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The persistence of poliovirus in activated sludge treatment

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SUMMARY

A model activated sludge treatment plant was used which was functionally very similar to a full scale plant. It was inoculated with poliovirus either continuously or with a single dose and the amounts of virus in the influent, mixed liquor and effluent were monitored regularly. The distribution of the virus in the liquid and solid phases of the mixed liquor was very unequal with about 85% of the virus associated with the suspended solids fraction. Only small amounts of virus were recovered from the effluent and after inoculation was stopped virus rapidly became indetectable. The efficiency of the plant with regard to removal of virus was closely related to its capacity to remove suspended solids and the adsorption of virus to solids and its inactivation is briefly discussed.

INTRODUCTION

The limited work on the distribution and survival of viruses in full scale and laboratory models of activated sludge sewage treatment plants has indicated that much of the virus disappears. In full scale studies the amount of virus recovered in the effluent ranges from 10 to 50% of that observed in the influent (Kelly & Sanderson, 1959; England, Leach, Adame & Shiosaki, 1967; Lund, Hedstrom & Jantzen, 1969; Berg, 1973) but such variations could easily be accounted for by differences in the various operating conditions, for instance the length of the retention time, the concentration of the suspended solids, and the temperature of the plant.

Studies on laboratory models of activated sludge treatment also show that much of the virus is not recovered but it is difficult to assess these results because each model was different. Carlson, Ridenour & McKhann (1943) used a tank of mixed aerated activated sludge and observed that the infectivity of poliovirus for mice was greatly reduced within 6–9 h. They did not feel justified in making a quantitative evaluation because the mice they used for the assay died at different times and with different symptoms. Kelly, Sanderson & Neidl (1961) used a similar model except that it was periodically harvested and replenished with fresh raw settled sewage. They assayed the poliovirus in cell cultures and recovered only about 22% of the influent virus but in experiments with T_2 phage they observed that 60% was recovered. Clarke, Stevenson, Chang & Kabler (1961) adopted the model devised by Ludzak (1960) in which aeration and settling of sewage was carried out in one subdivided tank and reported losses of coxsackievirus A-9 as high as 99% and losses of 90% for poliovirus I.

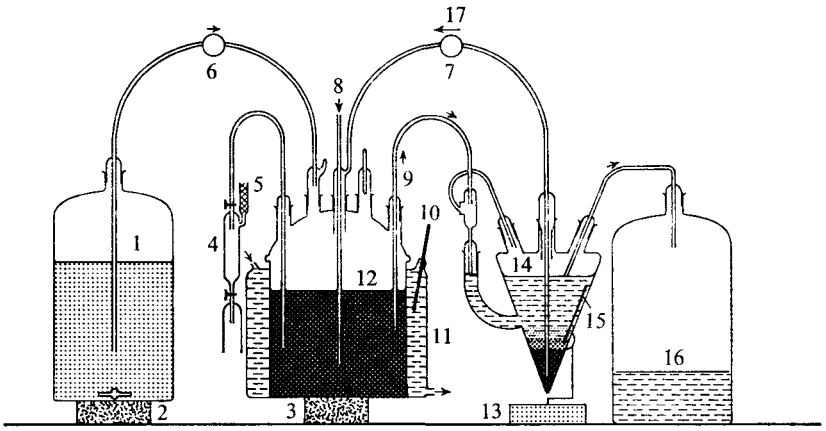

Fig. 1. Laboratory model activated sludge plant (modified from Curds & Fey, 1969). 1, Raw settled sewage reservoir; 2, magnetic stirrer; 3, magnetic stirrer; 4, sampling device; 5, air filter; 6, pump; 7, pump; 8, air diffuser; 9, mixed liquor overflow; 10, thermometer; 11, water jacket; 12, aeration tank; 13, motor; 14, settling tank; 15, scraper; 16, effluent reservoir; 17, return sludge.

All of these models were only partially representative of systems used in the field so it was decided to use the continuous flow model of activated sludge treatment devised by Curds & Fey (1969). This model obviously had potential advantages over the other models because not only was it functionally similar to the field system but it provided ready access to different and distinct regions of the treatment process. Its application to the study of survival of poliovirus is reported below.

