APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1981, p. 1023-1028
0099-2240/81/121023-06$02.00/0
Vol. 42, No. 6

# Recovery of Indigenous Enteroviruses from Raw and Digested Sewage Sludges

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Received 23 June 1981/Accepted 20 August 1981

We examined different types of raw sewage sludge treatment, including consolidation, anaerobic-mesophilic digestion with subsequent consolidation, and aerobic-thermophilic digestion. Of these, the most efficient reduction in infectious virus titer was achieved by mesophilic digestion with subsequent consolidation, although a pilot-scale aerobic-thermophilic digester was extremely time effective, producing sludges with similarly low virus titers in a small fraction of the time. Although none of the treatments examined consistently produced a sludge with undetectable virus levels, mesophilic digestion alone was found to be particularly unreliable in reducing the levels of infectious virus present in the raw sludge.

It has been well established that very large numbers of potentially pathogenic human enteric viruses may be present in raw sewage (8, 13) and that a large proportion of these viruses are associated with the solids fraction (19), much of which is deposited in the primary settling tanks to constitute the raw sludge. This sludge is generally disposed of to land after a treatment regime, typically of anaerobic digestion and subsequent consolidation, which does not necessarily result in the inactivation of viruses with any degree of consistency; indeed quite high levels have often been isolated from the treated sludge (1, 3). However, some forms of treatment, such as high-temperature anaerobic digestion or dewatering by evaporation, have produced sludges from which only low levels of infectious virus could be recovered (1, 16). There are difficulties involved in obtaining reliable and sensitive assays of infectious virus in sludge, and consequently evidence for the incidence and range of enteric viruses in different types of sludge has not been widely sought. Some of these problems have now been overcome (7), and this report provides data on frequent samples taken from different treatment plants over a period of several months.

## MATERIALS AND METHODS

**Sludges.** Samples of raw, consolidated (thickened) raw, anaerobic-mesophilic digested, aerobic-thermophilic digested, and consolidated digested sludges were selected from three plants, each following slightly different treatment regimes (Fig. 1 and Table 1). The samples were single-dip samples or pooled-dip samples collected over a 24- to 48-h period and held in 500-ml plastic bottles. A few milliliters of chloroform was added to each sample to inhibit bacterial growth, and the bottles were housed in an insulated box which kept the temperature at about 7°C. The samples were transported by rail, delivered on the day of sampling, and processed immediately on arrival at the laboratory.

**Isolation of viruses.** The technique was based on that of Glass et al. (7) and incorporated the organic flocculation concentration method of Katzenelson et al. (9), modified by the use of skim milk as the elutant (2). Skim milk was used in preference to beef extract as elutant, partially because of its easier availability and lower cost, and also because the pH required for its precipitation is pH 4.5. This is 1 U higher than that required for beef extract, thus subjecting the viruses to less ionic stress during the flocculation stage of the isolation procedure.

Sample volumes of 20 ml (raw and consolidated sludges) or 40 ml (digested sludges) were diluted to 200 ml with a 1% (wt/vol) solution of skim milk (Oxoid Ltd.). These were shaken rapidly (HKV orbital shaker) for 30 min at 20°C, and the solids were then removed by centrifugation at 23,000 × *g* for 30 min. The supernatant was flocculated at pH 4.5 with 2 N HCl, and the floc was collected by centrifugation at 23,000 × *g* for 20 min. The pellet obtained was redissolved in 5 ml of 0.15 M disodium hydrogen phosphate, and this solution was mixed with an equal volume of dithizone in chloroform (0.08 g/liter). After centrifugation at 30,000 × *g* for 15 min, the supernatant was transferred to a sterile bottle, exposed to a sterile airflow to remove any residual chloroform present, and finally stored at −190°C before assay.

**Cell cultures.** Buffalo green monkey kidney (BGM) cells between passage levels 112 and 130 were used for viral assays. They were grown in equal quantities of Leibovitz L15 and Eagle minimal essential medium with Hanks salts (Flow Laboratories, Inc.), containing 10% (vol/vol) fetal calf serum, 1% (vol/vol) 200 mM L-glutamine, and 0.11% (wt/vol) sodium bicarbonate. Cells were confluent after 3 days, at which time they were changed onto maintenance medium consisting of 0.5% (wt/vol) lactalbumin hydrolysate in Earles balanced salt solution with 3% (vol/vol) fetal calf serum and 0.22% (wt/vol) sodium bicarbonate. Both media also contained penicillin (100 U/ml) and

