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Survival of Enteroviruses and Coliform Bacteria in a Sludge Lagoon†

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Enteroviruses associate with aerobically and anaerobically digested sludge were determined before the addition of the sludge to a sludge lagoon. The fate of sludge-associated viruses was followed during detention of sludge in the lagoon and after application of sludge to land for disposal. While digested sludge was being added to the lagoon, enteroviruses were readily detected in grab samples of sludge from the lagoon. Sludge-associated viruses dropped to low or undetectable levels after disposal of sludge on land and during periods when addition of digested sludge to the lagoon was suspended. Changes in the levels of fecal coliforms in the lagooned sludge paralleled changes in the numbers of enteroviruses. Enteroviruses were not detected in water from deep wells located on the sludge disposal site or near the lagoon. During the initial part of the study, poliovirus serotypes accounted for greater than 90% of the viruses identified. Later, poliovirus serotypes comprised less than 40% of the virus isolates, and echoviruses and Coxsackieviruses were the most common enteroviruses identified.

Wastewater treatment by the activated sludge process results in the production of large amounts of excess sludge. This sludge may contain parasitic helminth eggs, protozoan cysts, bacterial pathogens, and viruses (2, 9, 11). Since anaerobic sludge digestion is not sufficient for total virus inactivation (2, 6), the possible spread of viruses during disposal of sludge must be considered. In the United States, the Water Pollution Control Act of 1972 (PL 92-500), as recently amended, requires acceptable methods for the utilization and disposal of wastewater sludge. Among the various alternatives available, land application is an attractive one in many cases. Sludge disposal by lagooning amounts of 264,000 dry tons (c. 237,600 t) per year within the United States (P. K. Bastian, unpublished data). This amount represents 4.5% of the total sludge. However, sludge lagooning is at best a temporary disposal method since the lagooned sludge is often applied to land for disposal.

In the present study, the fate of viruses associated with sludge during lagooning and after disposal of sludge on land was determined. Survival of coliform bacteria during lagooning was also followed.

MATERIALS AND METHODS

Sludge source and disposal site. Aerobically digested sludge (15-day detention time) from the Montclair plant and anaerobically digested sludge (30-day detention time) from the Main Street Plant in Pensacola, Fla., were transported by tank truck to a sludge-holding lagoon located at the Agricultural Research Center of the University of Florida at Jay. The sludge lagoon was approximately 60 by 100 ft (ca. 18 by 30 m), contained sludge and water to a depth of approximately 6 ft (ca. 1.8 m), and contained approximately 10^6 gallons (ca. 3.8×10^6 liters) of sludge-water mixture. Addition and removal of sludge occurred at opposite ends of the lagoon. Sludge removed from the lagoon was spread on a field divided into 72 plots of 40 by 120 ft (ca. 12 by 36 m) which received from 0 to 15 acre-inches (0 to ca. 170 m^3) of sludge per year. At certain stages of crop growth, land application of sludge as well as addition of digested sludge to the lagoon were suspended.

Well water. Water from three wells at the sludge disposal site and one from near the sludge lagoon were tested for the presence of enteroviruses. The water table at these sites was 40 to 60 ft (ca. 12 to 18 m) below the surface. Turbidity was measured by using a Model DRT 100 turbidimeter (HF Instruments Limited, Bolton, Ontario). Turbidity is expressed in nephelometric turbidity units.

Virus concentration procedures. (i) Well water. Water from two wells was hand pumped into 100-gallon (ca. 384-liter) tanks. The water was adjusted to pH 3.5 by the addition of 0.2 N HCl and adjusted to 0.0005 M aluminum chloride. Water from the other wells was removed by using deep-well pumps, added to 100-gallon tanks, and adjusted as described above or adjusted to pH 3.5 and 0.0005 M aluminum chloride by in-line injection of acid and salts (16). The treated water was passed through a 10-in (ca. 25-cm), 0.45-μm

