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Methods for Virus Recovery from Solids

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ABSTRACT

Much work is still needed before one single method can be selected which ensures reliable quantitative methodology. The various types of solids and the composition of the samples influence the results and there is no technique available today which is 100% efficient under varied circumstances. Negative findings may be misleading, because there is no guarantee that the virus cultivation system employed will reveal all the virus types present in the sample.

For some sludges it is possible to detect viruses by direct inoculation in cell cultures. Such a method may give 10-50% recovery, essentially depending on a balance between the cell-toxicity of the samples, the tenacity of the virus to stick to the solids and the care given the cell cultures. In most methods viruses are extracted from the sample either by elution by high pH (8.5-11.5), by proteinaceous media, with chelating agents, agitation with solvents or by sonication. Adsorption to polyelectrolytes or precipitation at low pH, in both cases followed by elution by means of glycine, trisbuffer with EDTA, or beef extract has given good recovery from seeded samples and positive results from field work. Decontamination of samples is carried out in various ways.

KEYWORDS

Efficiency of recovery; sludge samples; soil samples; elution; concentration; decontamination.

INTRODUCTION

Our first examination of virus in treated and untreated wastewater
was in the years 1964 and 1965. At first we were quite contended to leave out a spontaneous sediment from our wastewater samples, but when the results from swab samples invariably came out with much more positive samples, as Melnick and co-workers (1958) had shown, we became suspicious. If a mere squeezing of a swab would do it, viruses in wastewater must be different from proper laboratory suspensions, in fact the viruses must be solid associated. We became very careful not to let any fraction escape no matter how disagreeable the samples were for inoculations (Lund and Hedström, 1966, 1969, Lund, Hedström and Jantzen, 1969). Wellings and co-workers (1975, 1976) showed that 70-90 per cent of influent and effluent samples are solid-associated and point out that the techniques being used fail to address the question of solids-associated virus, i.e. virus that is an integral part of a solid as opposed to merely being adsorbed thereon. Moore and co-workers (1974) reported among others that solid associated virus is infectious. It seems that in those years many workers become aware of the importance of the solid-associated virus and agreed with Schaub and Sorber (1976) when they concluded that "Many of the methods used to concentrate and assay viruses in natural waters and wastewaters do not recover those viruses adsorbed to suspended material. Although it complicates the problem of recovering viruses, virus adsorption to solids is real and must be considered in the assessment of any water". Thus most of the virus detection methods for water and wastewater published in those years must be quantitatively less than optimal.

**Adsorption of Viruses to Solids**

Clarke and co-workers (1961) showed that virus in wastewater adsorbs to settleable solids to 67-79% and Kelly and co-workers (1961) showed that around half of the virus is removed by settling. It seems that we might long ago have taken the consequences of these observations. For instance it seems quite reasonable that in the situation where virus examinations of various wastewaters and treatments are so difficult and expensive to carry out we might get valuable guidance from analysis of BOD and suspended solids. We are seeking suitable indicators because we realize that faecal coliforms are not very helpful and may be we already have the necessary data at hand for evaluating treatment efficiency by looking at the routine determinations of BOD and suspended solids. Balluz (1977) reports that the efficiency of a treatment plant to remove virus is closely related to its capacity to remove suspended solids.

Glass and O'Brien (1980) emphasize that enterovirus removal by activated sludge treatment cannot be equated with virus inactivation. They state that fluid volume reduction from mixed liquor to settled sludge could result in concentrations of infective viruses in sludge which exceeds the concentration in the original wastewater.

This statement can easily be confirmed. Lund (1971, 1973, 1976) found viruses in primary sludges in a number of cases where the corresponding raw wastewaters did not yield viruses. In various chemical treatments in a Copenhagen pilotplant (table I) the same was the case. In other cases a concentration procedure was required for virus demonstration from wastewater, when direct inoculation was enough for the corresponding sludge sample. When it is remembered that in primary settling about half the virus will go with the sludge and that the primary sludge has a volume of about 0.5 per cent of the raw
wastewater from which it originates these findings are not surprising. In fact before we get methods for virus demonstration in primary sludge that give more than 10-fold and preferably 100 fold as much virus from sludge samples than from wastewater samples our methods for solid associated virus are probably not good enough, even if the wastewater methods were.

From this kind of reasoning it may also be deducted that some sludge disposal practices may be hazardous from a virological point of view and that efforts to improve the methodology of virus detection from sludge samples may be justified, so that a more firm base for public health precautions may be obtained.

