SURROGATE VIRAL INDICATORS (ET 9208)

Final report to the Department of the Environment, July 1987 to June 1990.

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Author: T E Irving, M Barnes and N Ellis
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WRC plc, Henley Road, Medmenham, PO Box 16, Marlow, Buckinghamshire
SL7 2HD. Telephone: Henley (0491) 571531
PREFACE

Virological analysis is expensive and time-consuming, and it requires specialised skills and equipment. A simpler alternative would allow higher levels of monitoring.

In July 1987, the Department of the Environment placed a contract (Ref PECD 7/7/237) with the Water Research Centre (WRc) for a study to develop further and evaluate a specific bacteriophage test as a measure of the efficiency of water treatment for virus removal, and an assessment of the suitability of coliphages as indicators of sewage pollution under various conditions. The study ended 30 June 1990.

This final report summarises the work undertaken.
OBJECTIVES

1) To evaluate a specific bacteriophage test as a measure of the efficiency of water treatment for virus removal, and suggest necessary modifications.

2) To assess the value of coliphages as indicators of faecal contamination in waters of different quality.

3) To assess the suitability of coliphages as indicators of quality in water samples where long delays exist between sampling and analysis.

CONCLUSIONS

A method developed at RIVM, the Netherlands for enumeration of F-specific coliphages appeared to be effective, but did not prove to be completely reliable, owing to problems with the host organism, S. typhimurium WG49. This engineered host organism is genetically unstable, and requires careful handling. Experience suggests that some laboratory workers will have more success with the method than others.

An adsorption-elution method for concentrating bacteriophages from large volumes of water was developed, and proved reasonably effective, though unsuitable for saline waters. It was time-consuming, however, and results showed considerable variability. An alternative method of elution, which would have improved the methodology, was unfortunately unsuccessful.

In the first trial carried out at a sewage works, coliphages appeared to respond in a similar way to enteroviruses to the treatment processes studied, while the response of total and thermotolerant coliform bacteria was quite different. In the second trial, there appeared to be less removal of coliphages than bacterial indicators, but no useful virological results were obtained.
Coliphages were less numerous than bacterial indicators in river water and were sometimes found in the absence of enteroviruses. However, no samples were tested which did not contain coliphages, so it cannot be deduced whether or not they would be more sensitive indicators than bacteria.

Survey of a water treatment works suggested that enteroviruses, though present in only low numbers in the raw water, were not removed by the treatment processes to the same extent as bacteria, in percentage terms. Phages seemed to be removed to a greater extent than viruses, and were not detected in samples of slow sand filter effluent, when bacterial indicators and enteroviruses were found.

Use of coliphages as pollution indicators is included as a tentative method in APHA Standard Methods. In tests on recreational waters, somatic coliphages were found to correlate positively with thermotolerant coliform organisms and faecal streptococci, but correlation coefficients were not high (0.8 and 0.62 respectively). F-specific coliphages were below the limit of detection in all but one sample tested.

Coliphages would be no better than coliform bacteria as pollution indicators in situations where there is a long delay between sampling and analysis.

F-specific coliphages survived better than polioviruses when exposed to free chlorine sufficient to give a residual concentration of 0.3 mg/l after 30 minutes. However, the opposite was true with a higher residual. F-specific phages were very much more resistant to combined chlorine (pre-formed monochloramine) than were polioviruses. F-specific coliphages therefore have potential use as model viruses in disinfection trials.

In storage tests, the rate of inactivation of viruses in the dark was generally much less than for phages. Somatic coliphages were much more stable than F-specific ones, and their survival was affected by
increased temperature, but not salinity. F-specific phages were sensitive to both higher temperature and salinity, and their survival in sea water at 20 °C was very poor, even compared with that of thermotolerant coliforms.

Coliphages were generally more resistant than polioviruses to inactivation by sunlight, with somatic coliphages being consistently more resistant than F-specific types. Polioviruses survived exposure to sunlight better in fresh water, while coliphages survived better in saline water.

Bacteriophages may therefore be useful as model viruses in certain situations, but not in all. In particular, the comparative lack of stability of F-specific coliphages with increasing temperature, means that in many situations they would not persist in the environment for as long as mammalian viruses such as polioviruses.

III RECOMMENDATIONS

Improvements are needed in the techniques for enumerating mammalian viruses in environmental samples. It is difficult to assess the value of an indicator organism when the target organism for which it is a surrogate cannot be enumerated reliably.
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SECTION 1 - INTRODUCTION

In recent years the virological safety of public water supplies and recreational waters has been a topic of increasing interest and concern. Although new techniques are always being developed, virological analysis itself remains expensive and dependent on specialist skills and facilities, and is likely to remain so for some time.

The use of bacteriophages as 'model viruses' has often been suggested, but there have always been criticisms of the lack of specificity of the methods used, especially as many bacteriophages are quite unlike mammalian viruses in size and structure. However, resulting from the work of Havelaar (1986), a technique has been developed which is able to enumerate only one specific class of bacteriophages, the male-specific (or F-specific) RNA coliphages, which are similar in size to many enteric viruses. Enumeration methods for these coliphages do not require much additional expertise or specialist equipment than normal bacteriological tests. Validation of this technique would therefore make the use of bacteriophages as surrogate viral indicators more feasible.

1.1 ENUMERATION OF BACTERIOPHAGES

Bacteriophages are inert unless they can successfully infect a susceptible host cell. The host must be a bacterium with suitable binding sites on its outer surface, and with appropriate biochemical properties to allow the phage to take over the host's metabolic processes for the production of new phage particles. Attachment can occur without successful infection and replication, but the complete lytic cycle is required before phages can be detected by their effect on host cultures.

Such detection is usually done using a double-layer agar plate. The lower layer is a conventional nutrient base, while the upper layer is a thin film of semi-solid agar, with the phage-containing sample and host bacteria dispersed within it. The host grows to produce confluent
turbidity in the top layer, which is termed a bacterial 'lawn'. However, where a phage particle has infected a host cell and completed a lytic cycle, the progeny phages will be released, and will infect other host cells in the vicinity. Because of the low gel strength of the soft agar layer, the phages are able to diffuse outwards from the initial point of infection. The lysis of the host cells produces a zone of clearing in the bacterial lawn, and this is termed a 'plaque'. This may be thought of as analogous to a bacterial colony, and is theoretically the result of infection of a host cell by a single phage. However, as with bacteria, the assumption of randomly-distributed single particles is not always valid, and so concentrations of phages in a sample are usually reported in terms of 'plaque-forming units' (pfu) per unit volume. It is also possible to test for the presence of phages using a plate containing only a single thin layer of agar.

1.2 FACTORS AFFECTING THE ENUMERATION OF BACTERIOPHAGES

A number of factors can affect the successful formation of phage plaques, and their maximum size reached. The most obvious are the susceptibility of the host bacterium used, and the types of phages present in the sample. If the host organism is resistant to infection by a particular phage, then no plaque will be formed, and the phage will not be detected.

If all the host cells are susceptible to a particular phage, then a completely clear plaque will be obtained, while a turbid plaque will result if a proportion of the host cells are resistant to the action of the phage. The type of phage present may also affect the size of plaque produced. A slowly-replicating phage, or one which produces a small number of infective progeny particles, will tend to form a smaller plaque than one which replicates more quickly, or is more productive. Superimposed on this effect is that of the physical size of the phage. A small phage will diffuse through the semi-solid agar faster and more easily than a large one, and so will tend to form a larger plaque.
Other factors affecting the size of plaques include the volume of the lower (nutrient) layer in the assay plate. A thin lower layer produces smaller plaques, presumably through nutrient limitation of the host. The size of the host inoculum also has an effect, but here the proportionality is inverse. Enough host cells to produce a confluent lawn must be present, but if very large numbers are used, there may be insufficient phages produced to infect all the host cells in the vicinity. Any plaque formed will be small because the phages will not have to diffuse far before finding a susceptible host, and if sufficient host cells survive, they may grow and mask the plaque completely.

The concentration of agar in the top semi-solid layer also affects the size of phage plaques. High-strength gels inhibit phage diffusion and reduce plaque size. Therefore the lowest agar concentration is usually used which will allow the integrity of the layer to be maintained during normal handling of the plate.

SECTION 2 - LABORATORY METHODS

2.1 SELECTION OF HOST ORGANISM

Initial studies concentrated on the use of strains of *Escherichia coli* which were known to be susceptible to a variety of phage types. Classically, derivatives of *E. coli* types B, C, and K12 have been used for enumeration of *E. coli* bacteriophages ('coliphages'), and so these were tried first. *E. coli* B, National Collection of Industrial and Marine Bacteria (NCIMB) No 10243, is host for the range of virulent coliphages T1 to T7, and *E. coli* K12 (NCIMB 9481) is a 'male' strain carrying the wild type F, or sex, factor. This strain produces structures called F pili, which are involved in the transfer of genetic material between cells, and is thus susceptible not only to phages which attach to receptor sites on the cell envelope ('somatic coliphages'), but also to male-specific (or F-specific) phages which attach only to the F pilus. An American derived strain of *E. coli* C, American Type Culture Collection (ATCC) No 13706, was used, as this had been found by
Isbister and others (1982) to produce the highest plaque counts of all strains of E. coli which they tested.

It was decided to restrict initial studies to these three hosts, and to use them to study aspects of the phage enumeration technique. All the three hosts described produced plaques from river samples and wastewater, although numbers obtained were very variable between samples. E. coli C (ATCC 13706) consistently gave the highest plaque counts, as had been previously reported (Isbister and others 1982).

The effluent from a small sewage treatment works was periodically tested for coliphages, and counts obtained for the three hosts (pfu per ml) were in the ranges: E. coli C (ATCC 13706), 13 - >1000; E. coli B (NCIMB 10243), 12 - 380; E. coli K12 (NCIMB 9481), 10 - 17. Results for river Thames water, also in pfu per ml, were: E. coli C, 7 - 70; E. coli B, 4 - 8; E. coli K12, 3 - 12.