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pore size Filterite filter (Filterite Corp., Timonium, Md.). The filters were then treated with 800 ml of 0.05 M glycine (pH 11.5). The glycine solution was permitted to remain in contact with the filters for 1 min, removed, and neutralized by the addition of 1 M glycine (pH 2). The neutralized samples were stored on ice for 24 to 48 h during transporation to the laboratory. The samples were then adjusted to pH 3.5 by the addition of 1 M glycine (pH 2) and centrifuged at 800 × *g* for 10 min. The supernatants were passed through a series of two 0.45-μm Filterite filters in 47-mm holders. Next, 7 ml of 0.05 M glycine–2% fetal calf serum (FCS) at pH 9 was passed through the filters, neutralized, and used as the inoculum for cell cultures. The sediment remaining after centrifugation at pH 3.5 was mixed with 5 volumes of phosphate-buffered saline (PBS) with 10% FCS at pH 9, readjusted to pH 9 by the addition of 1 M glycine (pH 11.5), if necessary. After centrifugation at 15,000 × *g* for 10 min, the supernatant was removed, neutralized, and used for inoculation of cell cultures.

(ii) Sludge. For most of the study, a modified version of the procedure of Hurst et al. (10) was used to recover viruses from sludge. Dried sludge-soil mixtures were mixed with 5 ml of 0.05 M glycine (pH 11.5) per g (wet weight) of sludge. The samples were mixed for 5 min on a magnetic stirrer. The pH was checked during mixing and adjusted to pH 10.5 to 11.0 by the addition of 1 M glycine (pH 11.5), if necessary. After centrifugation at 4,000 × *g* for 5 min, the supernatants were removed and adjusted to pH 3.5 by addition of 1 M glycine (pH 2). The adjusted samples were centrifuged at 4,000 × *g* for 20 min. The supernatants were passed through a series of two 0.45-μm Filterite filters in 47-mm holders. Next, 7 ml of PBS–10% FCS (pH 9) was passed through the filters, neutralized, and used as the inoculum for cell cultures. The pellet remaining after centrifugation at pH 3.5 was mixed with 5 volumes of PBS–10% FCS, adjusted to pH 9 by the addition of 1 M glycine (pH 11.5), and centrifuged at 15,000 × *g* for 10 min. The supernatant was neutralized by the addition of 0.05 M glycine (pH 2) and represented the final sample. Aerobically digested, anaerobically digested, and lagooned sludge were first centrifuged at 800 × *g* for 20 min. The supernatants were adjusted to pH 3.5 by the addition of 1 M glycine (pH 2) and treated as described above for the supernatants obtained by treating dried sludge with glycine at a high pH. The floc remaining after the first centrifugation was treated as described above for the dried sludge-soil mixture. Later in the study, a procedure employing 4 M urea buffered with 0.05 M lysine (pH 9) to elute viruses associated with sludge flocs along with a two-stage concentration procedure for recovering eluted viruses (7a) was compared with the modified Hurst et al. (10) procedure for its ability to recover viruses from sludge applied to land.

(iii) Lagooned water. Four liters of water overlying the lagooned sludge was adjusted to pH 3.5 by addition of 1 M glycine (pH 2) and centrifuged at 15,000 × *g* for 10 min. The supernatant was passed through a series of two 0.45-μm Filterite filters in 47-mm holders; 7 ml of PBS–10% FCS (pH 9) was passed through the filters, neutralized, and saved as the final sample. The pellet remaining after centrifugation was mixed with an equal volume of PBS–10% FCS (pH 9), readjusted to pH 9 by the addition of 1 M glycine (pH 11.5), and centrifuged at 15,000 × *g* for 10 min. The supernatant was removed and neutralized by the addition of 0.05 M glycine (pH 2) before assay.

Bacteriological assays. Fecal coliforms were determined by using EC broth and a multiple-tube procedure (1).

