water, have prompted a search for a means of supplementing the groundwater aquifer with renovated wastewater effluents. Included among proposed methods are: spray irrigation; deep well injection; basin recharge; and "overland flow schemes" using natural land and aquatic ecosystems. Inherent in any scheme of wastewater reuse or recycling are the potential hazards posed by pathogenic microorganisms which inhabit the human alimentary tract, and are commonly associated with domestic wastewater. Included among the pathogenic and parasitic milieu of human wastes are a variety of fungal and protozoan species, bacteria (e.g. Salmonella, Shigella, Vibrio, etc.), and human viruses (enteroviruses, adenoviruses, hepatitis viruses, etc.). In recent years, major land treatment concern has been directed towards the human viruses by virtue of: 1) their enhanced potential for producing disease; 2) their ability to survive the rigors of conventional sewage treatment and subsequent disinfection; 3) their likely survival capacity through the various effluent receiving modes (e.g. fresh and saltwater discharge, recharge, etc.); and 4) the fact that, to data, few studies have been conducted to delineate the full impact of viruses on the various proposed land treatment methods.

The major human virus groups known to occur in human sewage include: 1) Enteroviruses - transient members of

the human alimentary tract which include over 150 serotypes included within the Poliovirus, Coxsackievirus and ECHOvirus groups; 2) Adenoviruses - upper respiratory viruses which can survive the rigors of the human alimentary tract and be shed in the feces; 3) Hepatitis virus and 4) Rotaviruses - Reovirus-like particles which cause diarrheal diseases in infants and adults. While only Hepatitis and Poliovirus have been conclusively proven to be transmitted via the water route, studies have indicated the likelihood of such transmission for many or all of the above groups. A partial list of sewage-associated viruses and their diseases is presented in Table 1.

A number of field studies have been discussed in the literature which indicated that viruses might be effectively removed from sewage effluents by movement through or over soil. At the Santee Water Reclamation Project, chlorinated sewage effluent percolated through 400 feet of sand and gravel was used to supply waters for a recreational lake (29). Out of 128 samples, only two showed positive viral isolations. After seeding treated wastewater with high concentrations of Polio type 3, no virus could be detected after passage through 200 feet of sand reclamation bed. It should be noted that the authors used swabs and gauze pads as water sampling devices, and these methods do not represent very effective means of recovering viruses under field conditions. Gilbert et al., (18) found that percolation through 60-90 cm of fine loamey sand was sufficient to remove over 99% of total coliforms, fecal coliforms, and fecal streptococci (Gilbert et al., 17).

TABLE 1

HUMAN VIRUSES COMMONLY FOUND IN SEWAGE

AND DISEASES ASSOCIATED WITH THESE DISEASES

GROUP	SUBGROUP	NO. OF SEROTYPES	TYPE OF NUCLEIC ACID	DISEASES
Enterovirus	Poliovirus	3	RNA	Mild-Severe Gastroenteritis Abortive Poliomyelitis Aseptic Meningitis Paralytic Polimyelitis
	Coxsackie-virus			
	A	24	RNA	Summer Minor Illness Herpangina Aseptic Meningitis Common Cold Hand, Foot & Mouth Disease Infant Diarrhea
	B	6	RNA	Aseptic Meningitis Common Cold Pleurodynia Neonatal Disease Sudden Infant Death Syndrome Myocarditis Pericarditis
	Echovirus	34	RNA	Aseptic Meningitis Mild Paralysis Febrile Illness Conjunctivitis Boston Exanthem Disease Infant Diarrhea Vaginitis & Cervicitis Pericarditis & Myocarditis
Hepatitis	A		RNA?	Infectious (viral) Hepatitis
	B		DNA?	Serum Hepatitis
Adenovirus		31	DNA	Acute Respiratory Disease Pharyngoconjunctival Fever Primary Atypical Pneumonia Epidemic Keratoconjunc-tivitis (shipyard eye) Intussusception Febrile catarrh
Rotavirus		?	RNA	Infant & Adult Diarrhea

A number of studies have detected the presence of viruses in groundwater following the recharge of sewage effluents through sand basins. Hori et al., (23) studying the movement of Poliovirus type 2 through Oahu Island soils found instances of viral contamination of groundwater despite the good removal characteristics of the soil. The authors concluded that the possibility of groundwater contamination existed if the underlying soil was interrupted by fissures and fractures, resulting in channeling of the percolating waters. In a study of the rapid infiltration of viruses through silty sand and fine gravel, Schaub and Sorber (34) demonstrated the sporadic occurrence of enterovirus in groundwater. Laboratory experiments confirmed the poor removal qualities of the test soil used in their field experiments.

The probable mechanisms of virus removal during movement through or across soils includes: adsorption, filtration, biomass utilization, photochemical reaction, and thermal inactivation (8, 32). Of these, adsorption appears to play the major role. The adsorption process is strongly influenced by a number of factors including: the pH of the recharged water; the chemical composition of the soil; the moisture content of the soil; and the rate of recharge (16). Since viruses are electrically-charged colloidal particles, consisting of an inner core of nucleic acid surrounded by a protein coat, the pH and ionic strength of the surrounding medium greatly influences the ability of the virus to adsorb to soil particles. Drewry and Eliassen (8) demonstrated this pH dependence in a study of bacteriophage adsorption to different types of soils. They found

that maximum adsorption occurred when pH values were below the isoelectric point of the virus particle. Under these conditions, the virus would be positively charged and electrostatically attracted to the negatively charged soil.

The ionic strength of the adsorbing environment was also found to be an important factor in the attachment of virus particles. Wellings et al., (47) studied the ability of soil within a cypress dome to remove enteric viruses present in treated sewage effluent. No isolations were observed during the first five months, however, three isolations of virus from groundwater were later reported following a period of heavy rainfall. The authors concluded that the rainfall resulted in an increase in the water/soil ratio which acted to desorb viruses, allowing them to move towards the aquifer. A similar desorption effect was seen when deionized water was added to 250 cm calcareous sand columns used to recharge sewage effluent (26). The virus, which had been previously adsorbed to the top cm of the soil column, moved down the column readsorbing at a lower level. Desorption was minimized by drying the columns one day between applications of the sewage, or by addition of cations to the effluent. The investigators concluded that desorption was due to a reduction in the ionic strength of the soil. In a similar study Duboise et al., (9) reported that a specific conductance of 700-800 microohms per cm (Mohms/cm) was necessary for maximum retention of virus to soil. The addition of distilled water to simulate rainfall diluted the ionic capacity of the soil and freed the virus. It should be noted here that much of the information concerning virus-soil interactions has been developed for relatively few

virus types. Recent studies have demonstrated significant variability when a wide variety of viruses are tested (13, 27).

