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Low-Temperature Stability of Viruses in Sludges

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Enteroviruses survived for up to 38 days without diminishing in numbers in extended-aeration sludges maintained at 5°C. In oxidation ditch sludges similarly maintained, enteroviruses survived for up to 17 days without diminishing in numbers. The pHs of the sludges in this study were well inside the pH 6 to 8 corridor in which destruction of enteroviruses by the detergents and ammonia present in sludges reportedly does not occur. Unexplained, however, was the survival of large numbers of enteroviruses in sludges at pH 3.5, a pH at which some anionic detergents commonly present in sewage are rapidly virucidal. The long survival of enteroviruses in these sludges at 5°C indicates that such sludges can probably be stored under refrigeration in the laboratory for extended periods while awaiting processing without suffering significant losses in enterovirus numbers.

Large quantities of sludge are generated by biological treatment of municipal wastewater. Such sludge contains large numbers of enteroviruses and other viruses pathogenic for humans. The treatment and safe disposal of sludge are thus of major importance to regulators and land developers in the United States.

In cold- and temperate-climate areas, temperatures in the near-freezing range may persist for long periods. Since viruses survive longer in cold temperatures than in warm temperatures, it is important to know how well viruses survive in sludges under cold conditions. Such information is also useful in the laboratory, because sometimes sludge samples cannot be processed on the days that they are collected, and it is important to know for how long virus numbers remain stable in cold storage.

The study presented here demonstrates that enteroviruses are stable for long periods at 5°C in extended-aeration sludges and in oxidation ditch sludges.

The sludges used in this study were taken from wastewater treatment plants that used extended-aeration and oxidation ditch processes. These processes are conceptually identical in that they bring about waste stabilization through relatively long hydraulic residence times (on the order of 24 h or more) at low food/microorganism ratios. Both are most commonly used to treat wastewater flows from small communities, developments, and institutions.

Extended-aeration sludges were obtained from the Williamsburg Sewage Treatment Plant in Williamsburg, Ohio. The plant operates at a dry-weather flow rate of about 0.175 million gallons per day (1 gal = 3.785 liters). Oxidation ditch sludges were obtained from the Frankfort Sewage Treatment Plant in Frankfort, Ky. The plant operates at an approximate food/microorganism ratio of 0.03 and at a dry-weather flow rate of about 4.0 million gallons per day. Samples of all sludges were processed by a virus recovery procedure on the day of sludge collection and later assayed for viruses. The sludges were then stored at 5°C (±1°C) and processed and assayed again periodically thereafter.

From one to four replicate samples of sludge were homogenized in a Waring blender for 60 s, and 800-ml quantities were poured into 1.5-liter beakers. Sludges were then treated by one of two variations of the U.S. Environmental Protection Agency virus recovery method (1–3). In either case, 8 ml of 0.05 M $AlCl_3$ (final concentration in sludge, 0.0005 M) was added to each sludge and, on a magnetic stirrer, the pH of each sludge was reduced to 3.5 with 5 M HCl. The salted, acidified sludges were then stirred for 30 min longer and centrifuged at 1,000 × *g* for 30 min. The supernatants were poured off, and the solids from each sludge sample were treated by one of two variations of the U.S. Environmental Protection Agency method. In the first variation, to elute the viruses from the sludge solids, 100 ml of a 1% solution of skim milk (lot 11915; BBL Microbiology Systems) in 0.15 M Na_2HPO_4 (containing 1 drop of Antifoam) was mixed with the solids from each sludge sample for 30 min on a magnetic stirrer, the mixture was centrifuged for 30 min at 1,000 × *g*, the solids were discarded, and the pH of each supernatant was reduced to 4.5 with 5 M HCl. Flocs formed. The acidified milk eluates were mixed for 30 min on a magnetic stirrer to allow fuller development of the flocs, and the flocs were sedimented by centrifugation for 30 min at 1,000 × *g*. The supernatants were stored overnight at 5°C, and the flocculation procedure was repeated at pH 3.5. The supernatants were discarded. Each floc was suspended in 5 ml of 0.15 M Na_2HPO_4 and mixed on a magnetic stirrer for 30 min to elute the viruses from the floc. The floc suspensions were centrifuged for 30 min at 1,000 × *g*, the precipitates were discarded, and the eluates were stored at −70°C until the day on which they were assayed. In the second variation, to elute the viruses from the sludge solids, 100 ml of 10% beef extract (lot 07037; GIBCO Laboratories) was mixed with the solids from each sludge sample on a magnetic stirrer for 30 min, the pH of each sludge mixture was adjusted to 7.2 with 5 M HCl or with 5 M NaOH, as appropriate, as mixing began, and the mixture was centrifuged for 30 min at 1,000 × *g*. The solids were discarded, and the beef extract eluates were frozen at −70°C until the day on which they were assayed. Although two methods for recovering viruses were used, all recoveries from any given sludge were always done with the same method.