2.2 INCUBATION CONDITIONS

All plates were normally incubated at 37 °C. Phages exist which can only form plaques within a certain temperature range, and three distinct classes of phages have been described on this basis (Seeley and Primrose 1980). Low temperature (LT) phages form plaques between 15 and 30 °C, mid temperature (MT) types between 15 and 41 °C, and high temperature (HT) types between 25 and 45 °C. LT phages are predominant in unpolluted water, while MT and HT types are predominant in faeces and faecally-polluted water. The use of incubation at 37 °C is thus reasonable, as both MT and HT types will be included. However, occasional tests incubated at 20 °C have shown larger numbers of plaques than were apparent at 37 °C.

Phage plaques on E. coli hosts at 37 °C are fully formed in about 8 hours. Continued incubation has been found to give lower counts, because of plaques being masked by the growth of other bacteria in the sample which are not susceptible to the phages. Initially, a dual temperature incubator was used, giving 8 hours incubation at 37 °C, then
lowering the temperature to 4 °C until the plates were read the following morning.

This gave satisfactory results, but was inconvenient, and subsequently methods employed by workers at the RIVM institute, the Netherlands, were used instead. These involve using a host which is resistant to the antibiotic nalidixic acid, and including this agent in the culture medium. This appears to work well in practice, by inhibiting growth of other bacteria in the sample, and preventing plaques from being obscured. The strains used at RIVM have been kindly donated by Dr Ir A H Havelaar (Head of Water and Food Microbiology). His strain of \textit{E. coli} WG5 has subsequently been routinely used for enumeration of somatic coliphages. This strain is a spontaneous nalidixic acid-resistant mutant of \textit{E. coli} C, ATCC 13706, and shows similar phage sensitivity.

2.3 FORMULATION OF CULTURE MEDIA

Coliphage assays are normally carried out using a non-selective nutrient medium, and the exact formulation of this seems less important than the fact that it should contain sufficient levels of divalent cations (calcium and/or magnesium), which are essential for attachment of the phage to the host.

The medium initially chosen was one which had already been successfully used for enumerating coliphages with various hosts, and also phages of \textit{Serratia marcescens}, another member of the Enterobacteriaceae. This contained, per litre of distilled water: tryptone, 10 g; yeast extract, 5 g; sodium chloride, 5 g; D-glucose, 1 g; and Oxoid Agar No 1, 12.5 g. No pH adjustment is required. After sterilisation for 15 minutes at 121 °C, and cooling to 50 °C, to each litre were added 5 ml of 0.5M calcium chloride and 5 ml of 0.5M magnesium sulphate, both aqueous solutions. The medium was dispensed in volumes of 15-20 ml in 90-mm petri dishes, and allowed to set.
The soft overlay contained, per 833 ml of distilled water: nutrient broth, 8 g; sodium chloride, 5 g; and Oxoid Agar No 1, 3 g. After melting, this was dispensed in 5-ml volumes and sterilised. This formulation has been devised for sample volumes of 1 ml, and other volumes would require adjustment of the quantity of distilled water used.

Previous findings were confirmed, that this medium combination gave good results with all the host organisms tried, but comparison was also made with four media selective for coliform organisms. The use of selective media has been suggested to reduce background growth of other bacteria present in the sample (Drury and Wheeler 1982). This seems dubious reasoning, since many sewage-derived organisms will be able to grow on such media. Nevertheless, tests were carried out using MacConkey Agar, Brilliant Green Bile Agar, Endo Agar and Violet Red Bile Agar in place of the lower nutrient layer. None of these appeared to offer any advantages. Brilliant Green Bile Agar gave lower phage counts than the standard medium, and all plates using selective media were considered difficult to interpret because of the colour, and in some cases the opacity, of the lower agar layer.

Another addition, claimed by Isbister and others (1982) to make plates easier to read, was the inclusion of 2,3,5-triphenyl tetrazolium chloride (TTC) in the upper soft agar layer. This compound is colourless in solution, but is reduced by most bacteria to give an insoluble red pigment. A range of concentrations was used, with 0.02 g/l being found sufficient to give a uniform red colour to the bacterial lawn. The clear plaques could then be counted by viewing the plate against a white background. Plaque counts were not significantly different with or without TTC, and plates with TTC were not considered any easier to count than the normal ones. The latter were normally viewed using a Gallenkamp colony counter, with illumination round the edge of the plate, and a black background. The light is scattered by the opaque lawn, and plaques are seen as dark areas.
2.4 USE OF SINGLE-LAYER PHAGE ASSAY PLATES

The use of a single layer of agar for visualisation of phage plaques has been reported by Isbister and others (1982) and Havelaar (1986). Initial attempts with this technique were unsuccessful, owing to the failure of the host to produce a satisfactory bacterial lawn. Later attempts using a much higher host inoculum gave better results, but plaque counts from river water samples were only about 25% of those obtained with the double-layer technique. Better results have been obtained using a slightly different method developed at RIVM in the Netherlands, but the double-layer method has generally been adopted as a standard procedure for all bacteriophage determinations, as it appears to be more reliable.

2.5 ENUMERATION OF F-SPECIFIC BACTERIOPHAGES.

Isolation of these particular phages was initially tried using the methods and host cultures of Havelaar (1986), who has proposed the F-specific phages as model viruses for assessing water treatment processes such as disinfection. The host is a mutant Salmonella typhimurium, WG 49, which is resistant to nalidixic acid and kanamycin, and which carries a plasmid from E. coli which causes it to produce F pili, and to ferment lactose. This host thus detects only those phages which adsorb to F pili, and somatic Salmonella phages, which normally occur only in small numbers in water and wastewater.

Initial attempts with the S. typhimurium host were unsuccessful, but further information gained at RIVM indicated the reasons for this. In particular the host has to be grown, used and stored under far more carefully-controlled conditions than do normal E. coli hosts.

The following procedure has been used for propagating the host strain. This differs slightly from the methods used at RIVM, and has been designed to make as sure as possible that the essential characteristics of the bacterial strain are maintained.
The host culture WG 49 is first grown overnight on a non-selective lactose agar, supplemented with nalidixic acid and kanamycin. This contains, per litre of distilled water: peptone, 10 g; lactose, 10 g; sodium chloride, 5 g; phenol red, 0.025 g; and agar, 12 g. Nalidixic acid, 0.1 g; and kanamycin sulphate, 0.02 g are added to the cooled medium after sterilisation. An acid-producing colony on this medium therefore shows the required antibiotic resistance pattern, and also has the gene for lactose fermentation, which is used as a marker for the F factor gene.

A suitable colony is then subcultured to 50 ml of tryptone-glucose-yeast extract (TCY) broth, also supplemented with the same concentrations of nalidixic acid and kanamycin, in a 250-ml Erlenmeyer flask. TCY medium referred to in this section has the following contents, per litre of distilled water: tryptone, 10 g; yeast extract powder, 1 g; and sodium chloride, 8 g. After sterilisation for 15 minutes at 121 °C, the medium is cooled and the following additions are made, per litre, from filter-sterilised stock solutions: glucose, 1 g; and calcium chloride, 0.3 g. The host culture is incubated overnight at 37 °C, with gentle orbital shaking (100 rev/min).

Sterile glycerol is then added to a final concentration of 20% (v/v), to protect the cells during freezing. Normally, the glycerol would be incorporated in the growth medium before incubation, but WG 49 will not grow under those conditions. After addition of the glycerol, the culture is distributed in 1.5-ml volumes in Eppendorf tubes, and stored in a freezer until required.

When required for phage analysis, a tube of the host is thawed, and inoculated into 50 ml of TCY broth, containing nalidixic acid, in a 250-ml Erlenmeyer flask. The flask is incubated at 37 °C on an orbital shaker running at 100 rev/min. Vigorous shaking must be avoided, so that the delicate F pilis are not damaged by shear forces.

The culture is grown to a density of about 10⁸ cells per ml. This corresponds to an absorbance of about 0.5 at 600 nm, and normally takes
about 5 hours. The flask is placed in melting ice, and the culture remains usable for about 2 hours.

For the assay, 1 ml of sample is added to 2.5 ml of TGY medium containing 1% w/v agar, which has been supplemented with nalidixic acid and calcium chloride, and held in a water bath at 45-47 °C. The host culture (1 ml) is added, and the mixture is immediately poured on to the surface of a plate of TGY agar (agar content 2% w/v). When the soft overlay has set, the plate is inverted and incubated overnight at 37 °C. Plaques of F-specific coliphages are of variable size, and turbid. Any completely clear plaques are likely to be due to somatic Salmonella phages. These have been seen very rarely in the present course of work.

This RIVM plating method also worked well using E. coli hosts such as WG5 and ATCC 13706, and was adopted for analyses for somatic as well as F-specific phages. The WRC method described in Section 2.3 is as effective as the RIVM one using E. coli hosts, but is not as satisfactory for tests for F-specific phages using the WG 49 host.

2.6 PROBLEMS ENCOUNTERED IN THE ANALYSIS OF F-SPECIFIC COLIPHAGES

On occasions work was severely disrupted by unexpected problems with the phage host WG 49, which was originally obtained from the RIVM Institute, the Netherlands. The manifestation of the problem was a sudden and complete loss of sensitivity to F-specific phages. Other properties of the organism which would indicate that the F-plasmid had not been lost (resistance to kanamycin, and ability to ferment lactose), were still present as normal.

Previous experience with the strain had shown that it could not be stored successfully in the frozen state for more than two to three months, and it was necessary to propagate the culture and re-freeze regularly. However, this process proved unsuccessful on one occasion. Sparse growth of the cultures was obtained on solid plating media, and no phage sensitivity was seen. Consequently, all available stocks of WG 49 were subcultured, and the most vigorous were screened for
sensitivity to MS2 phage (a strain of F-specific RNA coliphage). This required a considerable amount of time-consuming work, and the negative results obtained were extremely disappointing. Preparations from the original culture obtained from RIVM, which had been maintained in liquid nitrogen, were also resuscitated and screened in these tests, but in all cases, while the required pattern of antibiotic resistance and lactose fermentation could be obtained, no plaques were formed in plating tests with MS2.

