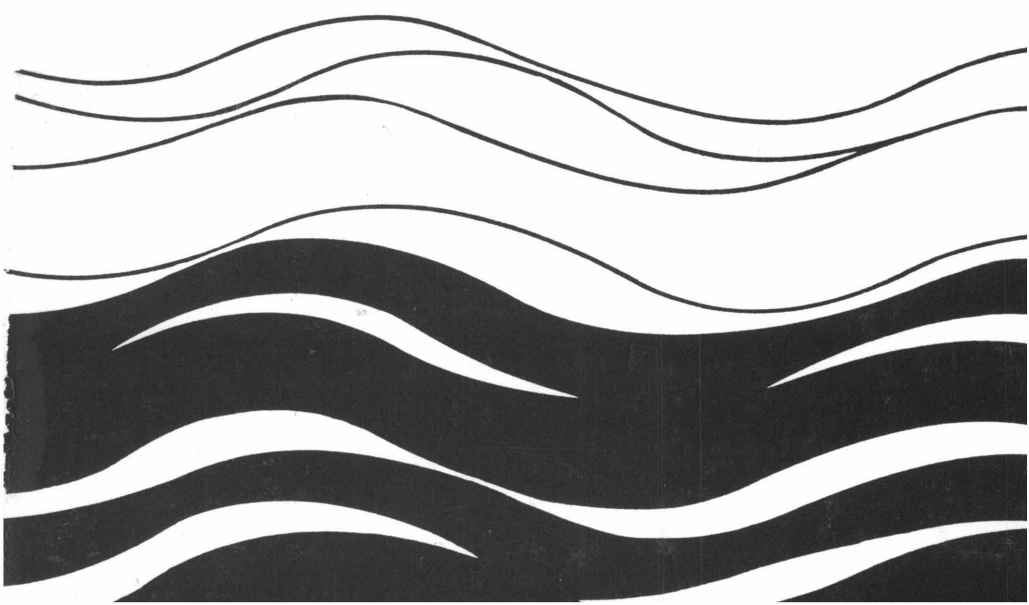
Virginia Water Resources Research Center Bulletin 131

Enumeration and Inactivation of Enteric Viruses in Sludge

Michael W. Nath & James C. Johnson



Bulletin 131
July 1980

Enumeration and Inactivation of Enteric Viruses in Sludge

Michael W. Nath
James C. Johnson

Department of Biological Sciences
Old Dominion University
Norfolk, Virginia 23508

The work upon which this report is based was supported in part by funds provided by the United States Department of the Interior, Office of Water Research and Technology, as authorized by the Water Research and Development Act of 1978 (P. L. 95-467).

Project A-086-VA
VPI-VWRRC-BULL 131
3.75C

A publication of the
Virginia Water Resources Research Center
Virginia Polytechnic Institute and State University
Blacksburg, Virginia 24060

Additional copies of this publication, while the supply lasts, may be obtained from the Virginia Water Resources Research Center. Single copies are provided free to persons and organizations within Virginia. For those out-of-state, the charge is $4.50 per copy if payment accompanies the order, or $6 per copy if billing is to follow.

TABLE OF CONTENTS

List of Figures iv

List of Tables iv

Acknowledgments vii

Abstract 1

Introduction 3

Materials and Methods 5
I. Cells and Viruses 5
II. Wastewater Sludges 5
III. Extraction of Virus from Sludge 5
IV. Determination of Solids 6
V. Determination of pH, Ammonia, and Conductivity 6
VI. Plaque Assay 6
VII. Growth and Purification of [^{14}C]-Poliovirus-2 7
VIII. Sludge Dewatering 8

Results 9
I. Extraction and Enumeration of Viruses from Wastewater Sludges 9
A. Identification of Virus-Plaquing Efficiencies 9
B. Physical and Chemical Parameters of Sludge 10
C. Determination of the Proportion of Virus-Bound to Sludge Solids 11
D. Desorption and Isolation of Virus from Primary Sludge . . . 12
E. Extraction and Concentration of Indigenous Virus in Sludge 13
II. Inactivation of Enteric Viruses in Sludge by Heat-Assisted Evaporation 16
A. Evaporation as a Virucidal Process 16
B. Dewatering of Primary Sludge at 22.5°C 17
C. Dewatering of Primary Sludge at 37°C 18

Discussion 19

References Cited 23

Figures 29

Tables 33

LIST OF FIGURES

1. Glycerol Gradient of [^{14}C]-Poliovirus-2 30

2. Autoradiogram of Glycerol Gradient of [^{14}C]-Poliovirus-2. 31

3. CsCl Centrifugation of [^{14}C]-Poliovirus 32

LIST OF TABLES

1. Human Enteric Viruses and Diseases 34

2. Relative Plaquing Efficiency of Several Viruses. 35

3. Sludge Parameters 36

4. Fraction of Poliovirus Bound to Primary Sludge Solids. 37

5. Effect of Dilution on the Amount of Sludge Particle-Bound Virus 38

6. Surfactant Structures 39

7. The Effect of Surfactant Concentration on Several Cell Lines. . . .40

8. Relative Efficiency of Virus Extraction from Seeded Primary Sludge 41

9. Multiple Extraction-Concentration of Poliovirus-2 Seeded Primary Sludge 42

10. Indigenous Virus Found in Primary Sludge 43

11. Effect of Evaporation of Poliovirus-2 Inactivation in Water at 22°C 44

12. Effect of Evaporation on Poliovirus-2 Inactivation in Primary Sludge at 22.5°C 45

13. Effect of Evaporation on Poliovirus-2 Inactivation in Primary Sludge at 37°C 46

ACKNOWLEDGMENTS

These experiments were supported by grant No. A-086-VA from the Virginia Water Resources Research Center and in part by grant No. GR-13 (RF 79-952) from the Old Dominion University Research Foundation.

The authors acknowledge the assistance of D. Wright, R. Dale, and D. Waltrip of the James River Wastewater Treatment Plant in the Hampton Roads Sanitation District for collection and transportation of sludge samples. We thank Donnie Wheeler, Barry Stern, and Sam Merritt for constructive assistance in the initial design of the experiments.

Special acknowledgment is made to the following who generously gave their time to a critical review of the manuscript: Anna M. Rule, South Shore Laboratory, Hampton Roads Sanitation District; Samuel R. Farrah, Department of Microbiology and Cell Science, University of Florida; and Samuel N. Merritt, Public Health Laboratory, Hampton Roads Sanitation District. Acknowledgment is made also to Sandra P. Milliken for editorial processing and typesetting and to Gretchen S. Bingman for layout composition.

ABSTRACT

The presence of large numbers of enteric viruses in wastewaters has resulted in concern regarding their significance and consequence for humans. This study describes virus isolation methods and a new and efficient multiple extraction-concentration procedure for the enumeration of viruses in wastewater sludges.

As an integral part of a comparison of isolation procedures, several anionic surfactants were examined for extraction of enteric viruses from primary wastewater sludges. Plaquing efficiencies for poliovirus-2 and coxsackievirus B-2 on several common cell lines were determined. The results demonstrate: (1) that the total poliovirus burden in primary sludge may be determined by means of an efficient extraction procedure followed by concentration by centrifugation, and (2) that dewatering by evaporation is virucidal.

The methods described should be useful in monitoring studies of wastewater treatment plant functions, sanitary landfill operations, or any other areas where health assessments are required for the disposal of wastewater sludges.

Key Words: Sludge, Enteric Virus, Inactivation, Evaporation, Enumeration, Plaquing, Efficiencies, Bound-Virus Sludge Solids, Desorption, Extraction and Concentration, Surfactants, Poliovirus-2, Coxsackievirus B-2, *Anatid* Herpesvirus, Ultracentrifugation, Monitoring, [^{14}C]-Poliovirus.

INTRODUCTION

The presence of viruses in wastewaters, treated wastewaters, sludges, and receiving waters has led to concern about the short-term and long-range effects of human viruses in the environment. These viruses are most often apparent in the locality of or in waters downstream from outfalls of wastewater treatment facilities, as well as on land subjected to sludge application or treated wastewater irrigation. An accurate evaluation or prediction of the health hazard consequences of viruses in return waters and sludges can be made only after precise determinations of virus type and amount, probable or possible modes of transmission, and other environmental and epidemiological considerations are formulated.

Despite advances in virology and cell culture, relatively few reports describe an effective technology for virus isolation and enumeration from wastewaters and sludges. Techniques vary from one laboratory to another. Various factors, including practice and experience as well as reagent or equipment availability, are involved in the choice of selected procedures. No set of standard procedures has been proposed; yet, studies reporting the presence of up to 1×10^2 infectious polioviruses per gram of fecal material and 5×10^5 enteric viruses per liter of raw wastewater have been published [Sabin, 1955; Nupen, 1974]. Frequently, the range of infectious viruses per liter has been reported as 10^2 to 10^4 with 7×10^3 plaque-forming viruses (pfv) as typical [Clarke et al., 1961; Grabow, 1968; Berg and Metcalf, 1978; Melnick, 1978]. For a 30-million-gallon-per-day wastewater treatment plant, the input burden of virus thus may reasonably bracket 1×10^{10} to 1×10^{12} infectious enteric virus particles per day.

The medical importance of viruses in wastewaters may be readily appreciated by an examination of the human "enteric" virus groupings listed in *Table 1.* The epidemiology relative to these enteric viruses has been reviewed recently [Berg, 1976; Katzenelson, 1978; Melnick, 1978]. Of the more than a hundred virus types listed, most are suspected of being transmitted by the water route, and many are known to have caused human disease outbreaks [Melnick, 1978].

This report describes virus isolation and a new and efficient multiple extraction-concentration procedure for the enumeration of viruses in wastewater sludges. As an integral part of a comparison of isolation procedures, we have examined several anionic surfactants for extraction

of enteric viruses from primary sludges and have determined plaquing efficiencies for poliovirus-2 and coxsackievirus B-2 on several common cell strains. The efficiency of a heat-assisted evaporation procedure for virus inactivation is explored with the emphasis on identifying low-cost virucidal procedures.