MATERIALS AND METHODS

Activated sludge treatment plant model

The model based on that devised by Curds & Fey (1969) is represented diagrammatically in Fig. 1. The main modification was the incorporation of a water-jacket around the aeration tank to enable temperature control, otherwise it was essentially as originally designed with the component vessels of Pyrex glass and the connexions made with silicone rubber tubing.

Raw settled sewage was pumped from a 2·5 l reservoir into the aeration tank (1 l capacity) which contained the mixed liquor maintained at constant temperature. The liquor was aerated with an aquarium air diffuser (3 l/min) and stirred with a magnetic stirrer (200 rev./min). Flow to the settling tank (0·5 l capacity) was facilitated by air pressure created by the air diffuser. The settling tank was mechanically scraped by a wire scraper encased in glass which was drawn around the inner surface of the vessel (1 rev./min) by an externally placed magnet operated by a motor. The effluent was discharged into a reservoir and the sludge was pumped back into the mixed liquor vessel. The raw settled sewage used in the model was obtained from the Guildford sewage treatment plant which serves a population of 80000. The sewage was collected as grab samples in 2·5 l volumes and if necessary was stored at 4 °C for up to 48 h. The performance of the model

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was standardized in that the mixed liquor in the aeration tank contained 2000 parts/10^6 suspended solids, the retention time was 10 h at a controlled temperature of 15 °C.

Chemical and physical analysis

Determinations of ammonia, nitrate, 5-day biochemical oxygen demand and total solids of the influent, mixed liquor and effluent were carried out according to the methods outlined by the Department of the Environment (1972).

Virus

Stock cultures of poliovirus type I LS-c,2ab (Sabin) were prepared in Vero cells. Infective fluid was harvested by disrupting the cells with three successive cycles of freezing and thawing (−20 °C, +20 °C). It was clarified and purified by shaking with an equal volume of 1,1,2-trichlorotrifluoroethane (arcton) and subjected to ultrasonic disintegration with an MSE 150 W ultrasonic disintegrator for one minute at 2 °C using a probe tip amplitude of 13 μm. The arcton was removed by centrifugation (1500 ***g***, 10 min, 20 °C) and the extraction procedure repeated twice more to provide a clear virus suspension which was stored at −20 °C in 2 ml volumes.

Cell culture

Stock cultures of Vero cells were grown as monolayers in 20 oz glass medical flat bottles in 40 ml medium 199 containing 0·11% sodium bicarbonate, 5% bovine fetal calf serum, penicillin (200 u/ml) and streptomycin (1 μg/ml). After 3 days' incubation at 37 °C, when the cultures were confluent, they were drained and washed with phosphate buffered saline and were re-fed with 40 ml maintenance medium which differed from the growth medium by containing double the concentration of sodium bicarbonate and half the serum concentration. At 7 days the monolayers were subcultured at a ratio of 1:4 and 2 oz bottles were seeded with 7·5 ml cell suspension for use in the plaque test. These cultures were used for the test when confluent at 3 days.

Infectivity assay

Samples of virus were assayed either by the plaque technique as modified by Poynter, Jones & Slade (1975) to provide infectivity values expressed as plaque forming units/ml (p.f.u./ml) or by the microtitre method providing infectivity values expressed as the 50% tissue culture infective dose/ml (TCID 50/ml). For this test 0·05 ml volumes of a suspension of Vero cells in growth medium containing double the normal serum concentration were added to each well of a microtitre plate. The test was completed by the addition of an equal volume of a dilution of virus prepared in growth medium without serum. Eight replicates of each dilution were tested. The plates were incubated at 37 °C and were examined 3 days later for signs of cytopathic effects which were taken as evidence of viral infectivity. The infectivity titre of the virus was calculated by the Kärber equation. Since

Table 1. *Chemical and physical analysis of the influent and effluent of the laboratory model and Guildford activated sludge sewage treatment plants*

Sample	Ammonia	Nitrate	5-day BOD	Suspended solids
Guildford and laboratory model influent	35 ± 11·8*	1·25 ± 0·7	270 ± 88	300 ± 52
Laboratory model effluent	0.9 ± 1·51	24 ± 11·1	11 ± 4·0	15 ± 3·5
Guildford effluent	1·34 ± 0·5	25 ± 4·1	15·4 ± 2·1	11 ± 2·3

* Mean and standard deviation (parts/10^6).

undiluted samples were not assayed, titres below about 10^2 TCID 50/ml were not detected by this method.

