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# Project Summary

# Quantitation of Viruses in Waste and Other Waters

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**During treatment of domestic sewage by the activated sludge process, large numbers of viruses are inactivated, principally by adsorption to the microbial floc. The numbers of viruses which are removed by adsorption vary over a wide range due to largely unknown factors. This study describes the interactions between the virions and exopolysaccharide surfaces of gram negative, urealytic floc bacteria and demonstrates adsorption of virus particles by extracellular polyhydroxybutyrate granules produced by *Zoogloea ramigera*, a common sludge floc organism.**

***Zoogloea ramigera* was used as a model system to study the kinetics of virus removal by adsorption. By varying carbohydrate levels, large amounts of extracellular polysaccharides or extracellular polyhydroxybutyrate granules were obtained; both materials avidly absorbed $^{125}$ I-labeled enteroviruses and some phages in a nonspecific fashion. Adsorbed viruses could be removed by alkaline extraction and/or displacement with organic competitors.**

**Factors which affect the virus-exopolysaccharide interactions include the species of organisms, presence of heavy metals and multivalent cations, bulking conditions of the sludge treatment, pH, and humic acid or other organic compounds. Some virions are released from these complexes as the sludge flocs disintegrate during various phases of sewage treatment. The presence of bacterial polysaccharide particles can be detected on the virus coats.**

**In the laboratory viruses can be transferred to protozoa which graze on the flocs in aerobic treatment processes and on the suspended solids in effluent. Purified entero-, reo-, adeno-, and SV40 viruses labeled with $^{125}$I by lactoperoxidase or chloroglycoluril methods were adsorbed to flocs of ultraviolet-irradiated bacteria and fed to ciliates. The zoomicrobes were isolated and their virus content was determined as $^{125}$I equivalents or plaque forming units. Appreciable quantities of viruses were acquired by several ciliates in axenic cultures and by mixed ciliates which were obtained by flotation from primary sludge. The $^{125}$I-virus counts in the ciliates were inversely proportional to the reduced number of bacteria. Retention of intact virions was determined by using viruses labeled with $^{3}$H-uridine and $^{125}$I. Retention times varied with different feeding regimes. Protozoa excreted 90 percent of the $^{125}$I in 2 to 72 hours and small numbers of viable poliovirus 1 were excreted. Attempts to transfer $^{125}$I-viruses from ciliate to ciliate or to cysts failed.**

***This Project Summary was developed by EPA's Environmental Monitoring and Support Laboratory, Cincinnati, Ohio, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).***

## Introduction

Microbiologists have demonstrated routinely the presence of enteroviruses in fecal contaminated water, sewage sludge, and treated effluent. The rate of these viruses' natural die-off in water and sewage has been determined, frequently with a comment aside that either the initial reduction in titer or even the entire loss could be ascribed to adsorption of the virions by colloidal material. Although

repeated attempts have been made to correlate both the type and quantity of the colloidal solids in sewage with the amount of virus infective units lost after seeding, the overall picture of what happens physically to the virus particles is not clear. The enteroviruses and their components are potentially long-lived. A large proportion of the viruses which survive the initial reduction at the treatment plant and then reach the outfall will exhibit half-lives measured in days or weeks instead of a few hours.

There appear to be two methods of virus adsorption which produce this viral presence. Reversible adsorption occurs when viruses are adsorbed to clays, inorganic precipitates and vegetable fiber. It occurs very quickly after virus is added to the water and responds to manipulation of pH and ionic strength. Irreversible adsorption is apparently a biological process with a rather long lag time, has a pronounced oxygen demand, is not clearly related to any particular bacterial or algal species which have been studied. Its response to pH, solvents, and ionic strength is unpredictable and not very pronounced. There are no definite indications as to whether this virus loss is due to irreversible adsorption or to some other factor(s). It is striking, however, that these losses correspond very well to the characteristics of the ciliate protozoan populations in wastewater. These populations build up exponentially during flock formation, show seasonal variations which might explain unusual peaks of virus occurrence, have slower generation times than bacteria, and maintain feeding activity which is very sensitive to changes in temperature and oxygen levels.

Sludge solids also contribute to the presence of virus in fecal contaminated water, sewage sludge, and treated effluent. Sludge solids are generated by methods which are largely nondegradative to virions and which effectively concentrate dilute viruses. Consequently the types of virus pathogens and their virus content must be considered in the disposal of the sludges. There also exists the possibility that after activated sludge has concentrated viruses by adsorption, it may release them continuously into the effluent. If the sludge becomes overloaded with organic solutes that compete for binding spots, it may provide bursts of free virus with high titers.

