

## Demonstration of Virus in Groundwater after Effluent Discharge onto Soil

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The survival of virus present in secondary effluents discharged into a cypress dome was studied. Isolations were made from concentrates of water drawn from 10-foot (304.80 cm) deep wells. Data presented show vertical and lateral virus movement as well as survival within the dome for 28 days during a period of heavy rains when no effluent was being applied. Due to the inefficiency of virus concentration procedures, it is proposed that much of the virus present was probably not demonstrated. A rapid, relatively inexpensive concentration technique for sewage influents and effluents is discussed.

There is relatively little data available on the survival of human pathogenic virus in soils under natural conditions. Mack et al. (12) isolated type II poliovirus from a well, 100 feet (3,048 cm) in depth, located about 300 feet (9,144 cm) from the edge of a wastewater drain field. In general, laboratory data derived from soil-packed column studies utilizing tagged bacteriophage (2) have concluded that virus is trapped in the upper mantle of the soil, provided that clays and silt are an integral part of the soil composition. These studies have contributed to the understanding of soil adsorption mechanism(s). Unfortunately, such studies do not produce information which can be applied to soils in their natural state. Lateral movement and its subtle changes over time is precluded in column studies as is heterogeneity within the soil structure.

The first study undertaken in St. Petersburg, Fla. (20) demonstrated that virus present in wastewater used for spray irrigation of a test plot readily percolated through 5 feet (152.40 cm) of sandy soil. After the project had been in progress for about 2 years, 28 inches (71.12 cm) of rainfall in a 3-month period resulted in saturation of the semipermeable organic layer. As these waters receded, a burst of virus was demonstrated in both the 10- and 20-foot (304.8 and 609.60 cm) monitoring wells. Why this occurred is not clear but may indicate desorption of attached virus resulting from solution of a portion of the organic layer.

This communication describes a current study in which effluents from a package treatment plant serving a 154-unit mobile home park are being discharged onto a cypress dome.

Determining the fate of virus present in the effluents is an integral part of a more comprehensive study of the effects of wastewater discharge into cypress swamps at the Center for Wetlands under the direction of H. T. Odum, University of Florida in Gainesville. The multiccosystem found in cypress domes was considered to offer a more efficient milieu for additional treatment of wastewaters than conventional soils. From a virological standpoint, two major questions were posed. First, would the wastewater from a package treatment plant serving a mobile home park be comparable to wastewater from an urban treatment plant serving a much larger number of residences? Secondly, would the high water-soil ratio found in cypress domes discourage virus attachment to soil particles and permit relatively free passage of virus to deeper levels?

### MATERIALS AND METHODS

**Research site.** The research site is a typical Florida flat lands cypress dome, so named because of the dome-like silhouette produced by older, taller trees grouped in the center. The size and age of trees decrease gradually toward the fringe area. The experimental dome covers an area of about 55,000 square feet (5060.0 m<sup>2</sup>) with a water holding capacity of about 378,000 gal (about 1,436,400 liters). The ecology of the site had been altered drastically. First, in March 1973, a drainage ditch was dug just southwest of the dome which lowered the water table sufficiently to dry up the natural dome pond. Secondly, in December 1973, all the undergrowth in the dome was destroyed by fire, as were most of the pine and a few of the medium sized cypress trees.

The first 2 feet (ca. 60.96 cm) of the dome depression is a black organic material containing 12% clay (5) at the surface and decreasing to 4% in the

underlying natural surface sand which may vary but is about 4 feet (about 122 cm) in depth. An organic, semipermeable layer containing 32% clay interspersed throughout represents the first confining layer between 6 and 7 feet (ca. 183 to 213 cm). Below this, a 6-foot thick layer of tan to gray sand gives way to blue-gray, sandy clay with increasing (5 to 19%) clay content. At about 17 to 18 feet (518.2 to 548.64 cm), solid blue clay is evidenced. Permeability (6) of the gray-blue, sandy clay was measured at  $3 \times 10^{-5}$  to  $3 \times 10^{-6}$  cm/min, so low as to preclude significant vertical flow. The major groundwater flow therefore occurs in the sandy layer immediately above, which has a permeability of  $3 \times 10^{-2}$  to  $3 \times 10^{-3}$  cm/min.