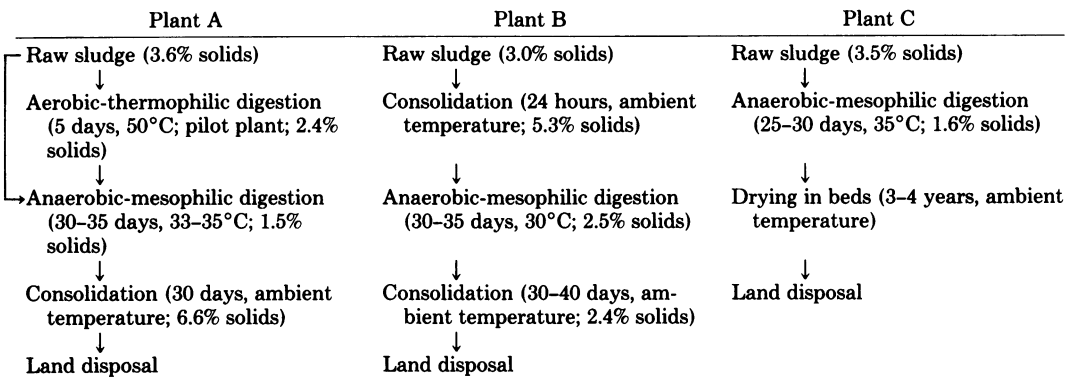


FIG. 1. *Flow diagrams of sludge treatments employed at three wastewater treatment plants. Raw sludge = primary sludge.*

streptomycin (100 μg/ml).

**Viral assay.** Viruses were assayed by a method based on that developed by Cooper (4, 5) and modified by Slade (14). A 10-ml amount of cells, at a concentration of $1.2 \times 10^7$ cells $ml^{-1}$, was mixed with 40 ml of overlay medium consisting of 10% (vol/vol) medium 199 with Hanks salts, 2% (vol/vol) fetal calf serum, 1% (vol/vol) 200 mM L-glutamine, 0.22% (wt/vol) sodium bicarbonate, 0.5% (wt/vol) magnesium chloride, 0.003% (wt/vol) neutral red, antibiotics (neomycin, amphotericin B, polymyxin B sulfate, kanamycin, penicillin, and streptomycin, used at the recommended levels [GIBCO-Biocult]), and 1.2% (wt/vol) Difco purified agar. A 1- or 2-ml amount of virus concentrate was mixed with the cell suspension, and the whole volume was poured into a triple-vented 14-cm petri dish (Sterilin, bacterial grade). Plates were inverted and incubated at 37°C in 5% carbon dioxide in air. Plaques were counted daily after 2 days, and the results were expressed in relation to the dry weight of the sample (plaque-forming units per gram) because the sludges varied widely in their solids content (Fig. 1) and the association of virus with the solids fraction was thought to be of greater importance than their level per unit volume of sludge.

**Identification of viruses.** A maximum of five plaques were removed from each plate, and each was used to inoculate a tube culture of BGM cells. When a cytopathic effect had developed, the culture was subjected to freeze-thawing (−20°C, +20°C) three times, and the harvest was centrifuged to remove cell debris. The supernatant was used to infect a second tube culture, and the centrifuged cell lysate of this was used for identification. Cultures not exhibiting cytopathic effect during the first passage were treated in the same way to complete one blind passage. If this test was negative, the culture was abandoned. Isolates were identified with rabbit neutralizing antisera (Microbiological Associates) to polioviruses 1, 2, and 3; coxsackieviruses B1 to B6; and pooled antisera to the echoviruses. Equal volumes (25 μl) of a $10^{-3}$ dilution of virus suspension and each antiserum (1/20 dilution) were added to 100 μl of cells ($5 \times 10^5$ $ml^{-1}$) in wells of a microtiter plate, and the plates were incubated at 37°C in an atmosphere of 5% $CO_2$ in air.

TABLE 1. *Operational parameters at three wastewater treatment plants*

| Plant | Operational parameter | | |
|---|---|---|---|
| | Mean flow ($m^3$ per day) | Population equivalent | % Industrial input |
| A | 40,000 | 150,000 | 10–15 |
| B | 154,000 | 620,000 | 17 |
| C | 450,000 | 1,500,000 | 15 |

## RESULTS

The levels of infectious virus isolated from the various sludges ranged widely, with particularly high values recorded in July and from October to November, whereas low titers occurred in June, September, and late November to December (Fig. 2 and 3). Virus levels were consistently highest in plant C, which handled the largest sewage input (450,000 $m^3$ per day), with the maximum level of 1,429 plaque-forming units $g^{-1}$ recorded in the raw sludge and levels up to 210 plaque-forming units $g^{-1}$ recorded in the digested sludge.