Although viruses are readily adsorbed to soils during the process of recharge, they can remain viable for significant periods of time. Moore et al., (30) found that the Poliovirus adsorbed to organic and inorganic particulates was still infective. Schaub and Sagik (33) reported that clay-adsorbed virus retained its infectivity in tissue culture monolayers, and in mice. Bagdasaryan (3) studied the survival of enteric viruses in soil and concluded that survival was dependent on the pH of the soil, its moisture content, the nature of the soil and its temperature. Sandy soil at a pH of 7.5 provided the best conditions for virus survival, with Polio type 1 surviving for 170 days at 3-10 C. Wellings et al., (47) reported the isolation of Poliovirus in a groundwater well below a recharge basin 28 days after application of a sewage effluent was terminated. Dubois et al., (9) found poliovirus capable of surviving 84 days in soil at 3 C. Increasing the temperature to 20 C resulted in a 99% inactivation in 84 days. A similar study by Tierney et al., (38) detected poliovirus after 96 days in irrigated soils during the winter. Summer survival in soil was significantly shorter, the test organisms lasting only 11 days.

Much of the virus-soil interaction data which has been produced over the past 15 years is somewhat extrapolable to overland-flow type land-treatment systems. A recent study conducted by Schaub et al., (32) dealt directly with virus removal in an overland flow system (comparable to our Meadow System - see Materials and Methods). The authors observed

removal rates of 30-60% of a poliovirus seed. They concluded that while overland flow appeared to be a relatively effective method for removal of this virus, it would be unwise to assume similar removal efficiencies for other virus types.

The virus-removal efficiency of natural aquatic ecosystems (as a method for sewage renovation) cannot be similarly extrapolated from an appropriate surrogate system, and little direct data on the subject currently exists. Ideally, natural treatment systems should be studied individually in order to accurately predict the relative impact of viruses on each. This document presents data accumulated during a two-year study of a lowland sewage recharge system which made use of both land (i.e. meadows) and aquatic (marsh, ponds) ecosystems for the treatment and renovation of wastewater. The primary concern of the study was to provide information on the movement and survival of human viruses through each pilot system in an effort to delineate its overall virus-removing capability. The study represented the first concerted effort to relate human virus fate to such varied natural treatment ecosystems.

II. METHODS AND MATERIALS

A. Study Site

The site chosen for the study included two artificial wetlands sewage reclamation semi-works operated at Brookhaven National Laboratory. Their precise design and history have been described previously (48, 49, 36, 20). Briefly, the systems were designed as follows*.

*System design summary adapted from Small (37). Reprinted here with permission of the author.

The Meadow/Marsh/Pond (M/M/P) System contained two Reed Canary Grass meadows of 0.08ha each, followed by a 0.08ha Typha marsh followed by a .08ha pond about 1.5m deep. The Marsh/Pond (M/P) was similar, but omitted the meadows. Both were underlain by a barrier membrane. Each system received screened, comminuted, aerated, raw, unsedimented sewage. Both systems polished their effluent by surface application to a wooded, sandy-soil aquifer recharge area. Schematics of both are shown in Fig. 1.

The wetlands began operation in the Spring of 1973. Since Spring 1975, both have been in continuous, on-line operation, reclaiming water from blends of septage and sewage, septage and sewage alone, sludge from septage lagoons and from conventional primary plants and, raw solids from the BNL sewage system clarifier. The wetlands operated throughout the year, without producing odor or vermin (with no regular maintenance), at application rates between 420 and 835 m^3/ha-d. Both ponds were stocked with fish and freshwater clams. Wildlife have frequently been attracted to these natural settings.

No permanent sediment build-up has been noticed over the five-year operation of either system, despite the fact that no sludge has been removed from the sewage at any point. Hay is harvested from the meadows at the rate of 18 Mg/ha y dry weight. Duckweed is harvested from the M/M/P ponds. Both crops are fed to horses. The M/P is not cropped. The characteristics of the influent sewage and pond effluent are presented in Table 2.

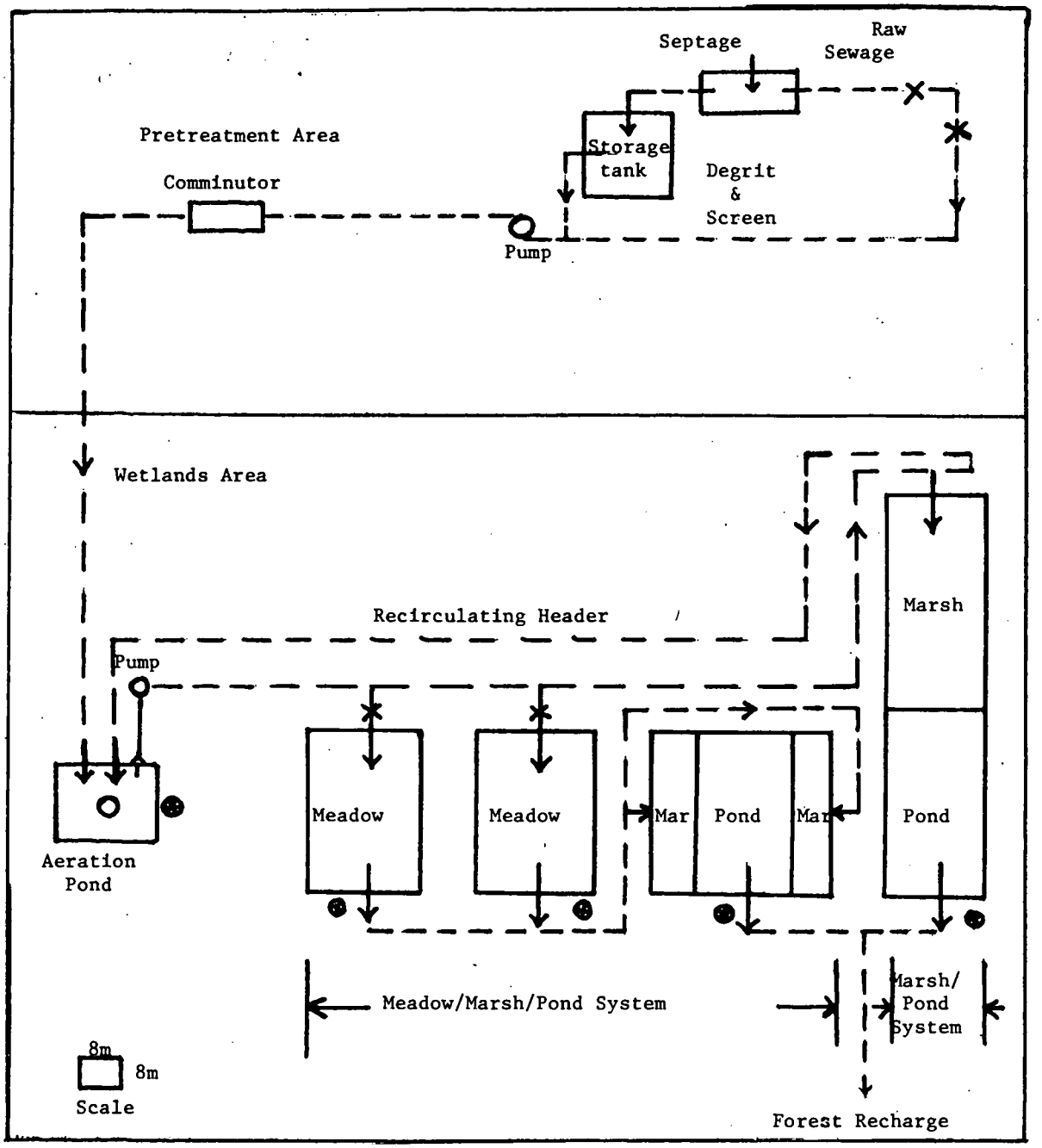

Figure 1. Land and Aquatic Ecosystem Schematic

TABLE 2

CHARACTERISTICS OF INFLUENT SEWAGE AND POND EFFLUENTS

(in mg/ℓ except for pH and as noted)