Wells were constructed of 1.25-inch (ca. 3.22 cm) polyvinyl chloride (PVC) pipe with slotted screen terminals. They were installed with the aid of a mobile gasoline powered drillrig with a 1.5-inch (ca. 3.22 cm) drill bit. The tops were covered with PVC well caps to prevent overt external contamination.

**Epidemiological approach.** To determine pertinent characteristics of the population served by the treatment plant our public health nurse made monthly visits to the park. The number of mobile homes occupied, total population, and number of children were recorded. In addition, families having children were interviewed to determine the number of children having had polio vaccine and the number and types of illnesses experienced during the month. Stools were obtained when possible from individuals experiencing acute illness.

**Virus concentration.** All monitoring well waters were processed for virus concentration by the membrane adsorption technique (3, 13, 14, 16-19). Samples varied in size from 50 to 100 gallons (ca. 190-380 liters). Test waters were pretreated by adding  $\text{Al}^{3+}$  in the form of  $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$  to a concentration of 0.0005 M and 12 N HCl, sufficient to achieve a pH 3.5. Celite (Sigma Chemical Co., Diatomaceous Earth Grade I, St. Louis, Mo.) was added to a concentration of 0.0001% to reduce rapid membrane clogging (8). The test sample was placed in a pressure tank and forced through an AP-20 prefilter and a 142-mm diameter cellulose nitrate membrane, 0.45- $\mu\text{m}$  porosity, held in a stainless-steel membrane filter (Millipore Corp.) holder by using compressed nitrogen at 5 to 50 lb/in<sup>2</sup>. Rate of filtration was 0.5 to 5.0 liter/min.

**Virus elution.** The Celite-coated prefilter and cellulose nitrate membrane were processed separately. In the field each was placed in a separate plastic bag containing 20 to 50 ml of eluting medium [5% beef extract buffered to pH 9.5 with tris(hydroxymethyl)aminomethane and NaOH] and transported to the laboratory on wet ice. There they were either triturated with mortar and pestle in the presence of alundum or homogenized in a Waring blender. After sonication (SoniFier, model W-350, Ultrasonic, Inc.) at 100 W for 15 min in a rosette cooling cell, the material was centrifuged at  $1,200 \times g$  for 30 min. Supernatant was removed and further concentrated as described below.

**Concentration of supernatants, influent and effluent.** Original concentration of influents and effluents and final concentration of supernatants were

carried out by polyethylene glycol dehydration (4, 15). The material was placed in dialysis tubing (24 nm average pore size) and exposed to polyethylene glycol (Carbowax 20,000) overnight at 4 C. The following morning sufficient eluting medium containing 2,000 U of penicillin per ml and 2,000  $\mu\text{g}$  of streptomycin per ml was added to the dialysis bag to make a final volume of 5 ml. Concentrates were centrifuged at  $20,000 \times g$  for 30 min before storage at  $-70$  C in the Revco.

**Virus assay.** Buffalo green monkey kidney cells in passage 148 were kindly supplied by Gerald Berg, Environmental Protection Agency, Cincinnati, Ohio. This continuous cell line has been shown to be more sensitive for the recovery of viruses from water than primary Rhesus and African green monkey kidney cells (7). Stock bottles were trypsinized, centrifuged, and resuspended in sufficient Eagle minimal essential medium-Hanks 10% fetal calf serum to give a final cell concentration of  $6.6 \times 10^4$ /ml. Six milliliters of the cell suspension were seeded into 30-ml Falcon plastic flasks (Falcon Plastic, Division of Bio Quest, Los Angeles, Calif.), and incubated at 36 C for 7 days at which time monolayers were confluent. These were inoculated with 0.5 ml of the test materials. Adsorption proceeded for 2 h at 37 C with rocking every 15 min to facilitate adsorption. Six milliliters of Eagle minimal essential medium, Earle's salts with 5% fetal calf serum and 1% agarose overlay were added after adsorption and allowed to solidify at room temperature. Bottles were inverted and incubated at 36 C. At 72 h a second agarose overlay containing 1:66,000 neutral red was applied. Observations for plaque forming units (PFU) were made daily for 14 days. Individual plaques were picked and inoculated into tube cultures for antigen production.