MATERIALS AND METHODS

I. Cells and Viruses

Certified Cell Lines 1(L-929), 2.2(HeLa), 7.1(LLC-MK_2), 75(WI-38), 81(VERO), and 136(RD) were obtained from the American Type Culture Collection. Buffalo green monkey kidney cells were a gift from Dr. T. Metcalf (Department of Microbiology, University of New Hampshire, Durham). Duck embryo fibroblast cultures were prepared from 15-day embryonated eggs obtained from the Barnhardt Farms, Urbanna, Virginia. All cells were maintained in minimal essential medium (AP-MEM; Flow Laboratories, Inc.) supplemented with 5 or 10 percent fetal bovine serum(FBS). Viral reagents VR-684 *(Anatid* herpesvirus, Holland strain [AHV]), VR-29 (coxsackievirus B2, Ohio-1 strain [Cox B-2]), and VR-301 (poliovirus 2, W-2 strain [Po-2]) were obtained from the American Type Culture Collection and stored at -85°C.

II. Wastewater Sludges

Raw sludge was obtained from the primary settling facility of the James River Wastewater Treatment Plant (JRWTP) in the Hampton Roads Sanitation District. Digested sludge was obtained from the outlet of the anaerobic digestors from the same facility. The sludge (1-liter samples) were maintained at 4° C until all assays for viral contents were completed.

III. Extraction of Virus from Sludge

A multiple extraction-concentration procedure dependent upon centrifugation was found effective for virus enumeration from primary sludge. The sludge samples (4 to 100 ml) were diluted, when necessary, to $\leqslant 1$ percent solids using deionized water and adjusted to 0.1-M tris (hydroxymethyl) aminomethane (Tris-HCl, pH 7.5), 0.2-M NaCl, 5 percent v/v glycerol, and 10 percent v/v FBS in final concentration. The mixtures were stirred on a reciprocating mechanical shaker for 10 minutes at 22°C. The mixtures were then centrifuged at 20,000 rpm for 30 minutes at 4°C in a JA-20 Beckman rotor. The supernatant fraction was decanted and saved, and an equivalent volume of extraction mixture (as above) was added to the pellets. The pellets were resuspended, agitated for 10 minutes, and centrifuged as before. The supernatant fractions were combined, and the pellets were again extracted in the same manner combining all

supernatant fractions. The combined supernatants were centrifuged at 45,000 rpm for 3 hours at 4°C in a Beckman 45-Ti rotor. The pellets were resuspended by sonication in 1.0 ml of minimal essential growth medium containing 10 percent FBS and 20mM HEPES (Sigma). The resuspended material was either assayed for virus content immediately or stored at -85°C.

IV. Determination of Solids

Triplicate 100-ml samples of primary or digested sludge were weighed, then dried in convection ovens at 100°C under partial vacuum until a constant final weight was reached. The average percent solids were calculated.

V. Determination of pH, Ammonia, and Conductivity

The pH was determined directly using a calibrated and standardized combination electrode (Beckman 39505) and a Beckman Expandomatic IV pH meter. The supernatant fractions of primary and digested sludges (10,000 rpm, 20 minutes, 4°C) were analyzed for ammonia with an ion-selective electrode (Beckman, ammonia sensor, 39565) and for conductivity with a Barnstead PM-70CB conductivity bridge with a 1-cm cell.

VI. Plaque Assay

Dilutions of virus-containing mixtures, including sludges, were made in minimal essential growth medium containing 10 percent FBS and 20mM HEPES buffer, pH 7.4. Monolayer cultures of RD cells (passages 36 to 41), BGM cells (passages 122 to 136), VERO cells (passages 124 to 134), WI-38 (passages 15 to 20, LLC-MK_2 (passage 10 as derivative to 23), HeLa (3 laboratory passages), and DEF (passages 2 to 6) were aspirated. The virus inoculum (0.5 ml) was added, and adsorption was for 1.0 hour at 37°C with rocking at 15-minute intervals. The inoculum was removed by aspiration, and the cell sheets rinsed with 1 ml of complete growth medium. The cells were overlayed (8 ml) with minimal essential growth medium (AP-MEM) supplemented with 50 I.U./ml penicillin, 50 μg/ml streptomycin, 1 μg/ml fungizone, and 0.1 μg/ml gentamycin, 5 or 10 percent v/v FBS, and 1.2 percent methylcellulose (4,000 cps). Plaques were counted 48 to 105 hour postinfection, either microscopically at 25-fold magnification or macroscopically with or without the aid of

crystal violet staining of the cell sheets. The linearity of cytopathic response for each virus using each counting procedures was confirmed.

VII. Growth and Purification of [^{14}C]-Poliovirus-2

The procedure used was a modification of that described by Ward and Ashley [1976]. Confluent monolayers of BGM cells were infected with poliovirus-2 (moi=3) in minimal essential medium containing 5 or 10 percent FBS. Adsorption was for 3 hours, after which the inoculum was removed and the cells washed once with low amino acid medium [Eagle's MEM (modified) with 1/20 the normal concentration of amino acids and supplemented with an equivalent concentration of MEM non-essential amino acids (Flow Laboratories, Inc.), 20 mM HEPES, pH 7.4, 50 I.U./ml penicillin, 50 μ/ml streptomycin, and 10 percent dialyzed FBS]. Five μCi to 7.5 μCi of an L-amino acids [^{14}C] mixture ($\geqslant$100mCi/mM each amino acid, New England Nuclear) were added per ml of low amino acid medium. The mixture (10 ml) was incubated with the cell monolayers at 37°C until complete cell lysis was observed (17 to 19 hours). The radioactive lysates were freeze-thawed three times and centrifuged at low speed (100 x g) for 10 minutes to remove cellular debris. The lysate was centrifuged at 16,000 rpm for 20 minutes at 4°C in a Beckman JA-20 rotor. The supernatant was centrifuged at 50,000 rpm for 1.5 hours at 4°C in a 60-Ti rotor to pelletize the virus. The virus pellets were resuspended in a small volume of a buffer (0.1-M NaCl, 0.01-M tris (hydroxymethyl) aminomethane (Tris-HCl), pH 7.5 and 0.003-M $MgCl_2$). A double volume of freon-113 (1,1, 2-trichlorotrifluoroethane; Matheson) was added. The mixture was vortexed for 2 minutes, then centrifuged at low speed (450 x g) to separate phases. The aqueous phase was further purified by sedimentation through 15 to 30 percent glycerol gradients in NTE buffer (0.1-M NaCl, 0.01-M Tris-HCl, pH 7.5, 0.001 M ethylenediaminetetraacetate [EDTA] at 27,000 rpm for 4 hours at 4°C in an SW-27 rotor. Fractions were collected from the tube bottom and analyzed for radioactivity *(Figure 1).* The fractions from the peak of radioactivity migrating at 150 to 160S were pooled and dialzed against 0.01-M Tris-HCl, pH 7.4, 0.1-M NaCl and 0.001-M EDTA. The yield of plaque-forming viruses (pfv) through dialysis was virtually 100 percent.

Further purification of the glycerol gradient isolated virus was unnecessary. Sodium dodecyl sulfate (SDS) polyacrlamide gel electrophoresis of glycerol gradient virus revealed only 4 component polypeptides—VPI, VPII, VPIII, and VPIV in the proper molecular weights for poliovirus

proteins *(Figure 2).* Isopycnic banding of glycerol gradient virus in CsCl did not significantly improve purity *(Figure 3)* and resulted in the loss of about 70 percent of the total pfv.

The specific pfv/cpm activity for glycerol virus was 2.6×10^4 to 4.4×10^3 depending upon the initial level of labeled amino acids.

VIII. Sludge Dewatering

Samples (50 ml) of sludges to be dewatered were mixed with a 1/100 dilution of a 1.1×10^9 pfv/ml poliovirus-2 stock prepared from culture lysates by differential centrifugation and pelletization. The seeded sludges were placed into open petri dishes (150 cm^2) or in sealable polypropylene tubes and incubated at 22.5 or 37°C for various times until dehydration by evaporation was complete. At times during incubation, 1.0-ml samples were removed and diluted to 1.0 percent solids, mixed with SDS (0.2 percent final concentration) and centrifuged (16,000 rpm, 4°C, 20 minutes). The supernatant fractions were assayed for infectivity.

RESULTS

I. Extraction and Enumeration of Viruses from Wastewater Sludges

A. Identification of Virus-Plaquing Efficiencies

The efficiency with which a virus can initiate infection and produce a detectable cytopathic effect is partially the result of the nature, susceptibility, and type of the host cells, and the conditions for cell growth and assay. A standard set of cells and conditions may be established to maximize the number of plaques for any given virus; however, this is difficult in situations such as exist in wastewaters where neither the type nor the number of viruses can be predicted. Accordingly, the plaquing efficiencies for poliovirus type 2 strain W-2, coxsackievirus B2 strain Ohio-1, and the *Anatid* herpesvirus strain Holland Duck Plaque were determined for several different certified cell lines, duck embryo fibroblasts (DEF), and buffalo green monkey cells (BGM) *(Table 2).*

Poliovirus-2 was found to be most efficiently titered on monolayer cultures of CCL-136 (RD) followed by BGM, CCL-7.1 (LLC-MK_2), CCL-81 (VERO), and CCL-75 (WI-38), respectively. Coxsackievirus B-2 was found to be most efficiently plaqued on BGM cells followed by CCL-75, CCL-81, and CCL-7.1. Coxsackievirus infections of RD cells resulted in diffused, incomplete cytopathic effects, which were not easily detected in counting; therefore, RD cells were inefficient for the enumeration of the coxsackievirus B-2 under the conditions chosen for assay. Predictably, duck embryo fibroblasts were not susceptible to either picornavirus. The *Anatid* herpesvirus has not been found infectious for any cell line of mammalian origin. AHV will produce a characteristic and typical plaque in cell cultures of ducks, geese, and swans, as well as in cell cultures derived from *Galliformes* [Attanasio, 1977].

The results demonstrate that virus susceptibility is a function of the choice of host cells with RD and BGM cells found most efficient for the enumeration of poliovirus and coxsackievirus B-2, respectively. The efficiency of the BGM cell line relative to primary cells of rhesus and African green monkey kidney has been reported [Dahling et al., 1974].

