Method for Determining Virus Inactivation during Sludge Treatment Processes

FRANZ TRAUB,† STEFANIE KATHARINA SPILLMANN, AND ROBERT WYLER*

Institute of Virology, University of Zurich, CH 8057 Zurich, Switzerland

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A simple and reliable method is described which allows determination of virus inactivation rates during sludge treatment processes in situ. Bacteriophage f2 was adsorbed onto an electropositive membrane filter which was then sandwiched between two polycarbonate membranes with pores smaller than the virus diameter. The resulting sandwich was fixed in an open filter holder, and several such devices were connected before being exposed in sludge-digesting tanks. The device described prevented uncontrolled virus escape, but allowed direct contact of the various inactivating or stabilizing substances present in the environment tested with the virus adsorbed to the carrier membrane. After exposure to an environment, the surviving fraction of virus was eluted from the inner filter and determined by plaque counting. By using polycarbonate membranes without pores for sandwiching, the influence of temperature alone on virus inactivation could be measured. Thermophilic fermentation at 60°C and at 65 kPa pressure led to a bacteriophage f2 titer reduction of 3.5 log10 units per h, whereas during thermophilic digestion at 54.5°C titers decreased 1.2 log10 units per h. During mesophilic digestion an inactivation rate of only 0.04 log10 units per h was observed. Under these latter conditions, temperature had only a minor effect (19%) on virus inactivation, whereas at 54.5°C during thermophilic digestion heat accounted for 32% of the total inactivation, and during thermophilic fermentation at 60°C temperature and pressure were 100% responsible for virus denaturation.

Survival of pathogens in the environment is gradually endangering human and animal health. Therefore, the application of sewage sludge to agricultural lands with the inherent possibility of pathogen spread through contamination of food and drinking water must be carefully evaluated with respect to public health. Hygienic concerns have led to rather strict regulation of this practice in Switzerland. Among others, these regulations require that wastewater sludge has to undergo some decontamination before certain agricultural use. Sludge is considered hygienically safe when no more than 10 enterobacteriaceae per gram and no viable eggs of parasitic worms are present. Viruses as important pathogenic agents are not mentioned simply owing to the lack of appropriate methods for determining the inactivation rates, although it is well known that viruses endogenous to wastewater are concentrated in the sludge during wastewater treatment (23). Therefore, the Swiss Environmental Protection Agency has initiated a program to examine whether sludge fulfilling the above-cited hygienic criteria is also safe with respect to viruses.

Present knowledge suggests that various environmental factors operate on virus inactivation with complex mutual interdependence. Virus inactivation during sludge treatment, for instance, is on the one hand the result of the inactivating effect of temperature and on the other hand dependent on substances with denaturing or stabilizing effects (48, 49). Ammonia (46), detergents (48, 49), heavy metals (2), solvents, and extracellular proteases and nucleases of microorganisms have been identified or suspected as virucidal agents. Stabilizing effects on viruses are ascribed to inorganic ions (43, 44), detergents (49), polypeptides, and other organic material (21, 31, 41). Protection from inactivation may be due to coating by certain substances and by adsorption onto, or incorporation into, solid particles (13, 34).

Determination of virus inactivation rates in a particular environment is a difficult task. In most cases virus suspensions have been mixed with sludge samples under controlled conditions in the laboratory, but extrapolation from results thus obtained to reality is often not convincing (24, 45). Membrane diffusion chambers have also been used to expose virus to a particular environment. But such a system suffers from several disadvantages such as (i) fouling or destruction of the membrane by microbial activity or through mechanical stress, (ii) loss of virions through adsorption to the membrane or chamber walls, and (iii) slow inside-to-outside equilibration (19).

It was the aim of this work to develop a simple method by which virus inactivation could be reproducibly determined in large-scale systems. For reasons of easy and fast handling, hygienic aspects, and low costs, we chose bacteriophage f2 as a model virus (17). This virus resembles the human enteroviruses in many aspects and is characterized by a marked thermal stability (5, 10).

The experiments showed that temperature is not necessarily the major factor responsible for virus inactivation during sludge treatment processes but that denaturing substances present in the sludge also play an important role.

MATERIALS AND METHODS

Virus and bacteria. The strain of the bacterial virus f2 used in this study was kindly provided by Y. Kott, Israel Institute of Technology, Haifa, and the host bacterium, Escherichia coli K-12 Hfr CSH, was a gift from M. Billeter, Institute of Molecular Biology, University of Zurich, Switzerland.