**Identification of isolates.** Depending upon the rapidity of appearance, morphology of the plaque and type of cytopathic effect on passage, agents were serotyped by utilizing pooled immune sera after Lim and Benyesh-Melnick (11). On a small number of isolates, confirmation of the pooled sera findings was obtained by using the indicated specific immune serum in a neutralization test. Reovirus identification was by hemagglutination-inhibition.

## RESULTS

Total capacity of the mobile home park is 154 units. At the initiation of the study only 133 spaces were occupied and after 6 months, 145 were filled. The total population varied between a low of 310 and a high of 337. The percentage of children ranged from 19.1 to 31.0. During each month of the study reported here at least one and as many as four children had received oral polio vaccine.

Three stools were obtained from children exhibiting gastrointestinal symptoms. One collected 9 September was negative. Coxsackie B4 was isolated from a stool obtained on 5 August and a Coxsackie A (to be typed) from one obtained on 17 October. Coxsackie B4 had been

circulating in the park for at least 3 months as evidenced by the isolation of the virus from plant influent on 15 May.

Since this Coxsackie A isolate does not plaque in cell culture there is no evidence as to the time of its appearance in this community. None of the environmental samples are assayed in newborn mice, whereas, stool specimens are routinely inoculated into both mice and cell culture.

Table 1 shows the number of isolations made from 500 ml of plant influent concentrated by polyethylene glycol. Although these represented grab samples taken at various times during the day, 86.4% (19/22) yielded virus. Only a portion of the counted plaques were picked and of these 95.9% (466/486) passed. Applying this percentage to the  $\geq 1,851$  plaques counted, 22 samples yielded  $\geq 1,775$  PFU or  $\geq 80.65$  per sample ( $\geq 161.2$ /liter). Polio type 1, 2, and 3 accounted for 40.4% (71/176) of the isolates identified, Coxsackie B3 or B4, 42.6% (75/176), and Echo 4, 7, or 11, 17.0% (30/176).

It required approximately 0.5 to 1.5 h for the chlorinated effluent to transverse the approxi-

mately 823-m distance to the dome outflow. None of the 14 polyethylene glycol concentrated samples yielded virus. Table 2 shows the virus isolation data from the large samples concentrated by membrane adsorption. Of the 15 samples, 8 (53.3%) yielded 79 PFU, 73 of which passed (92.4%). Of the 58 identified to date, 52 (89.7%) were polio type 1, 2, or 3, and 6 (10.3%) were Coxsackie B3 or B4. Even though these samples represent relatively large volumes, they must be considered grab samples in comparison to the volume of effluent (2,430,200 gallons [ca. 9,134,760 liters]) applied during the study period.

Three samples of the stagnant dome waters taken approximately 1 m from the inflow site were tested. One 20-gallon (ca. 760 liters) sample taken on 11 August 1974 yielded five Coxsackie B4 isolates.

Eighteen, 10-foot-deep monitoring wells were constructed. However, only 11 yielded water during this study period and one of these, well A-10, could be pumped in April only. Table 3 shows the results of virus isolation attempts from monitoring well waters. In April the six

TABLE 1. Virus isolations made from 500 ml<sup>a</sup> of plant influent April through September 1974