B. Physical and Chemical Parameters of Sludge

Primary and anaerobically digested sludges obtained from the James River Wastewater Treatment Plant (JRWTP) of the Hampton Roads Sanitation District were routinely collected and analyzed specifically for properties most likely to affect virus survival, isolation, and enumeration *(Table 3).* From analysis of the table, it may be observed that the pH of primary sludge was on the average 1 to 1.5 units lower than for digested sludge taken from the outlet of the digestor on the same day. The solids content varied from a low of 0.05 percent dry weight on 6-18-79 (primary sludge) to maximally 4.72 percent (9-4-79). This nearly 100-fold range of solids cannot be attributed to rainfall variations of other natural conditions and likely reflects sampling procedure variation. Digested sludge was more uniform in solids content with extremes of 1.7 to 3.14 percent during the 9-month observational period.

Ammonia, a compound found to be virucidal to polioviruses, coxsackieviruses, and reoviruses, is most effective in its virucidal capacity in digested sludge at a pH greater than 8.0 [Ward and Ashley, 1977a]. The mechanism for ammonia-induced inactivation was identified as a capsid alteration followed by destruction of the primary structure of the viral RNA [Ward, 1978]. The levels of the primary sludges from the JRWTP were found to be between 24 and 293 mg/l. For digested sludges, these values increased to between 690 and 2,000 mg/l. In conjunction with the consistently higher pH found in digested sludge, RNA virus inactivation would more likely occur in the digestion process, a conclusion reached by others [Ward and Ashley, 1976; Ward et al., 1976; Ward and Ashley, 1977a].

The conductivities of both primary and digested sludges obtained during the test period were each found to vary less than twofold. Digested sludge contained a five to seven times greater conductance than found in raw sludge. The ionic environment is known to affect the binding affinity for viruses to different matrices, and in some cases, these binding interactions can be used for the purification of the virus [Philipson, 1967]. The conductance may be an indicator of the proportion of viruses bound to sludge solids; however, the small differences determined in these studies suggest that little or no differences in virus binding characteristics could be expected.

C. Determination of the Proportion of Virus-Bound to Sludge Solids

Recent studies suggest that >90 percent of the virus in raw wastewater and sludge is particle bound [Lund, 1973; Wellings et al., 1976; Sattar and Westwood, 1976a; Subramanyan, 1977; Hurst et al., 1978]. The nature of this association probably involves both ionic and entrapment phenomena. Whatever the nature or extent of the phenomena, Ward and Ashley [1979a] determined for poliovirus, the association with sludge solids would not permit the virus to enter cells other than by means of the normal receptor-mediated route. This implies that the infectivity of a population of sludge-bound viruses is "masked" until a stable virus-cell surface receptor contact is made.

The fraction of poliovirus-2 rapidly bound by sludge solids was examined using highly purified, [^{14}C]-amino acid labeled poliovirus. Despite greater than tenfold differences in the amounts of solids in samples collected more than 8 months apart, more than 85 percent of the input virus (1.1×10^7pfv) was adsorbed or bound by the particulate fraction of primary sludge *(Table 4).* Even for sludges with a 0.44 percent solids content, 90 percent of the virus was removed from the supernatant fraction of seeded sludge. Addition of [^{14}C]-poliovirus-2 to dilutions of a primary sludge with an initial solids concentration of 4.72 percent discerned further the adsorptive nature of sludge solids. A reduction in the fraction of bound virus was observed; however, this bound fraction was not directly proportional to the sludge dilution *(Table 5).* Furthermore, with a 40-fold reduction in solids, there still was over 50 percent of the [^{14}C]-poliovirus bound. The results infer (1) that a variety of virus adsorbants, each with differing affinities for poliovirus, likely exists in the sludge material, and (2) that there are interactions between sludge fractions capable of adsorbing virus and inhibitors of adsorption capable of blocking or modulating adsorption affinities.The extensive adsorption of the population of virus to solids was likely responsible for the failure to detect indigenous virus in the supernatant fractions from any of the sludge samples listed in *Table 4.* Infrequently, infectious activities were detected by inoculation of an unconcentrated and noneluted sludge sample. Viral titers calculated from direct inoculation or supernatant inoculation studies were at the level of minimal detection, less than 1 to 3×10^3 pfv/l under these conditions, and were considered statistically insignificant (data not presented).

D. Desorption and Isolation of Virus from Primary Sludge

Virus desorption from sludge solids has been accomplished with varying efficiencies by mechanical agitation [Sattar and Westwood, 1976a; Hurst et al.,1978], using proteinaceous materials [Sattar and Westwood, 1976a; Wellings et al., 1976], using high pH (≥11.0) buffers [Hurst et al., 1978], using the surfactant SDS (sodium dodecyl sulfate) [Ward and Ashley, 1976], by fluorocarbon treatment [Wellings et al., 1976], or by combinations of the above. Few reports have added adequate controls to determine whether the virus was irreversibly inactivated or occluded by sludge particles and thereby prevented from infecting assay cells. Still fewer have considered elution parameters strictly as a function of virus-bound sludge particles. Hurst et al. [1978] prepared a sludge solids-virus fraction by centrifugation. The removal of organics and other materials in the supporting liquid by centrifugation and resuspension, modifying the nature of the sludge solids-virus interaction, was not considered. Many of the necessary controls for recovery calculations in these experiments may be obtained using radioactively labeled, purified virus.

Characteristically, nonenveloped enteric viruses are known to be resistant to surfactants. Ward and Ashley [1976] employed SDS in a poliovirus purification scheme with high recovery of infectivity. The surfactants employed in this current study included: Tween-20, Tween-80, Triton X-100, Triton X-200, Nonidet P-40, and SDS, the structures of which are presented in *Table 6.* Triton X-200 and SDS are anionic in nature due to the presence of a sulfonic acid group. The others are neutral in charge character at intermediate pH.

The cytotoxicity for each surfactant on each of eight separate cell lines was determined *(Table 7).* Each surfactant or NaCl was added to confluent monolayers of the indicated cell types during a one-hour mock virus adsorption period, removed, and replaced with an overlay medium not containing the surfactant. By means of a careful microscopic examination of the cell monolayers at 24-hour intervals following mock infection, maximal concentrations of each surfactant found to be noncytotoxic vary between 0.01 and 0.05 percent (v/v). Each cell line tested was tolerant of NaCl at 0.36 percent. In control experiments, plaque formation was not inhibited by poliovirus-2 inoculated in the presence of the surfactants SDS, Nonidet P-40, and Triton X-100 or NaCl. The data suggests that surfactants in low concentrations may be tolerated by these cells and that poliovirus infections are not inhibited or prevented.

The relative extraction efficiency of seeded primary sludge with the surfactants, bovine embryo extract (BEE), fetal bovine serum (FBS), or NaCl was determined using either seeded poliovirus-2 or coxsackievirus B-2 *(Table 8).* SDS was found to be most efficient in the recovery of either poliovirus (77 percent) or coxsackievirus (96 percent) from sludge in a single extraction protocol. Least efficient were the Tweens (Tween 20, 18 percent and 5.3 percent, and Tween 80, 15 percent and 9.9 percent for poliovirus and coxsackievirus, respectively). These efficiencies were less than or equal to those of the unextracted sludge control (17 percent and 24 percent for poliovirus and coxsackievirus, respectively). This result implied either an enhanced virus-solids binding or virus inactivation. In these studies, indigenous virus-induced plaques were not observed, probably due to the relatively low numbers of indigenous virus found in this particular sample and the large dilution required for plaque assay of the seeded samples. NaCl, at 79 percent efficiency for the recovery of poliovirus, was not found equally efficient for the extraction of coxsackievirus B-2 (23 percent) nor was NaCl found effective for virus extraction from primary sludge with solids content above 0.5 percent. Both beef embryo extract and FBS were equally efficient in desorbing poliovirus and coxsackievirus, yet they were only 50 percent as efficient as SDS. The efficiency with which SDS reduces the virus-sludge solids interaction and prevents virus readsorption probably stems from its detergency action as a surfactant, the size of the hydrophobic region, and the imposition of net negative charge to both the surface of the viral capsid and the virus-adsorbing sludge particle. Triton X-200, also an anionic surfactant , but with a larger hydrophobic region relative to SDS, was not effective in releasing either virus. The reasons for the differences in the actions of these two compounds are unclear. Ward and Ashley [1978] found significant differences in the behavior of ionic detergents during virus heat inactivation studies. Many of the detergents that were found to reduce the heat required to inactivate reovirus were found to protect poliovirus and other enteroviruses. It was suggested that the hydrophobic region of anionic detergents must be of a minimal size to affect heat protection. These protective effects were found to be critically dependent upon the pH of the mixture [Ward and Ashley, 1979b]. It is not certain whether any of these inactivation parameters effect sludge particle-virus disassociation.

E. Extraction and Concentration of Indigenous Virus in Sludge

Although several enteroviruses, chiefly among the family *Picornaviridae,*

are resistant to the virucidal effects of SDS, many other enteric, non-enveloped viruses are rapidly inactivated at low SDS concentrations. Adenovirus at room temperature in the presence of 0.012 percent SDS is rapidly disrupted [Smith et al., 1965]. Reovirus is rendered sensitive to inactivation by means of SDS [Ward and Ashley, 1977b]. Enveloped viruses also cannot be expected to survive SDS treatment because of the disruptive action surfactants have on lipid-containing membranes.

In enumeration studies requiring the concentration of SDS eluates, it was found that interference occured unless care was taken to remove SDS to concentration less than 0.02 percent w/v. Concentration of [^{14}C]-poliovirus seeded sludge was attempted by three different protocols. By chilling a centrifugation clarified extract to 0°C, an SDS floc developed which could be collected by centrifugation. This floc contained 70 to 90 percent of the labeled virus added to the sludge. A second procedure involved the addition of an equivalent molar amount of $CaCl_2$ to the SDS extracted and clarified sludge. The resultant precipitate (chiefly, Ca-dodecyl sulfate) was collected by centrifugation and found to contain 80 to 90 percent of the input labeled virus. In either of the above procedures, biological assays were impossible without prior removal of SDS. This was accomplished by extraction of the SDS using an equal volume of n-butanol; n-butanol extraction of SDS provided a 10- to 20-fold concentrated extract in which the SDS concentration was greatly reduced, but the total yield of [^{14}C]-poliovirus in this procedure was 40 to 60 percent of the amount originally seeded.