A letter was subsequently sent to RIVM, and another freeze-dried culture of WG 49 was very kindly supplied by Dr Ir A H Havelaar, Head of Water and Food Microbiology. When reconstituted, this culture gave plaques as normal with MS2, but after propagation of the host followed by freezing in small volumes at -20 °C, the sensitivity again appeared to be lost. This occurred again on re-testing, and eventually the new culture was re-plated, and the largest colonies formed were screened for phage sensitivity. Frozen preparations of two of these strains produced satisfactory plaques, and allowed work to proceed.

A letter received from RIVM in the Netherlands indicated that this sudden loss of phage sensitivity was not unknown to them, and that they have had similar experiences in the past. Their solution is to keep several lines of stock, preserved by freezing or freeze-drying, which may be resorted to when necessary. Dr Havelaar of RIVM is of the opinion that some change takes place in the F-plasmid, causing disruption to certain areas of it. As about one third of the plasmid codes for the gene responsible for F-pilus formation, there is a fair chance that this gene will be affected, while the others may not be.

Whatever the cause of this effect, it does detract from the reliability of the technique. The method is clearly not as robust as had originally been thought, and some staff using it have become discouraged, and lost confidence in the technique when failures have occurred. At a recent conference of the International Association on Water Pollution Research and Control, held in Tubingen, W Germany in April 1990, a number of workers commented during discussion sessions that they had experienced
similar problems to those described above, with the host organism WG 49. In reply, Dr Havelaar of RIVM indicated that many details of methodology are important in the test, and that slight differences in operating technique between workers may mean the difference between success and failure (J J Bryan, WRc Medmenham, personal communication).

SECTION 3 - CONCENTRATION OF BACTERIOPHAGES FROM LARGE VOLUMES OF WATER

3.1 USE OF ZETA-PLUS 60S ELECTROPPOSITIVE FILTERS

Initially the concentration technique described by Isbister and others (1982) was used. This involves the use of positively charged filters (AMF-Cuno Zeta-Plus 60S). The sample is adjusted to pH 6.0 before filtration, under which conditions the phages will adsorb to the filter. Elution is then carried out using a small volume of 3% w/v tryptone soya broth (TSB) at pH 9. The published method used a single-layer plating method for testing the concentrated sample, but this was found to give low recoveries, and the standard double-layer method was substituted.

The 60S filters are awkward to use, being pads of some 3 mm thickness, but the method proved reasonably successful, using both TSB as the eluant, and also 3% w/v beef extract, which is commonly used for elution of viruses from such filters. Overall recoveries of phages from river water were just under 60% using the concentration procedure with 100-ml samples. Isbister and others (1982) reported recovery efficiencies of 90 to 100% using spiked distilled water samples, and figures from 12.5 to 240% using natural samples.

Increasing the time of the elution stage did not affect recoveries, but varying the volume of sample filtered did. Using the same sample material, a 50-ml volume gave 66% recovery, while a 300-ml volume gave only 40%. The time required for filtering larger volumes increased considerably. A 50-ml sample took about 3 minutes, while 300 ml took 30 minutes, and 400 ml could not be filtered in an hour.
While this technique was effective, it was felt that it could be improved, and modifications were tried. One change attempted was the use of cellulose acetate membrane filters for concentration, with the sample adjusted to pH 3.5. This resulted in recovery of only 0.02% of the phages applied, and this may have been due to them not surviving the acid conditions. A subsequent experiment substantiated this suspicion. A 1-litre water sample was divided into 100-ml portions, and these were adjusted to various pH values with hydrochloric acid. A control portion was left untreated, and the numbers of recoverable phages were measured in each portion. This experiment showed that acidification to a pH value below 6 resulted in a marked decrease in phage recovery, and indicated that the cellulose acetate filter method was probably inappropriate for concentrating coliphages.

3.1.1 Studies on elution of bacteriophages from positively-charged filters

In an attempt to improve the efficiency of the concentration procedure, several different procedures for eluting bacteriophages from the Zeta-Plus 60S filters were investigated.

Four eluants were studied. These were solutions of Tryptone Soya Broth (Oxoid Ltd), Lab-Lemco Powder (Oxoid Ltd), Beef Extract Paste (Difco), and Skimmed Milk Powder (Oxoid Ltd). These were all prepared at a concentration of 3% (w/v), and were adjusted to pH 8.5 with sodium hydroxide solution. The skimmed milk was sterilised for 5 minutes at 121 °C, and the others for 15 minutes at the same temperature.

To assess the relative performance of these eluants, 100-ml volumes of Thames water were filtered through Zeta-Plus 60S filters. The eluant under test was then drawn through the filter in two separate 10-ml volumes. The filter was then washed with three 1-ml volumes of sterile distilled water. The concentrates produced, and a sample of unconcentrated river water, were analysed for bacteriophage content using the double-layer plating method. Using the results of these tests, the recovery efficiency of the concentration procedure could be expressed as a percentage. Initial tests showed that warming the eluant
to 37 °C improved the efficiency of elution, and so this was routinely carried out in the comparisons. The values obtained are shown in Table 1.

<table>
<thead>
<tr>
<th>Eluant</th>
<th>Mean Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone soya broth</td>
<td>59%</td>
</tr>
<tr>
<td>Lab-lemco powder</td>
<td>51%</td>
</tr>
<tr>
<td>Beef extract</td>
<td>63%</td>
</tr>
<tr>
<td>Skimmed milk</td>
<td>66%</td>
</tr>
</tbody>
</table>

Interestingly there was considerable difference between the performance of Lab-lemco powder and Difco beef extract paste. This confirms the opinion, widely held among virologists, that different types of beef extract, and even different batches of the same type, can vary considerably in their suitability for this sort of technique.

These results were confirmed in a later, similar set of experiments, carried out after a change in laboratory personnel. Here tryptone soya broth averaged 50% recovery, beef extract 53% and skimmed milk 71%. As a result of these trials, 3% skimmed milk at pH 8.5 appeared to be the best overall of the eluants tested.

3.1.2 Investigation of elution time

In the early stages of developing the concentration technique, when beef extract was being routinely used as the eluant, experiments were carried out to test whether leaving the eluant in contact with the filter for an extended period would improve the recovery efficiency. In fact, no difference was seen between the various treatment procedures, which
included contact times from 0 to 10 minutes. One possible reason for this is that the filter matrix is densely packed, and the eluant does not penetrate the depth of a wet filter until vacuum is applied. As a consequence, increasing the retention time of the eluant on the filter does not affect desorption of the bacteriophages.

3.2 USE OF VIROZORB 1-MDS ELECTROPOSITIVE FILTERS

As mentioned earlier, the Zeta-Plus 60S filters are awkward to use, owing to their thickness, which makes them difficult to seal into standard filtration funnels. An alternative filter from the same manufacturer was therefore tried. This type, called Virozorb 1-MDS, is physically thinner and more pliable, resembling a paper or glass-fibre filter. Its cost is half that of the 60S type, but the manufacturers recommend using two filters together to obtain the best results. A complete review of each step in the concentration procedure was performed, and the following results were obtained.

Virozorb filters either singly or in twos or threes, were compared in performance to the established 60S filter. Recovery efficiency was calculated, and the values are shown in Table 2.

<table>
<thead>
<tr>
<th>Filter combination</th>
<th>Mean recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeta-Plus 60S</td>
<td>80%</td>
</tr>
<tr>
<td>Virozorb 1-MDS x 1</td>
<td>82%</td>
</tr>
<tr>
<td>Virozorb 1-MDS x 2</td>
<td>74%</td>
</tr>
<tr>
<td>Virozorb 1-MDS x 3</td>
<td>55%</td>
</tr>
</tbody>
</table>

Table 2 - Percentage recovery of bacteriophages using different filter combinations
3.2.1 Investigation into filtration forces

Several experiments were performed to determine the effects of filtration forces on recovery. Havelaar (1986) recommends using gravity alone, which is feasible if only a single Virozorb filter is used. With two or more filters, vacuum is required, although this can be adjusted to control the speed of filtration. The values obtained from the experiments are summarised in Table 3.

<table>
<thead>
<tr>
<th>Filtration force</th>
<th>No of filters</th>
<th>Mean recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>High vacuum</td>
<td>1</td>
<td>29%</td>
</tr>
<tr>
<td>High vacuum</td>
<td>2</td>
<td>68%</td>
</tr>
<tr>
<td>Gravity</td>
<td>1</td>
<td>82%</td>
</tr>
<tr>
<td>Low vacuum</td>
<td>2</td>
<td>59%</td>
</tr>
</tbody>
</table>

3.2.2 Comparison of eluants

Skimmed milk eluant and beef extract eluant were compared using both single and double Virozorb filters, using low vacuum for recovery. The results are summarised in Table 4.

<table>
<thead>
<tr>
<th>Eluant</th>
<th>No of filters</th>
<th>Mean recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skimmed milk</td>
<td>1</td>
<td>60%</td>
</tr>
<tr>
<td>Skimmed milk</td>
<td>2</td>
<td>56%</td>
</tr>
<tr>
<td>Beef extract</td>
<td>1</td>
<td>34%</td>
</tr>
<tr>
<td>Beef extract</td>
<td>2</td>
<td>44%</td>
</tr>
</tbody>
</table>
3.2.3 Volume of eluant used

A smaller volume of eluant would be of considerable advantage, as long as no loss of recovery occurred. To investigate this 20-ml and 10-ml eluant volumes were compared, both being applied in two equal portions. The recoveries recorded are shown in Table 5.

Table 5 – Percentage recovery of bacteriophages using different eluant volumes

<table>
<thead>
<tr>
<th>Eluant volume</th>
<th>Mean recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 ml</td>
<td>55%</td>
</tr>
<tr>
<td>10 ml</td>
<td>61%</td>
</tr>
</tbody>
</table>

3.3 ALTERNATIVE METHOD OF ELUTING PHAGES FROM 1-MDS FILTERS

An alternative to the standard concentration procedure was investigated to try to improve the overall efficiency. This involved removing the filters from the funnel after filtering the sample, and placing them in a test-tube containing 10 ml of skimmed milk eluant. Initial recoveries were fairly poor ranging between 0.4-7.8%.