Date	No. of PFU counted	No. of PFU		Identifications made to date									
				Polio			Coxsackie		Echo				
		Picked	Passed	1	2	3	B3	B4	7	11	14		
4-11-74	13		13					4					
5-13-74	0												
5-14-74	4	4	4	4									
5-15-74	96	48	48										
5-15-74	40	40	40										
5-15-74	65	19	19		1			18					
5-15-74	74	18	18										
6-13-74	33	33	33	2	6			2		1			
6-13-74	$\geq 353^b$	0											
6-13-74	27	27	25		11					1			
7-11-74	40	40	29				2	2					2
7-12-74	37	37	33				1	7					
7-13-74	71	71	68	2			4	2					
7-14-74	168	35	35	1				16					
8-08-74	0												
8-09-74	89	20	20	9	3	2		6					
8-10-74	310	0											
8-11-74	353	0											
8-12-74	47	47	47			1		18					
9-11-74	28	28	28		2					26			
9-11-74	0												
9-13-74	6	6	6		5								
Total	$\geq 1,851$	486	466	19	39	13	4	71	26	2			2
%		26.2	95.9	10.8	22.2	7.4	2.3	40.3	14.8	1.1			1.1

<sup>a</sup> Concentrated by polyethylene glycol.

<sup>b</sup> Estimated since PFU were too numerous to count.

TABLE 2. Virus isolations made from large samples of effluent at dome discharge site April through September 1974

Date	Sample size (gal.) <sup>a</sup>	No. of PFU from		No. of PFU		Identifications made to date				
		Membrane	Celite and prefilter	Picked	Passed	Polio			Coxsackie	
						1	2	3	B3	B4
4-10-74	25	0	13	13	8		6		2	
5-13-74	10	0	0							
5-14-74	10	1	0	1	1			1		
5-14-74	10	2	27	29	29	7	6	16		
5-14-74	10	0	0							
5-15-74	10	0	0							
5-15-74	10	1	0	1	1			1		
5-15-74	10	0	0							
7-12-74	5	0	21	21	21	12		2		
7-13-74	5	8	0	8	8					
7-14-74	5	0	1	1	1			1		
8-11-74	5	0	0							
8-12-74	10	0	5	5	4					4
9-11-74	15	0	0							
9-12-74	15	0	0							
Total	155	12	67	79	73	19	12	21	2	4
%		15.2	84.8	100	92.4	32.8	20.7	36.2	3.4	6.9

<sup>a</sup> Conversions: 25 gallons (ca. 950 liters); 15 gallons (ca. 570 liters); 10 gallons (ca. 380 liters); 5 gallons (ca. 19.0 liters).

TABLE 3. Results of virus isolation attempts from monitoring wells April through September 1974

Month	Monitoring well numbers (No. of positive/No. of test)										
	A-4	A-5	A-8	A-9	A-10	A-11	A-12	A-15	A-16	A-17	A-18
April	0/2	0/1	0/3	0/2	0/1	0/1	— <sup>a</sup>	—	—	—	—
May	—	0/2	0/1	—	—	—	0/1	0/2	1/2	—	—
June	0/2	0/2	0/2	1/2	—	1/2	—	0/2	0/1	0/1	0/2
July	0/2	0/2	0/3	0/1	—	0/2	0/2	0/1	0/2	—	0/2
August	0/1	0/2	0/2	0/2	—	0/2	0/2	0/1	0/2	0/2	—
September	0/1	0/2	0/1	0/1	—	0/1	0/1	0/1	0/1	0/1	0/1

<sup>a</sup> —, Not tested.

wells tested were negative for virus. However, in May, a 50-gallon (ca. 1,900 liter) sample from well A-16 located approximately 7 m outside the southwestern rim of the dome pond (see Fig. 1) yielded a Coxsackie B4 isolate indicating both vertical and lateral movement of the virus in this cypress dome milieu. Coliform counts on the same specimen were two total and no fecals per 100 ml. On 14 June, 50-gallon samples from wells A-9 and A-11 were also positive. A-9 yielded a polio type 2 isolate and A-11 yielded a polio 1. Unfortunately, coliform counts were not done on the same specimens. Counts per 100 ml done on waters from these wells on 24 June were 33 total coliforms and 13 fecals in well A-9 and two total and no fecal in well A-11.