(From: Small, 37)

	INFLUENTS		M/P EFFLUENT		M/M/P EFFLUENT	
Contaminant	Mean	Max.	Mean	Max.	Mean	Max.
Total Solids	562	5,300	206	300	180	365
Total Volatile Solids	335	3,640	102	142	87	241
Total Suspended Solids	353	4,300	43	100	39	104
Total Volatile Suspended Solids	235	3,050	35	76	28	70
Total Dissolved Solids	208	1,000	163	242	140	308
Biochemical Oxygen Demand (BOD)	170	2,700	19	46	13	62
Chemical Oxygen Demand	495	7,900	58	120	48	120
Total Nitrogen (liquid + solid)	25	91	9.5	18	5.2	15.6
Kjeldahl Nitrogen (liquid + solid)	19.7	88	6.8	14	3.7	12.6
Ammonia Nitrogen (liquid)	8.4	18	3.5	11.5	1.2	6.8
$(NO_2 + NO_3)$-N (liquid)	5.5	17	2.6	6.7	1.5	3.2
Total Phosphorous (liquid + solid)	7.2	27.7	2.1	4	1.6	5.3
Orthophosphate-P (liquid)	4.8	22	1.3	3	1.1	4.6
Total Coliform (#/100ml)*	59.6	20,000	2	234	2.25	127
Fecal Coliform (#/100ml)*	1.6	1,000	.05	10.6	.03	4.5
pH	6.8	8.9	7.4	9.1	6.9	9.2
Turbidity (J.U.)	43	400	8.5	74	4.8	71
Water Temperature (°C)	10	22	11	24	10.6	24
Specific Conduc. (μ mhos)	464	6,600	262	340	224	315
MBAS (ABS)	.3	3	.24	1.4	.3	2.9
Calcium	20	72	14	26	14	47
Chloride	35	110	30	46	29	85
Chromium	.05	.5	.01	.03	.02	.3
Copper	.7	3.2	.03	.14	.04	.2
Fluoride	.5	1	.4	.6	.3	.5
Iron	3.6	20	1.2	5.5	1.5	3.9
Magnesium	4.3	8.5	3.6	6.3	3.3	4.4
Manganese	.14	.75	.1	.3	.1	.5
Potassium	5	11	4	9	3.1	11
Sodium	26	52	25	52	22.8	30
Zinc	1.3	4	.2	.6	.2	.7

*Multiply value by 10^3: Means are geometric.

B. Virus, host cell and bacterial stocks

Plaque-purified poliovirus type 1 (LSc-2ab) was propagated on low-passage Buffalo Green Monkey kidney cells (BGM, 6), and was prepared in monodispersed cultures by the procedure of Jakubowski et al. (24). Non-vaccine strains of polio type 2, obtained from field samples during an earlier study (42), were propagated and prepared in a similar manner. Cultures of bacteriophage MS-2, an RNA-containing coliphage, and its host bacterium Escherichia coli p4x6 were obtained from Dr. R. M. Zsigray, Department of Microbiology, University of New Hampshire. Cultures of E. coli B and Salmonella typhimurium, obtained from the American Type Culture Collection (ATCC), were propagated in nutrient broth, unless otherwise indicated.

C. Virus Assay

Samples (including concentrates) were treated with chloroform for 30 min to limit bacterial contamination, and were diluted in appropriate volumes of phosphate-buffered saline (pH 7.2). Sample volumes of 0.5 ml were inoculated onto monolayers of BGM cells in 25-cm^2 flasks. After a 60-min adsorption period with rocking, inocula were decanted and replaced with 4 ml of a neutral red agar overlay (22). Flasks were incubated at 36 C, and observed for plaque formation for a period of 7 days. Bacteriophage assays used the agar overlay technique of Adams (1).

D. Bacterial Assay

Enumerations of E. coli from survival experiments were carried out using a pour plate method. After dilution in appropriate volumes of phosphate buffer (pH 7.2), 0.5 ml volumes were pipetted into 100 x 17 mm Petri dishes.

Thirty-four ml volumes of molten (42 C) deoxycholate-lactose agar were added to each dish which was then rotated to allow uniform distribution of the sample. Dishes were incubated at 36 C and read for colony formation at 24 and 48 hr. Assay of samples containing Salmonella species involved the use of a spread plate technique. Appropriately diluted 0.5 ml sample volumes were pipetted to 100 x 15 mm Petri dishes containing solidified Salmonella-Shigella (SS) agar. Samples were then spread across the surface of the agar with a sterile glass rod. Plates were incubated at 36 C and checked for colony formation at 24 and 48 hr.

Total and fecal coliform determinations from field samples were carried out using standard membrane-filtration (MF) techniques (2).

E. Field Sampling

Routine sampling was carried out on a weekly basis for the period 1/77 to 11/78. Samples were collected from the points indicated in Figure 1, which included: holding pond waters; Meadow 1 effluent; Meadow 2 effluent; Marsh/Pond 1 effluent; and Pond 2 effluent (meadows were usually operated on an alternating basis, samples were therefore collected only from the operating meadow). Samples for coliform analyses were collected as 24-hr composites in sterile 1-gallon containers. Large volume samples for virus analysis were collected in 55-gallon containers. Between collections, containers were thoroughly rinsed with tap water, sanitized with 1.2 N hydrochloric acid for 30 min. and rinsed once again with tap water. Immediately before actual sample collection, containers were rinsed with 10 to 20 gal (cu. 37.9 - 65.7 ℓ) of sample water. Pumping equipment (impeller pumps, hosing)

was also subjected to acid rinse and flushing with sample water. These precautions were taken to obviate any chance of cross-contamination between samples.

Viruses in large-volume water samples were initially concentrated by means of an Aquella virus concentrator (Carborundum Corp.). Appropriate sample volumes were pumped through a series of prefilters to remove debris. Sample pH was then adjusted to 3.5, and aluminum chloride was added to a final concentration of 0.0005 M. The water was then passed through a virus-concentrating filter series consisting of a fiberglass depth cartridge (K27) and an epoxy-fiberglass-asbestos microfilter sandwich (0.45 and 1 μm, Cox-AA45 and AA100). Elution of adsorbed virus was carried out with 2-liter volumes of 0.1 M glycine at pH 11.5. Eluates were then neutralized to pH 7.5 with an equal volume of pH 2.0 glycine. The concentration procedures routinely yielded a final volume of 4 ℓ, which was reconcentrated in the laboratory by means of an inorganic flocculation procedure (10) to a final volume of 25-50 ml. After the addition of 10% fetal calf serum, samples were stored at -72 C to await assay.