INFORMATION OBTAINED ABOUT THE ADSORPTION OF VIRUSES UNDER LABORATORY CONDITIONS AND IN THE FIELD

A number of studies have been carried out on the adsorption of viruses to various materials in the laboratory and under field conditions. Some studies have to do with virus detection methods from water and others with soil adsorption etc. As the reversible nature of such adsorption is often demonstrated the information thus obtained should be useful also when the opposite i.e. the desorption of solid-associated virus is desired.

We know that low pH, proper ionic strength and polyvalent cations are important for adsorption and on the other hand alkaline conditions, presence of organic substances, some detergents and physical means of disintegration favours desorption. Lefler and Kott (1973) showed that the ion concentration influenced virus adsorption to sand and also that proteinaceous matter protected virus. It seemed that solid-associated virus survived better than free virus. Bitton (1975) points out that adsorption is inhibited by competition by organic substances and reversible. When Dryden and co-workers (1979) studied detection limits at each stage of sample processing they found that recovery efficiency is sacrificed to obtain more sensitivity. They did attempt to demonstrate virus in precipitates. Results are obtained by subtracting results from liquid parts of the samples.

Drewry and Eliassen (1968) studied virus movement in groundwater. They studied various soils in batches and in columns. These models may seem remote for the present subject of methods, but an important conclusion may be drawn: The ability of a soil to adsorb virus particles cannot be judged solely on the basis of the various tests which normally are employed to characterize a soil. The adsorption increases with increasing clay content, silt content, ion-exchange capacity and glycerol-retention capacity, but a soil could be ranked lower than other soils in any one or all of these properties and still rank highest in virus-adsorption ability. The conclusion must thus remain that although we know of a number of factors important also for desorption, there are a number of unknowns and interactions take place, so that various solids may behave differently under different circumstances. Many times the disagreeable solid-associated virus situation has been to some degree avoided by the use of experimental columns and lysometers. This has for instance been done by Scheuerman and co-workers (1979), when they study the transport of viruses through organic soils and sediments. They point out that humic materials interfere significantly with
the adsorption capacity of poliovirus to a sandy soil. CaCl₂ may restore the sorptive capacity and so does removing of the humic materials. Humic acid might thus be a useful eluent for the detection of viruses. This approach has apparently never been used.

Vilker and Burge (1980) have attempted mathematical models for virus adsorption, but so many data seem needed before models relevant for the field situation can be established reliably, that we have still to rely on empirically obtained results.

METHODS FOR VIRUS RECOVERY FROM SOLIDS

The following is by no means an attempt to cover all published procedures or variants of techniques. Many publications may not be mentioned, but it is attempted to take up the principal features of a number of available methods. It seems unfortunate that so many descriptions do not contain any indication why this particular procedure were preferred rather than another. Comparisons and evaluations are difficult because so many variables are at play and few publications contain results obtained with different methods used on the same sample materials. The methods and reported results deal with viruses from various types of wastewater treatment sludges, sediments, soils, mixed liquors, plants and other solid containing materials. The samples examined contain indigenous viruses or seeded virus.

Decontamination. Many publications do not contain any information on contamination problems. This seems somewhat surprising, as the samples often must contain bacteria and fungi even after sample treatment. In connection with direct inoculations ether (e.g. Lund, 1971, Lund and Rønne, 1973, Eisenhardt and co-workers, 1977) or chloroform (e.g. Subrahmanyan, 1977, Glass and O’Brien, 1980) have been reported. If ether was used on beef extract eluates samples can become toxic to cell cultures (Nielsen and Lydholm, personal communication), but for eluted samples filtrations seem to be the natural way of decontamination with little risk of losing virus. Some publications, e.g. Bertucci and co-workers (1977), report the use of a high amount of antibiotics. The methods employing sonication or blending this treatment apparently is sufficient to decontaminate, but Lund and Hedström (1966) found that 10 per cent of samples were still contaminated after sonication.

Direct inoculation. Bertucci and co-workers (1977) seeded samples with high concentrations of virus, so that they could expect proper recovery even if not quantitative method was available for concentration. They sonicated the samples and found 80-120 per cent recovery. Sanders and co-workers (1979) centrifuged their samples, tested the supernatant directly and resuspended the solids. No real estimation of the efficiency is given. Penters and co-workers (1979) prefer centrifugation at 20,000 g to obtain sludge supernatants that were employed as seeded samples. Lund (1971), Damgaard-Larsen and co-workers (1977) and others have preferred direct inoculations. Samples have often been toxic to cell cultures and required special care, but in many cases at least as high virus yield has been obtained with direct inoculations than with other methods.
Blending or sonication. Foliquet and Doncoeur (1972) homogenized their samples and inoculated the supernatant after centrifugation at 3,000 rpm/min. They did not find any toxic effects, but do not discuss efficiency of recovery. Moore and co-workers (1978) homogenized, centrifuged and rehomogenized, but they found 90 per cent of the virus in the first run.