Thus, the overall objectives of the following study have been three-fold: to determine more precisely those elements in sludge solids which remove or inactivate virions; to improve the technology for recovering and assaying adsorbed, infectious viruses; and to devise techniques for controlling and improving the removal of viruses by precipitable sludge solids or other methods related to the recovery of wastewaters.

## Results

Centrifugation profiles of $^{125}$I-poliovirus seeded into anaerobic sludge samples showed that the virus was largely concentrated in the *supra-colloidal* fraction of the Rickert-Hunter classification (Table 1). This fraction contains individual bacterial cells and protozoa.

The research conducted makes clear that some portion of the microbial population in activated sludge can produce exocellular polymers (glycocalyx) with a high avidity for unenveloped viruses. Experimental evidence further asserts that even when adsorbed to fecal solids and other colloids, viruses often are infectious and should be assayed when the sedimentable solids of water supplies are examined for microbial pathogens. Adsorption may even increase infectivity by concentrating multiple virions on particles which can be retained at sensitive sites in the host.

Representative viruses from all the major unenveloped viruses adhere to bacterial flocs from activated sludge. The sludge bacteria and yeast can be divided into two groups based on their ability to adhere to polystyrene plates or not. All adherent bacteria adsorb poliovirus type 1 and other viruses to the surface of colonies and individual cells in the presence of $Al^{3+}$ and $Mg^{2+}$; nonadhering bacteria frequently do not adsorb viruses or do so less efficiently.

Sludge solids were fractionated by differential centrifugation and sucrose gradient density centrifugation. When $^{125}$I-labeled PV-1 was mixed with the solids before centrifugation, most of the label was associated with either fractions containing broken bacterial fragments and single cells or with fractions containing large clumps and filaments, paper debris, vegetable fragments, and so on. The largest number of counts were associated with fragments 1 $\mu$ diameter.

Polyhydroxybutyrate (PHB) granules from *Zoogloea ramigera* are avid virus adsorbents. Large quantities of PHB granules occur as a result of lysis induced by the activated sludge process. Treatment of the native granules with proteinases markedly reduced adsorption; treatment of adsorbed viruses under nondegradative conditions caused the release of viruses. It appears that the adsorption sites are located on the proteinaceous PHB granule membrane. The extent to which cations are involved is unknown at this time.

Exocellular polymers were extracted from four strains of *Zoogloea ramigera* and one *Klebsiella pneumoniae* type 3 and purified. The purified exopolysaccharides reacted very strongly with $^{125}$I-poliovirus type 1 and either precipitated the virions or neutralized them.

The adsorption of viruses by *Zoogloea ramigera* and its cell exopolysaccharides was mediated by the addition of $Al^{3+}$ and $Mg^{2+}$. $Ca^{2+}$ cannot be substituted. These large ions are involved in a complex interaction of the viruses and the cell surface. The data suggest that the first interaction of the virion and the bacterial surface is electrostatic and results in a destabilization of the bacterial surface. This is sometimes

***Table 1.*** *Comparison of Virus-Containing Sucrose Gradient Profiles of Four Digested Sludge Samples, Metropolitan Syracuse Treatment Plant.*

| *Fraction From Differential Centrifugation** | *Recovered Radioactivity** in Seeded, 5-Day Anaerobic Sludge Samples* | | | |
|---|---|---|---|---|
| | *Sample 1* | *Sample 2* | *Sample 3* | *Sample 4* |
| Soluble | *702* | *1214* | *693* | *1298* |
| Colloidal | *778* | *2849* | *1035* | *2152* |
| Supra-colloidal | *4831* | *6084* | *4212* | *4958* |
| Settleable | *1314* | *1154* | *1742* | *672* |

* Soluble: *resists pelleting at 62,500 x g, 15 min*
Colloidal: *pellets at 62,500 x g, 15 min*
Supra-colloidal: *pellets at 4,800 x g, 30 min*
Settleable: *pellets at 1 x g, hr*

** *Disintegrations per min ($^{125}$I) corrected for background*

represented as a "reversible sorption" and is sensitive to EDTA interference or stripping. At the end of 60 to 180 minutes the virus particles can no longer be stripped with EDTA and/or pH 11 glycine buffer. The reversible sorption with the cells was analogous to the reversible sorption of viruses which has been observed with membrane filters in the presence of aluminum ion. The secondary binding is broken only with 4 percent beef extract, pH 9, suggesting that metal hydroxides are being hydrolyzed and specific superficial "domains" of virus protein coat substructures no longer interdigitate with exopolysaccharides or glycoproteins.