The above concentration, reconcentration method was used during most of the field sampling phase of the project. During the latter portion of the study, however, a new method was tested and incorporated into the remainder of the virus monitoring program. A description of the new method and its efficiency of virus recovery can be found in "G" of this section and in the Results Section.

F. Survival of Microorganisms in Aquatic Ecosystems

In order to determine the stability of various microorganisms in natural aquatic ecosystems, a series of survival experiments were carried out in the waters of Pond #2. Unless otherwise indicated, the procedure for each survival study was as follows. Autoclave-sterilized, filter-sterilized, or untreated volumes (usually 100 ml) of Pond #2 water were inoculated with various concentrations of appropriate microorganisms (survival studies were carried out on cultures of *E. coli* B, *Salmonella typhimurium*, bacteriophage MS-2 and Poliovirus type 1 LSc-2ab). Seeded volumes were then placed in Membrane Diffusion Chambers (28) fitted with either 0.45 μm cellulose acetate membrane filter media for bacterial studies, or 15 nm porosity Nuclepore membrane filters for virus studies. Chambers were then immersed in the center of Pond #2, hung from a floating platform. At intervals predetermined for each experiment, samples were collected from each chamber and the number of surviving bacteria or virus determined using one of the assay methods described above. When possible, several studies were carried out on each test organism during various seasons of the year in an effort to assess the overall effects of ambient temperature on the survival of the respective organisms.

G. Improvements in Virus Concentration - Reconcentration Methods

Virus recoveries from M/M/P systems using the filter adsorption-elution system with a 0.10 M glycine (pH 11.5) eluant were often inconsistent (see Results). Experiments conducted

in the laboratory with poliovirus-seeded Pond #2 effluent also yielded erratic data. It became obvious that some characteristic of the pond waters (and meadow effluents) was interfering with the virus-recovery process. In an effort to increase the recovery efficiency, experiments were conducted with an elution-reconcentration system using beef extract as described by Katzenelson et al., (25) for recovery of virus from tap water. Known concentrations of poliovirus (LSc-2ab) were added to 25 gal volumes of wastewater. First stage virus concentrations were carried out as before with the exception that no clarifying filters were used (this was done on the recommendation of Dr. F. M. Wellings during a Rockefeller Foundation supported visit to her laboratory). Viruses were eluted from concentrating filters with two 600-ml volumes of 3% beef extract (pH 9.0). The pH of the eluate was then lowered to 3.5 causing the formation of a virus-adsorbing protein precipitate. The precipitate was collected by centrifugation at 5,000 x g for 10 min. The resulting pellet was dissolved in a small volume (20-25 ml) of 0.15 M $Na_2 HPO_4$ (pH 9.0) and adjusted to a final pH of 7.2. Samples were stored at -70 C until assayed. A schematic of the modified technique is presented in Fig. 2.

An additional modification made to the concentration procedure was the replacement of the Cox filter with a pleated cartridge filter (Duo-Fine). This latter type combined good virus recovery efficiency with high sample flow rate. By using these filters, larger sample volumes could be processed through the concentrator in a fraction of the time required by the Cox filter.

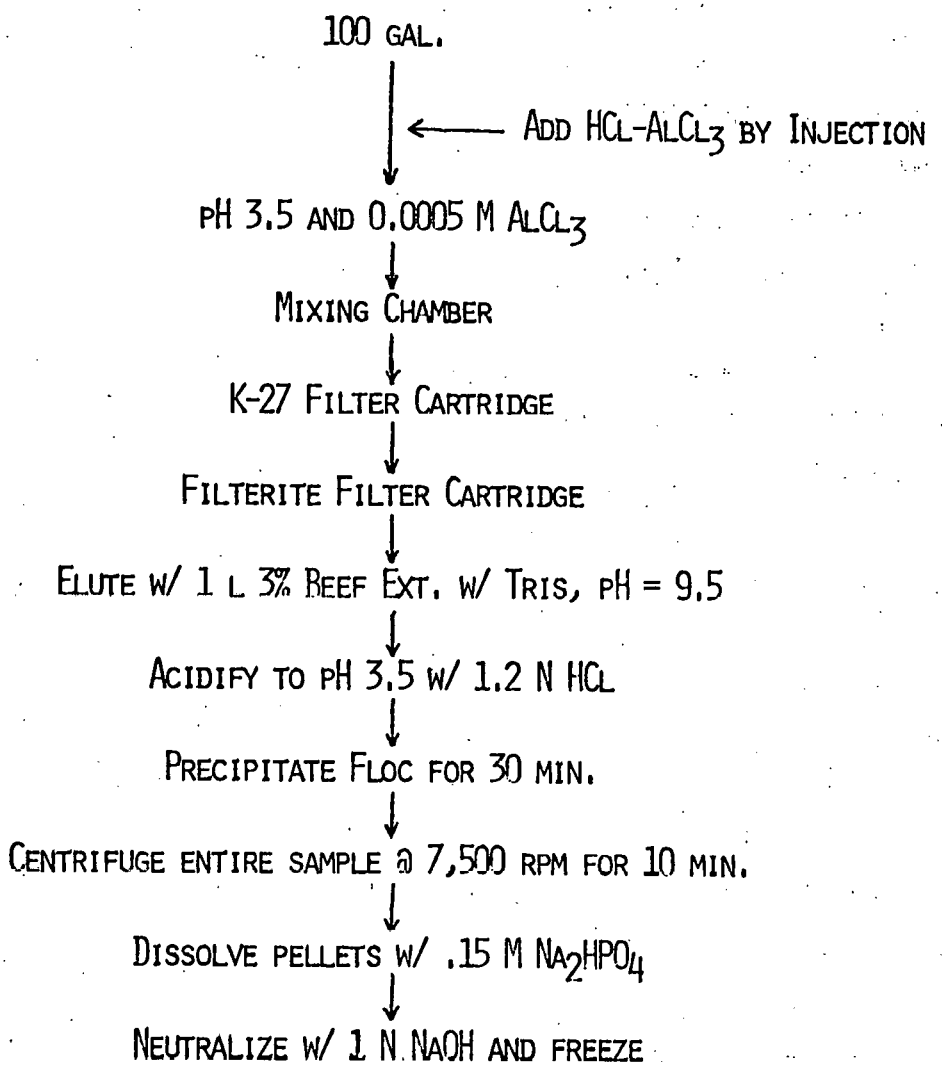

Figure 2. Concentration of Virus from Groundwater and Wastewater

H. Virus Removal in a Marsh Ecosystem

In order to determine the efficiency of virus removal during passage of wastewater across marsh systems, a 1/100 scale "mini-marsh" model was constructed to the appropriate specifications of the BNL marsh ecosystems. The units, built out of wood and underlain with Polyvinyl chloride (PVC) plastic, contained two 2' x 8' x 3' troughs containing either a clean pebble (control), or muck-sediment bottom (test cell). Both troughs were planted to cattails (Typha latifolia) and various Scirpus species. Also present were numerous varieties of zooplankton. Freshwater (control), or holding pond sewage was discharged into the appropriate trough from separate 55-gallon plastic barrels. A water level of 1-2 inches was maintained in both troughs (approximate flow rate - 1 gal/hr). Trough effluents were collected in large volume composite samplers. During experimental runs, freshwater and sewage input barrels were seeded with poliovirus type 1 (LSc). Waters were then discharged through the appropriate control (freshwater) or sewage system. Experiments were carried out for 3-day periods during which input barrels were replenished with virus seeded freshwater or sewage. Two-hour composite effluent samples (1 liter) were collected at intervals. Viruses in samples were concentrated by an organic flocculation method. One-liter volumes were first supplemented with beef extract (3%). Sample pH was then adjusted to 3.5 which caused the formation of a virus-adsorbing organic precipitate which was collected by centrifugation (5000 x g/10 min). Pellets were dissolved in 15 ml volumes of dibasic sodium phosphate (pH 9.0). Concentrates, adjusted to