For the virus assay, the agar overlay method of Adams (1) was used. The growth medium for the host bacteria contained (per liter of distilled water): 10 g of tryptone (Difco Laboratories, Detroit, Mich.), 1 g of yeast extract (BBL
Microbiology Systems, Cockeysville, Md.), 1 g of glucose, 8 g of NaCl, and 294 mg of CaCl₂. For bottom agar, 14 g of Bacto-Agar (Difco) per liter, and for top agar, 14 g of agar per liter was added to the growth medium. For plaque counting, 0.1 ml of indicator bacteria and 0.1 ml of virus dilution were thoroughly mixed with 2.5 ml of molten top agar and poured onto the bottom agar. The plates were incubated at 37°C, and the plaques were counted after 12 to 16 h. Appropriate dilutions showed a minimum of 30 and a maximum of 300 plaques per plate. Two plates per dilution step were routinely scored.

The buffer (pH 7.0) used as dilution medium for virus titration consisted of 7 g of Na₂HPO₄·2H₂O, 3 g of KH₂PO₄, 4 g of NaCl, and 5 g of beef extract (powdered; E. Merck AG, Darmstadt, Federal Republic of Germany) per liter. The buffer was filtered through cellulose nitrate filters of 0.45-μm porosity, and after autoclaving, 5 × 10⁻³ M MgSO₄ was added aseptically.

Virus stock solutions were prepared by the method of Kott (16).

**Sandwich technique for virus exposure in situ.** Zetapor positively charged filters (AMF, Cuno Div., Meriden, Conn.) with a nominal porosity of 0.2 μm, a thickness of 70 μm, and a diameter of 25 mm were used as virus carriers. The filters were placed in filter holders and sterilized by autoclaving. Virus stock suspension was diluted 1:10 into 50 mM phosphate buffer at pH 6.0 (loading buffer). With a syringe, 5 ml of this virus suspension was filtered through the Zetapor membrane. The virus-carrying membrane was then sandwiched between two sterile Nuclepore polycarbonate membranes (Nuclepore Corp., Pleasanton, Calif.). These membranes of 6-μm thickness were used with pores of 15-nm diameter or without pores to determine the effect of temperature-dependent inactivation. Care was taken to avoid any air bubble formation between the filter layers. Each sandwich was locked within a Nuclepore Swin-lok polycarbonate filter holder which had been modified by removing the inlet and outlet connections, leaving openings 15 to 16 mm in diameter. Some holders for aerosol filtration were also used by removing the outlet connections.

The virus-containing sandwich devices were stored at 4°C in phosphate buffer at pH 7.0. Although we always prepared the sandwich devices fresh for each experiment, preliminary tests showed that they could be stored for up to 2 months without a measurable drop in titer (data not shown). For mailing, the devices were put into small plastic containers without adding buffer.

Holes drilled in both lateral base parts of the sandwich devices allowed their connection in rows. Such rows were held by stainless-steel cable ties during exposure in the sludge container. Four specimens sandwiched between polycarbonate membranes with pores and four specimens sandwiched between membranes without pores were exposed and scored for each time point.

After exposure, the sandwich devices were washed in phosphate buffer at neutral pH before transport to the laboratory, where they were carefully dried with soft paper towels. Thereafter the virus-containing Zetapor membrane was transferred to a sterile filter holder for elution. Elution was performed by filtering 5 ml of elution medium (4% beef extract, 0.5 M NaCl [pH 8.5] [11, 36]) dropwise and at room temperature through the membrane. An even contact of the elution medium with the whole membrane surface was assured by slight pumping movements with the syringe used for elution. Elution proceeded after an initial contact time of approximately 1 min. Rinsing with a 2-ml volume of neutral phosphate buffer completed elution.

**Statistical analysis.** Four sandwich specimens were scored for each time point. The curves obtained were statistically analyzed on a semilogarithmic base by linear regression. An inactivation curve is characterized by the formula \( p_x/p_0 = 10^{-k'x} \), in which \( k' \) stands for the slope of the curve. Standard deviations for the \( k' \) values were determined as described by Sachs (33).

**Inactivation studies in the laboratory.** For determination of temperature-dependent virus inactivation in the laboratory, sandwich devices were shaken in the dark in an Erlenmeyer flask containing phosphate buffer (pH 7.0; 5 × 10⁻³ M MgSO₄) at 170 rpm in a water bath equipped with an electronic temperature control.