The following modifications were investigated. Firstly the tube and filter were vortex mixed for approximately 1 minute, to try to release the phages. Secondly the tube was submitted to brief treatment in an ultrasonic cleaning bath, with and without a small amount (0.05% v/v) of the wetting agent Tween 80 (polyoxyethylene sorbitan mono-oleate). The results are summarised in Table 6.
Table 6 – Mean percentage recovery of F-specific and somatic coliphages by alternative elution method using various treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Recovery ($F^+$)</th>
<th>Recovery (somatic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vortex mixing</td>
<td>24%</td>
<td>32%</td>
</tr>
<tr>
<td>Ultrasonication + Tween</td>
<td>5%</td>
<td>2%</td>
</tr>
<tr>
<td>Ultrasonication - Tween</td>
<td>0.4%</td>
<td>4%</td>
</tr>
<tr>
<td>No treatment</td>
<td>1%</td>
<td>20%</td>
</tr>
</tbody>
</table>

3.4 DISCUSSION

From the results it appears that the use of a single Virozorb 1-MDS filter is the most efficient method. However, by statistically comparing mean recoveries obtained in the various experiments, it emerges that there is no significant difference in recovery between the use of a single Virozorb filter, two Virozorb filters or indeed the 60S filter. However the single Virozorb filter is more economical than the other combinations. The triple Virozorb filter combination gives a significantly lower recovery, and it seems likely that this reduction with increasing numbers of filters is caused by an inability to elute phages from the filter, rather than poor binding of the phage particles. In fact the efficiency of binding should be increased by using extra filter layers.

Havelaar (1986) reported gravity to be the best filtration force to use with Virozorb filters. The results confirm this, provided that speed of processing is not a major requirement. If speed is necessary, and vacuum must be used, then better results are obtained by using two filters together, as recommended by the manufacturers.

Statistical analysis of mean recoveries obtained in a number of experiments shows that better results are obtained by using 3% skimmed milk, pH 8.5, warmed to 37 °C, than with the other eluant systems tested.
The concentration procedure developed by Ibister and others (1982) recommends a 20-ml eluant volume. However, the results show no significant difference between the recoveries recorded for 20 and 10 ml, and so the smaller volume may be used without compromising the efficiency of the technique.

The discouraging results obtained during the investigation of removing filters from the filtration apparatus and eluting in a tube are unfortunate, as this method would have considerably simplified and speeded up the concentration procedure. It may be that shear forces produced by passage of the eluant through the filter are necessary for successful release of the phage particles, and that this can only be achieved by drawing the eluant through the filter by vacuum. This may also explain why elution is more efficient from a single filter than from two used together. Treatment with ultrasonics, which has been used before to release bacteria from sediments and particulates, was unsuccessful in releasing phages from the Virozorb filters, and may even have caused some inactivation. Vortex mixing was the most effective method used, but was still less efficient than the standard technique.

SECTION 4 - STUDIES ON NATURAL POPULATIONS OF BACTERIOPHAGES, VIRUSES AND INDICATOR BACTERIA

4.1 EXAMINATION OF VARIOUS TYPES OF WATER

This study was undertaken to make a preliminary assessment of the relative incidence of faecal indicator bacteria, bacteriophages, enteroviruses and rotaviruses in various types of samples. In consultation with Dr R Morris of Severn Trent Laboratories, Coventry, a sampling programme was devised to include a wide spectrum of waters. However, since at the time the method for concentrating phages from large volumes had not been fully developed, it was decided to examine only a small number of samples of treated water, in which the concentration of bacteriophages was likely to be low.
A total of 79 samples were analysed, 40 from sewage treatment works, 25
from rivers, and 14 from a water treatment works. All samples were
collected by Severn Trent Water. A portion of each sample was
transported immediately to WRC Medmenham, where analysis for F+ and
somatic coliphages, and total and thermotolerant coliform organisms, was
carried out without delay. The remainder of each sample was taken to
the virology unit of Severn Trent Laboratories, where it was frozen
until analysis for enteroviruses and rotaviruses could be carried out.

4.2 METHODS OF ANALYSIS

4.2.1 Total and thermotolerant coliform organisms

Test portions, or decimal dilutions of the sample made in 0.1% peptone
solution, were filtered through 47-mm diameter cellulose acetate
membrane filters with a nominal pore size of 0.45 μm. The membranes
were then placed on sterile absorbent pads (Whatman No 17) soaked with
‘Membrane lauryl sulphate broth’ (DoE and others 1983). For
determination of total coliform organisms the plates were incubated for
4 h at 30 °C followed by 14 h at 37 °C. For thermotolerant coliform
organisms, the equivalent conditions were 4 h at 30 °C followed by 14 h
at 44 °C. Yellow (acid-producing) colonies formed on the filters were
counted.

4.2.2 F-specific and somatic coliphages

The double-layer plating method, already described, was used.
*S. typhimurium* WG 49 was used as host for F-specific phages, and
*E. coli* C WG5 for somatic coliphages.

Samples of treated water were concentrated by the method described in
Section 3.1, using Zeta-Plus 60S filters, and elution with beef extract.

4.2.3 Enteroviruses and rotaviruses

Sewage and effluent samples were analysed by a direct inoculation
procedure, but samples from surface waters and the water treatment plant
were concentrated by an adsorption-elution process.
The concentration procedure involved filtering 10 litres of water, adjusted to pH 3.5, through a Balston glass-fibre cartridge filter with a nominal retention pore size of 8 µm. Viruses adsorbed to the filter were eluted with 400 ml of 3% (v/v) beef extract at pH 9.5. The eluant was then flocculated by adjusting the pH to 3.1. The precipitate produced was recovered by centrifugation (6000 g for 30 minutes) and then was dissolved in 0.15 M Na₂HPO₄. At this stage the concentrates were usually frozen at -20 °C to await analysis. When required, the concentrates were thawed, then clarified by centrifugation (3000 g for 10 min). The volume was then adjusted to 10 ml by the addition of Na₂HPO₄ (0.15 M). Using this procedure, 1 ml of concentrate was equivalent to 1 litre of original sample.

Enterovirus assays were conducted using a suspended plaque assay, and the BGM (Buffalo Green Monkey Kidney) cell line. Rotaviruses were detected with an immunoperoxidase intracellular antigen detection system, with MA-104B cells.

4.3 RESULTS

Table 7 shows the means of the total coliform, thermotolerant coliform, bacteriophage, enterovirus and rotavirus counts obtained from the samples taken at the sewage treatment works. Rotavirus concentrations were consistently below the limit of detection of the analytical technique used (that is less than 10 per 100 ml). As expected, enteroviruses, and both F+ and somatic coliphages were consistently found in the samples.

For each treatment process examined, the ratios of the mean counts of total and thermotolerant coliforms to those of enteroviruses were extremely variable. The ratio of total coliforms to enteroviruses was greatest (about 400,000:1) in samples from the effluent of the activated sludge plant, and lowest in those from the effluent of the rapid sand filter (4700:1). The thermotolerant coliform to enterovirus ratio varied from about 2000:1 (effluent from percolating filters) to about 62,000:1 (activated sludge effluent). The ratios of bacteriophage counts to
<table>
<thead>
<tr>
<th>Source of samples</th>
<th>Total coliforms</th>
<th>Thermotolerant coliforms</th>
<th>F-specific coliphages</th>
<th>Sonotic coliphages</th>
<th>Enteroviruses</th>
<th>Rotaviruses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Partial activated sludge - influent</td>
<td>$1.1 \times 10^7$</td>
<td>$2.4 \times 10^6$</td>
<td>$1.0 \times 10^5$</td>
<td>$2.3 \times 10^5$</td>
<td>77</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Partial activated sludge - effluent</td>
<td>$9.7 \times 10^7$</td>
<td>$1.5 \times 10^7$</td>
<td>$1.7 \times 10^5$</td>
<td>$7.3 \times 10^5$</td>
<td>240</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Percolating filters - influent</td>
<td>$1.1 \times 10^7$</td>
<td>$3.0 \times 10^6$</td>
<td>$1.3 \times 10^5$</td>
<td>$2.2 \times 10^5$</td>
<td>135</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Percolating filters - effluent</td>
<td>$6.6 \times 10^5$</td>
<td>$1.3 \times 10^5$</td>
<td>$4.4 \times 10^4$</td>
<td>$9.9 \times 10^4$</td>
<td>64</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Rapid sand filters - influent</td>
<td>$1.4 \times 10^6$</td>
<td>$4.1 \times 10^5$</td>
<td>$5.1 \times 10^4$</td>
<td>$2.0 \times 10^5$</td>
<td>70</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Rapid sand filters - effluent</td>
<td>$5.0 \times 10^5$</td>
<td>$3.0 \times 10^5$</td>
<td>$7.1 \times 10^4$</td>
<td>$1.6 \times 10^5$</td>
<td>105</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

All counts are expressed per 100 ml.
enterovirus counts were less variable, with a maximum range of about a factor of two. This suggests that the response of the bacteriophages to the treatment processes was similar to that of the enteroviruses. Although such a conclusion is rather tenuous when based on so little data, it is supported by examining the apparent response of each type of organism to the three treatment processes studied. In Table 8 the concentration of each of the organisms in the effluents from each of the treatment processes have been expressed as a ratio of the equivalent influent concentrations. From these ratios it is apparent that the response of total and thermotolerant coliforms to the treatment processes was very different from that of the enteroviruses. However, the ratios obtained for both the F+ and somatic coliphages were broadly similar to those of the enteroviruses.

The results obtained from the examination of river water samples are shown in Table 9. Sampling stations were chosen upstream (a) and downstream (b) of discharges of sewage works effluents. The impact of these effluents on river water quality is demonstrated by the increased concentration of bacteria and bacteriophages in samples collected downstream of the point of discharge. Although the same trend is evident in the enterovirus results, virus concentrations (particularly rotavirus) were often below the limit of detection of the analytical technique used.