Frozen skim milk and frozen beef extract viral eluates were thawed in cold water. Beef extract eluates were centrifuged at 9,000 × *g* for 30 or 60 min. The Na_2HPO_4 eluates

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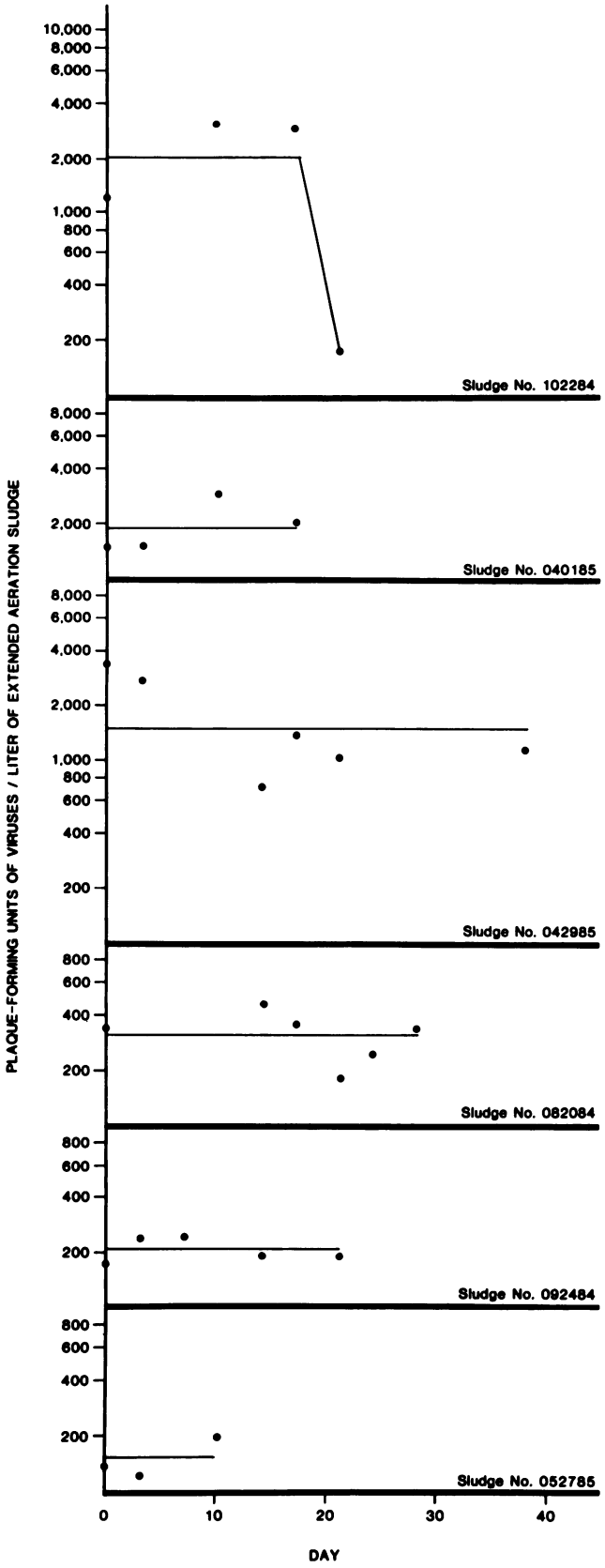

FIG. 1. Stability of viruses in extended-aeration sludge at 5°C.