A third procedure involved adjustment of the SDS extract to pH 11.0, followed by the addition of $CaCl_2$ to form a $CaOH_2$-dodecyl sulfate floc. The floc could be collected by centrifugation and dissolved by adjusting the pH to 3.5. Following dialysis against Tris-HCl, pH 7.5 (0.01 M), the yield of labeled material was 60 percent. The pfv yield was less than 0.1 percent. This contrasting result infers that although the yield of label was high, the virus population was extensively inactivated. Virus inactivation by low concentrations of SDS at high pH levels has been reported [Ward and Ashley, 1979b]. Of the three procedures employing SDS as the extractant, none could be expected to recover enveloped viruses.

Multiple sludge extractions in the absence of SDS were used to improve over all virus yields. Adsorption processes are likely charge dependent.

Neutralization of the charge network in the microenvironment surrounding a virus-particle complex should favor desorption. In the case of poliovirus-1, two isoelectric points (pH 7.0-7.2 and 4.5) have been identified [Mandel, 1971]. The data was interpreted in terms of resonating conformational states (*A* and *B*) of the viral capsid protein. At a pH of 7 to 7.5, all of the virus is stabilized in conformational state *A* and remains infectious. Desorption may be favored, provided that the microenvironment surrounding each poliovirus is adjusted to a pH near the *A* conformational state and sufficient agitation is provided to break up clumps of matter. Accordingly, three extractions of primary sludge containing 0.1-M Tris-HCl, pH 7.5, 0.2-M NaCl, 10 percent v/v FBS, and 5 percent v/v glycerol in final concentrations were used to extract [^{14}C]-poliovirus-2 seeded sludge (multiple extraction-concentration procedure) *(Table 9).* When the sludge solids were 0.7 percent or less in the extraction mixture, greater than 70 percent recovery of virus was obtained in a resuspended high-speed centrifugation pellet. It was found, for these conditions of extraction and concentration, that an equivalent percent of poliovirus infectivity was obtained. Maximal recovery was obtained (98.7 percent) using primary sludge with the least amount of solids (0.44 percent).

The efficiency of extraction was found to be dependent upon the amount of sludge solids in the extraction mixture. In a single extraction using 0.2 percent SDS (final concentration) as the extractant, 4.7, 11.3, 16.1, 21.3, 20.9, and 32.3 percent of added [^{14}C]-poliovirus were recovered in a primary sludge with solids adjusted to 2.84, 2.37, 1.89, 1.42, 0.95, and 0.47 percent dry weight, respectively. The data suggests that the efficiency of extraction and concentration should be determined for each sample of sludge and especially when the solids content exceeds one percent by weight. The total amount of poliovirus-2 recovered from several different samples of seeded, primary sludge, using 0.2 percent SDS and under conditions in which greater than 80 percent of the virus was bound to sludge solids, was examined. For primary sludges having 3.2, 2.84, 0.93, or 0.50 percent solids, the recoveries of [^{14}C]-poliovirus were 10.4, 5.3, 29.5, and 89.5 percent, respectively. It may be concluded that the sludge nature, amount of solids, and other factors enter into any prediction of the efficiency of virus recovery. The addition of a radioactively labeled virus to a sludge sample serves as an easily measured positive control for all aspects of the virus enumeration and also serves as a reference point when comparing methodologies.

Using the multiple extraction-concentration procedure and the efficiencies determined from *Table 9*, the set of primary sludges was examined for indigenous virus content on different indicator cell lines *(Table 10).* With the 20-fold concentration achieved in these extractions, there was little difficulty in detecting virus-induced cytopathic effects in confluent BGM or VERO cells. The range of virus contents as assayed on VERO cells was 0 pfv/l to 1.9×10^4 pfv/l and for BGM cells 1.0×10^3 pfv/l to 4.5×10^3 pfv/l. None of the eight sludge samples were found to be free of viruses specific for eukaryotic cells. As the multiple extraction-concentration procedure is relatively gentle,analyses were conducted for the presence of coliphage. From *Table 10,* it may be observed that the range of coliphage varied between 4.6×10^4 pfv/l and 3.4×10^7 pfv/l. The coliphage to enterovirus ration varied from a maximum of 28,000:1 to a minimum of 13:1. Although the coliphage recovery efficiencies were not determined in this study, it does appear that no relationship exists between the numbers of the two very different types of viruses. This finding is strongly supported in principle in the review by Scarpino [1978] which attempts to place in perspective the controversy over the usefulness of the bacteriophage indicator as a measure of enteroviruses in contaminated waters.

In light of these results, it is clear that there is a large variation in the viral burdens of primary sludge in the James River Wastewater Treatment Plant (JRWTP). Applying the extremes of enterovirus content determined during the 7-month observational period to a 30-million-gallon-per-day operation, the estimate may be made that between 1.1×10^{11} and 2.2×10^{12} pfv of enteric viruses enter the plant per day. The fate of these viruses is not clear. A significant fraction appears to be inactivated during anaerobic digestion [Ward and Ashley, 1976, 1978]. At a rate of 1-log inactivation of virus content per day, more than a week of digestion would be required to remove most of a static population of virus. Since digestion is by nature dynamic, considerably more time for complete inactivation would be required.

II. Inactivation of Enteric Viruses in Sludge by Heat-Assisted Evaporation

A. Evaporation as a Virucidal Process

Sludge dewatering is frequently employed in the processing of wastewater solids. The high cost of transportation of the frequently more than 90 to 95 percent water contained in sludge makes dewatering procedures

attractive. Under certain circumstances, evaporation of water in sludge may be the most cost-effective procedure [Ward and Ashley, 1977a].

Exposure of several viruses to liquid-air interfaces by shaking, blending, or aeration causes rapid inactivation [de Jong et al., 1975, 1976; Dixon et al., 1966; Trouwborst and de Jong, 1973; Trouwborst et al., 1972, 1974]. It has been surmised that the increased exposure of viruses to liquid-air interfaces may be in part responsible for the virucidal action that takes place in sludge during dewatering by evaporation [Ward and Ashley, 1977b]. These investigators concluded that inactivation was not the result of the ratio of surface area to volume, thus implying that air was not the virucidal component.

To examine the relationship between the rate of evaporation and virus inactivation, deionized water (50 ml) was seeded with purified poliovirus-2. Equal amounts of pfv were placed into open petri dishes (surface area 150 cm^2) and into sealable polypropylene tubes. The vessels were incubated at 22.5°C in a forced air incubator operating under slight negative pressure and in total darkness. Samples of the vessel contents were removed periodically, analyzed for virus content, and compared to the total evaporative weight loss *(Table 11).* Through the 23.5 hours necessary to achieve a 19.7- fold decrease in initial weight, there was only a 4.7-fold in virus concentration. During the same time period, there was no change in virus titer in the control. The total decrease in infectivity in the open vessel was 73 percent, which suggests that the process of evaporation renders poliovirus-2 noninfectious. At dryness (27 hours) the open petri dish was rinsed in 0.2 percent SDS in pH 9.0 Tris-HCl (0.1M), a procedure found to liberate poliovirus which could have adsorbed to the container. The total amount of adsorbed virus was 3.1×10^6pfv/ml or 2.4 percent of the total initial virus content. This finding implies that the loss of viral titer in the open dish was not the result of poliovirus adsorption to the container material.

B. Dewatering of Primary Sludge at 22.5°C

Results reported in the previous section suggest that dewatering of sludge by evaporation results in extensive viral inactivation with nearly 1-log of infectivity lost during a 24-hour period under conditions in which the rate of evaporation was about 2.0 grams per hour. The effects of primary sludges on the inactivation rate of poliovirus-2 were examined *(Table 12).* At up to about 55 percent solids obtained during the first 23.5 hours of

dewatering, there occurred less than 2-logs of virus inactivation, a result consistent with the rate determined for deionized water alone *(Table 11).* Above 55 percent solids and during a 1.5 hour period to achieve 90.9 percent solids, a rapid inactivation occurred with a total loss of over 3-logs infectivity. Control studies, with primary sludge in the absence of evaporation, failed to show any decrease in titer through the course of the experiment. At dryness, or 27.0 hours following the initiation of dewatering, the sludge solids were resuspended to 1.0 percent with deionized water and residual virus extracted using 0.2 percent SDS. No infectivity was detected. These results suggest that partial dewatering of sludge results in a situation highly conducive for poliovirus inactivation which is distinct or additive to the effects resulting exclusively from dewatering by evaporation.

C. Dewatering of Primary Sludge at 37°C

Although the rate of evaporation is a function of temperature, the second component of virus inactivation, present at above 55 percent solids and responsible for the rapid decrease in virus infectivity, may either be temperature dependent or independent. To examine this possibility, seeded sludge was dewatered by evaporation as before at 37°C *(Table 13).* Dewatering to 43.3 percent solids resulted in less than a 2-log inactivation of poliovirus-2 (93 percent). A 3-log drop in titer again occurred between 43.3 percent and 79.8 percent solids during a 1-hour period. In the evaporation negative-control, little or no virus losses were observed. At 87 and 92 percent solids, as well as in sludge dried at 37°C for 25 hours (98.5 percent solids), no infectivity was detected.

The occurence of a breakpoint in the viral inactivation study at about 55 percent solids for either temperature of study suggests that inactivation by processes other than evaporation may occur in raw primary sludge undergoing dewatering. Ward and Ashley, [1979b] have found that ionic detergents in sludge may stabilize or destabilize viruses to inactivation as a function of the ambient pH. It is not known whether detergents alone or along with other compounds, with a totally different set of properties, affect this rapid inactivation in primary sludge. Further experimentation will be required to resolve this point. From the set of experiments presented in this study, it does appear that the temperature of sludge incubation and dewatering does not affect the breakpoint in poliovirus inactivation.