The relationships between the various treatments and the titers of infectious virus which could be recovered from the sludges were compared by the calculation of regressions from all of the data available, assuming constant consolidation and digestion times (Fig. 4). Due to the high variability in virus content, including indetectable levels, it was necessary to normalize the data using a ln $(n + 1)$ transformation, and the plots of the calculated regressions (Fig. 4) revealed quite strikingly different trends in virus recovery after the various treatments. For instance, the consolidation of raw sludge for 24 h at plant B, which increased the solids content from 3 to 5.3%, resulted in a 63% increase in the isolation of infectious virus per g (dry weight) of


FIG. 2. *Levels of infectious virus isolated from sludge samples taken from wastewater treatment plant A between May and December 1980.*

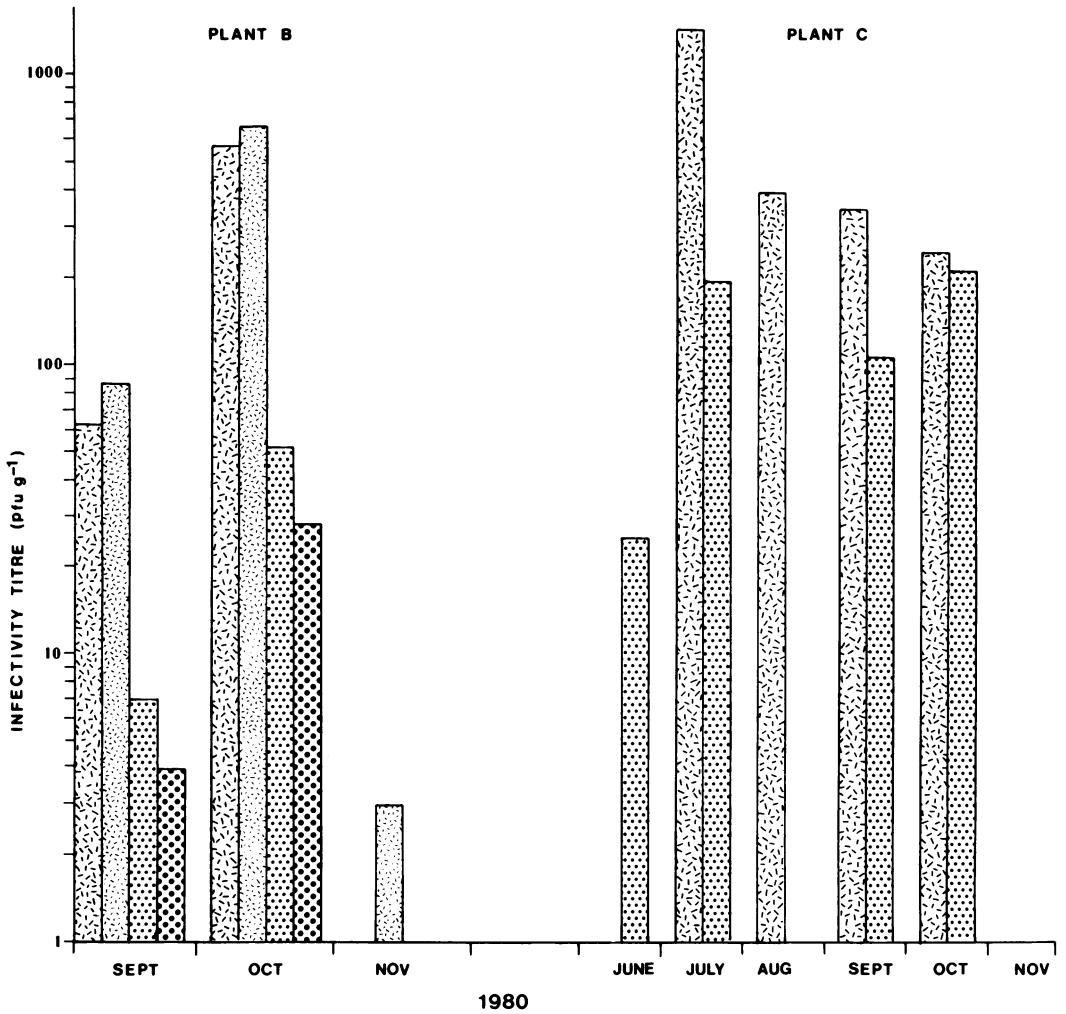


FIG. 3. *Levels of infectious virus isolated from sludge samples taken from wastewater treatment plants B and C between June and November 1980. See Fig. 2 for key.*

sludge. However, after anaerobic digestion at 30°C for 35 days, 88% of this virus could not be detected, an overall reduction of 6.2% per day having occurred. This represented a far higher loss of recoverable viral infectivity after mesophilic digestion than was observed in plants A and C, where the equivalent reductions were 3 and 0.8% per day, respectively.