pH 7.2, were stored at -70 C to await assay. In addition to virus assays, temperature, turbidity plus pH values were determined during each sampling interval. Experiments were carried out with environmentally realistic virus input concentrations (0.01 - 0.1 pfu/ml) to better ascertain the likely performance of the large marsh systems.

III. RESULTS

A. Field Sampling

The use of recently developed virus concentrator technology has greatly facilitated the isolation of human viruses from large volumes of water. However, the variability of conditions encountered during field sampling, such as turbidity, presence of organics and variations in ion concentration, tend to obviate a 100% efficiency of virus concentration. As a result, the data presented below likely represent the minimum virus numbers in each sample. The inability to detect viruses within the constraints of the testing system used during this study (1977-1979) could not preclude the possibility of virus presence. Greatest difficulties were encountered with Holding Pond and Pond #1 and #2 samples (during periods of algal bloom), where high turbidity likely accounted for considerable loss of virus recovery efficiency.

Virus recoveries from field samples are presented in Tables 3 through 7. As expected, highest recoveries were obtained from holding pond samples (Table 3). Virus concentrations in these samples ranged from 8.5 to 1,224 pfu/gal.

TABLE 3

VIRUS AND COLIFORM ISOLATIONS - HOLDING POND WATERS

Sample #	Date	Total Coliform/100 ml	Fecal Coliform/100 ml	Virus PFU[a]/gal
1	4/7/77	8,400,000	>100,000	NI[b]
2	4/14	65,000	1,800	784.2
3	4/21	47,000	800	NT[c]
4	5/5	83,000	11,000	154.7
5	6/17	NT	NT	NI
6	6/23	830,000	49,000	NT
7	6/30	>10,000,000	>100,000	556.2
8	7/7	89,000	1,400	NT
9	7/14	NT	NT	1,224.0
10	7/21	3,300,000	83,000	NI
11	8/4	NT	NT	74.0
12	8/18	NT	NT	228.0
13	8/25	NT	NT	NI
14	9/1	1,140,000	10,100	NT
15	9/8	240,000	1,500	NT
16	9/15	5,900,000	122,000	100.0
17	9/22	840,000	33,000	547.0
18	9/29	540,000	15,800	123.0
19	10/6	NT	NT	275.0
20	10/13	8,500,000	164,000	NT
21	10/20	560,000	39,000	NT
22	10/27	470,000	8,700	NT
23	11/3	1,090,000	76,000	NT
24	11/17	440,000	3,600	NT
25	12/1	NT	NT	NI
26	1/5/78	320,000	27,000	201.6
27	1/19	540,000	0	19.2
28	2/2	890,000	0	110.6
29	2/24	NT	NT	NI
30	3/2	340,000	>100,000	322.8
31	3/10	NT	NT	10.6
32	3/23	NT	NT	NI
33	4/13	NT	NT	NI
34	4/21	NT	NT	9.8
35	5/4	NT	NT	NI
36	5/25	NT	NT	NI
37	6/8	NT	NT	NI
38	7/20	NT	NT	8.5
39	8/10	NT	NT	35.7
40	8/31	NT	NT	NI
41	9/14	NT	NT	80.4
42	10/19	NT	NT	NI

a, pfu - plaque forming units

b, none isolated

c, not tested

TABLE 4

VIRUS AND COLIFORM ISOLATIONS - MEADOW #1 EFFLUENT

Sample #	Date	Total Coliform/100 ml	Fecal Coliform/100 ml	Virus PFU/gal
1	4/14/77	19,400	1,370	NI
2	6/30	>1,000,000	>100,000	NI
3	7/7	10,000	6,300	NT
4	8/4	NT	NT	NI
5	8/18	NT	NT	NI
6	8/25	NT	NT	8.4
7	10/13	100,000	1,560	NT
8	10/27	11,000	72	NT
9	11/17	3,400	60	NT
10	12/1	NT	NT	NI
11	1/5/78	44,000	5,500	NI
12	1/19	>1,000,000	8,200	NI
13	3/2	10,200	5,300	NT
14	4/21	NT	NT	NT
15	5/4	NT	NT	4.0
16	6/22	NT	NT	NI
17	7/20	NT	NT	NI
18	8/31	NT	NT	NI
19	9/14	NT	NT	NI
20	9/28	NT	NT	4.4
21	10/19	NT	NT	NI

TABLE 5

VIRUS AND COLIFORM ISOLATIONS - MEADOW #2 EFFLUENT

Sample #	Date	Total Coliform/100 ml	Fecal Coliform/100 ml	Virus PFU/gal
1	4/7/77	244,000	14,300	NT
2	4/21	1,300	80	NT
3	5/5	55,000	5,600	NT
4	6/9	>100,000	3,600	NT
5	6/23	>100,000	>10,000	NT
6	7/14	NT	NT	NI
7	7/21	>100,000	10,000	NI
8	9/1	>100,000	>10,000	7.3
9	9/8	>100,000	5,400	NT
10	9/15	>100,000	1,410	NI
11	9/22	48,000	3,400	NI
12	9/29	2,000	1,270	NI
13	10/6	NT	NT	NI
14	11/3	119,000	12,100	NT
15	2/2/78	1,500	270	NI
16	4/14	NT	NT	NI
17	5/25	NT	NT	NI
18	7/6	NT	NT	NI
19	8/10	NT	NT	NI