Temperature-dependent inactivation of virus in suspension was studied by shaking 50 ml of a virus suspension in a 150-ml Erlenmeyer flask at 170 rpm. To assure homogeneity, the virus stock suspension was diluted 1:1 into elution medium, sonicated for 30 s (microtip output setting, 50 W), and filtered through a Zetapor filter (0.2-μm nominal porosity). A 0.5-ml volume of the filtered virus suspension was diluted into 49.5 ml of phosphate buffer (pH 7.0; 5 × 10⁻³ M MgSO₄).

**Field experiments in the wastewater treatment plant.** The field experiments for in situ determination of virus inactivation during sludge treatment were performed at the wastewater treatment plant at Altenrhein, Switzerland. The hydraulic capacity of this plant is designed for 110,000 population equivalents, and at present the amount of water equals 80,000 to 90,000 equivalents. Since about 40% of the wastewater is of industrial origin, the plant was planned for a pollution load of 185,000 population equivalents. The treatment scheme is conventional and includes mechanical, biological, and chemical treatment (phosphate elimination). Sludge digestion is under anaerobic conditions at 34 to 35°C. To study partial thermophilic fermentation followed by either mesophilic or thermophilic digestion as alternative sludge treatments, a large-scale pilot system was established at the plant and connected with the conventional system. To permit comparison of relevant process parameters, the raw sludge was mixed and ground before being subjected to one of the various treatment processes listed in Table 1.

**Table 1. Process and sludge data of the wastewater treatment plant at Altenrhein, where the in situ experiments were performed**

<table>
<thead>
<tr>
<th>Process</th>
<th>Date</th>
<th>TS feed (g/liter)</th>
<th>OS feed (g/liter)</th>
<th>pH of reactor</th>
<th>Reactor temp (°C)</th>
<th>Reactor vol (m³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaerobic mesophilic digestion</td>
<td>March 6, 1985</td>
<td>4.88</td>
<td>3.04</td>
<td>7.25</td>
<td>34.5</td>
<td>1,800</td>
</tr>
<tr>
<td>Anaerobic thermophilic digestion</td>
<td>February 28, 1985</td>
<td>4.41</td>
<td>2.84</td>
<td>7.65</td>
<td>54.5</td>
<td>8.2</td>
</tr>
<tr>
<td>Aerobic thermophilic fermentation</td>
<td>May 17, 1985</td>
<td>4.48</td>
<td>2.54</td>
<td>6.75</td>
<td>60.3–61°C</td>
<td>7.2</td>
</tr>
</tbody>
</table>

* TS, Total solids.  
* OS, Organic solids.  
* Process under 0.65 bar pressure.
sandwich method enabled us to assess the inactivation effect on viruses of each sludge treatment step.

RESULTS

Appropriateness of the sandwich method. Electropositive filter materials were originally introduced for virus recovery from water by Sobsey and Jones (37, 38). The electropositive Zetapor nylon filter membranes served as efficient virus carriers. By filtration of 5 ml of virus suspension containing $3.2 \times 10^8$ to $9.0 \times 10^{10}$ PFU/ml more than 99.9% of phage particles were retained by the filter membrane at pH 6.3. The adsorbed virus particles could be readily eluted by a solution containing 4% beef extract and 0.5 M NaCl at pH values above 8.0 (11, 36). Preliminary tests showed that at pH values of $>10$ bacteriophage f2 is rapidly inactivated in the elution medium and that pH values higher than 8.5 did not lead to a significant increase in elution efficiency. A single elution step resulted in desorption of 50 to 60% of the virus load, but by consecutive elution steps with the same or additional elution volumes the percentage could be raised to virtually 100% (data not shown). Loss of viral particles owing to leakiness of the filter device was practically nonexistent. When the sandwich devices were shaken at 4°C for 97 h in elution medium, less than 1% of the elutable virus fraction could be detected in the medium, provided membranes without pores or with pores of 15-nm diameter were used for sandwich device assembly. After the virus carrier layer was sandwiched between polycarbonate membranes of 30-nm pore size, 10 to 30% of the phage were eluted into the medium.

No systematic determination of virus stability within the sandwich device was performed beyond the duration of the experiments. However, by single measurements of elutable virus titers after storage of specimens in buffer at 4°C, no drop in titer was recognizable after either 12 or even 66 days.

To check whether the elutable titer of infectious viruses corresponds to the surviving fraction, we determined viral inactivation in parallel experiments in sandwich devices and in suspensions in a water bath at 60°C. The inactivation rates were not significantly different (Table 2, lines 6 and 7).