Inoculation of the plant and sampling procedure

(*a*) Continuous inoculation method: virus was introduced via the settled sewage reservoir which was inoculated each day with sufficient stock virus to give an initial titre of $10^{4·5}$ p.f.u./ml. (*b*) Single inoculation method: sufficient stock virus suspension was introduced directly into the aeration tank to provide a titre of $10^{5·6}$ TCID 50/ml in the mixed liquor.

Samples (5 ml) of settled sewage, mixed liquor, and effluent were collected at various intervals. The settled sewage and effluent samples were immediately stored at −20 °C while the samples of mixed liquor were centrifuged at 1500 *g* for 10 min at 20 °C to provide a deposit or suspended solids fraction. The supernatant or liquid fraction was removed and kept and the solids fraction was resuspended in 10% fetal calf serum in phosphate buffered saline to produce a final volume of 1 ml. It was then subjected to ultrasonication (13 μm, 1 min, 2 °C) and stored, along with the liquid fraction at −20 °C. Since the suspended solids fractions were derived from 5 ml samples of mixed liquor but were resuspended in only 1 ml, the titre of the virus was divided by 5 to bring it to equivalence with the titre of the liquid fraction which was unconcentrated.

RESULTS

Chemical and physical performance of the model

The model was functionally very similar to that of the Guildford activated sludge sewage treatment plant and produced a similar high-quality effluent (Table 1).

Virus studies

Before each experimental run uninoculated influent, mixed liquor and effluent were assayed for evidence of naturally occurring enteroviruses and none were detected. Also, the virus-free mixed liquor solids fraction was shown to exert no adverse affect on the Vero cells in either the plaque assay or in the microtitre test.

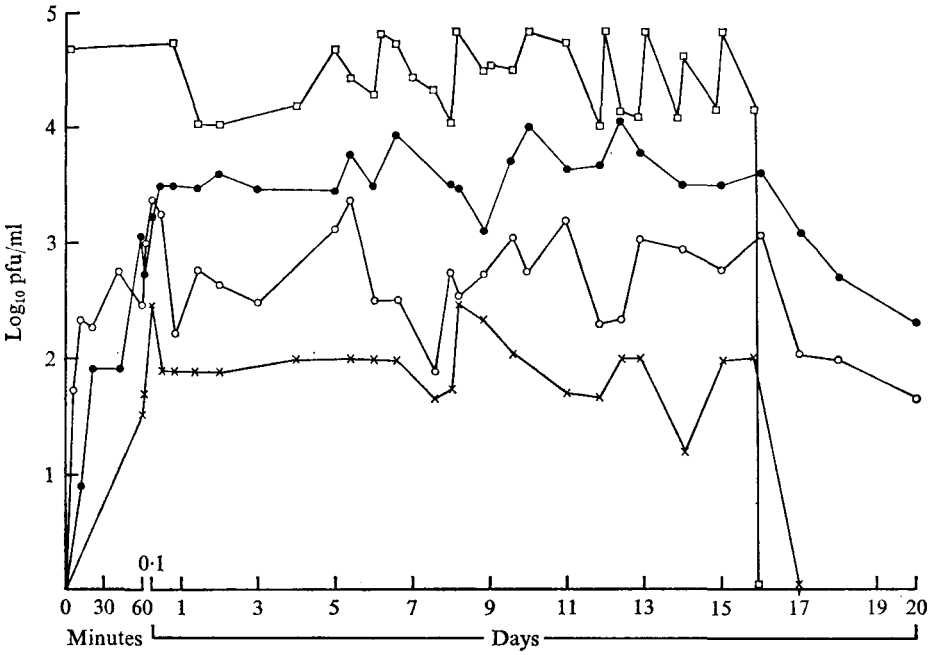

Fig. 2. Distribution of virus in the model during and after cessation of continuous inoculation with virus. Virus in raw settled sewage (□—□), mixed liquor solids fraction (●—●), mixed liquor liquid fraction (○—○) and effluent (×—×).