DISCUSSION

The results of this study demonstrate: (1) that the total poliovirus burden in primary sludge, and presumably most of the total enterovirus burden, may be determined by means of an efficient extraction procedure followed by concentration by centrifugation, and (2) that dewatering by evaporation is virucidal, both from the evaporation process and from the probable concentration of virucidal components in primary sludge.

Procedures for virus concentration by centrifugation are not all new to virology. The purification of most nonenveloped viruses and several enveloped viruses is aided by centrifugation, whether it is by pelletization or by zonal or isopycnic procedures. Ultracentrifugation studies are not new as applied to wastewater studies. Cliver and Yeatman [1965] found pelletization efficient for the concentration of two enteroviruses but not for a reovirus. Gravelle and Chin [1961], Gibbs and Cliver [1965], and Pittler et al. [1967] found centrifugation efficient for virus isolations from wastewaters, but in these early studies centrifugation procedures were often laborious and time consuming. Anderson [1966] and Anderson et al. [1967] reported the use of a continous flow centrifuge and zonal or isopycnic banding for the isolation of virus from contaminated water. The major advantage of continous flow procedures is the increased amount of material which may be processed. The continuous flow procedure was found to be 95 percent efficient in removing poliovirus from seeded wastewater at a flow of 2 to 3 liters per hour.

The multiple extraction and concentration procedure, as reported in this study, used approximately 20-ml quantities of sludge with a suspended solids content of 1 percent or less. Without modifying procedures or performing multiple centrifugations, the procedure is limited to about 200 ml of sludge resulting from the capacity of the Beckman 45-Ti rotor. With existing continuous flow techniques, the scale-up of procedures from one to five liters should not be difficult. It may not be necessary to increase volumes examined in view of the large numbers of viruses generally found in sludge and wastewaters. The multiple extraction and concentration procedure is furthermore convenient in terms of time expenditure. From the initial extraction to inoculation, a total elapsed time of six hours is invested; most of this is in centrifugation, a process involving little hands-on effort.

The efficiency with which poliovirus-2 and coxsackievirus B-2 could be

extracted from sludge was found to be dependent upon the nature of the extraction, yields of ⩾77 percent were obtained for poliovirus-2 and 95 percent for coxsackievirus B-2. With the multiple extraction-concentration procedure, 71 to 99 percent recoveries were obtained depending upon the particular sludge sample and extraction procedure. Using SDS in a single extraction, yields of ⩾77 percent were obtained for poliovirus-2 and 95 percent for coxsackievirus B-2. With the multiple extraction-concentration procedure, 71 to 99 percent recoveries were obtained depending upon the particular sludge sample. These efficiencies are as high or higher than that reported by Hurst et al. [1978] using a high pH, glycine-NaOH elution and flocculation procedure.

Since >85 percent of the virus in wastewater appears to be bound to solids *(Table 4),* extraction procedures must be used to separate the virus from sludges and their solids. The studies of Sattar and Westwood [1975, 1976a, and 1976b] examined proteinaceous materials for the efficacy of elution of virus from sludge solids. Fetal calf serum was found more efficient than BEE, lactalbumin hydrolyzate. or casein. Elutions were aided by mechanical mixing, but for poliovirus, the yield was calculated at only 0.4 percent maximal recovery. Wellings et al. [1976] reported that sludge, which was treated either by sonication in the presence of 3 percent BEE, mechanical stirring for extended periods, or by fluorocarbon extraction, resulted in equal recoveries of indigenous virus. They also reported the detection of echovirus type 7 (24 pfv) from 250 grams of caked sludge exposed for 13 days to high ambient temperatures. The significance of Welling's finding in light of all of Ward and Ashley's results and our own, which suggest that few if any poliovirus type 1 or type 2 remain in dehydrated primary sludge, suggests that further studies need to be performed. Specifically, composting operations, agricultural usages, or landfill operations should be monitored for the presence of enteric viruses.

Concentration of viruses from large volumes and elution of virus from wastewater solids have been reviewed recently [Sobsey, 1976]. Despite the large number of comparative reports for virus concentration, no one method appears superior in all wastewater situations. There remains a need for direct comparative studies to advance methodology for virus isolation and enumeration.

The efficiency of indigenous virus recovery is enhanced by the proper selection of indicator cells. Cooney [1963] studied several cell lines for susceptibility to primary isolates from fecal and nasal swabs. Human embryonic kidney cells were found more useful for adenovirus, polio-

virus, and coxsackievirus; WI-38 cells were found more efficient for herpesviruses and echoviruses and were required for cytomegalovirus and rhinoviruses. Ludovici et al. [1970] reported that primary human amnion cultures gave variable results in plaquing several different virus isolates. Cultures of WI-38 and HEp-2 were utilized for coxsackievirus and poliovirus; however, the coxsackievirus were not found to cause distinct cytopathic effects on the WI-38 cell cultures. Sigel et al. [1976] have reviewed systems for detecting viruses identifying both advantages and disadvantages of several current technologies. Schmidt et al. [1978] have studied the efficacy of plaque formation versus quantal and enumerative procedures. They have concluded that quantal procedures have certain advantages in the precise determination of total virus in wastewaters.

The results of this study demonstrate that as much as a fourfold variation in enumerative titer may be achieved with poliovirus-2, depending upon the choice of a susceptible cell line. Somewhat less of a variation was observed for coxsackievirus B-2. BGM and VERO cells, because of high-plaquing efficiencies under the methylcellulose overlay medium, were the superior indicator cells. Not all of the plaques that appear on initial inoculation of indicator cell monolayers are of virus origin. These non-viral plaques generally do not passage, as the agent or chemical is diluted upon subcultivation. We subcultured 47 plaques initially appearing on RD and BGM cells and positively passaged the virus onto RD and BGM cells, respectively. None of the isolates was positively identified.

The thermo-evaporation studies in which a breakpoint in inactivation occurred at about 55 percent solids both at 22.5°C and 37°C fully support the earlier studies of Ward and Ashley [1977b] using poliovirus-1. In light of the study by Hurst et al. [1978] in which sludge deposits on land were found to stabilize between 50 and 70 percent solids, it is possible that an inactivation breakpoint had been exceeded, and that the residual virus populations detected were resistant species. Effects similiar to those found in sludge may also take place in soils, although it could not be expected that the virucidal compounds predicted to exist in sludge, which aid in viral inactivation at 55 percent solids or above, would be the same as found in soils [Bagdasr'yan, 1965; Lance et al., 1976; and Moore et al., 1976]. Further experimentation will be required to clarify the nature of the virucidal materials in soils.

In this study, procedures for elution and concentration of virus from sludge and sludge solids were assessed. The indigenous population of virus

in primary sludge was determined at monthly intervals over a 7-month period was found to be in the range of 1.0×10^3 to 1.9×10^4 pfv/l enteric viruses and 4.6×10^4 to 3.4×10^7 pfv/l coliphage. The inactivation of virus by evaporation was measured as a function of the change in solids content of primary sludge. The methods described should be useful in monitoring studies of wastewater treatment plant functions, sanitary landfill operations, or any other health assessments are required for the disposal of wastewater sludges.

REFERENCES CITED

Anderson, N. G., 1966. "An Introduction to Particle Separation in Zonal Centrifuges." In *The Development of Zonal Centrifuges, Journal of the National Cancer Institute Monographs* 21:9-40.

Anderson et al., 1967. "Isolation of Virus Particles from Large Volumes." In *Transmission of Viruses by the Water Route,* G. Berg, editor, pp. 75-88. New York: Wiley Interscience.

Attanasio, R., 1977. "Duck Plaque Virus: An Investigation of Cytopathic Effects *in vitro,* Host Range, and Inhibition by Phosphonoacetic Acid." M.S. Thesis. Old Dominion University, Norfolk, Virginia.

Bagdasr'yan, G. A., 1965. "Survival of Viruses of the Entervirus Group (Poliomyelitis, ECHO, Coxsackie) in Soil and on Vegetables." *Journal of Hygiene and Epidemiological Microbiology and Immunology* 7:497-505.

Berg, G., 1976. "Microbiology—Detection Occurrence and Removal of Viruses." *Journal of the Water Pollution Control Federation* 48(6):1410-1417.

Berg, G., and T. G. Metcalf, 1978. "Indicators of Viruses in Waters." In *Indicators of Viruses in Water and Foods,* G. Berg, editor, pp. 267-296. Ann Arbor, Michigan: Ann Arbor Science Publishers.

Clarke, N. A., R. E. Stevenson, S. L. Chang, and P. W. Kabler, 1961. "Removal of Enteric Viruses from Sewage by Activated Sludge Treatment." *American Journal of Public Health* 51:1118-1129.

Cliver, D. O., and J. Yeatman, 1965. "Ultracentrifugation in the Concentration and Detection of Enteroviruses." *Applied Microbiology* 13: 387-392.

Cooney, M. K., 1973. "Relative Efficiency of Cell Cultures for Detection of Viruses." *Health Laboratory Science* 10:294-302.

Dahling, D. R., G. Berg, and D. Berman, 1974. "BGM, A Continuous Cell Line More Sensitive than Primary Rhesus and African Green Monkey Kidney Cells for the Recovery of Viruses from Water." *Health Laboratory Science* 11:275-282.

de Jong, J. C., M. Harmsen, and T. Trouwborst, 1975. "Factors in the Inactivation of Encephalomyocarditis Virus in Aerosols." *Infection and Immunity* 12:29-35.

de Jong et al., 1976. "Inactivation of Semliki Forest Virus in Aerosols." *Applied and Environmental Microbiology* 32:315-319.

Dixon, G. J., R. W. Sidwell, and E. McNeil, 1966. "Quantitative Studies on Fabrics as Disseminators of Viruses. II. Persistence of Poliomyelitis Virus on Cotton and Wool Fabrics." *Applied Microbiology* 14:183-188.

Gibbs, T., and D. O. Cliver, 1965. "Methods for Detecting Minimal Contamination with Reovirus." *Health Laboratory Science* 2:81-90.