TABLE 6

VIRUS AND COLIFORM ISOLATIONS - MARSH/POND #1 EFFLUENT

Sample #	Date	Total Coliform/100 ml	Fecal Coliform/100 ml	Virus PFU/gal
1	4/7/77	41,000	780	NT
2	4/14	1,640	116	NI
3	4/21	2,100	328	NI
4	5/5	3,700	180	NI
5	6/9	>100,000	12,500	NT
6	6/17	NT	NT	56.3
7	6/23	>100,000	580	NT
8	6/30	26,000	4,200	NI
9	7/7	6,000	176	NT
10	7/14	NT	NT	NI
11	7/21	6,000	12	NI
12	8/4	NT	NT	NI
13	8/18	NT	NT	NI
14	8/25	NT	NT	20.8
15	9/1	110,000	1,040	NT
16	9/8	>100,000	108	NT
17	9/15	1,600	12	NI
18	9/22	29,000	1,180	NI
19	9/29	2,100	32	NI
20	10/6	NT	NT	42.3
21	10/13	520	72	NT
22	10/20	11,000	64	NT
23	10/27	820	<3	NI
24	11/3	4,000	132	NT
25	11/17	56,000	<3	NT
26	12/1	NT	NT	NI
27	12/8	NT	NT	NI
28	1/5/78	>100,000	<3	3.9
29	1/19	>100,000	<3	NI
30	2/2	>100,000	<3	NI
31	2/24	NT	NT	NI
32	3/2	14,000	2,000	NT
33	3/10	NT	NT	NI
34	3/23	NT	NT	NI
35	4/14	NT	NT	NI
36	4/21	NT	NT	NI
37	5/4	NT	NT	NI
38	5/25	NT	NT	NI
39	6/8	NT	NT	NI
40	6/22	NT	NT	NI
41	7/6	NT	NT	NI
42	7/20	NT	NT	NI
43	8/10	NT	NT	NI
44	8/31	NT	NT	NI
45	9/14	NT	NT	NI
46	9/28	NT	NT	NI
47	10/19	NT	NT	NI

TABLE 7

VIRUS AND COLIFORM ISOLATIONS - POND #2 EFFLUENT

Sample #	Date	Total Coliform/100 ml	Fecal Coliform/100 ml	Virus PFU/gal
1	4/7/77	79,000	500	NI
2	4/14	2,100	160	NI
3	4/21	36,000	1,000	NI
4	5/5	6,500	1,120	NI
5	6/9	37,000	5,000	NT
6	6/17	NT	NT	NI
7	6/23	11,500	>1,000	NT
8	6/30	21,000	2,500	12.9
9	7/7	19,000	>10,000	NT
10	7/14	NT	NT	NI
11	7/20	4,100	700	NI
12	8/4	NT	NT	NI
13	8/18	NT	NT	NI
14	8/25	NT	NT	NI
15	9/1	135,000	5,500	NT
16	9/8	70,000	<3	NT
17	9/15	<3	<3	NI
18	9/22	1,500	424	NI
19	9/29	320	32	NI
20	10/6	NT	NT	NI
21	10/13	7,000	340	NT
22	10/20	27,000	190	NT
23	10/27	>1,000,000	22	NI
24	11/3	13,000	660	NT
25	11/17	1,800	20	NT
26	12/1	NT	NT	NI
27	1/5/78	2,000	16	NI
28	1/19	55,000	280	NI
29	2/2	>100,000	175	NI
30	2/24	NT	NT	NI
31	3/2	14,000	>10,000	NT
32	3/10	NT	NT	NI
33	3/23	NT	NT	2.1
34	4/14	NT	NT	NI
35	4/21	NT	NT	NI
36	5/4	NT	NT	NI
37	5/25	NT	NT	NI
38	6/8	NT	NT	NI
39	6/22	NT	NT	NI
40	7/6	NT	NT	NI
41	7/20	NT	NT	NI
42	8/10	NT	NT	NI
43	8/31	NT	NT	1.4
44	9/14	NT	NT	NI
45	9/28	NT	NT	NI
46	10/19	NT	NT	NI

In general, viruses were recovered from samples yielding high coliform counts. This, however, was not consistent, as viruses were often not detected in other samples which also had high coliform counts. The apparent contradiction was likely the result of one or more of the factors cited above (especially turbidity). On the basis of the available Holding Pond isolation data, two facts became apparent: 1) human viruses could be routinely isolated from holding pond waters (60.6% - Table 8); and (2) while isolation frequency was high, detectable virus concentrations were lower than those normally found in raw domestic sewage (3,000 - 10,000 PFU/gal [39]. The fact that the sewage input was "virologically weak" must be considered in any estimation of the system's virus removing capabilities.

Virus isolation frequencies were significantly reduced in the renovated waters (Table 8). In general, there was little correlation between coliform numbers and virus recoveries in these waters. Meadow #2 was apparently more effective than Meadow #1 in reducing the frequency of virus isolation (9% - Meadow #2 *vs.* 20% - Meadow #1). This difference may have been the result of the extensive use of Meadow #1 during the study period. With the longer drying periods occurring in Meadow #2, viruses would have had more opportunity to interact (*i.e.* adsorb) with soil particles (Note: Loading and drying cycles have been proposed as a method for enhancing virus removal during groundwater recharge operations through soil basins [26].

Treatment through both aquatic systems also resulted in reduced frequencies of virus isolation (Tables 6, 7 and 8). Of the two, Marsh/Pond system #2 appeared to be most

TABLE 8

COMPILED VIRUS RESULTS FOR M/M/P FIELD SAMPLING

Sample Type	No. Samples Taken	No. Positive	Percent Positive
Holding Pond	33	20	60.6
Meadow #1 Effluent	15	3	20.0
Meadow #2 Effluent	11	1	9.0
Marsh/Pond #1 Effluent	36	4	11.1
Pond #2 Effluent	36	3	8.3

successful with a virus isolation frequency of only 8.3% versus 11.1% for Marsh/Pond #1. Total virus numbers recovered from system #2 were also lower than those encountered in system #1. The observed differences in virus removal efficiencies between the two systems may have been related to the larger functional marsh area in system #2. (When built, both marshes measured 0.2 acres in area. Because of the design of Marsh/Pond #1 [Fig. 1]), large areas of Marsh #1 were often flooded, reducing the functional area of the system.) The mechanics most involved in virus removal in these systems were adsorption to marsh soils and adsorption-sedimentation in the ponds (experiments discussed later addressed the likelihood of other "removal" mechanism - i.e. survival). Of the two, adsorption to marsh soils appeared to play the greater role in removing viruses.

Within the limitations imposed by the wastewater input (i.e. the "weak" viral character of the sewage), the field sampling program indicated the Marsh/Pond approach (i.e. the design used in system #2) to be most effective in removing viruses. Moreover, with the exception of periods of phytoplankton blooms in pond waters, effluent turbidities from this system (indeed, from all systems tested) were sufficiently low (usually below 10 NTU - Table 2) to allow efficient effluent disinfection (chlorination, ozonation) prior to recharge. While no current treatment scheme can assure virus-free effluents, a properly designed and managed Marsh/Pond system may very well produce effluents of similar or superior quality (in terms of virus occurrence) to those produced by secondary and tertiary plants currently operating

on Long Island (41, 39). It is strongly recommended that comparative studies with conventional treatment modes be initiated in order to precisely delineate the effectiveness of aquatic ecosystems for treating "normal" raw wastewater inputs.

As expected, little correlation was noted between virus and coliform recoveries in all samples tested. The inappropriateness of the coliform-based standard for predicting virus occurrence has been noted by several authors (44, 14, 19, 11, 45).

B. Survival Experiments

Data from field sampling studies suggested that significant virus loss occurred during the passage of wastewater through the Marsh/Pond systems. While adsorption and adsorption-sedimentation were considered to be the major mechanisms of removal, viral (and to a lesser extent bacterial) survival in pond waters also required consideration.