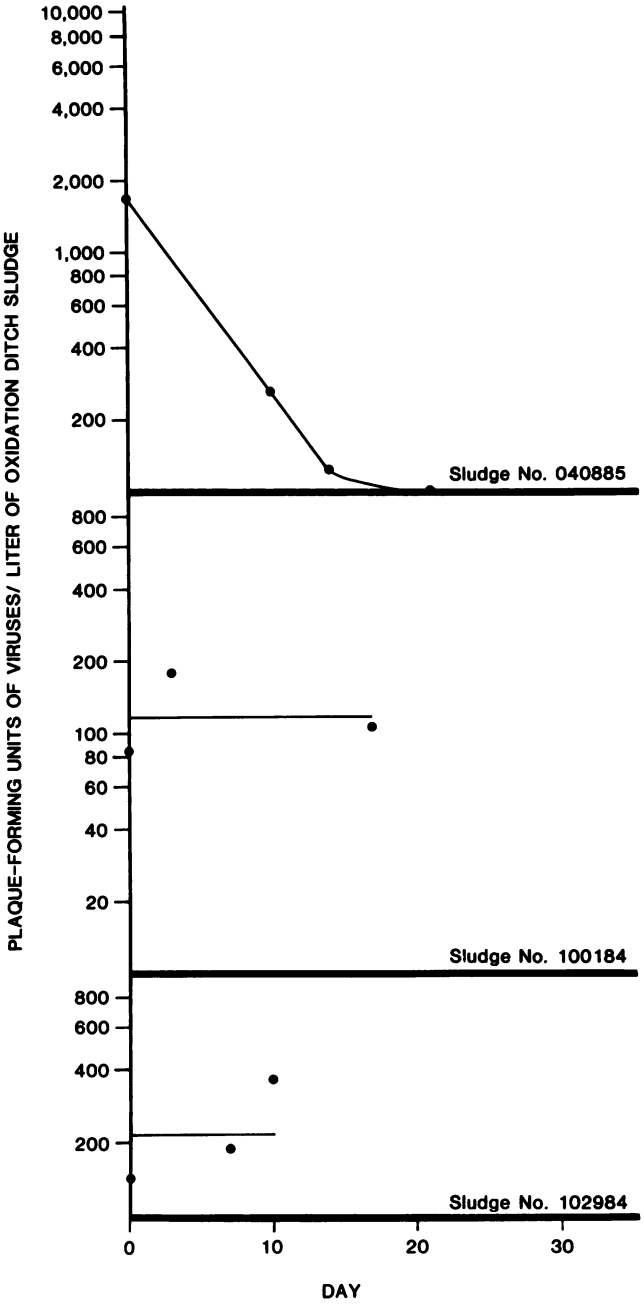

FIG. 2. Stability of viruses in oxidation ditch sludge at 5°C.

derived from the milk precipitations at pHs 4.5 and 3.5 were passed through filter packs (2) and assayed separately. Viruses were assayed by the plaque technique (6). Each Na_2HPO_4 eluate (1 ml) was inoculated onto each of four cell cultures, and viral counts obtained for each pair of eluates from the same sludge sample were added together. Each beef extract eluate (1 ml) was inoculated onto each of 10 cell cultures. All viral assays were done in BGM cells, a continuous line of cells derived from African green monkey (*Cercopithecus aethiops*) kidneys. The methods for preparing cell monolayer cultures and the plaque overlay technique were described earlier (5).