Grabow, W. O. K., 1968. "The Virology of Wastewater Treatment." *Water Research* 2:675-701.

Gravelle, C. R., and T. D. Y. Chin, 1961. "Enterovirus Isolations from Sewage: A Comparison of Three Methods." *Journal of Infectious Disease* 109:205-209.

Hurst et al., 1978. "Development of Quantitative Methods for the Detection of Enteroviruses in Sewage Sludges During Activation and Following Land Disposal. *"Applied and Environmental Microbiology* 36:81-89.

Katzenelson, E., 1978. "Survival of Viruses." In *Indicators of Viruses in Water and Food,* G. Gerb, editor, pp.39-50. Ann Arbor, Michigan: Ann Arbor Science Publishers.

Kirk, R. E., and D. F. Othmer, 1969. "Surfactants." In *Enclyclopedia of Chemical Technology, Volume 19,* pp. 507-593. New York: John Wiley and Sons, Inc.

Laemmli, U. K., 1970. "Cleavage of Structural Proteins During Assembly of the Head of Bacteriophage T4." *Nature* 227:680-685.

Lance, J. C., C. P. Gerba, and J. L. Melnick, 1976. "Virus Movement in Soil Columns Flooded with Secondary Sewage Effluent." *Applied and Environmental Microbiology* 32:520-526.

Ludovici, R. R., R. Riggin, and M. L. Moore, 1970. *"Plaquing and Immunological Methods for Detection and Identification of Enteroviruses."* OWRR Project Q-015-Ariz. Department of Microbiology and Medical Technology, Arizona University.

Lund, E., 1973. "The Effects of Pretreatments on the Virus Contents of Sewage Samples." *Water Research* 7:873-879.

Mandel, B., 1971. "Characterization of Type 1 Poliovirus by Electrophoretic Analysis." *Virology* 44:554-568.

Melnick, J. L., 1978. "Water as a Reservoir of Virus in Nature and Means for Control." In *Viruses and Environment,* E. Kurstak and K. Maramorosch, editors, pp. 203-226. New York: Academic Press.

Moore, B. E., B. P. Sagik, and C. A. Sorber, 1976. "An Assessment of Potential Health Risks Associated with Land Disposal of Residual Sludges." *Proceedings of the Third National Conference on Sludge Management, Disposal and Utilization.* Rockville, Maryland: Miami Information Transfer, Inc.

Nupen, E. M., 1974. "The Reduction of Viruses by the Various Unit Processes Used in the Reclamation of Sewage to Potable Waters." In *Virus Survival in Water and Wastewater Systems,* J. F. Malina and B. P. Sagik, editors, pp. 107-114. Austin, Texas: Center for Research in Water Resources.

Philipson, L., 1967. "Chromatography and Membrane Separation." In *Methods in Virology, Volume II,* K. Maramorosch and H. Koprowski, editors, pp. 179-235. New York: Academic Press.

Pittler, H., W. Höpken, and K. W. Knocke, 1967. "Enteroviren und Adenoviren im Abwasser. Methode des Nachweises und epidemiologische Studie 1963-1965." *Zentralblatt fuer Bakteriologie Parasitenkunde, Infektions-krankheiten und Hygiene Arbteilung* 204:33-39.

Sabin, A. B., 1955. "Behavior of Chimpanzee-avirulent Poliomyelitis Viruses in Experimentally Infected Human Volunteers." *American Journal of Medical Science* 230:1-9.

Sattar, S. A., and J. C. N. Westwood,1975. "A Method for the Isolation of Indigenous Human Viruses from Sludge." *IRCS Journal of Medical Science* 3:431-438.

———,1976a. "Comparison of Four Eluents in the Recovery of Indigenous Viruses from Raw Sewage." *Canadian Journal of Microbiology* 32:1586-1589.

———,1976b. "Comparison of Talc-Celite and Polyelectrolyte 60 in Virus Recovery from Sewage: Development of Technique and Experiments with Poliovirus (Type 1, Sabin) — Contaminated Multilitre Samples." *Canadian Journal of Microbiology* 22:1620-1627.

Scarpino, P. V., 1978. "Bacteriophage Indicators." In *Indicators of Viruses in Water and Food,* G. Berg, editor, pp. 201-227. Ann Arbor, Michigan: Ann Arbor Science Publishers.

Schmidt, N. J. et al., 1978. "Comparative Sensitivity of Various Cell Culture Systems for Isolation of Viruses from Wastewater and Fecal Samples." *Applied and Environmental Microbiology* 36:480-486.

Sigel, M. M. et al., 1976. "Systems for Detecting Viruses and Viral Activity." In *Viruses in Water,* G. Berg, editor, pp. 139-151. Washington: American Public Health Association, Inc.

Smith, K. O., W. D. Gehle, and M. D. Trousdale, 1965. "Architecture of the Adenovirus Capsid." *Journal of Bacteriology* 90:254-261.

Sobsey, M. D., 1976. "Methods for Detecting Enteric Viruses in Water and Wastewater." In *Viruses and Water,* G. Berg, editor, pp.89-127. Washington: American Public Health Association, Inc.

Subramanyan, T. P., 1977. "Persistence of Enteroviruses in Sewage Sludge." *Bulletin of the World Health Organization* 55:431-434.

Trouwborst, T., and J. C. de Jong, 1973. Interaction of Some Factors in the Mechanism of Inactivation of Bacteriophage MS2 in Aerosols."*Applied Microbiology* 26:252-257.

Trouwborst, T., J. C. de Jong, and K. C. Winkler, 1972. "Mechanism of Inactivation in Aerosols of Bacteriophage T1." *Journal of General Virology* 15:255-242.

Trouwborst,T. S. et al., 1974. "Inactivation of Some Bacterial and Animal Viruses by Exposure to Liquid-Air Interfaces." *Journal of General Virology* 24:155-165.

Ward, R. L., 1978. "Mechanism of Poliovirus Inactivation by Ammonia." *Journal of Virology* 26:299-305.

Ward, R. L., and C. S. Ashley, 1976. "Inactivation of Poliovirus in Digested Sludge." *Applied and Environmental Microbiology* 31-921-930.

———, 1977a. "Identification of the Virucidal Agent in Wastewater Sludge." *Applied and Environmental Microbiology* 33:860-864.

———, 1977b. "Inactivation of Enteric Viruses in Wastewater Sludge Through Dewatering by Evaporation." *Applied and Environmental Microbiology* 34:564-570.

———,1978. "Identification of Detergents as Components of Wastewater Sludges That Modify the Thermal Stability of Reovirus and Enteroviruses." *Applied and Environmental Microbiology* 36:889-897.

———, 1979a. "Mode of Initiation of Cell Infection with Sludge Associated Polioviruses." *Applied and Environmental Microbiology* 38:329-331.

———, 1979b. "pH Modification of the Effects of Detergents on the Stability of Enteric Viruses." *Applied and Environmental Microbiology* 38:314-322.

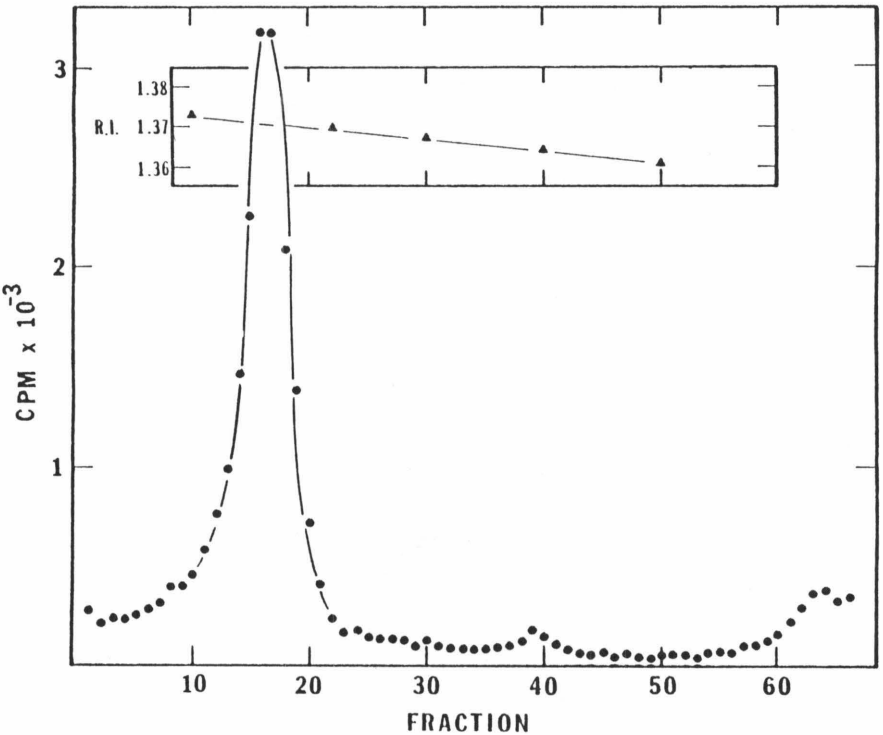
———, 1976. "Heat Inactivation of Poliovirus in Wastewater Sludge." *Applied and Environmental Microbiology* 32:339-364.

Weber, K., and J. Osborn, 1969. "The Reliability of Molecular Weight Determination by Dodecyl Sulfate Polyacrylamide Gel Electrophoresis." *Journal of Biological Chemistry* 244:4406-4412.

Wellings, F. M., A. L. Lewis, and C. W. Mountain, 1976. "Demonstration of Solids-Associated Virus in Wastewater and Sludge." *Applied and Environmental Microbiology* 31:354-358.