Wellings and co-workers (1975, 1976) pretreat with beef extract at pH 9, sonicate, stir for 18 hours and fluorocarbon treat in a blender. Ward, Ashley and Moseley (1976) and Ward and Ashley (1977) employ sonication with 0.1-0.2 sodium dodecyl sulphate. No loss in recovery was found, when virus was mixed in raw sludge, but a loss of 25-50% occurred with digested sludge. Balluz and co-workers (1977) centrifuged at 1500 g. The supernatant was tested and the solids resuspended and sonicated.

Elution at high pH. Moore and co-workers (1974) eluted with borate buffer at pH 9.0. Subrahmanyan (1977) found that pH 9.5 was the best because virus became inactivated at higher pH and virus was not recovered at lower pH. Tierny and co-workers (1977) studied virus contaminated vegetables. They were extracted at pH 8.5 and clarified through glass wool and membrane filtered.

Hurst and co-workers (1978) demonstrated a very high adsorption to sludge of seeded enterovirus and confirmed that elution with glycine buffer at pH 11.5 is highly efficient for enteroviruses. Their method calls for two elution steps with an acid flocculation or aluminum flocculation in between. With seeded preparations 80 per cent recovery could be obtained.

Gerba and co-workers (1979) studied virus recovery from marine sediments. As direct inoculation would only work with heavily polluted sample they eluted with glycine at pH 11.5, concentrated at pH 3.5 and eluted the filtered material with glycine at pH 11.5.

Some examples of comparative studies. Wellings and co-workers (1976) found, as is shown in table 2, quite varied results with variations in methodology. Lydholm and Nielsen (1980) compared direct inoculation, precipitation at pH 3 followed by beef extract elution and alum flocculation followed by trisbuffer elution. Their results on some sludge samples are shown in table 3. The direct inoculation failed on these materials, but it seems indicated that more than one method should be employed on unknown samples.

As also described in the review by Hajemion and Butler (1979) poly-electrolytes like PEGO have been employed for the concentration of viruses (e.g. Wallis and co-workers, 1969, Chandhury and Westwood, 1972). The PEGO apparently gave very varied results and varied in quality, so gradually its use seems discontinued. In connection with studies of drying bed sludges and land application of sludges it was found that the sludges to which sludge thickeners have been added more frequently yielded viruses than other sludges. Nielsen and Lydholm (unpublished results) studied the use of these polyacrylamid sludge thickeners and found it very satisfactory to employ them for virus detection in the following way: To a 20 ml aliquot the polyacrylamid (e.g. 100 ppm of Zetag, molecular weight $10^5$) was added at pH 5.5. The floc formed was filtered through a 1 mm mesh.
sieve or a loose cotton plug. Elution was performed at pH 9 using glycine buffer or beef extract. With seeded samples the recovery was very high (60-120%) with less than one per cent of the virus in the filtrate. With natural samples the eluates were concentrated by polyethylene glycol hydroextraction. In table 4 some results obtained using this method are compiled. The samples are collected in August-September 1978 from three treatment plants at Copenhagen and a number of technical data were obtained for the sludges.

**SOME OF THE PROBLEMS AND LIMITATIONS CONNECTED WITH THE AVAILABLE METHODS**

None of the methods described are yet supported by large amounts of data and few studies are available that compare the efficiency of one method with another under the same conditions. There are many indications that solid-associations vary in different types of materials and that components in the samples influence desorption and also the sensitivity of the cell cultures.

Determinations of plaque forming units are in some cases less than optimal and although some methods give good quantitative recovery with known amounts of exogenously added viruses, it is most difficult to evaluate whether they are quantitative when employed on field samples. It is suspected that they may be much less efficient for some types of samples. A virus isolation cannot be regarded finished before identification is made. There is a danger that reported virus plaques are false positives. In a report (Jakubowski, 1979) where alkaline (pH 10.5) elution is used followed by freon treatment and hydro extraction and a final sonification is employed all the 58 plaques demonstrated in the material proved to be negative on passage. It seems to be a warning signal that even a very careful procedure may lead into a very difficult situation.

Direct inoculation often seems advantageous and probably always should be carried out. The virus level may, however, be so low that a concentration procedure is required. From for instance the work of Tierny and co-workers (1977) the relation between virus level and recovery efficiency is pointed out: A good recovery (80%) was reported when soil and plants were inoculated with $10^7$ PFU per 100 g, but the efficiency of the method, which includes filtering of samples through 0.45 um filter membranes, but did not include elution of any kind, decreased with decreasing virus load. It is reported that the method worked well at 100 PFU per 100 g. Below that level the efficiency decreased and at 10 PFU per 100 g no virus was detected.