The compiled results of a number of survival studies are shown in Table 9. The greatest survival capacity was demonstrated by poliovirus, followed by MS-2 bacteriophage, E. coli B and Salmonella. A seasonal influence was noted in the case of the bacterial organism where survival was enhanced during colder months. With the exception of MS-2 phage, all test organisms appeared to be more stable in unsterile, raw pond waters than in filter-sterilized waters. It may be that filtration removed organisms or particles which exerted a protective influence, thus, the decreased survival. The most important general conclusion arising from the data was that the bacteriocidal-virucidal activity of the pond water was not sufficient to provide significant

TABLE 9

COMPILED DATA: SURVIVAL OF BACTERIA AND VIRUS IN AQUATIC ECOSYSTEMS

Season	Suspending Medium	Organism	Final Day Test Organisms Detected	Percent Survival - Final Day
Spring	Sterile Pond Water	E. coli B	24	0.000056
"	Unsterile Pond Water	E. coli B	24	0.00012
Summer	Sterile Pond Water	E. coli B	12	0.000014
"	" " "	Salmonella	12	0.0000016
"	" " "	MS-2	19	0.0088
"	Unsterile Pond Water	E. coli B	12	0.0043
			16	0.0000004
"	" "	Salmonella	12	0.000034
			5	0.0049
"	" "	MS-2	19	0.0000011
Fall	Sterile Pond Water	MS-2	14	0.000023
"	" " "	Polio LSc	18	12.4
"	Unsterile Pond Water	MS-2	20	0.00017
"	" "	Polio LSc	18	30.0
Winter	Sterile Pond Water	E. coli B	25	0.00069
"	" " "	Salmonella	20	0.000088
"	Unsterile Pond Water	E. coli B	25	0.000081
"	" "	Salmonella	20	0.00017

bacterial/viral removal during the normal residence time of these organisms through the pond systems. Removals reflected in field data must therefore have been the result of an adsorption-sedimentation mechanism. Organisms thus trapped in the lower regions of the ponds would remain for sufficient periods to affect more extensive inactivation. The rate at which this inactivation would take place could not be determined as sediment survival studies were not carried out.

C. Improvements in Virus Concentration/Reconcentration Methods

1. Efficiency of beef extract in the recovery of virus

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 25-gallon volumes of 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 μm porosity). The results of duplicate experiments (Table 10) indicated beef extract elution followed by organic flocculation to be superior to a glycine elution-inorganic flocculation method in the recovery of seeded poliovirus. 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 order to insure reproducibility, a series of additional seeding experiments were carried out using the techniques previously described. The data, presented in Table 11, show a consistently higher recovery of poliovirus from wastewater using

TABLE 10

COMPARISON OF POLIOVIRUS RECOVERY: 3% BEEF EXTRACT (pH 9.0) VERSUS 0.10 M GLYCINE (pH 11.5)

Experiment	Total PFU[a] Recovered From			
	Beef Extract Elution		Glycine Elution	
	Input	Recon.	Input	Recon.
1	3.03×10^5	3.16×10^5 (>100)[b]	5.68×10^5	1.80×10^5 (31.69)
2	3.31×10^6	3.18×10^6 (96.07)	2.27×10^6	9.30×10^5 (40.96)

[a] PFU, plaque forming units

[b] Values in parentheses are percent recoveries based on input values.

TABLE 11

THE OVERALL EFFICIENCY OF POLIOVIRUS RECOVERY
USING 3% BEEF EXTRACT (pH 9.0) ELUTION-RECONCENTRATION

Experiment	Total PFU[a] 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

[a] PFU, plaque forming units

beef extract. In 7 trials, the mean recovery efficiency was 85%, with recoveries ranging from 68 to 100%.

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 25-gallon 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. Immediate elution resulted in the recovery of 1.34×10^7 PFU (87%), while the extended elution method yielded a recovery of 1.24×10^7 total PFU (80.5%). These results suggested that the elution was likely instantaneous, and also that poliovirus was not inactivated by prolonged contact (30 min) with beef extract at pH 9.0.

Recently, a number of investigators have adopted pleated cartridge filters for use in the recovery of enteroviruses from large volume water samples (10, 12, 31). The increased surface area of these filters eliminates clogging problems, allowing higher volume samples to be processed. Experiments were therefore performed to insure that 3% beef extract (pH 9.0) was equally effective in systems using these filters. Virus-seeded sewage samples were processed through a filter series consisting of a fiberglass K27 filter and a 0.45 μm pleated filter (Duo-Fine; Filterite, Timonium, Maryland). The results, presented in Table 12, indicated that an average of 65% of the input polioviruses were recovered following concentration and reconcentration.

TABLE 12

EFFICIENCY OF POLIOVIRUS RECOVERY FROM PLEATED CARTRIDGE FILTERS USING 3% BEEF EXTRACT (pH 9.0)

Experiment	Total PFU[a] Recovered From	
	Input	Reconcentrate
1	3.28×10^6	2.04×10^6 (62.19)[b]
2	2.22×10^6	1.53×10^6 (68.91)
Mean % Recovery		65.55

[a] PFU, plaque forming units

[b] Values in parentheses are percent recoveries based on input.

2. Efficiency of beef extract in second-stage reconcentration

Katzenelson et al. (25) recently reported that an organic flocculation procedure, utilizing 3% beef extract, was superior to an inorganic floc method using glycine for the recovery of seeded poliovirus from tapwater during second-stage reconcentration. To determine whether a similar occurrence could be observed in seeded wastewater, poliovirus was inoculated into 1 ℓ volumes of treated sewage effluent. The sample was divided into 250 ml aliquots and the poliovirus concentrated using either organic or inorganic flocculation. The results (Table 13) support the findings of Katzenelson et al., (25) regarding the superiority of beef extract in the second-step reconcentration process. Eighty-five percent of the input viruses were recovered with the organic method, while inorganic flocculation yielded an average recovery of 64%.

Since all previous reconcentration experiments utilized 3% beef extract, it was of interest to determine if lower concentrations of beef extract would yield similar virus recoveries. Initial experiments were conducted using small volumes of 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 BY INORGANIC OR ORGANIC FLOCCULATION METHODS

Experiment	Total PFU[a] Recovered after	
	Inorganic Flocculation	Organic Flocculation
1	2.35×10^5 (68.11)[b]	3.04×10^5 (88.11)
2	2.07×10^5 (60.00)	2.87×10^5 (83.18)
Average	64.05	85.64

[a] PFU, plaque forming units

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

TABLE 14

RECONCENTRATION EFFICIENCY OF VARIOUS CONCENTRATIONS OF BEEF EXTRACT

Percent Beef Extract	Total PFU[a] Recovered From		Mean Recovery (%)
	Exp 1[b]	Exp 2[b]	
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

[a] PFU, plaque forming unit

[b] 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.