In all six of the studies with extended-aeration sludges

TABLE 1. pHs of sludges over the test periods

Sludge	No.	pH range
Extended-aeration	102284	7.0–7.4
	040185	7.0–7.2
	042985	6.4–7.1
	082084	6.8–7.0
	092484	6.5–6.8
	052785	7.0–7.3
Oxidation ditch	040885	6.3–6.8
	100184	6.6–6.8
	102984	6.5–6.9

(Fig. 1) and in two of the three studies with oxidation ditch sludges (Fig. 2), viruses were stable for at least 10 days. With one exception (sludge no. 102284), viruses were stable in extended-aeration sludges for as long as the sludges were assayed. In one sludge sample (sludge no. 042985), viruses were stable for at least 38 days. Viral die-off appeared to occur in one extended-aeration sludge sample (sludge no. 102284) after 17 days at 5°C and in one oxidation ditch sludge sample (sludge no. 040885) almost immediately. There is no way to know, however, if the 17-day sample point in sludge no. 102284 was aberrant. If the zero-day sample point for sludge no. 040885 was aberrant, moreover, a case could be made for the long-term stability of viruses in this sludge sample, too. It is also possible, however, that a virucidal agent was present in sludge no. 040885. The pHs in the extended-aeration sludges ranged from 6.4 to 7.4; the pHs in the oxidation ditch sludges ranged from 6.3 to 6.9 (Table 1). The pH levels in sludges are important, because they affect the level of nonionized ammonia (NH_3) present and the susceptibility of viruses to some detergents present. Ammonia is virucidal, and under appropriate pH conditions, certain detergents are also virucidal.

The lack of homogeneity among all sludges contributed greatly to error in viral and probably in all other assays in sludges. Nonetheless, the data suggest that at 5°C viruses usually survive in extended-aeration sludges and in oxidation ditch sludges for at least several weeks without a measurable loss in numbers (Fig. 1 and 2). The inability of viruses to multiply in sludges gives further credence to this interpretation of the data, because outside of experimental error, no point on any death rate curve can be higher than the lowest point preceding it. It might be argued that virus recovery procedures break up clumps of viruses and increase counts in so doing, but since all samples of each sludge were treated by the same virus recovery procedure, the same changes would have been encountered for all sample points for each sludge.

The pHs of the extended-aeration and oxidation ditch sludges may be important in the stability of the viruses present in these sludges. Detergents and NH_3 are present in most domestic sludges. Under certain pH conditions, both are highly destructive to enteroviruses, the viruses most likely to be recovered in the BGM cells used in this study for recovering viruses. The anionic detergent sodium dodecyl sulfate, for example, at 4°C rapidly destroys poliovirus 1 at pHs below 6 but not at higher pHs (8). At 47°C the antipoliovirus 1 activity of sodium dodecyl sulfate increases rapidly as pHs increase above 8. Between pHs of about 6 and 8, poliovirus 1 is stable, and the detergent, in fact, is reported to protect against temperature inactivation (8). At neutral and alkaline pHs, the cationic detergent dodecyltrimethylammonium chloride also reportedly protects enteroviruses against heat (8). Ammonia is a powerful virucide, but the ammonium ion (NH_4^+) appears not to be enterovirucidal. Although significant quantities of NH_3 and NH_4^+ occur in most sludges, there is little NH_3 below pH 8 (7). Both the extended-aeration sludges and the oxidation ditch sludges, collected during many different months of the year, occurred in a pH corridor (between pHs 6 and 8) that reportedly protects the enteroviruses present from potentially virucidal constituents in the sludges (7–9). The extended-aeration sludges ranged in pH from 6.4 to 7.4. The oxidation ditch sludges ranged in pH from 6.3 to 6.9. Yet to be explained, however, is the survival of large numbers of enteroviruses in sludges treated for about 1 h at pH 3.5 (as were all sludges in this study). In comparative studies (3, 4), the adsorption of virions in suspension onto sludge solids at pH 3.5 (as we did in this study) before elution of the virions from the solids yielded greater numbers of virions than did the same method without the pH 3.5 adsorption step. Anionic detergents (such as sodium dodecyl sulfate) commonly found in domestic sewage are extensively virucidal in 1 h at pH 3.5 (8).

The long survival of enteroviruses in extended-aeration sludges and in oxidation ditch sludges maintained at 5°C indicates, of course, that such sludges must be appropriately treated before discharge to the environment. It also indicates that such sludges usually may be stored in the laboratory under refrigeration for extended periods of time when necessary without affecting the numbers of viable enteroviruses present.

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