The results obtained in the analysis of samples from various points in a water treatment works are shown in Table 10. The variability observed in the concentrations of organisms suggests that more intensive sampling programmes would be needed for a reliable indication of removal efficiencies of the processes to be obtained. It is notable that enteroviruses and coliforms were isolated from the effluent of the slow sand filter, but bacteriophages were not. However, as mentioned above, the concentration technique for bacteriophages had not been fully researched when this study was undertaken.
Table 8 - Concentration of each organism in process effluents as a ratio of the equivalent influent concentration.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total coliforms</th>
<th>Thermotolerant coliforms</th>
<th>F-specific coliphages</th>
<th>Somatic coliphages</th>
<th>Enteroviruses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Partial activated sludge</td>
<td>8.8</td>
<td>6.4</td>
<td>1.7</td>
<td>3.2</td>
<td>3.1</td>
</tr>
<tr>
<td>Percolating filter</td>
<td>0.06</td>
<td>0.04</td>
<td>0.34</td>
<td>0.45</td>
<td>0.47</td>
</tr>
<tr>
<td>Rapid sand filter</td>
<td>0.36</td>
<td>0.73</td>
<td>1.39</td>
<td>0.8</td>
<td>1.5</td>
</tr>
</tbody>
</table>
Table 9 - Average counts of coliform bacteria, bacteriophages and viruses in samples from rivers above and below sewage treatment works discharges.

<table>
<thead>
<tr>
<th>Source of samples</th>
<th>Total coliforms</th>
<th>Thermotolerant coliforms</th>
<th>F-specific coliphages</th>
<th>Somatic coliphages</th>
<th>Enteroviruses</th>
<th>Rotavirus</th>
</tr>
</thead>
<tbody>
<tr>
<td>River Soar - a</td>
<td>3.3x10^4</td>
<td>4.6x10^3</td>
<td>500</td>
<td>2.0x10^3</td>
<td>0.9</td>
<td>ND</td>
</tr>
<tr>
<td>River Soar - b</td>
<td>6.8x10^4</td>
<td>1.2x10^4</td>
<td>1.1x10^3</td>
<td>4.0x10^3</td>
<td>4.6</td>
<td>ND</td>
</tr>
<tr>
<td>River Swift - a</td>
<td>3.3x10^3</td>
<td>1.3x10^3</td>
<td>80</td>
<td>1.9x10^3</td>
<td>0.1</td>
<td>ND</td>
</tr>
<tr>
<td>River Swift - b</td>
<td>1.4x10^5</td>
<td>4.7x10^4</td>
<td>2.8x10^3</td>
<td>4.5x10^4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>River Avon - a</td>
<td>2.9x10^4</td>
<td>6.4x10^3</td>
<td>800</td>
<td>3.2x10^3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>River Avon - b</td>
<td>4.1x10^4</td>
<td>2.9x10^3</td>
<td>1.6x10^3</td>
<td>6.7x10^3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>River Sowe - a</td>
<td>4.7x10^4</td>
<td>4.6x10^3</td>
<td>800</td>
<td>2.3x10^3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>River Sowe - b</td>
<td>1.2x10^5</td>
<td>3.3x10^4</td>
<td>2.3x10^4</td>
<td>7.2x10^4</td>
<td>2.7</td>
<td>ND</td>
</tr>
<tr>
<td>River Avon - a</td>
<td>3.2x10^4</td>
<td>4.4x10^3</td>
<td>8.4x10^3</td>
<td>3.1x10^4</td>
<td>6.4</td>
<td>ND</td>
</tr>
<tr>
<td>River Avon - b</td>
<td>7.3x10^4</td>
<td>1.7x10^4</td>
<td>1.2x10^4</td>
<td>4.8x10^4</td>
<td>8.7</td>
<td>ND</td>
</tr>
<tr>
<td>River Tame - a</td>
<td>2.2x10^5</td>
<td>5.6x10^4</td>
<td>6.3x10^3</td>
<td>1.7x10^4</td>
<td>5.9</td>
<td>0.1</td>
</tr>
<tr>
<td>River Tame - b</td>
<td>2.5x10^5</td>
<td>7.6x10^4</td>
<td>7.3x10^3</td>
<td>3.0x10^4</td>
<td>9.2</td>
<td>1.6</td>
</tr>
</tbody>
</table>

All counts are expressed per 100 ml
a = above and b = below sewage works discharge  ND = none detected
Table 10 - Average counts of coliform bacteria, bacteriophages and viruses in samples from water treatment processes.

<table>
<thead>
<tr>
<th>Source of samples</th>
<th>Total coliforms</th>
<th>Thermotolerant coliforms</th>
<th>F-specific coliphages</th>
<th>Somatic coliphages</th>
<th>Enteroviruses</th>
<th>Rotavirus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw intake</td>
<td>7.3x10³</td>
<td>1.7x10³</td>
<td>900</td>
<td>1.4x10³</td>
<td>1.8</td>
<td>ND</td>
</tr>
<tr>
<td>Post flocculation</td>
<td>2.3x10⁴</td>
<td>1.9x10³</td>
<td>12</td>
<td>9</td>
<td>0.1</td>
<td>ND</td>
</tr>
<tr>
<td>Post settlement</td>
<td>6.6x10³</td>
<td>1.2x10³</td>
<td>20</td>
<td>900</td>
<td>1.3</td>
<td>ND</td>
</tr>
<tr>
<td>Post rapid gravity sand filter</td>
<td>3.1x10³</td>
<td>550</td>
<td>7.6</td>
<td>25</td>
<td>1.5</td>
<td>ND</td>
</tr>
<tr>
<td>Post slow sand filter</td>
<td>12</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
<td>0.4</td>
<td>ND</td>
</tr>
<tr>
<td>Before chlorine contact tank</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Post chlorination</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

All counts are expressed per 100 ml. ND = none detected.
Arrangements were made with a water authority to obtain samples from a large sewage treatment works. The Public Health Laboratory at the Royal Berkshire Hospital, Reading, was also contracted to carry out analysis for enteroviruses on these samples. The sampling programme was necessarily limited, owing to the expense of the virological analysis, and to the number of samples which could be handled by the virology laboratory.

Samples of crude sewage were examined, as well as effluent samples from various parts of the works. The sewage treatment works studied is useful in that it uses two separate types of treatment. Some of the influent, after grit removal, passes through two banks of trickling filters, each followed by settlement tanks, while the remainder is treated by an activated sludge process. This is itself divided into two parts, having both a conventional plant, and also a Wimpey Unox plant, where the mixed liquor is treated with oxygen, extracted from the air on site.

Samples of crude sewage were collected using an automatic battery powered sampler (Instrument Specialties Company, Lincoln, Nebraska, USA). This was placed at a suitable position at the inlet to the works, and was set to take discrete samples every two hours. The machine has an insulated sample chamber, and this was filled with ice to keep the contents as cool as possible until collection 24 hours later.

It was not feasible to take effluent samples using an automatic sampler, as the volumes required for virological analysis were too large. Grab samples were therefore taken at four points in the works, at the same time as the automatic sampler was retrieved. The effluent samples were all taken from the outlets of settlement tanks. The four types of settled effluents collected were from the primary trickling filters, the secondary trickling filters, the conventional activated sludge plant, and the Unox plant. Two-litre volumes of these samples were taken to the Public Health Laboratory, and smaller portions were returned to WRC.
Medmenham for analysis for somatic and F-specific bacteriophages, and for total and thermotolerant coliform organisms. On each occasion four samples of crude sewage, spread over the 24-hour sampling period, were also selected for analysis.

4.4.1 Methods

Analysis for somatic and F-specific coliphages was carried out by the double-layer plating method already described. The host bacteria used were *E. coli* C strain WG 5 for somatic coliphages, and *S. typhimurium* strain WG 49 for F-specific phages. Tests for total and thermotolerant coliform organisms were carried out using the membrane filtration technique, as described in Report 71 (DoE and others 1983).

For virological analysis, 100-ml volumes of sewage, or 2-litre volumes of effluent were concentrated using a membrane adsorption technique, followed by elution with skimmed milk to give a final concentrate volume of 10 ml. Various cell types were inoculated with 0.1-ml volumes of the concentrates, and incubated under liquid medium for 17-21 days at 37 °C. Three human cell types were used (He1, F1, and HeLa), and two monkey types (BGM and Vero). Any cytopathic effect was noted in the cultures, and these cells were passaged to new cells. Viruses were then identified by serum neutralisation tests.

4.5 RESULTS

Table 11 gives average counts per ml of indicator bacteria and bacteriophages over the whole sampling period, and shows the kind of variation in concentration which was seen.
Table 11 – Average counts per ml of indicator bacteria and bacteriophages in samples from a sewage treatment works.

<table>
<thead>
<tr>
<th></th>
<th>Coliphages:</th>
<th></th>
<th>Coliform organisms:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Somatic</td>
<td>F+</td>
<td>Total</td>
</tr>
<tr>
<td>Crude sewage:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.4x10^4</td>
<td>1.0x10^4</td>
<td>1.5x10^6</td>
</tr>
<tr>
<td>2</td>
<td>1.2x10^4</td>
<td>9.5x10^3</td>
<td>1.5x10^6</td>
</tr>
<tr>
<td>3</td>
<td>7.3x10^3</td>
<td>5.6x10^3</td>
<td>1.2x10^6</td>
</tr>
<tr>
<td>4</td>
<td>5.3x10^3</td>
<td>3.0x10^3</td>
<td>8.6x10^5</td>
</tr>
<tr>
<td>Effluents:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.1x10^3</td>
<td>1.3x10^3</td>
<td>7.3x10^4</td>
</tr>
<tr>
<td>2</td>
<td>2.3x10^3</td>
<td>9.9x10^2</td>
<td>2.2x10^4</td>
</tr>
<tr>
<td>3</td>
<td>430</td>
<td>98</td>
<td>5.7x10^3</td>
</tr>
<tr>
<td>4</td>
<td>63</td>
<td>68</td>
<td>5.7x10^3</td>
</tr>
</tbody>
</table>

Crude sewage, collected at 1: 1030, 2: 1630, 3: 2230, 4: 0430
Effluents. 1: Primary filter, 2: Secondary filter, 3: Unox plant
4: Conventional activated sludge plant.

There is a certain variation in the concentrations of bacteria and bacteriophages with time, in the crude sewage, but this is not as marked as is sometimes seen, probably owing to the large geographical area from which the sewage is gathered. Lower concentrations are present in the effluents, and in general the activated sludge processes have resulted in greater removal of microorganisms than have the two banks of trickling filters.