Cell cultures and viral assays. BGM or primary rhesus monkey kidney cells (Flow Laboratories, Inc., McLean, Va.) were used for isolation of indigenous viruses. Serial dilutions of samples were made in PBS–2% FCS and used to inoculate cell cultures. The cells were examined for cytopathic effects for up to three weeks. Samples showing cytopathic effects were frozen, thawed, diluted 1:100 to 1:10,000 to separate possible mixtures of viruses, and used to reinoculate cell cultures. Samples that produced cytopathic effects on the second passage were frozen. The titer of these isolates was determined by using MA-104 cells with a methyl cellulose overlay (4). Neutralization with specific antisera was used to identify isolates (12). The 50% tissue culture infective dose was determined by the method of Reed and Muench (14). The ability of randomly selected poliovirus isolates to form plaques at both 37 and 40.5°C was determined. Poliovirus 1 strains LSc and Mahoney were used as controls.

Ultracentrifugation. Samples with excessive final volumes were centrifuged at 120,000 × *g* for 90 min in a T1-60 rotor with a Beckman Model L3-50 ultracentrifuge (Beckman Instruments, Fullerton, Calif.). The pellets were suspended in 1 to 4 ml of FCS and assayed.

Chlorophyll *a*. Chlorophyll *a* was determined by the trichromatic method of Weber (17).

RESULTS

A modification of the procedure of Hurst et al. (10) was used in most of this study to recover viruses associated with sludge particles. The procedure for elution of virus associated with sludge flocs by using 0.05 M glycine at approximately pH 11 and concentration of eluted viruses on in situ-formed flocs at pH 3.5 or on membrane filters was similar to that of Hurst et al. (10). In the modified procedure, the supernatant fraction remaining after the collection of sludge flocs by centrifugation was also examined for viruses. Adjusting glycine solutions that had been used to elute virus associated with sludge flocs to pH 3.5 led to the formation of 1 to 2 ml of floc when activated sludge was used (10). In this study with digested and lagooned sludge, floc volumes in excess of 21 ml were produced. To reduce the volume of FCS required, these flocs were treated with 5 volumes of 10% FCS in PBS (pH 9) rather than the 5 volumes of whole FCS previously used. Elution of viruses adsorbed to the floc could be accomplished by either solution, but the cost could be greatly reduced by using 10% FCS. To reduce the exposure of virus to glycine (pH 11 to 11.5), virus adsorbed to membrane

filters was eluted with 10% FCS in 0.05 M glycine (pH 9) rather than 0.05 M glycine (pH 11.5). Using this modified procedure, Pancorbo et al. (O. C. Pancorbo, P. R. Scheuerman, S. R. Farrah, and G. Bitton, Can. J. Microbiol., in press) recovered approximately 55% of poliovirus added to the lagooned or anaerobically digested sludge and 25% of the virus added to the aerobically digested sludge used in this study.

At the Jay Agriculture Research Center, digested sludge from two wastewater treatment plants serving Pensacola, Fla., was added to a sludge lagoon before disposal on land. As shown in Table 1, aerobically digested sludge had a greater number of viruses associated with it than did the anaerobically digested sludge. Although aerobically digested sludge accounted for approximately 30% of the total sludge added to the lagoon, it provided most of the viral load. Digested sludge was added to the western end of the lagoon and removed from the eastern end for land application. At certain stages of crop development, sludge application to land as well as the addition of sludge to the lagoon were suspended. Viruses were readily recovered from grab samples of lagooned sludge during periods when digested sludge was added to the lagoon. After the addition of digested sludge was stopped, viruses were recovered in relatively high numbers from a sample of lagooned sludge obtained 3 weeks later. For the next 6 months, viruses were found in low numbers or were undetected in sludge samples from the lagoon (Fig. 1). After the addition of digested sludge to the lagoon was resumed, viruses could again be easily detected in the lagooned sludge. Changes in the numbers of fecal coliforms associated with the lagooned sludge paralleled changes in enterovirus numbers (Fig. 2).

TABLE 1. *Viruses associated with digested sludge added to the sludge lagoon*

Date obtained	Type of sludge digestion used[a]	Viruses ($TCID_{50}/g$)[b]
17 February 1978	Aerobic	260
17 February 1978	Anaerobic	7
6 December 1978	Aerobic	41
6 December 1978	Anaerobic	2
12 February 1979	Aerobic	14
12 February 1979	Anaerobic	4

[a] Aerobically digested sludge was obtained from the Montclair Plant, and anaerobically digested sludge was obtained from the Main Street Plant, Pensacola, Fla.