Continuous inoculation experiments. Within the first few hours the amount of virus recovered from the plant steadily increased to reach a plateau at about 8 h, and the virus became distributed very unequally between the liquid and solids fraction of the mixed liquor (Fig. 2). The bulk of the virus, about 85%, was associated with the suspended solids fraction and the rest, 15%, was in liquid fraction. The percentage values were calculated from the mean of the titres in the solids and liquid fractions (21 samples) observed between 8 h and 16 days. The titre of virus in the effluent was never more than 0·4% of that recorded for the mixed liquor. Separation of the effluent into liquid and suspended solids fractions was not thought necessary because the total solids constituted only 15 parts/10^6 which was 0·75% of that in the mixed liquor.

As soon as the introduction of virus into the system was stopped by replacing the settled sewage reservoir with fresh uninoculated material the virus titres declined and within a day no virus was detected in the effluent. However, the relative difference in virus content of the suspended solids and liquid fractions of the mixed liquor was maintained throughout the following 4 days although the total yield of infectious virus steadily declined.

The rate of decline of virus in the mixed liquor after the cessation of inoculation was critically examined in order to differentiate between losses due to dilution on the one hand and inactivation on the other. Dilution alone would obviously account for some of the fall in titre but the dilution factor was complicated by the fact that solids heavily loaded with virus were constantly recycled to the mixed liquor.

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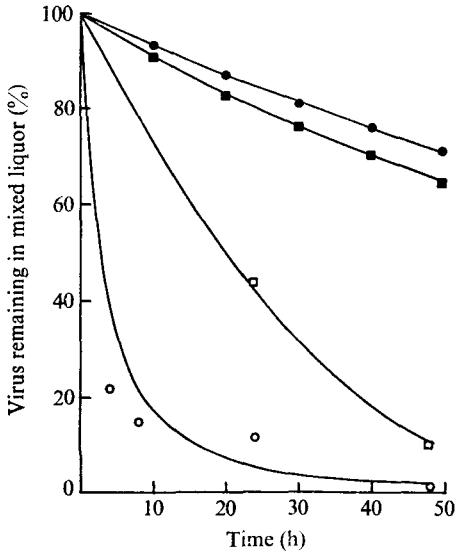

Fig. 3. Calculated and observed rates of decline of virus in mixed liquor after single and continuous inoculation of the model. Calculated decline in single inoculation experiment (Vs = 88, V_L = 12, Se = 15, Sm = 2000) (●—●) and observed decline (○—○). Calculated decline in continuous inoculation experiment (Vs = 85, V_L = 15, Se = 15, Sm = 2000) (■—■) and observed decline (□—□).

Thus in one retention time (RT) defined as the time required at influent flow rate (F, l/h) for the volume of the plant (A, l) to be equalled (RT = A/F), the expected loss of infectivity due to dilution alone was calculated according to the formula:

$$Ve = \frac{V_L}{2} + Vs\frac{Se}{Sm},$$

where Ve = percentage of virus infectivity lost in one retention time; V_L = percentage of virus in the mixed liquor liquid fraction (in one retention time half the original virus will remain assuming complete mixing of the liquid components of the aeration tank); Vs = percentage of virus adsorbed to the mixed liquor suspended solids fraction; Se = concentration of solids in the effluent (parts/10^6); Sm = concentration of solids in the mixed liquor (parts/10^6).

At 48 h the observed and calculated percentages of the remaining virus were 10 and 65 respectively (Fig. 3), which indicated that some factor or factors other than dilution might be involved in the decline of viral infectivity.

Single inoculation experiment. As expected, highest virus titres were found in the mixed liquor almost immediately because virus was directly introduced therein (Fig. 4). Within the first 15 min the titre of virus in the liquid and solids fractions was about the same but by 4 h the bulk of the virus (92·6 %) was associated with the suspended solids fraction and this differentiation was maintained for the following 20 h even though the absolute levels of detectable virus in the mixed liquor declined steadily. Virus in the effluent soon reached indetectable levels (after 8 h) after a brief peak at 20 min, when the values were about 16 % of those in the mixed liquor. After 48 h only 0·5 % of the virus was recovered from