FIGURES

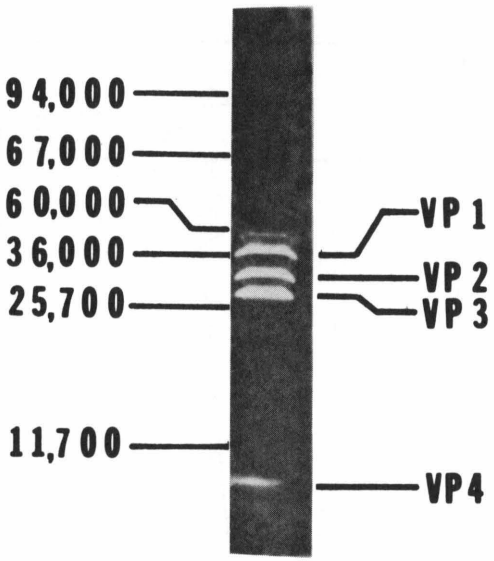
FIGURE 1
Glycerol Gradient of [^{14}C]-Poliovirus-2*



*Continuous glycerol gradients (15-30% v/v) in 0.01-M Tris-HCl, pH 7.5, 0.10-M NaCl, and 0.001-M EDTA (35 ml) were overlaid with the aqueous layer from the freon-113 extraction and centrifuged at 27,000 rpm for 4 hours at 4° in an SW-27 rotor. Fractions (0.05 ml) were collected from the bottom of the tube and analyzed for radioactivity. Direction of movement is from right to left. Inset: refractive index for each fraction.

FIGURE 2

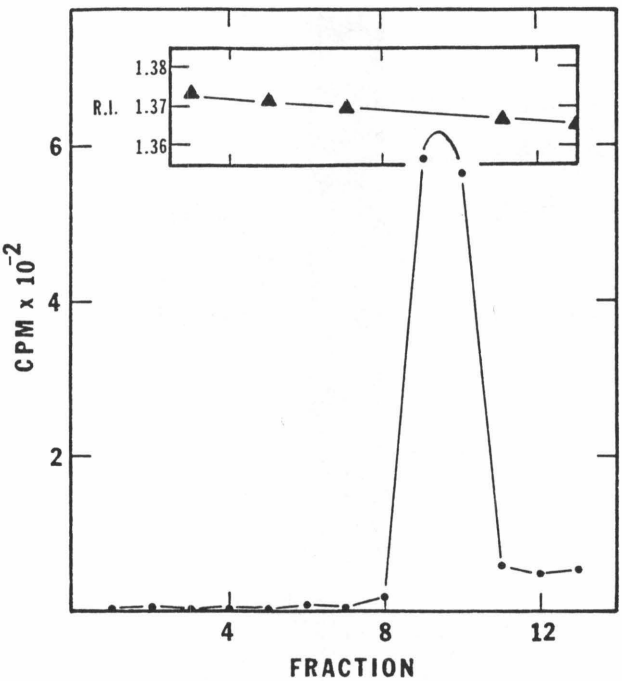
Autoradiogram of Glycerol Gradient Purified [^{14}C] -Poliovirus-2*



*SDS polyacrylamide gel electrophoresis of glycerol gradient purified poliovirus-2 was performed as described by Laemmli [1979] in a slab gel apparatus containing a 10 percent w/v acrylamide gel. Marker proteins were phosphorylase alpha (94,000d), bovine serum albumin (67,000d), catalase (36,000d), lactate dehydrogenase (25,000d), and cytochrome C (11,700d) [Weber and Osborne, 1969]. The slab gel was dried and exposed to Kodak, no screen, x-ray film. Film development was as described by Kodak Corp., Inc. The abbreviation "VP" is equivalent to virus-specfic polypeptide.

FIGURE 3

CsCl Centrifugation of [^{14}C] -Poliovirus-2*



*A sample of pooled glycerol gradient purified poliovirus was made to 1.340 mg/ml in CsCl and centrifuged at 35,000 rpm for 24 hours at 4°C in 60-Ti rotor. Following reorientation, 0.6-ml fractions were collected from the tube bottom. Samples of each fraction were analyzed for radioactivity. Sedimentation was from right to left. Inset: refractive index.

TABLES

TABLE 1
Human Enteric Viruses and Diseases

Virus Group and Subgroup	No. of Types	Disease
Picornaviridae		
Poliovirus	3	Paralytic poliomyelitis aseptic menginitis
Coxsackievirus		
Group A	24	Herpangina, aseptic meningitis, paralysis, fever
Group B	6	Aseptic meningitis, acute infantile myocarditis, pleurodynia, fever
Echovirus	34	Diarrheal disease, respiratory illness, rash, fever
Reoviridae	3	Fever, respiratory infections and diarrhea
Adenoviridae	30+	Respiratory infections infectious conjunctivitis
Ungrouped Enteric Viruses		
Hepatitis A	1	Infectious hepititis
Gastroenteritis A	2	Epidemic vomiting and diarrhea, fever
Rotavirus	1+	Epidemic vomiting and diarrhea, chiefly of children or elderly
Gastroenteritis B	1+	Vomiting, fever

TABLE 2
Relative Plaquing Efficiency of Several Viruses*

Virus	Cell Culture					
	VERO CCL-81	BGM	LLC-MK_2 CCL-7.1	RD CCL-136	WI-38 CCL-75	DEF
Po-2	1.0	1.4	1.1	3.0	0.80	0
Cox-B2	1.0	2.2	0.9	ID†	1.05	0
AHV	0	0	0	0	0	1.0

*A single stock of each virus was assayed on the indicated cell lines and titers were computed. The relative efficiency is the titer of a given virus on a cell line compared to the titer for the same Virus on VERO cells.
† ID plaques were indistinct.

TABLE 3
Sludge Parameters

Date of Sludge	Primary Sludge pH	Dry Weight* (% w/w)	Ammonia† (mg/l)	Conductivity‡ (mMho)	Digested Sludge pH	Dry Weight (% w/w)	Ammonia (mg/l)	Conductivity (mMho)
12-15-78	5.60	1.58	152	1.17	7.50	1.83	690	3.45
1-2-79	6.05	1.28	28	0.70	7.21	1.74	960	3.52
1-15-79	6.86	0.50	152	1.09	7.27	1.70	840	3.50
2-1-79	5.49	1.64	80	0.80	7.27	2.23	810	3.71
2-15-79	5.50	0.93	56	0.76	7.20	1.78	768	3.92
3-1-79	6.35	0.88	132	0.80	7.04	2.21	920	5.01
3-15-79	5.43	2.84	293	1.74	7.13	2.10	760	4.29
4-2-79	6.15	0.77	68	0.76	7.33	2.51	810	5.28
4-16-79	5.99	3.20	220	0.76	7.10	2.50	920	3.82
5-1-79	6.26	0.44	50	0.58	7.12	2.71	920	4.42
5-15-79	5.81	2.27	34	0.82	7.22	2.70	760	4.07
6-1-79	5.75	1.07	28	0.78	7.25	2.68	2000	5.04
6-18-79	6.48	0.05	24	0.58	7.17	2.82	1900	4.77
7-2-79	5.84	1.84	150	1.19	7.14	3.14	1800	4.75
7-19-79	6.07	0.44	135	0.64	7.13	2.49	1550	3.68
8-1-79	6.15	0.89	68	0.71	7.12	2.40	1650	4.28
8-15-79	6.10	2.40	74	0.96	7.05	2.31	1450	3.33
9-4-79	5.79	4.72	140	1.17	6.93	1.92	1150	2.92

* Average of triplicate determinations.
† Determined in clarified supernatants using an ammonia probe.
‡ As † above using a conductivity meter.

TABLE 4
Fraction of Poliovirus Bound to Primary Sludge Solids*

Date of Sample	Solids (% w/w)	Virus Bound (% of Input)
2-1-79	1.64	94.5
3-1-79	0.88	85.9
4-2-79	0.77	95.7
5-1-79	0.44	90.2
6-1-79	1.07	97.2
7-2-79	1.84	97.2
8-1-79	0.89	92.5
9-4-79	4.72	98.4
Control (No Sludge)	0.00	0.00

*Glycerol gradient purified [^{14}C]-poliovirus-2 (2000 cpm) was mixed with a 5.0-ml amount of each sludge, incubated at 22°C for 15 minutes, and centrifuged (16,000 rpm, 4°C, 30 minutes). The supernatant was assayed for radioactivity using a phase combining scintillator (Amersham Corp.). The results are expressed as 1.0 minus the relative amount of [^{14}C] material determined to remain in the supernatant fraction.

TABLE 5

Effect of Dilution on the Amount of Sludge Particle-Bound Virus*

Sludge Solids (% w/w)	Virus Bound (% of Input)
4.72	98.4
2.36	98.2
0.94	82.5
0.47	65.7
0.23	60.5
0.12	53.7

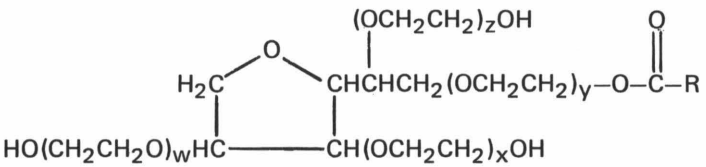
*A primary sludge sample (9-4-79) was diluted with deionized water to the percent w/w solids as shown. [^{14}C]-poliovirus (glycerol gradient fraction-2200cpm) was added to 5.0-ml samples of diluted sludge and incubated 15 minutes at 22°C. Centrifugation and radioactivity analysis were performed as in *Table 4.*

TABLE 6
Surfactant Structures*

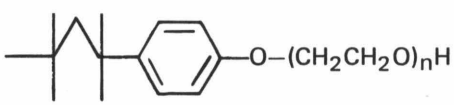
Tween 20, R = monolaurate

$w + x + y + z = 20$

Tween 80, R= monooleate



Triton X-100 series

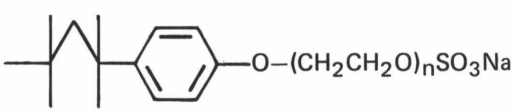


n = 9, 10

NP-40

$C_8H_7–(OCH_2CH_2)_nOH$ n = 40

Triton X-200 series



n = 20

Sodium Dodecyl Sulfate

* As obtained from Kirk and Othmer [1969].