Laboratory tests have further shown that stain permeation through the polycarbonate membranes with 15-nm pores was evident in less than 1 h.

Field experiments in the wastewater treatment plant. The virus content of wastewater is generally reduced after activated sludge treatment, and therefore this process was studied by means of the sandwich method. Three time points were chosen: 3.6, 8.4, and 21 h corresponding to the minimum, average, and maximum detention time in relation to the actual hydraulic load of the plant. The water temperature was 11°C, and the pH was 7.15 to 7.20. The filter-adsorbed phage particles were not significantly inactivated, and the influence of temperature on viral inactivation was negligible (Table 2, line 1).

Anaerobic mesophilic digestion was also tested. The conventional sludge treatment process at the wastewater plant was tested for its effect on viral inactivation. The process temperature of 34.5°C had only a weak effect on virus titers within 2 weeks (Fig. 1 and Table 2), but inactivating factors present in the sludge led to a slow but steady virus inactivation. The $k'$ values were 0.0375 ± 0.003 for total inactivation and 0.007 ± 0.0006 for temperature-dependent inactivation alone. Thus, temperature contributed only up to 19% to virus inactivation in the sludge digester.

In a further experiment the virus-inactivating potential of anaerobic thermophilic digestion at 54.5°C was analyzed. The inactivation curve reflecting thermal inactivation alone (Fig. 2) was linear, and the inactivation rate $k'$ was 0.384 ± 0.0129. The sludge environment exerted a strong synergistic effect on virus inactivation (Fig. 2). The curve was again linear with a $k'$ value of 1.189 ± 0.0192, i.e., temperature was up to 32% responsible for total virus inactivation (Table 2, line 3).

In a control experiment, virus inactivation at 55°C was also studied in the laboratory (see Materials and Methods). The inactivation rate obtained by the sandwich method in the water bath equaled that in the sludge digester, as indicated by similar $k'$ values (Table 2, line 3 and 5).

One of the goals of this study was to gain information about the decontaminating effect of partial thermophilic sludge fermentation. In the large-scale pilot system at Altenrhein this process preceded anaerobic sludge digestion, and it was expected (i) to yield hygienically safe sludge, (ii) to allow shortening of the digestion time without substantial gas reduction, and (iii) to result in stable sludge of higher solid content than conventionally treated sludge. The experiments indeed confirmed a rapid virus inactivation during this process by which the temperature of 60°C and the pressure of 65 kPa were solely responsible for virus inactivation (Fig. 3). Although pressure was simply due to the technical design of the fermentor and to the aeration, it exerted a marked effect, since inactivation rates measured at a similar temperature in the laboratory were inferior to those obtained in the reactor (Table 2, lines 4 and 6). The $k'$ value characterizing temperature and pressure inactivation in situ was determined to be 3.60 ± 0.207, compared with a value of 2.234 ± 0.0664 obtained for the respective curve in the laboratory.

DISCUSSION

The sandwich device presented in this paper for the determination of virus inactivation in real-scale environ-
ments has several advantages. It is an easy, fast, and reliable method. The results obtained are reproducible, comparable, and may be analyzed statistically. The method may also be suitable for inactivation studies of microorganisms, spores, and parasite eggs. Even the influence of a particular environment on macromolecules could be tested by choosing adequate filter membranes. The adsorbed state of the virions during exposure copies in a standardized manner the situation in nature, where a large, or even predominant, fraction of viruses is solid associated (22). This aspect is important since adsorbed virus might be inactivated at different rates compared with virus in suspension (20, 32, 34, 40), and viral particles retain their infectivity during adsorption (25, 32, 35), which is known to be a reversible process (42). It has been shown that sludge solids carry factors influencing virus stability, e.g., detergents (47), but the solid content could not be correlated with virus inactivation in sludge samples (6, 28). Although the level of solids may alter the distribution of virus inactivation factors between the adsorbed and solute state, the solids may influence virus inactivation merely by physical protection of the virions. The most extreme case, in which viruses would be completely embedded within solid material and thus would no longer be accessible to dissolved or solid-associated inactivation factors, is reflected by the sandwich device equipped with membranes without pores. Such a device allows determination of thermal inactivation alone, another important advantage of the method.