[b] $TCID_{50}$, Fifty percent tissue culture infective dose.

To determine whether viruses were being eluted from the lagooned sludge and transferred to the overlying water, samples of lagoon water were obtained while sludge addition to the lagoon was suspended and after it was resumed. Enteroviruses were found in the water when sludge was being added to the lagoon and were not found when sludge addition was suspended.

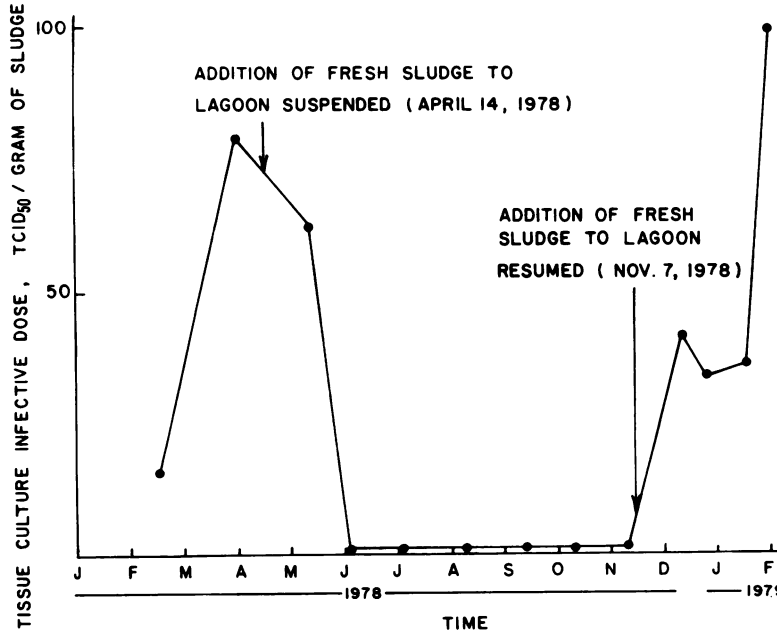

FIG. 1. *Survival of enteroviruses associated with lagooned sludge.*

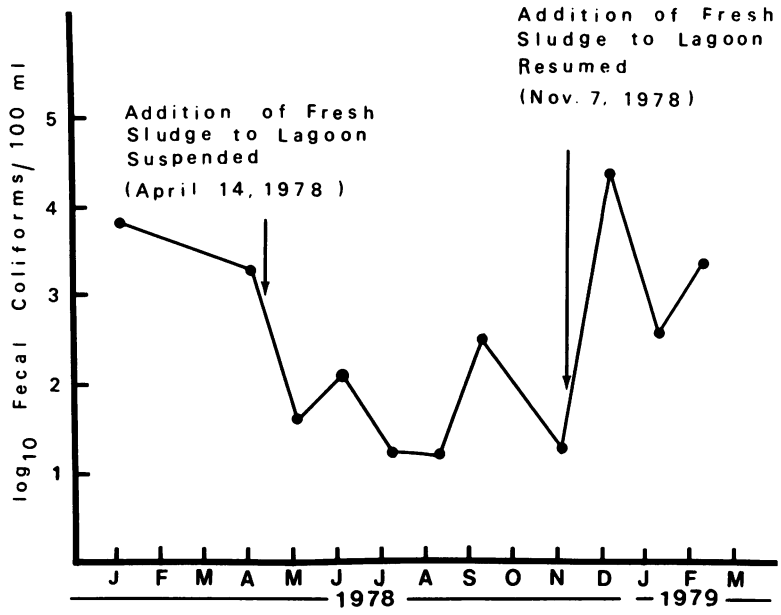

FIG. 2. *Survival of fecal coliforms associated with lagooned sludge.*

The lagoon water had a large number of algae as shown by the concentration of chlorophyll *a* (Table 2).