After digestion, the sludges from plants A and B were consolidated for 30 to 40 days in static open tanks at ambient temperature, and further losses of recoverable infectious virus of 3 and 0.9% per day, respectively, were recorded. The consolidated sludges had virus contents which were 34.6 and 68.4% of the respective digested sludges, giving total reductions of recoverable infectious virus of 88.5% for plant A and 84.2% for plant B before these consolidated sludges were disposed of to land. Land disposal of the sludge from plant C followed a 3- to 4-year period in drying beds.

The experimental aerobic-thermophilic digester operated at plant A produced a sludge with an infectious virus content of only 23% of that found in the raw sludge. This was similar to the degree of reduction in infectivity achieved by mesophilic digestion when followed by consolidation, except that in the thermophilic digester results were achieved in about 7% of the time. With both of these treatments, virus was only detected in the final sludge in less than 50% of the samples tested.

Virus isolates identified from the sludge samples were restricted to those which both repli-

cate and plaque in BGM cells, and most plaques were identified as poliovirus 1, 2, and 3 and coxsackievirus B3 and B5, although a few coxsackievirus B1 and B4 isolates were identified (Table 2).

## DISCUSSION

The levels of infectious virus which could be detected in the different sludges on any one day could not be compared directly, because they represented widely different intakes of raw sewage, which not only varied temporally but also varied in their indigenous viral population. However, titers of raw sludge samples could be related to those of digested and consolidated sludge samples taken at the same plant 30 and 60 days later, respectively, as these were the duration times of these processes, but a correlation was apparent on only a few such occasions (Fig. 2 and 3).

At certain times, all of the sludges from one plant had either high titers, as in plant B during October, or low titers (plant A, September). One reason for this variability could have been variation in assay sensitivity, but there were no trends in high or low values obtained in any one batch of assays of samples from different plants, so assay sensitivity was discounted, and the variation in values was assumed to reflect trends in virus excretion within the local population.

The various sludge treatments nearly all resulted in decreases in the number of infectious virions which could be recovered, although consolidation of raw sludge over a 24-h storage period at plant B resulted in an increased isolation of infectious virus. However, after digestion of this sludge, the level of recoverable virus was similar to that of digested raw sludge which had not been consolidated. None of these decreases in recovery of virus necessarily reflect inactivation, as loss or gain of virus could have been caused by variations in the strength of association between virus and sludge particles. Nevertheless, it would be reasonable to assume that some inactivation of virus did occur during digestion.

In general, mesophilic digestion of raw sludge did not appear to be a reliable process for reducing infectious virus titers, which confirmed findings reported previously (1, 11, 12), and Lund (10) was even able to detect virus after digestion for 50 to 60 days at 50°C. Studies on the mechanisms involved in viral inactivation during digestion (15, 17, 18) have demonstrated that ammonia causes irreversible inactivation of poliovirus, whereas cationic detergents increase the susceptibility of the reoviridae to heat inactivation. Explanation of the failure of mesophilic digestion to inactivate infectious virus probably reflects on the inadequate mixing of the daily addition of raw sludge; indeed, Eisenhardt et al. (6) observed that some influent sludge may appear in the effluent in as little time as 1 day. Inadequate mixing of the sludge will also result