Table 12 shows the average ratios of indicator bacteria to F-specific phages for these samples. Although there is some variation, the ratios of indicator bacteria to phages remains of the same order throughout the sampling period for the crude sewage. However, these ratios are much reduced for the effluent samples, indicating that there is greater loss of bacteria owing to the treatment processes, than there is of bacteriophages.
Table 12 - Average ratios of total coliforms (TC) and thermotolerant coliforms (TTC) to F-specific bacteriophages in samples from a sewage treatment works.

<table>
<thead>
<tr>
<th></th>
<th>Ratio TC:F+</th>
<th>Ratio TTC:F+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude sewage:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>150</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>160</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>210</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>290</td>
<td>28</td>
</tr>
<tr>
<td>Effluents:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>56</td>
<td>2.6</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>1.3</td>
</tr>
<tr>
<td>3</td>
<td>58</td>
<td>3.3</td>
</tr>
<tr>
<td>4</td>
<td>84</td>
<td>8.1</td>
</tr>
</tbody>
</table>

The virological analysis showed a variety of enterovirus types, including poliovirus type 1, 2 and 3, and Coxsackievirus types A7, B4, B5 and B6. No echoviruses were isolated, but the laboratory reports that none of these viruses had been isolated from any local clinical specimens during the previous month.

Overall though, few viruses were isolated, especially from the crude sewage samples, where the concentration procedure used does not seem to have been of sufficient sensitivity. The results supplied only gave the numbers of virus positive cultures out of the total number inoculated. Virus densities were therefore estimated using a most probable number calculation (Hurley and Roscoe 1983). The limit of detection for the method used for the sewage samples was roughly 70 viruses per litre, and for the effluent samples was 4 viruses per litre. Of 17 samples of crude sewage, only 7 contained detectable levels of viruses, and the highest number reported was 220 per litre. Effluents contained between 4 and 44 viruses per litre, only one sample being below the limit of detection.
These results were extremely disappointing, and do not allow any meaningful comparison to be made between numbers of viruses, bacteria and coliphages. Also it is not possible to deduce the degree of removal of viruses by the various treatment processes from these results. The virology laboratory commented that "the method of sample concentration was a recently-described one, which would seem to need further evaluation".

4.6 COLIPHAGES AND INDICATOR BACTERIA IN RECREATIONAL WATERS

Towards the end of the contract period, a study was undertaken to assess the relative numbers of indicator organisms and bacteriophages in inland waters used for recreational purposes.

Samples were collected on a number of occasions from several points at each of three sites, and transported to the laboratory in refrigerated containers. They were then analysed for thermotolerant coliform organisms and faecal streptococci using membrane filtration procedures as described in Report 71 (DoE and others 1983). The tests for faecal streptococci were incubated at the lower of the two recommended temperatures (37 °C). The same samples were also tested for somatic and F-specific coliphages by the methods already described. As numbers were expected to be low, the samples were all concentrated using the electropositive filter method described above. This gave limits of detection of 4 pfu per 100 ml for the coliphages, and 1 cfu per 100 ml for the bacterial indicators.

A wide range of counts were obtained from these samples. Thermotolerant coliform numbers ranged from undetectable to $1.2 \times 10^5$ per 100 ml, faecal streptococci from undetectable to 4300 per 100 ml, and somatic coliphages from undetectable to 1700 per 100 ml. No F-specific coliphages were detected in any of the samples tested, with the single exception of the one which yielded the highest density of thermotolerant coliforms. Therefore no results are shown for F-specific phages, and indeed in the latter half of the study they were not tested for.
Figures 1 to 3 show relative numbers of the three remaining organisms. Figure 1 shows results for streptococci plotted against thermotolerant coliform organisms for all samples where positive results for both were obtained. Logarithmic scales have been used for both axes, and there is a clear trend in the results. There is considerable scatter within the data, but the regression line shown gives a reasonable description of the trend, with a correlation coefficient of 0.87. As the slope of this line is not unity, this indicates that there is not a simple relationship between the numbers of the two organisms. In the more polluted samples the TTC:FS ratio is roughly 10, while in the less polluted samples it is closer to unity, presumably reflecting different survival rates with distance from the polluting source.

Figures 2 and 3 show similar plots, but with somatic coliphages plotted against thermotolerant coliforms and faecal streptococci. Here there is more scatter than in Figure 1, and calculated correlation coefficients are 0.8 (Figure 2), and 0.62 (Figure 3). Again there is a discernible trend in the results, but it is less clear, especially in Figure 3. The two Figures are rather similar to many given by Atlantic Research Corporation (1978), for results of many large-scale trials carried out in the USA on use of coliphages as pollution indicators. Indeed, many graphs presented in that report showed very poor correlation between coliphages and bacterial indicators, although the results led to the use of coliphages being recognised as a tentative method for pollution detection by the American Public Health Association (APHA and others 1985, 1989).

The slopes of the regression lines in Figures 2 and 3 are again considerably less than unity. If this observation is valid, it would indicate that somatic coliphages are surviving better than the bacterial indicators in situations remote from the source of pollution. Results of storage experiments described below would tend to support this hypothesis.
COLIPHAGES AS POLLUTION INDICATORS WHERE LONG DELAYS EXIST BETWEEN SAMPLING AND ANALYSIS

It is good practice to analyse samples as soon as possible after collection, and as a general rule delays of up to 6 hours are considered acceptable. However, in remote locations it may not be possible to deliver samples to the testing laboratory within this period, and the validity and accuracy of the analysis may be compromised. In such circumstances alternatives to bacterial pollution indicators may give a more representative indication of the sanitary quality of the sample, and one possibility is to use bacteriophages.

Experiments were carried out to test the survival of indicators under different conditions of transit. To simulate unrefrigerated transport, water samples were placed, after initial analysis, in an incubator which was set to cycle between 10 °C and 20 °C to represent night and daytime temperatures. To represent the kind of refrigeration which might be achievable in transit, a second set of samples was stored in a cool box containing ice packs. Organisms studied were somatic coliphages, coliform bacteria and faecal streptococci.

4.7.1 Results

Figure 4 shows the mean survival rates of the various organisms over 48 hours under the two sets of conditions. Each point is the mean of either 6 or 7 separate determinations in the case of coliform bacteria or coliphages, while only two experiments were carried out using faecal streptococci. The temperature in the cool box was maintained at 6 °C by the ice packs for about 10 hours, then rose at a rate of about 0.7 °C per hour. After 24 hours, when the temperature had risen to about 15 °C, the ice packs were replaced with new ones.

Statistical analysis of the rates of inactivation observed in the individual experiments showed that refrigeration increased the survival of coliform bacteria, but the improvement was only marginally significant at the 90% confidence level. There was no significant
effect of refrigeration on the survival of the other organisms. Coliform organisms survived better than coliphages under both storage conditions, and the differences were again statistically just significant at the 90% confidence level. Data for faecal streptococci were insufficient for meaningful calculations to be made.

These experiments are somewhat similar to others described later in this report, but were designed to simulate particular conditions. The survival experiments described in Section 5.4 would suggest that little mortality would be expected during the first two days of storage, except in the case of F-specific coliphages, which were not considered in the present experiments. The results described here thus indicate that storage of samples for 24 hours would probably not have a dramatic effect on the results of subsequent analyses, especially if efforts were made to keep the samples as cool as possible. It is assumed that samples would also be kept in the dark. The main conclusion to be drawn is that there would be no advantage in using bacteriophages rather than bacterial indicators. Under the conditions tested here, coliform bacteria showed better overall survival than somatic coliphages.

SECTION 5 - STUDIES ON THE RELATIVE SURVIVAL OF VIRUSES AND BACTERIOPHAGES

5.1 INTRODUCTION

A large program of study was undertaken to assess the relative survival of viruses and bacteriophages under a variety of conditions. Virology work was subcontracted to Severn Trent Laboratories, who also undertook to produce high-titre stocks of a suitable enteric virus and rotavirus, so that these could be used in spiking experiments. High-titre bacteriophage suspensions were produced at WRC by the method described in Section 5.2.

Experiments were carried out to assess the response of the various organisms to disinfectants, solar radiation, and long-term storage in various types of water. Samples were tested at WRC Medmenham by the
methods already described. Initially it was intended that the viruses would be added to the same reaction mixtures as the other organisms, but safety considerations and other problems prevented this. In particular, the virology laboratory had trouble in producing large quantities of rotavirus suspensions. It is only comparatively recently that tissue culture lines have been found which will grow rotaviruses at all, and their growth is slow compared with most culturable viruses. Because of delays, experiments were started before the virus stocks were available, and experiments with viruses were carried out separately. This allowed greater economy in the use of the suspensions, and facilitated isolation and safe disposal of virus-contaminated material. The viruses used were a vaccine strain of poliovirus type 1, and a bovine rotavirus.

5.2 PREPARATION OF HIGH-TITRE STOCKS OF F-SPECIFIC BACTERIOPHAGES

For subsequent survival experiments, it was necessary to produce high-titre stocks of F-specific coliphages. Initial attempts at propagation by infection of logarithmic phase cultures of _Salmonella typhimurium_ WG 49 were unsuccessful. To overcome the problems, a simpler plate-propagation method was used. Initially this was also unsuccessful, no infective phage particles being isolated from the propagation plates. This seemed to be a shortcoming of the host organism. While this is effective as the host for enumeration of F-specific phages, it appears to be unsuitable for propagation by plating or liquid culture methods. This problem was subsequently overcome by using two different host bacteria at different stages of the propagation procedure.

5.2.1 Propagation method

F-specific phages were isolated from natural samples using the double-layer plating method, and the _F+ Salmonella typhimurium_ WG 49 as the host. After overnight incubation at 37 °C, individual plaques were picked by removing a small portion of the agar overlay, using a sterile glass Pasteur pipette. Each piece of phage-containing agar was then resuspended in 0.5 ml of sterile nutrient broth. These suspensions

34
usually contained at least several thousand phage particles per millilitre. A ten-fold serial dilution of this stock was then made, and each dilution was plated using the standard double-layer method. The host used for this plating was a stable natural male strain of *Escherichia coli* (strain K12 F+, NCIMB 9481). The plates were then incubated overnight at 37 °C.