## DISCUSSION

Polyethylene glycol concentration of small samples (500 to 1,000 ml) in our hands has been shown to be both inexpensive and efficient. Although Cliver (4) and Shuval et al. (15) have reported relatively poor recoveries, 10 to 30% and 24 to 61%, respectively, control runs in our laboratory have shown 100% recovery of input virus. The major difference in the techniques used was use of 3% beef extract in tris(hydroxymethyl)aminomethane at pH 9.0 to rinse the dialysis tubing which most probably desorbed any attached virus. In neither report was the average pore size of the dialysis tubing mentioned which would also be a factor in the divergent findings. The dialysis tubing used in

this study had an average pore size of 24 nm which should preclude attachment of virus to the membrane matrix.

When virus was concentrated by membrane adsorption, it was of particular interest to note that 84.8% of the virus isolations stemmed from the prefilter and Celite fractions when effluents were tested. Since this would indicate that virus was adsorbed to or an integral part of a suspended solid, the majority of our isolates could have been lost had the prefilter and Celite not been processed. It is true that in situ elution would release surface adsorbed virions but those which are integral parts of solids could not be desorbed. Well waters would be expected to contain fewer and much smaller suspended solids. Therefore, isolation from the membrane of two of the three isolates obtained from these waters was not unexpected. Even so, the third isolate may not have been demonstrated if in situ elution had been relied upon. When testing waters in which a very low virus multiplicity is anticipated, it would appear that multiple filters, as described by Wallis et al. (18), may lead to erroneous results if the virus present is,

indeed, an integral part of minute suspended solids.

The quantity of virus present in sewage varies extensively throughout the world and is related to the strength of the influent. The average PFU/liter ( $\geq 161$ ) demonstrated in the mobile home sewage falls within the limits of 75 to 1,663 PFU/liter in urban sewage as demonstrated by Kelly and Sandersen (9). In Israel where the strength of influents is much greater than in the United States, Buras (1) demonstrated an average of 56,000 PFU/liter in secondary effluents by her direct inoculation technique.

Based on the quantity of virus demonstrated in the chlorinated effluents discharged onto the dome, at least  $12.9 \times 10^4$  PFU ( $0.5 \text{ PFU/gal} \times 257,850 \text{ gal}$  [ca. 979,830 liters]) had been applied to the dome between 26 March and 19 April. This virus load must be considered a minimum in view of the approximate 25% efficiency of the membrane concentration technique with these waters.

The first isolation made from the A-16 well was of particular interest because it demon-