The number of viruses associated with the sludge declined after the sludge was applied to land for disposal (Table 3). However, viruses were detected 9 days after the sludge had been applied. The modified glycine elution procedure yielded larger final sample volumes than did the urea-lysine procedure. Such large final sample volumes (30 to 140 ml) were not produced when viruses were recovered from activated sludge (10). Since heterogenous samples containing relatively low numbers of viruses were examined, it is difficult to compare recovery of viruses by the two methods. The fact that the urea-lysine procedure gave slightly high titers in 4 of 6 samples, whereas the glycine procedure gave a higher titer in 1 of 6 samples, suggests that lower final sample volumes can be obtained without sacrificing efficiency of virus recovery.

Polioviruses, echoviruses, and Coxsackieviruses B were isolated from sludge water samples (Table 4). A variation was observed in the relative number of isolates obtained from samples collected from 17 February 1978 to 24 January 1979. Poliovirus 1 was the most common serotype isolated from the samples collected from 17 February to 6 November 1978. From 6 December 1978 to 24 January 1979, poliovirus serotypes represented less than 40% of the isolates obtained, whereas greater numbers of echoviruses and Coxsackieviruses B were recovered.

TABLE 2. *Viruses in water overlying lagooned sludge*

Date obtained	Sludge addition	Viruses ($TCID_{50}$/liter)[a]	Chlorphyll *a* (mg/m^3)
3 October 1978	No	<0.5	2,370
6 November 1978	No	<0.5	1,508
14 December 1978	Yes	9	387
11 January 1979	Yes	55	172

[a] $TCID_{50}$, Fifty percent tissue culture infective dose.

None of the 20 poliovirus 1 isolates was capable of producing plaques at 40.5°C, but all 20 could form plaques at 37°C. A virulent strain of poliovirus 1 (Mahoney) used as a control was capable of plaque formation at both temperatures.

Samples of well water were obtained at bimonthly intervals for 1 year. Well water samples generally had turbidities of 1 to 5 nephelometric turbidity units. Viruses were not detected in these water samples (Table 5).

DISCUSSION

Sludge lagoons have been used for years to store wastewater solids. They often provide a temporary means for disposing of sewage sludges. Although they may require relatively large areas, they allow for long-term storage of sludge and hence its further digestion. Lagoons make sludge production independent from its ultimate disposal (7). There is little information on the impact of sludge lagooning on virus sur-

TABLE 3. *Survival of viruses in sludge after application to land*

Date obtained	Days after spreading on land	% Water	$TCID_{50}/g$[a] by method:		Final sample volume (ml) by method:	
			A[b]	B[c]	A[d]	B
3 October 1979	0	91	1.4	4.6	55	2.5
4 October 1979	1	39	<0.01	0.16	130	1.8
5 October 1979	2	40	0.72	0.10	140	1.8
8 October 1979	5	ND[e]	<0.01	<0.01	30	1.5
10 October 1979	7	15	0.05	0.06	30	1.5
12 October 1979	9	19	<0.01	0.02	30	1.5

[a] $TCID_{50}$, Fifty percent tissue culture infective dose.
[b] The modified procedure of Hurst et al. (10).
[c] The urea-lysine procedure of Farrah et al. (7a).
[d] Virus in these samples was further concentrated by ultracentrifugation before assay.
[e] ND, Not determined.

TABLE 4. *Virus types recovered from sludge and water samples*

Sample	Sampling period	Total no. of isolates identified	% of isolates identified as:								
			Poliovirus 1	Poliovirus 2	Poliovirus 3	Echovirus 1	Echovirus 4	Echovirus 7	Echovirus 15	CB-4	Nontypable
Digested sludge[a]	17 February to 6 November 1978	32	81	9	9	0	0	0	0	0	0
Lagooned sludge		27	63	33	0	0	0	4	0	0	0
Total		59	73	20	5	0	0	2	0	0	0
Digested sludge[a]	6 December 1978 to 12 October 1978	10	20	0	30	20	0	20	0	10	0
Lagooned sludge		21	24	19	0	0	0	19	5	33	0
Water overlaying lagoon sludge		16	12	25	0	19	0	6	0	31	6
Sludge applied to land		10	10	0	0	30	30	10	0	10	10
Total		57	17	14	5	14	5	14	3	24	3