Ciliated protozoa have been identified as a major factor in the control and removal of viruses from wastewater. They are the dominant protozoa present in activated sludge and their numbers are associated with the quality of the effluent. As the principal grazing population, they consume much of the adsorbed materials in sludge, including viruses. The transfer of virus to cultured protozoans as well as to wild ciliates has been demonstrated in the laboratory when ciliated protozoans graze on sludge flocs with adsorbed virions. The virus particles are internalized and shed over a 12-to 48-hour period by ciliates. Protozoa only acquire viruses which are adsorbed to bacteria. There is no indication that ingested viruses multiply in the ciliate cells during this period.

Virions are released into the effluent as the floc is comminuted by violent aeration in activated sludge treatment. The viruses, still adsorbed to small pieces of capsular polysaccharide, are washed out in the overflow. Thus, under bulking conditions more viruses are to be expected in the effluent. The virus particles released in this manner from the floc exhibit altered physical and biological characteristics: they may occur singly or in clusters; their surface is antigenically altered; their affinity for surfaces of rocks, water plants, algae and debris is greatly increased; and they may be protected to some degree against drying and subsequent inactivation. A reduction in suspended solids by settling may reduce the effluent viral count but will not eliminate single virions and small clusters which are shed by ciliates or released by dissolution of the floc.

## Conclusions and Recommendations

Three of the major factors that control the distribution and survival of viruses in sewage treatment are: (1) the exopolysaccharides of floc-forming bacteria which synthesize an abundant glycocalyx capable of removing more than 90 percent of the viruses by adsorption; (2) the protein receptors and specifically charged groups on the surface of the virus nucleocapsid which react with the exopolysaccharides; and (3) the virophagy and excretion of viruses by protozoans grazing on bacterial flocs which have virions adsorbed to their exopolysaccharide coats.

A quantitative screening method has been devised to select organisms from sludge flocs which produce heavy glycocalyx and adhere to polystyrene surfaces. Thus, subcultures can be chosen which have a high avidity for viruses. $^{125}$I- labeled viruses can be adsorbed by standardized layers of such adherent cells--typically species of nonpigmented pseudomonads and *Bacillus*--and be used to determine the most avid organisms. Similarly nonadherent isolates should also be characterized for their avidity for metals and viruses.

An effort should be made to extract relatively large quantities of glycocalyx from different types of sludge solids and to determine the feasibility of using it to adsorb viruses, heavy metals, organometallic compounds and selected materials from the items on the USEPA hazardous substances list. It may be possible to bind the sticky polymers to beads, chips, plates, and so on, and to use them as a means of scrubbing treated effluents free of viruses or other hazards.

Purified glycocalyx should be isolated form high- and low-avidity isolates and electrophoretic, biochemical and/or serological comparisons made. *Klebsiella pneumoniae* is frequently found in sludge solids. The chemical structures of *Klebsiella pneumoniae* serotypes are well known and would be useful models.

The fate of 0.1 to 0.5 $\mu$m polyhydroxybutyrate granules in activated sludge treatment needs to be described. It is unclear whether they are digested during sewage treatment or if they end up in the effluent as virus carriers. The kinetics of virus removal need to be studied in the laboratory with activated sludge units which have been heavily seeded with the high avidity bacteria. Variables such as pH, metal ion, temperature, aeration rate, C/N ratio, etc., need to be studied systematically.

The importance of ciliates as potential vectors for viruses should be investigated further using both pure cultures of *Tetrahymena* and wild mixed types isolated by flotation methods from sludges. Furthermore, an attempt should be made to follow the fate of the ciliate-virus combinations up the food chain. Autoradiographic methods can be used to see if the double-labeled virus on ciliates or *Zoogloea* are transferred to higher forms such as nematodes, rotifers, and fish. The cytology and possibly the cytopathology of the host recipients will be intriguing. The demonstrated capacity of virus to be transferred to cultured protozoans as well as wild ciliates opens up new and exciting possibilities for vector transmission, virus alteration, and kinetic studies of virus removal. Certainly critical studies must be made of the virus types which can be ingested and their survival rate. Numerous questions remain. What is the longevity of viruses relative to the protozoa's life cycle and activity? Is the virus actually ingested or merely adsorbed to the pellicle? And finally, the questions which originated the study. And protozoa actually responsible for the irreversible loss of viruses during waste treatment? What percentage of the $1 \times 10^4$ PFU/L commonly found in sewage are ingested by the ciliates?

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*The complete report, entitled "Quantitation of Viruses in Waste and Other Waters," (Order No. PB 83-190 306; Cost: $11.50, subject to change) will be available only from:*

*National Technical Information Service*
*5285 Port Royal Road*
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