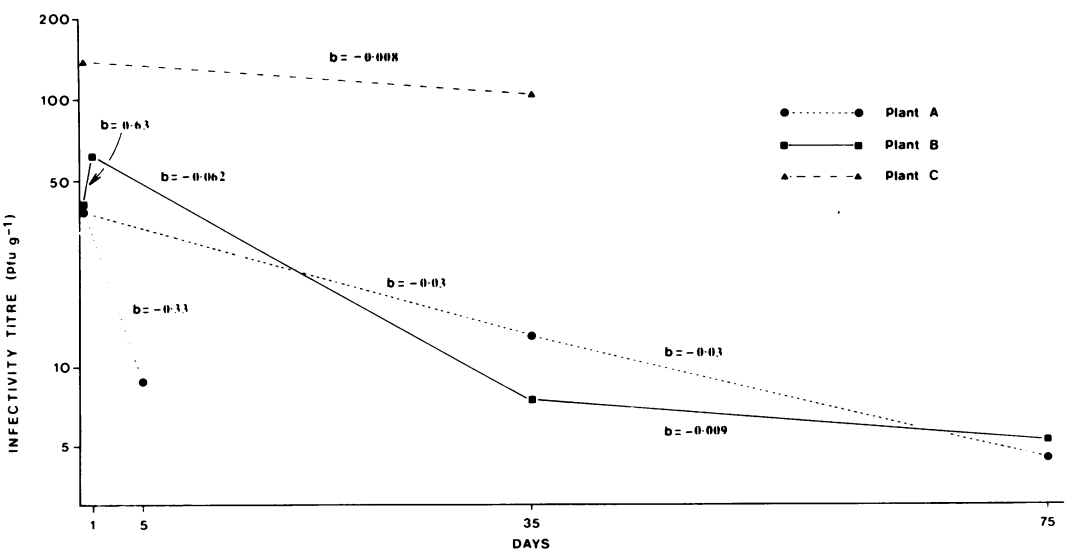


FIG. 4. *Survival of viral infectivity in sludges after various treatment processes. Intervals between points represent one form of treatment (1 day = consolidation of raw sludge; 5 days = aerobic-thermophilic digestion; 35 days = anaerobic-mesophilic digestion; 75 days = consolidation of digested sludge). The slope, b, of the regression line is indicated for each stage of treatment. pfu, Plaque-forming units.*

TABLE 2. *Type and frequency of enteroviruses isolated from sewage sludge from three wastewater treatment plants, May to December 1980*

| Plant | Virus | No. (%) of positive raw samples | No. (%) of positive consolidated raw samples | No. (%) of positive mesophilic digested samples | No. (%) of positive consolidated digested samples | No. (%) of positive thermophilic digested samples |
|---|---|---|---|---|---|---|
| A | Polio 1 | 2 (13) | | 3 (20) | 1 (7) | 1 (20) |
| | Polio 2 | 1 (7) | | 4 (27) | 2 (13) | 0 (0) |
| | Polio 3 | 1 (7) | | 2 (13) | 0 (0) | 1 (20) |
| | Coxsackie B3 | 5 (33) | —[a] | 2 (13) | 1 (7) | 2 (40) |
| | Coxsackie B4 | 0 (0) | | 0 (0) | 1 (7) | 0 (0) |
| | Coxsackie B5 | 6 (40) | | 6 (40) | 2 (13) | 3 (60) |
| B | Polio 1 | 1 (33) | 0 (0) | 0 (0) | 0 (0) | |
| | Polio 2 | 1 (33) | 0 (0) | 1 (33) | 1 (33) | |
| | Polio 3 | 2 (66) | 1 (33) | 1 (33) | 1 (33) | |
| | Coxsackie B3 | 0 (0) | 1 (33) | 0 (0) | 0 (0) | — |
| | Coxsackie B4 | 0 (0) | 1 (33) | 1 (33) | 0 (0) | |
| | Coxsackie B5 | 1 (33) | 2 (66) | 2 (66) | 1 (33) | |
| C | Polio 1 | 1 (17) | | 2 (33) | | |
| | Polio 3 | 1 (17) | | 2 (33) | | |
| | Coxsackie B1 | 0 (0) | — | 1 (17) | — | — |
| | Coxsackie B4 | 1 (17) | | 1 (17) | | |
| | Coxsackie B5 | 2 (33) | | 3 (50) | | |

[a] —, Not applicable.

in failure to achieve the optimum digestion temperature. It is also likely that association of virus with the sludge solids may protect it from inactivation by heat or the virucidal aqueous ammonia produced during digestion (15).

Consolidation of digested sludges, in contrast to that of raw sludges, resulted in an overall decrease in infectious virus. In plant A this decrease was at a similar rate to that recorded during mesophilic digestion, and it is probable that the inactivating mechanisms of digestion continue during the consolidation period.

Thermophilic-aerobic digestion was without doubt the most efficient treatment for reducing the titer of infectious virus in that it was far less time consuming than the combination of mesophilic digestion and consolidation, which ultimately gave similar reductions in virus titer.

## ACKNOWLEDGMENT

This work was supported by a grant from the Thames Water Authority.

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