The next day the plates were examined, and the ones where the plaques were almost confluent over the whole surface of the plate were selected for harvesting. Each of these plates was flooded with 10 ml of sterile nutrient broth, and the top layer of soft agar was carefully scraped off using a sterile spatula, and placed in a 30-ml universal container. This was mixed periodically over the next 15 minutes, then centrifuged at 1500 g for 10 minutes at 10 °C, to sediment the agar pieces. The supernatant fluid was then assayed for F-specific phages using *S. typhimurium* WG 49 as the host. This procedure resulted in phage suspensions containing between 1 and 3 × 10^{11} plaque-forming units (pfu) per millilitre.

To check that RNA phages had been isolated, the suspensions were also plated in the normal way, but with 100 µg of ribonuclease per plate added to the agar overlay. As ribonuclease only inactivates RNA phages, plaque formation should be inhibited under these conditions. If plaques are formed on the plates, the isolate can be assumed to be a DNA phage.

To obtain bacterium-free preparations, the phage suspensions were filtered through cellulose acetate membranes of 0.22 µm pore size. This resulted in a small loss of phages in some cases.

These phage suspensions were stored at 4 °C, and after storage for more than 2 months, were still found to contain over 10^{11} infective phage particles per ml.
5.2.2 Efficiency of plating of F-specific bacteriophages

The efficiency of plating of the F-specific phages isolated as described above, was tested using E. coli K12 F+ (NCIMB 9481) as the reference host. This strain is a natural host for these bacteriophages, as it possesses a stable wild-type F factor. The efficiency of plating (EOP) is calculated as follows:

\[
\frac{\text{pfu per ml using test host}}{\text{pfu per ml using reference host}} \times 100\%
\]

When carried out using WG 49 as the test host and 9481 as the reference, the EOP values obtained ranged from 89 to 139%, with a mean of 112%. This indicates that WG 49 is at least as efficient as the natural E. coli host for enumerating F-specific phages.

5.3 EXPERIMENTS ON INACTIVATION BY DISINFECTANTS

These were carried out in water with low disinfectant demand, to assess the effects of disinfectant in the absence of substances which would tend to protect the phages or viruses. Thus they are more appropriate to potable water disinfection than to wastewater processes.

5.3.1 Experimental procedure

The experiments were carried out in a 5-litre stirred vessel, immersed in a large thermostatically-controlled water bath. A vacuum operated sampling system was used, enabling 250-ml volumes of the reaction mixture to be withdrawn quickly, and in rapid succession when necessary.

For each experiment, 5 litres of water were placed in the vessel and allowed to equilibrate to the temperature of the water bath (10 °C). The vessel was then spiked with the phage suspension (0.1 ml) and any other organisms which were being tested at the same time, and a sample was taken to check the initial concentrations. A stock chlorine solution was then standardised using a colorimetric DPD method, measured
with a spectrophotometer. An appropriate dose of chlorine was then added to the reaction vessel, and sampling was commenced immediately. The sample bottles all contained sodium thiosulphate to neutralise the chlorine immediately. Non-neutralised samples were also taken before, during and after the experiment to test for free and combined chlorine residual.

Spiking the 5-litre volume with 0.1 ml of phage suspension resulted in an initial concentration in the reaction mixture of about $10^6$ pfu per ml. This allowed inactivation to be followed over 6 log cycles without having to resort to concentration procedures. The sampling programme was usually carried on for 30 minutes, with the most intensive sampling immediately after addition of the disinfectant. Later in the study a number of experiments were carried out in smaller-scale apparatus, and smaller samples were taken using pipetting devices. The basic method remained the same, however. Samples for virological analysis were immediately frozen, and transported to Severn Trent Laboratories in that state for analysis.

5.3.2 Results

All samples tested for rotaviruses gave completely negative results, including the pre-disinfection controls. It appeared that the original suspension of rotaviruses was at fault, and all the experiments were repeated. Here positive results were obtained from the pre-disinfection samples, but all the others, with one exception, failed to yield detectable rotaviruses. This may indicate that the viruses were particularly sensitive to the disinfectants, but this effect was possibly caused by the experimental conditions. A fundamental problem in using this viral preparation was that, as the density of particles was comparatively low (about $10^5$ per ml), the reaction vessel had to be spiked with a fairly large amount of suspension, and this exerted a considerable disinfectant demand. As the experiments were planned on the basis of achieving a particular residual concentration of disinfectant at the end of the 30-minute contact period, quite high initial doses had to be used to overcome the demand in the system. It
is therefore possible that these high disinfectant concentrations in the first few seconds of each test may have reduced the rotaviruses to undetectable levels. The same problem would not have been expected using polioviruses, as the density of particles in the suspension was much higher (over $10^8$ per ml). Consequently, only very small amounts of the suspension were needed to give an adequate starting concentration, and problems of excessive disinfectant demand did not occur.

Figure 5 shows inactivation by a chlorine dose sufficient to give a free residual of 0.3 mg/l after 30 minutes contact time. As inactivation was rapid, only the first 5 minutes of the contact period are shown. The plot for the poliovirus ends abruptly, as the next point on each curve was below the limit of detection for the initial inoculum applied. It would appear that on average the polioviruses survived less well than the F-specific phages.

The results for a dose to give a free chlorine residual of 1.0 mg/l after 30 minutes are shown in Figure 6. Here no phages or viruses were detectable after a minute. The longer decay lines shown for the F-specific phages reflect the different limits of detection for the two types of analysis. The mean decimal reduction time for F-specific phages was 12 seconds, and for polioviruses was 35 seconds. The relative resistance of the two organisms is in contrast to the tests using a 0.3-mg/l residual, and suggests that there may be a threshold concentration of free chlorine, below which the F-specific phages survive better than polioviruses, but above which the poliovirus shows the greater resistance.

Figure 7 shows two plots each for F-specific coliphages and polioviruses treated with pre-formed monochloramine dosed to give a residual concentration of 1.0 mg/l after 30 minutes contact. The plots are normalised to the same scales, and have been separated to avoid confusing overlap. Monochloramine is clearly much less effective than free chlorine for inactivating both phages and polioviruses, and relatively little inactivation is apparent over the 30-minute time scale of the plots. A higher dose of monochloramine, to give a 30-minute
residual concentration of 2 mg/l considerably affected the survival of F-specific phages, but had relatively little effect on the poliovirus. What is clear is that both F-specific coliphages and polioviruses would be expected to survive for considerable periods of time in situations where only combined chlorine residuals were present.

A simple calculation of linear regression lines is perhaps dubious considering the scatter of the points in some plots, but this process will allow rough comparison of the rates of survival of F-specific phages and polioviruses. Table 13 shows mean decimal reduction times calculated from the experiments described in this Section. As noted above, F-specific phages survived the lower free chlorine dose better than polioviruses, but the opposite was true with the higher dose. F-specific phages were markedly more resistant to monochloramine than the polioviruses.

Table 13 - Mean decimal reduction times for F-specific coliphages and polioviruses for various disinfection treatments.

<table>
<thead>
<tr>
<th></th>
<th>Poliovirus</th>
<th>F+ phage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free chlorine - 0.3 mg/l</td>
<td>57 sec</td>
<td>95 sec</td>
</tr>
<tr>
<td>Free chlorine - 1.0 mg/l</td>
<td>35 sec</td>
<td>12 sec</td>
</tr>
<tr>
<td>Monochloramine - 1.0 mg/l</td>
<td>45 min</td>
<td>420 min</td>
</tr>
<tr>
<td>Monochloramine - 2.0 mg/l</td>
<td>40 min</td>
<td>100 min</td>
</tr>
</tbody>
</table>

5.4 LONG-TERM STORAGE EXPERIMENTS

These were carried out to gain information on the basal mortality levels likely to be seen in various types of water. The organisms under test were suspended in river or sea water (salinity 35 g/kg), and stored in the dark at 10 or 20 °C. Sewage was used as the source of thermotolerant coliform organisms (TTC). Samples were taken periodically, and tested using the methods already described. Samples for virological analysis were frozen immediately, and transported in this state to the Severn Trent Coventry Laboratories.
Figures 8 to 11 show the results obtained for TTC, and somatic and F-specific coliphages, while Figures 12 and 13 show the results of similar experiments using polioviruses and bovine rotaviruses, which were carried out separately. In most cases the numbers of organisms declined with time, but there is some scatter and non-linearity. This is to be expected with bacterial indicators such as TTC, which can employ survival strategies in adverse conditions. Also, presumably because of the presence of nutrients from the sewage used as an inoculum, an initial rise in numbers of TTC was seen in the river water experiments.

The scatter of data in some cases means that linear regression analysis will not provide a very precise description of the results, but it will be adequate to give a picture of the relative survival of the different organisms.

Table 14 shows values of $T_{90}$, the time in days for 90% reduction in observable numbers, calculated from regression analysis of all the results obtained. It shows that the survival of TTC organisms is not very much affected by either salinity or temperature. Somatic coliphages consistently survived better than TTC, but there is greater reduction in numbers at the higher temperature. There appears to be no effect of salinity. F-specific coliphages survived better than TTC in river water at 10 °C, but less well under all other conditions tested. Their survival appears to be affected by both increased salinity and higher temperature. Somatic coliphages were consistently more stable than the F-specific types.

Rotaviruses appeared to survive better in sea water than in river water, but the counts in the seawater suspension at the beginning of the storage period were rather low, indicating that some viruses may have been inactivated immediately on contact with the saline water. This result must therefore be viewed with suspicion. Polioviruses survived better in river water than in sea water, proving by far the most stable of the test organisms in non-saline conditions. In general polioviruses survived better, usually very much so, than bacteria or coliphages,
Table 14 – $T_{90}$ values in days, for various test organisms, held in river or sea water at 10 or 20 °C in the dark.