Both membrane types used for the sandwich device are of marked chemical, thermal, and mechanical stability and are also biologically inert. According to the manufacturer, the Zetapor filters are not damaged by autoclaving at 135°C for 3 h, and high concentrations of chemicals are needed for their decomposition. We have no indication that the membranes were affected under the conditions used. Since polycarbonate has little adsorptive capacity (15) neither the outer membranes nor the filter holder tended to become encrusted even after being kept for 3 weeks in sludge digesters. By testing virus retaining ability, we checked 11 pairs of such membranes after they were exposed for 12 days at 35°C in an anaerobic sludge digester and 6 further pairs after they were

FIG. 1. Virus inactivation during anaerobic mesophilic sludge treatment at a temperature of 34.5°C. Total inactivation (●) was measured with sandwich devices equipped with polycarbonate membranes of 15-nm pore size. By using polycarbonate membranes without pores, the thermal inactivation (○) could be assessed separately.

FIG. 2. Virus inactivation during anaerobic thermophilic sludge treatment at a temperature of 54.5°C.
kept for 12 days in a digester run at 55°C. No encrustation and no leakiness could be observed. Although some clogging of pores did occur, pore-containing membranes were always found to remain permeable after 12 days of sludge exposure, as revealed by stain penetration experiments.

It was not fully clear from previous work whether activated sludge treatment would inactivate virus to any significant degree or would primarily remove virus by adsorption to flocs and sedimentation to the sludge fraction (9, 39, 45). Our measurements clearly indicate that there is no f2 virus inactivation during activated sludge treatment at ambient temperature (11°C), irrespective of the contact time up to 21 h.

During anaerobic mesophilic digestion we found a bacteriophage f2 titer reduction of 0.9 log_{10} units per day. Higher inactivation rates on the order of 1 to 2 log_{10} units per day have been reported for various viruses (4, 8, 30, 46). From a number of studies the conclusion can be drawn that this process does not sufficiently inactivate viruses (14, 26, 27) and that therefore sludge digested under mesophilic conditions often contains virus (26, 27).

Anaerobic thermophilic digestion of sludge was much more effective in reducing virus titers than mesophilic digestion. Our results suggest that 1 day of thermophilic sludge digestion at a temperature of 54 to 55°C would be sufficient to remove enteric viruses. However, Berg and Berman (3) in a similar study obtained only 99 to 99.9% reduction in enteric virus titers after 20 days at 49°C, and Lund (22) also reported that thermophilically digested sludge did occasionally contain virus. In both cases virus recovery was probably caused by short circuiting in the sludge digesters. We found in contrast to Nielsen and Lydholm (29) no protective effect of thermophilically digested sludge.

During aerobic thermophilic fermentation at 60°C, heat and pressure alone were responsible for virus inactivation, as can be concluded from the practically identical curves for total inactivation and thermal inactivation. A contribution of pressure to virus decay is suggested by the observation that in the laboratory at normal pressure 2.2 log_{10} units of virus activity are removed per hour, compared with 3.6 log_{10} units in the fermentor at the same temperature but under 65 kPa pressure (Table 2, lines 4 and 6). Inactivation in situ was not significantly altered whether porous polycarbonate membranes were used for the test or not. Thus, chemical factors present in the sludge did not contribute to a measurable extent to virus inactivation during this treatment.

The relative effect of heat on virus inactivation was clearly dependent on the temperature range at which the process was performed. Heat was only 19% responsible for virus inactivation at 34.5°C and 32% responsible at 54.5°C, but in combination with pressure, it was 100% responsible at 60°C. Therefore, temperature is not always the most important factor responsible for virus inactivation during sludge treatment, as has been proposed earlier (34). Proper design of sludge treatment processes, e.g., by technical measures preventing short circuiting, may lead to hygienically safe sludge at moderate temperature (and moderate costs) by taking advantage of the effect of inactivating factors present in the sludge.

Bacteriophage f2 used for this study belongs to the Leviridae, a group of RNA bacteriophages which correspond in morphology, genome type, and structure, as well as with regard to the target organ (intestine) to the enterovirus group of the Picornaviridae, except for the host cells which are procaryotes. The resistance of f2 virus to heat (5), ammonia (7), and chlorine dioxide (12) is similar or better than that of enteroviruses. Therefore, the results obtained with this model virus are of predictive value (10, 18).

The work presented here will be continued by studying inactivation of some human or animal viruses of special health importance or of extraordinary heat stability.

The potential of the method described in this paper is not restricted to virus inactivation studies, but may offer new possibilities and broad application for inactivation and decomposition studies in the environment.

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LITERATURE CITED


FIG. 3. Virus inactivation during aerobic thermophilic sludge treatment at a temperature of 60 to 61°C.