None of the methods studied have employed more than a few virus types. Gayal and Gerba (1979) observed a great deal of variability in the way various viruses adsorb to soil, even strain differences were found. It must be assumed that viruses may vary in a corresponding way when elution is attempted. In our efforts to obtain good recovery from solids we should, however, stop and think if it matters too much if we get 30 per cent or 99.8 per cent recovery. As long as legislation mentioning exact limits for this or that is forced upon us we can probably accept such a situation.

A grave problem is the fact that it is only a few virus types or families we have methods for. Goyal and Gerba (1979) have worked a little with a simian rotavirus and Smith and Gerba (1980) have de-
tected rotaviruses in wastewater. That is a beginning. We are at a point where we have to admit that from many points of view the relatively easily grown enteroviruses are may be not the most important ones to detect. We now know that we have a need for methods for hepatitis virus, coronavirus, parvovirus in addition to the ones working for reo and adenoviruses. Suitable cultivation methods are not yet ready, but the importance of a number of newer viruses for gastroenteritis troubles in man and animals is recognized.

TABLE 1 Demonstration of enteric viruses (enteroviruses and adenoviruses) in urban wastewater (Copenhagen) in connection with chemical treatment (alum, lime or ferric chloride)

| Type of sample            | No. of positive samples over total No. of samples |
|---------------------------|---------------------------------------------------|
| Raw wastewater            | 16/28                                             |
| Treated wastewater        | 8/28                                              |
| Chemical precipitate      | 21/28                                             |

TABLE 2 No. of plaques and type of virus isolated from 500 ml of digested sludge (from Wellings and co-workers (1976))

| Sample | Sonication | Sample treatment | Freon |
|--------|------------|------------------|-------|
| 1      | 6 polio 1  | 1 reo            | 0     |
| 2      | 0          | 2 reo echo 22/23 | 0     |
| 3      | 0          | 0                | 1 polio 1 |
| 4      | 17 polio 3 | 0                | 3 polio 2 |
|        |            |                  | 4 echo 22/23 |
### TABLE 3 Demonstration of viruses in sludge samples employing different methods (from Lydholm and Nielsen (1980))

| Sample                  | Direct inoculation | Acid precipitation with beef extract elution | Alum flocculation with trisbuffer elution |
|-------------------------|--------------------|--------------------------------------------|------------------------------------------|
| Raw sludge 1            | -                  | Polio 2                                    | Cox B5                                   |
| Raw sludge 2            | -                  | Cox B5                                     | Polio 1                                  |
| Raw sludge 3            | +                  | Cox B5, Adeno 2                            | Cox B2, Adeno 1                          |
| Raw sludge 4            | -                  | Cox B2                                     | Polio 3                                  |
| Digested sludge         | -                  | Cox B5                                     | -                                        |
| Land applied sludge     | -                  | -                                          | Adeno 2                                  |
| Land applied after 4     | -                  | -                                          | -                                        |
| months                  |                    |                                             |                                          |

### TABLE 4 Demonstration of virus in sludge samples (from Lydholm and Nielsen (unpublished results))

| Sample                  | with Zetag flocculation | Direct inoculation |
|-------------------------|-------------------------|--------------------|
|                         | with Zetag flocculation |                    |
|                         | followed by hydroextraction |                    |
| Raw sludge              | 33 Cox B3               | 32 Echo 11         |
| "                       | 33 Echo 7               | 22 Cox B3          |
| "                       | 22 Echo 11              | 0                  |
| "                       | 5 Echo 11               | 0                  |
| "                       | 22 Cox B4               | 5 Echo 27          |
| "                       | 5 Adeno 1               | 0                  |
| "                       | 5 Echo 11               | 0                  |
| "                       | 33 Cox B5               | 10 Cox B5          |
| "                       | 22 Echo 11              | 32 Cox B3          |
| "                       | 0                       | 5 Echo 7           |
| "                       | 10 Echo 11              | 0                  |
| Corresponding           |                         |                    |
| digested sludge         |                         |                    |
|                         | 1                       | 1 Echo 27          |
| "                       | 2                       | 0                  |
| "                       | 3                       | 0                  |
| "                       | 4                       | 1 Echo 15          |
| "                       | 5                       | 1 Echo 27          |
| "                       | 6                       | 5 Echo 15          |
| "                       | 7                       | 0.5 Echo 3         |
| "                       | 8                       | 5 Echo 3           |
| "                       | 9                       | 0                  |
| "                       | 10                      | 0.5 Cox A18        |
| "                       | 11                      | 2 Cox B3           |

*lowest detectable concentration for Zetag flocculation and direct inoculation was 5 TCID$_{50}$ per ml and for hydroextraction 0.5 TCID$_{50}$ per ml.*
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