[a] From the Montclair and Main Street treatment plants.

vival and ground water contamination. Because sludge addition to the lagoon at Jay was suspended for several months, an opportunity was provided to study the survival of indigenous enteroviruses under climatic conditions prevailing in north Florida. Enterovirus numbers declined to low or undetectable levels after the suspension of sludge addition to the lagoon (Fig. 1). The failure to detect viruses in the sludge and in the water overlying the lagoon suggests that the viruses were inactivated. Also, no enteroviruses were detected in the groundwater beneath the lagoon. Sludge is known to seal off effectively lagoon contents from surrounding soils (7), and this may help to prevent groundwater contamination. Therefore, lagooning may provide a means for further reduction of viruses present in digested sludge before land application. Sattar and Westwood (15) detected viruses in 39% of lagoon sludge samples. They also reported that viruses could be detected after 7 months in a sludge lagoon in Ottawa, Canada. It is worth noting that the sludge lagoon was frozen for a 6-month period.

Few investigators have studied the fate of indigenous viruses during sludge application to land under field conditions (10, 13). Such investigations should include studies on the survival of viruses as well as transport of viruses through the soil matrix (3, 4, 8). At the Jay site, sludge is generally mixed with the top soil within 15 days after application. Indigenous viruses in the

TABLE 5. *Analysis of well water for the presence of enteroviruses*

Well	Site	Total volume of water sampled (liters)[a]	Viruses detected
1	Northeast quadrant	1,100	0
3	Southeast corner[b]	1,100	0
9	East central section	1,100	0
New lagoon	Ca. 10 ft (3 m) from lagoon	2,650	0
Total		5,950	0

[a] Six samples of approximately 180 liters were obtained over a 1-year period from wells 1, 3, and 9. Six samples of 180 to 700 liters were obtained from the well near the new lagoon over the same period.

[b] In the direction of the groundwater flow.

TABLE 6. *Mean maximum and minimum temperatures and precipitation at Jay for January 78 through February 79*

Date	Mean daily maximum temperature (°C)	Mean daily minimum temperature (°C)	Total monthly precipitation [in (cm)]
January 1978	10.6	0.6	12.8 (32.5)
February 1978	13.1	1.1	3.8 (9.7)
March 1978	20.2	7.4	6.0 (15.2)
April 1978	27.1	13.2	6.1 (15.5)
May 1978	29.4	17.8	9.6 (24.4)
June 1978	32.6	21.6	11.3 (28.7)
July 1978	32.4	22.5	12.1 (30.7)
August 1978	32.6	21.5	6.8 (17.3)
September 1978	32.2	20.6	4.2 (10.7)
October 1978	27.4	12.3	0.1 (0.3)
November 1978	24.8	11.7	4.1 (10.4)
December 1978	17.9	6.3	3.8 (9.7)
January 1979	13.6	1.8	6.5 (16.6)
February 1979	15.2	4.6	9.2 (23.4)

sludge were still detectable after 9 days of exposure to environmental conditions. This represented approximately a 2-$\log_{10}$ reduction in viral numbers. Controlled experiments using added virus and columns with cores of undisturbed soil have shown that 35 days were required for a 4-$\log_{10}$ reduction of poliovirus-1 or echovirus 1 during the hot and wet summer in Gainesville. During the hot and dry fall, 21 days were required for a similar reduction in virus numbers (Pancorbo et al., manuscript in preparation).

Virus movement through soils to the groundwater did not appear to occur since no viruses were detected in wells after a 1-year bimonthly monitoring program. These data are in agreement with the lysimeter studies of Damgaard-Larsen et al. (5) and with our own studies with columns containing cores of undisturbed soil (Pancorbo et al., in preparation).

Based on our results and the results of others (5) it appears that enteroviruses are efficiently retained by sludge-soil mixtures. The conditions prevailing in north central Florida at the time of this study are shown in Table 6. Under these conditions viruses are inactivated relatively fast in lagoon sludge or after land application. Future studies should attempt to determine whether other viruses such as rotaviruses and Norwalk-type agents are similar to enteroviruses in their survival and transport patterns.

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