TABLE 7

The Effect of Surfactant Concentration on Several Cell Lines

Cells	Surfactant* (% v/v)					
	SDS	T-20	T-80	NP-40	X-100	X-200
CCL-1	0.025	0.05	0.05	0.01	0.01	0.05
CCL-2.2	0.010	0.05	0.05	0.01	0.01	0.05
CCL-7.1	0.025	0.05	0.05	0.01	0.01	0.05
CCL-75	0.025	0.05	0.05	0.01	0.01	0.05
CCL-81	0.025	0.05	0.05	0.01	0.01	0.05
CCL-136	0.025	0.05	0.05	0.01	0.01	0.05
BGM	0.025	0.05	0.05	0.01	0.01	0.05
DEF	0.010	0.05	0.05	0.01	0.01	0.05

* The surfactants were diluted into minimal essential medium (AP-MEM) containing 10 percent FBS and 0.5 ml was added to confluent monolayers in mock assay protocol (see Materials and Methods — Plaque Assay).

† The results are expressed as the amount of surfactant in which no cell or monolayer cytopathic effects were observed.

TABLE 8

Relative Efficiency of Virus Extraction from Seeded Primary Sludge*

	Relative Efficiency of Extraction			
	Po-2		Cox-B2	
Extractant	Total Infectivity Recovered (pfv x 10^{-6})	% of Control	Total Infectivity Recovered (pfv x 10^{-6})	% of Control
SDS (0.2%)†	9.02	77.1	3.27	95.6
FBS (5.0%)	5.24	44.8	1.32	38.6
T-20 (0.5%)	2.15	18.4	0.18	5.3
T-80 (0.5%)	1.78	15.2	0.34	9.9
NP-40 (0.1%)	3.98	34.0	0.76	22.2
X-100 (0.1%)	3.66	31.3	0.61	17.8
X-200 (0.1%)	3.89	33.2	1.08	31.6
NaCl (2.5%)	9.20	78.6	0.79	23.1
BEE (2.0%)	6.26	53.5	1.64	47.9
Control (No Sludge)	11.7	100.0	3.42	100.0
None	1.95	16.7	0.82	23.9

* A 1/100 dilution of purified poliovirus-2 and purified coxsackievirus B-2 stocks were added to Corex centrifuge tubes containing 5.0ml of 1-15-79 primary sludge and incubated at 22°C for 15 minutes. The extractants were added to the indicated final concentration. The tubes were sonicated 1 minute (Branson sonifier) and centrifuged at 10,000 rpm for 30 minutes at 4°C (poliovirus-2) or 22°C (coxsackievirus B-2). The amounts of viral infectivities in the supernatant fractions were determined and compared to a buffer control (0.01-M Tris-HCl, 0.1-M NaCl, and 0.003-M $MgCl_2$) treated identically but without sludge. Plaque-forming units are expressed as the numerical averages of duplicate plates in tenfold series dilutions where assay linearity was achieved between 10 and 500 plaques on an 87-mm diameter plate.
† Percent in extraction assay.

TABLE 9

Multiple Extraction-Concentration of Poliovirus-2 Seeded Primary Sludge*

Sludge Sample (Date)	Solids Content (% w/w)	Solids Content in Extraction Mixture (% w/v)	Volume		[^{14}C]-Poliovirus†				Average Efficiency (%)
					Trial I		Trial II		
			Initial (ml)	Final (ml)	cpm	(% of input)	cpm	(% of input)	
3-1-79	0.88	0.55	18.8	1.31	2146	97.6	1978	89.9	93.7
4-2-79	0.77	0.48	18.8	1.40	1904	86.6	2173	98.8	92.7
5-1-79	0.44	0.28	18.8	1.23	2211	100.6	2173	96.9	98.7
6-1-79	1.07	0.67	18.8	1.24	1680	76.4	2181	99.3	87.8
7-2-79	1.84	0.58	9.9	1.21	1049	93.2	1754	78.8	86.5
8-1-79	0.89	0.56	18.8	1.21	1673	76.1	1914	87.0	81.5
9-4-79	4.72	0.59	3.8	1.17	1414	64.3	1697	77.2	70.7‡

* Glycerol gradient purified [^{14}C]-poliovirus was seeded into duplicate samples of the indicated amounts of primary sludge, incubated at 22°C for 15 minutes, then subjected to extraction by the multiple extraction-concentration procedure (Materials and Methods).

† The results are expressed for each trial as the amount of virus in the supernatant fraction relative to that amount seeded.

‡ Double extraction only.

TABLE 10

Indigenous Virus Found in Primary Sludge*

Sludge (Date)	Amount Extracted (ml)	Efficiency of Extraction (%)	Infectivity Observed (pfv/plate)		Titer† (pfv/liter)	Coliphage to Enteric Virus (Ratio)
3-1-79	18.8	93.7	VERO	C‡	C	–
			BGM	4	1.0×10^3	2.5×10^2
			E. coli	671	2.5×10^5	–
4-2-79	18.8	92.7	VERO	58	1.9×10^4	1.2×10^2
			BGM	13	3.6×10^3	6.4×10^2
			E. coli $80^{(-2)}$ §		2.3×10^6	–
5-1-79	18.8	98.7	VERO	4	1.2×10^3	1.1×10^2
			BGM	4	1.2×10^3	1.1×10^2
			E. coli $10^{(-2)}$		1.3×10^5	–
6-1-79	18.8	87.8	VERO	11	2.9×10^3	1.6×10^1
			BGM	13	3.5×10^3	1.3×10^1
			E.coli $3^{(-2)}$		4.6×10^4	–
7-2-79	9.9	86.5	VERO	9	4.3×10^3	3.0×10^2
			BGM	4	2.1×10^3	6.2×10^2
			E. coli $45^{(-2)}$		1.3×10^6	–
8-1-79	18.8	81.5	VERO	4	1.2×10^3	2.8×10^4
			BGM	18	5.4×10^3	6.3×10^3
			E. coli $13^{(-4)}$		3.4×10^7	–
9-4-79	3.8	70.7	VERO	0	0	–
			BGM	3	5.0×10^3	1.6×10^3
			E. coli $81^{(-2)}$		8.0×10^6	–

* As extracted from the multiple extraction and concentration procedure.
† Calculated from the total dilution, plating factor, and efficiency of extraction for poliovirus-2 as obtained in *Table 9.*
‡ Contaminated
§ Number of plaques observed on a lawn of bacteria plated at the indicated 10-fold dilution.

TABLE 11

Effect of Evaporation on Poliovirus-2 Inactivation in Water at 22°C*

Elapsed Time (hours)	Sample Weight (grams)	Sample Concentration (fold)	Open Container		Sealed Container	
			Po-2 Total (pfv x 10^{-5})	Concentration (fold)	Po-2 Total (pfv x 10^{-5})	Concentration (fold)
0	49.32	1.0	128	1.0	117	1.0
11.0	31.25	1.58	99	1.3	125	1.1
17.0	15.69	3.14	78	1.7	112	1.0
22.5	6.81	7.24	50	2.6	122	1.1
23.5	2.50	19.73	31	4.2	125	1.1
27.0†	0.0	–	3	–	120	1.0

* A differentially centrifuged and pelletized, purified stock of poliovirus-2 in NTE buffer was diluted 1/100 into deionized water and incubated for the indicated times in an open petri dish (150 cm^2) or a polypropylene tube (sealed). At the indicated times samples of each vessel were taken for assay of infectivity. Infectivity is expressed as in *Table 8.*

† At dryness (27.0 hours) 1.0 ml of 0.2% SDS in Tris-HCl, pH 9.0 was added and agitated in the petri dish. The virus yield was 3.1 x 10^5 pfv total.

TABLE 12

Effect of Evaporation on Poliovirus-2 Inactivation in Primary Sludge at 22° C*

Elapsed Time (hours)	Sludge Solids (% w/w)	Open Container		Sealed Container	
		Infectivity (pfv total)	Inactivation (%)	Infectivity (pfv total)	Inactivation (%)
0	2.27	8.5×10^6	0	1.0×10^7	100.0
12.0	2.58	6.0×10^6	29	N.D.†	–
18.0	4.80	2.4×10^6	71	N.D.	–
22.0	24.0	9.8×10^5	88	0.98×10^7	5.8
23.0	41.6	2.8×10^5	97	N.D.	–
23.5	55.0	9.0×10^4	99	N.D.	–
24.0	71.6	$<1.0 \times 10^2$	99.999	1.1×10^7	0.0
25.0	90.9	$<1.0 \times 10^2$	99.999	N.D.	–

* the protocol was the same as for *Table 11* only 2-15-79 primary sludge was used.
†N.D.=not done.

TABLE 13

Effect of Evaporation on Poliovirus-2 Inactivation in Primary Sludge at 37.0°C*

Elapsed Time (hours)	Sludge Solids (% w/w)	Open Container		Sealed Container	
		Infectivity (pfv total)	Inactivation (%)	Infectivity (pfv total)	Inactivation (%)
0	2.27	9.1×10^6	0.0	9.0×10^6	0.0
12.0	2.63	7.3×10^6	20	N.D.†	–
16.0	9.21	2.5×10^6	73	N.D.	–
17.0	20.1	1.1×10^6	77	9.5×10^6	0.0
18.0	43.3	7.2×10^5	93	N.D.	–
19.0	79.8	9.0×10^2	99.991	N.D.	–
19.5	87.9	$<1.0 \times 10^2$	>99.999	N.D.	–
20.0	92.5	$<1.0 \times 10^2$	>99.999	7.1×10^6	21

*the protocol was the same as described for *Table 11* only 2-15-79 primary sludge was used.
† N.D.=not done.

The Virginia Water Resources Research Center is a federal-state partnership agency attempting to find solutions to the state's water resources problems through careful research and analysis. Established at Virginia Polytechnic Institute and State University under provisions of the Water Research and Development Act of 1978 (P.L. 95-467), the Center serves five primary functions:

- It studies the state's water and related land-use problems, including their ecological, political, economic, institutional, legal, and social implications.
- It sponsors and administers research investigations of these problems.
- It collects and disseminates information about water resources and water resources research.
- It provides training opportunities in research for future water scientists enrolled at the state's colleges and universities.
- It provides other public services to the state in a wide variety of forms.

More information on programs and activities may be obtained by contacting the Center at the address below.

Virginia Water Resources Research Center
617 North Main Street
Blacksburg, Virginia 24060
Phone (703) 961-5624