Since beef extract concentrations of less than 3% appeared to be effective in a reconcentration process, it was of interest to test their relative efficiencies of virus elution from concentrating filters. In a series of preliminary experiments, poliovirus was seeded into 25-gallon wastewater samples, and concentrated using a fiber-glass cartridge-pleated filter series. Elution was accomplished using either 1% or 3% beef extract (pH 9.0). Preliminary results indicated that 1% beef extract was as efficient as 3% beef extract in the recovery of poliovirus. Elution with 3% beef extract resulted in the recovery of 68% of the input virus, while 75% of the viruses were recovered using 1% beef extract elution.

D. Virus Removal in Marsh Test System

The "Mini-Marsh" experiment was carried out over a four-day period during the month of December. The turbidity, pH and temperature measurements which were taken throughout the experiment are presented in Table 15. No unusual fluctuations were noted in any of these data. Virus recovery data (Table 16) indicated that significant virus removal occurred during passage through both marsh systems. The only samples yielding recoverable virus numbers were those taken on the first two days from the sewage test system (here removals averaged between 82 and 94%). After day two, no viruses could be detected in any of the samples.

We had initially assumed that greater numbers of viruses would have been recovered from the fresh water system. This assumption was based upon the theory that the more turbid sewage would offer more opportunity for virus-particulate interactions, resulting in the sedimentation, or

TABLE 15

TURBIDITY, pH AND TEMPERATURE DATA:

"MINI-MARSH" COMPOSITE SAMPLES

Day	Sample #[a]	Turbidity (NTU)	pH	Temperature (°C)
1	A1[b]	2.2	6.3	6.0
	B1	6.6	6.8	7.5
	A2	1.8	6.3	6.0
	B2	7.2	6.4	7.5
	A3	1.5	6.6	6.0
	B3	7.1	6.3	7.5
2	A4	1.2	6.7	6.5
	B4	6.5	6.7	7.0
	A5	1.4	6.4	6.5
	B5	8.0	6.7	7.0
	A6	0.9	7.1	6.5
	B6	6.6	6.9	7.0
3	A7	2.0	6.4	4.0
	B7	7.5	7.0	5.0
	A8	0.75	6.7	4.0
	B8	6.8	6.7	5.0
	A9	2.0	6.7	4.0
	B9	7.0	6.5	5.0
4	A10	nt[c]	nt	4.5
	B10	8.5	7.1	5.5
	A11	nt	nt	4.5
	B11	8.5	6.5	5.5

[a] A denotes samples from freshwater test system; B denotes samples from sewage test system.

[b] Three composite samples collected from each system on each test day: first sample collected from 9:30 - 11:00 a.m.; second sample, 12:00 - 1:00 p.m.; third sample, 2:00 - 4:00 p.m.

[c] nt = not tested.

TABLE 16

POLIOVIRUS REMOVAL IN "MINI-MARSH" SYSTEM

Day	Sample #[a]	Virus Recovery (PFU/ℓ)
0	Input (A+B)	264
1	A1	0
	B1	16
	A2	0
	B2	32
	A3	0
	B3	0
2	A4	0
	B4	32
	A5	0
	B5	48
	A6	0
	B6	0
3	A7	0
	B7	0
	A8	0
	B8	0
	A9	0
	B9	0
4	A10	0
	B10	0
	A11	0
	B11	0

a, A denotes freshwater test system; B = sewage test system.

sieving-out of virus-laden particles. The virus input concentration, however, was sufficiently low (264 PFU/ℓ) to obviate a major role for this mechanism. Recent work by Seidel (35), Czerwenka and Seidel (5), and DeJong (7) indicated that various aquatic plants had some bacterial removing potential reflected both in their offering of additional adsorption sites around root systems, as well as their possible production of bactericidal by-products. This latter effect, while unsubstantiated by other workers, may provide some explanation for the somewhat unexpected results of the "Mini-Marsh" experiment. Were nonspecific virucidal components also being produced by one or more of the plant species, their effects would be compromised by the protective effect imparted to viruses by the particles and organic milieu of the raw sewage. Such enhancement of virus survival by particulates and organic material has been noted previously (46, 21, 15, 4). Since the fresh water system offered no such protective potential, virus isolates were recovered throughout the duration of the experiment.

In the above, we offer some speculation on the reasons for data differences occurring between the fresh water and sewage systems. The most significant aspect of this experiment, however, is its demonstration of notable virus removal in the marsh test system. The data are consistent with observations made during the field analyses portion of the research project and underscore the importance of the marsh system in the overall scheme of virus removal in lowland treatment systems.

IV. CONCLUSIONS

Recently developed and improved methods for the recovery and enumeration of human viruses have greatly facilitated the testing of environmental systems and the conduct of field-appropriate low virus input experimentation. However, the variabilities of conditions encountered during sampling and the nonexistence of a single concentration-enumeration system for all virus types likely to occur in sewage obviate the likelihood of a 100% virus recovery efficiency. Data from field studies must, therefore, be considered to represent the minimum number of viruses present in each sample. In addition to methodological limitations, it must be remembered that the test systems were operated using what must be described as a comparatively "weak" sewage blend, especially with respect to the number of viruses detected. The conclusions presented below have been developed with the above constraints in mind, but have been based primarily upon the results arising from each study element of the project (i.e. the observed data). As this rationale is used throughout the report, the dangers of "out-of-context" misinterpretation by the reader cannot be underestimated.

In summary, the following were developed during the study:

1) Field sampling data indicated that moderately effective virus removal occurred in all systems. The general clarity of the respective effluents would easily lend themselves to efficient disinfection prior to groundwater recharge (preferably at low infiltration rate on Long Island [41, 43]).

2) The Marsh-Pond #2 system provided the most consistent virus reductions, with the majority of removal apparently occurring in the marsh. The virucidal potential of the raw Pond water appeared to be limited owing to the relatively brief residence time of viruses in the system (however, sedimented, particulate-bound viruses would likely remain in the system long enough for inactivation to occur).

3) New and improved techniques for the concentration and enumeration of viruses from sewage-polluted fresh water were developed. The methods should prove extremely useful in all future related environmental testing.

4) No clear-cut correlations could be made between the occurrence of human viruses and indicator bacteria in the systems. Indeed, there was some evidence that coliform organisms could grow in some of the systems during warmer months.

It was not possible to perform virus seed-removal studies in the meadows. However, we feel confident that such a study, if carried out, would have corroborated the recent findings of Shaub et al., (32) in which 68-85% removals were realized.

One of the most important questions indirectly addressed by this study concerned the overall performance of the natural treatment mode vs. that of a conventional secondary treatment system. Based upon a comparison of system inputs and effluents, which drew upon the field results reported above and data resulting from extensive testing at several local secondary sewage treatment plants (39, 42), the overall virus removals in the natural systems were as good, if not better, than those in the conventional system. Obviously, the only way to corroborate this extrapolated comparison would be to monitor the performance of

both systems "side by side" where each receive identical amounts and blends of raw sewage. It is highly recommended that such a study be conducted, for it alone will provide sufficient "real world" data upon which appropriate wastewater management decisions may be made. Owing to the rather poor quality primary and secondary effluents produced by some existing local treatment plants (42), it is further recommended that the feasibility of the installation of marsh "polisher" systems at appropriate treatment plants be considered.

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