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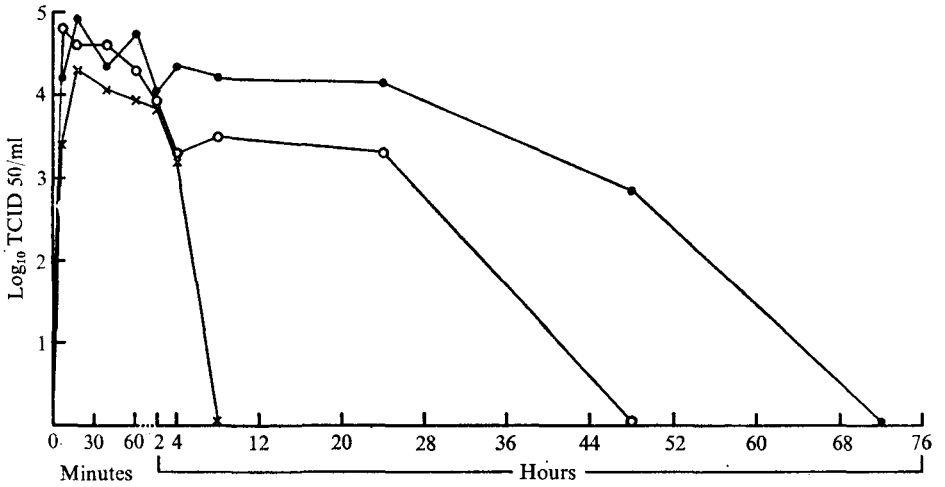

Fig. 4. Distribution and decline of virus in the model after the addition of a single dose of virus. Virus in the mixed liquor solids fraction (●—●), mixed liquor liquid fraction (○—○) and effluent (×—×).

the mixed liquor (Fig. 3), but according to the formula given above 72% of the virus would have remained if dilution alone had been responsible for the decline of virus, thus inactivation evidently played a significant role.

DISCUSSION

In essence, the model we used has confirmed the general observation that virus declines during the process of sewage treatment. However, what it has also made possible is a better understanding of the nature of the distribution of virus across the plant, the reasons for the persistence of virus and the ability to differentiate between losses in infectivity due to simple dilution and inactivation. From this it is possible to conclude that the efficiency of the plant in removing virus may be closely related to its capacity to remove suspended solids and therefore that effluent with a low solids content would be likely to contain very little virus. An interesting corollary of this which is worth commenting on is that the careless disposal of untreated sludge might be responsible for the transmission of pathogenic viruses in sewage the effluent of which was apparently free from such viruses.

The strong adsorption of viruses to the solids fraction of sewage has been reported by several others (Lund, 1971; Lund & Rønne, 1973; Malina, Ranganathan, Moore & Sagik, 1974) and the influence of the nature of solids on the adsorption of viruses has been thoroughly reviewed by Bitton (1975). What remains speculative is the mechanism of inactivation of viruses adsorbed to such solids but our studies do little to illuminate this matter. It was clear that inactivation takes place but the differences in the rates of decline in virus after the cessation of inoculation of the two experimental systems cannot easily be accounted for. We suspect that the slower decline of virus infectivity in the continuous inoculation experiment may be an artifact associated with the loading of the solids with virus which, in the case

of the continuous inoculation experiment might be expected to be greater. However, it might not be possible by our methods to detect all the virus in a heavily loaded solids fraction because the mechanism for separating virus from the solids, by ultrasonication, was probably dependent on the concentration of the virus, thus as the real virus titre declines the apparent titres remained high. Such an artifact would also lead to a lower apparent value for the percentage of virus adsorbed to the suspended solids fraction in the continuous inoculation model, which was the case.

The mechanism of inactivation of virus in sewage is not understood and has been a subject of much speculation because evidently it could have its origins in physical, chemical as well as biological activities. Clarke *et al.* (1961) claim that the loss of virus was due mainly to physical forces. Kelly *et al.* (1961) suggested that biological mechanisms were mainly responsible because after the application of metabolic inhibitors to the activated sludge there was much less loss of virus. It might be expected with poliovirus, which has a high tolerance for a range of physical and chemical factors, that direct or indirect biological activity could be the most important factor in its inactivation. However, there is no doubt that there are considerable problems related to monitoring virus in such a complex system as sewage treatment and therefore the interpretation of data concerning the recovery and disappearance of virus must be cautiously undertaken.

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