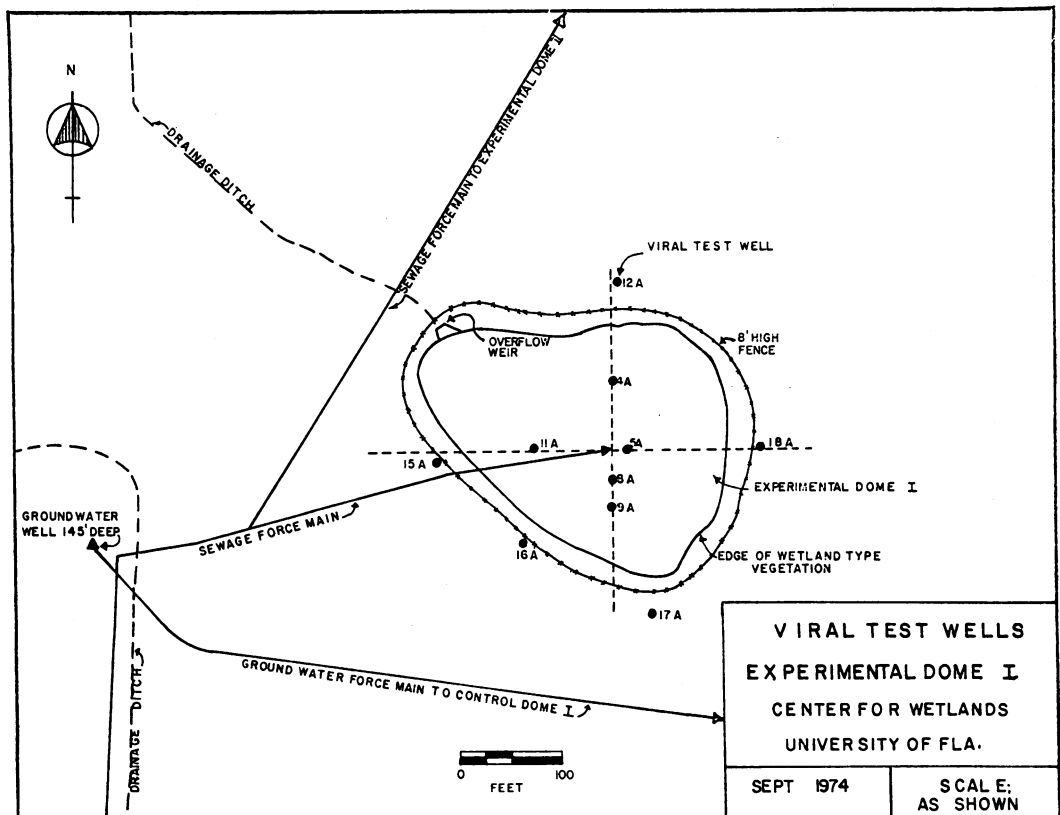


FIG. 1. Viral test wells, experimental dome I, Center for Wetlands, University of Florida, Gainesville.

strated percolation to a 10-foot level with lateral flow. Effluent was discharged into the center of the dome in which circulation is very limited. Over time, some lateral surface movement may have occurred. However, the A-16 well is located about 7 m outside of the dome pond (Fig. 1) and no effluent was applied to this surface area, therefore, subsurface lateral movement of at least 7 m was evidenced by this isolation. On 19 May four heavy pilings for supporting an observation tower had been sunk into the center of the dome cutting through the clay confining layers. This possibly permitted rapid passage of virus into the deeper water-bearing zones. If this were the case, then lateral transport of at least 38 m occurred to permit virus isolation from the A-16 well.

In spite of possible damage to the confining layer occasioned by the installation of the observation tower, the demonstration of virus on 14 June in wells A-11 and A-9 is vitally important. No effluent was applied to the site between 19 May and 2 July. Survival of virus in the dome for at least 28 days is evidenced by the two isolations made on 14 June. Whether or not this represented virus desorbed by heavy rains which fell during May and June can only be conjectured. However, the pattern is similar to that seen in St. Petersburg (19) where a burst of virus in the 10- and 20-foot deep wells occurred after 28 inches (71.12 cm) of rain fell in a 3-month period.

Our demonstration of virus survival for at least 28 days in the field and possible elution with rain water parallels the recently published laboratory data of Lefler and Kott (10) where they report survival of polio-virus type 1 in dry sterile sand for 77 days. In sand moistened with oxidation pond effluent, virus was detected on day 91 but not on day 105. Additionally, they showed that virus adsorbed to sterile sand could be eluted by washing with distilled or tap water. Although most of the virus was eluted in the first wash, virus could be found in the sand after 10 washings.

This study has again demonstrated the inadequacy of the coliform count in determining virus contamination. The total coliform counts in wells A-11 and A-16 (2 per 100 ml) would normally be discounted particularly in view of the negative fecal coliform counts. Yet, virus contamination was demonstrated with techniques recognized as being most inefficient.

Effluent has been applied daily to the test site but no additional isolations have been made nor have we experienced any heavy rains during this

time. Even so, in view of the infrequent sampling, once a month, with techniques having an efficiency of approximately 25%, it would appear that a great deal of undetected virus may be present in the groundwaters. The low numbers of demonstrated virus must be considered as a minimum.

This dome is being abandoned and will receive no additional effluents. However, we will continue to routinely monitor this site and increase our sampling during and after heavy rains to determine if long-term survival of virus can be demonstrated. It is unfortunate that the erection of the observation tower precluded an unbiased evaluation of the virus adsorption capabilities of this high water-soil ratio milieu. However, a virgin dome will be used for effluent application and every effort will be made to prevent any artificial breakthrough of the confining clay layer of this second experimental dome, so that this particular problem may be addressed.

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