<table>
<thead>
<tr>
<th>Test organism</th>
<th>River 10 °C</th>
<th>River 20 °C</th>
<th>Sea 10 °C</th>
<th>Sea 20 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTC</td>
<td>4.4 - 5.0</td>
<td>5.0</td>
<td>3.8 - 5.7</td>
<td>3.4</td>
</tr>
<tr>
<td>Somatic phages</td>
<td>14 - 19</td>
<td>9.9</td>
<td>10 - 20</td>
<td>8.0</td>
</tr>
<tr>
<td>F-specific phages</td>
<td>6.3 - 9.0</td>
<td>1.2</td>
<td>2.0 - 3.1</td>
<td>0.6</td>
</tr>
<tr>
<td>Polioviruses</td>
<td>NR *</td>
<td>26</td>
<td>69</td>
<td>5.6</td>
</tr>
<tr>
<td>Bovine rotaviruses</td>
<td>6.9</td>
<td>3.7</td>
<td>250</td>
<td>10</td>
</tr>
</tbody>
</table>

* NR: No detectable reduction during the experiment. $T_{90}$ tending towards infinity.

although they were less able than somatic coliphages to withstand the combination of saline water and the higher temperature of 20 °C. Thus under nearly all the conditions tested in these experiments, somatic coliphages would give a misleading picture of enterovirus survival, if used as model viruses. F-specific coliphages, on the basis of these results, would generally be of less use than bacterial indicators such as thermotolerant coliform organisms.

5.5 LIGHT MORTALITY EXPERIMENTS

These were carried out in a similar way to the storage experiments, but the reaction mixtures were placed in open pyrex beakers on a black surface, and exposed to sunlight. A Kipp and Zonen solarimeter, and a digital volt-time integrator were used to measure the solar radiation received, and experiments were continued for periods of 2 to 5 hours.

While the use of glass beakers for such experiments is far from ideal, this straightforward approach does nevertheless allow direct comparison of the rates of apparent mortality of the organisms under test. For a discussion on the effects of the reaction vessel in light mortality experiments, see Irving (1977) and Gameson and Gould (1985).
Figures 14 to 19 show the results of the various experiments. Where different organisms were analysed in the same reaction mixture at the same time, the data have been displayed on a single graph. However, at times this was not possible, and in particular the poliovirus experiments were conducted separately from the others, for reasons of safety and practicality.

A set of experiments was also carried out using bovine rotaviruses. Unfortunately, when analysed, all samples gave completely negative results. Controls run at the same time indicated that the original rotavirus suspension was at fault, and it did not prove possible to repeat the experiments.

Counts of all organisms tested declined with exposure to sunlight, usually in a more or less logarithmic manner, as has normally been observed in similar experiments (Irving 1977, Gameson and Gould 1985). Linear regression analysis of the logarithmically transformed data generally gave a good fit, and the calculated lines are shown in the Figures. There is greatest scatter in the poliovirus results, but nevertheless the general trend is evident, and the correlation coefficients for the regression lines are all 0.9 or above.

All figures show the incident solar radiation on the x-axis. All experiments were carried out in continuous bright sunshine, and to give an idea of the equivalent time scale, the radiation received was of the order of 2 to 2.5 MJ/sq m per hour.

The reciprocals of the slopes of the regression lines calculated from the logarithmically transformed data give values of $S_{90}$, the solar radiation required for 90% reduction in detected numbers. The range of values obtained for the various organisms is shown in Table 15. These figures are not corrected for endogenous mortality not caused by light, as concurrent dark mortality experiments were not carried out. However, results in Section 5.4 indicate that these corrections would be insignificant.
Table 15 – Values of $S_{90}$ recorded in light mortality experiments. All values in MJ/sq m.

<table>
<thead>
<tr>
<th>Test organism</th>
<th>River water</th>
<th>Sea water</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTC</td>
<td>1.5</td>
<td>1.4 – 1.5</td>
</tr>
<tr>
<td>Somatic coliphages</td>
<td>7.6</td>
<td>9.4 – 19.7</td>
</tr>
<tr>
<td>F+ coliphages</td>
<td>2.1</td>
<td>6.7 – 9.1</td>
</tr>
<tr>
<td>Poliovirus</td>
<td>4.5 – 5.6</td>
<td>3.3 – 4.3</td>
</tr>
</tbody>
</table>

Overall thermotolerant coliform organisms are seen to be more susceptible to inactivation than any of the viral types. In almost all cases the coliphages were more resistant to inactivation than the poliovirus, with the single exception of the F-specific coliphages in river water, whose numbers were reduced unexpectedly quickly. In all cases the somatic coliphages survived better than the F-specific types. In general the poliovirus survived better in river water than in sea water, whereas the reverse was true of the coliphages. Salinity did not seem to have any effect on the survival of E. coli.

While the results of such experiments cannot be taken as absolute values of mortality or inactivation, they can nevertheless indicate comparative survival. On the basis of these tests, use of coliphages, especially somatic types, would be expected to give a conservative estimate of enterovirus inactivation.

SECTION 6 – CONCLUSIONS

6.1 ENUMERATION OF F-SPECIFIC COLIPHAGES

The RIVM method which has been used for enumeration of F-specific coliphages appears to be effective. However, it has not proved completely reliable, owing to problems with the host organism, S. typhimurium WG 49. This engineered host organism is genetically
unstable, and requires careful handling. Experience suggests that some laboratory workers will have more success with the method than others.

6.2 CONCENTRATION OF BACTERIOPHAGES FROM LARGE VOLUMES OF WATER

The preferred method involves adjusting the sample to pH 6, adsorbing phages to Virozorb 1-MDS electropositive filters, and eluting them with 3% skimmed milk, pH 8.5, warmed to 37 °C. This method is effective, but adds a time-consuming step to the analysis. Reasonably efficient recovery has been obtained, but there has been considerable variability in the results. It is expected that this technique would be more effective with some types of water than with others, and in particular it cannot be used with saline waters.

6.3 PHAGES AND OTHER ORGANISMS IN ENVIRONMENTAL SAMPLES

In the first trial carried out at a sewage works, coliphages appeared to respond in a similar way to enteroviruses to the treatment processes studied, while the response of total and thermotolerant coliform bacteria was quite different. In the second trial, there appeared to be less removal of coliphages than bacterial indicators, but no useful virological results were obtained.

Coliphages were found in river water samples when enteroviruses were not, although they were less numerous than bacterial indicators. No samples were examined which did not contain coliphages, so it cannot be deduced whether they would be more sensitive indicators than bacteria.

Survey of a water treatment works suggested that enteroviruses, though present in only low numbers in the raw water, were not removed by the treatment processes to the same extent as bacteria, in percentage terms. Phages seemed to be removed to a greater extent than viruses, and were not detected in samples of slow sand filter effluent, when bacterial indicators and enteroviruses were found.
Use of coliphages as pollution indicators has been advocated in the past, and the test is included as a tentative method in APHA Standard Methods. In tests on recreational waters, somatic coliphages were found to correlate positively with thermotolerant coliform organisms and faecal streptococci, but correlation coefficients were not high (0.8 and 0.62 respectively). F-specific coliphages were below the limit of detection in all but one sample tested.

Coliphages would be no better than coliform bacteria as pollution indicators in situations where there is a long delay between sampling and analysis.

6.4 STUDIES ON SURVIVAL OF COLIPHAGES AND VIRUSES

F-specific coliphages survived better than polioviruses when exposed to free chlorine sufficient to give a residual concentration of 0.3 mg/l after 30 minutes. However, the opposite was true with a 1 mg/l residual. F-specific phages were very much more resistant to combined chlorine (pre-formed monochloramine) than were polioviruses.

The endogenous rate of inactivation of viruses in the dark was generally much less than for phages, although the rotavirus tested did not survive well in river water. Somatic coliphages were much more stable than F-specific ones, and their survival was affected by increased temperature, but not salinity. F-specific phages were sensitive to both higher temperature and salinity, and their survival in sea water at 20 °C was very poor, even compared with that of thermotolerant coliform bacteria.

Coliphages were generally more resistant than polioviruses to inactivation by sunlight, with somatic coliphages being consistently more resistant than F-specific types. Polioviruses survived exposure to sunlight better in fresh water, while coliphages survived better in saline water.
Bacteriophages may therefore be useful as model viruses in certain situations, but not in all. In particular, the comparative lack of stability of F-specific coliphages with increasing temperature, means that in many situations they would not persist in the environment for as long as mammalian viruses such as polioviruses.

6.5 VIROLOGICAL ANALYSIS

It has become apparent during the course of this work that some methods of enumerating mammalian viruses in environmental samples are not as effective as might have been wished. This complicates the assessment of surrogate viral indicators, as the viruses themselves cannot be enumerated with great reliability. Rather than concentrating on an indicator organism whose enumeration method itself is not completely reliable, it may be more useful to direct research effort into improving techniques in environmental virology.

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REFERENCES


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Figure 1. Relationship between thermotolerant coliform organisms (TTC) and faecal streptococci in environmental samples. Correlation coefficient $r = 0.87$.

Figure 2. Relationship between thermotolerant coliform organisms (TTC) and somatic coliphages in environmental samples. Correlation coefficient $r = 0.80$. 

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Figure 3. Relationship between faecal streptococci and somatic coliphages in environmental samples. Correlation coefficient $r = 0.62$.

Figure 4. Survival of indicator organisms under simulated transit conditions. Key: R = refrigerated, NR = not refrigerated, TC = total coliform organisms, FS = faecal streptococci.
Figure 5. Disinfection using free chlorine dosed to give a residual concentration of 0.3 mg/l after 30 minutes contact. Upper plot: F-specific coliphages. Lower plot: Poliovirus.
Figure 6. Disinfection using free chlorine dosed to give a residual concentration of 1.0 mg/l after 30 minutes contact. Upper plot: F-specific coliphages. Lower plot: Poliovirus.
Figure 7. Disinfection using pre-formed monochloramine dosed to give a residual concentration of 1.0 mg/l after 30 minutes contact. Upper plots: F-specific coliphages. Lower plots: Poliovirus.
Figure 8. Long term survival of coliphages and thermotolerant coliform organisms (TTC) in river water at 10°C.
Figure 9. Long term survival of coliphages and thermotolerant coliform organisms (TTC) in river water at 20°C.
Figure 10. Long term survival of coliphages and thermotolerant coliform organisms (TTC) in sea water at 10°C.
Figure 11. Long term survival of coliphages and thermotolerant coliform organisms (TTC) in sea water at 20°C.
Figure 12. Long term survival of polioviruses (upper plot) and rotaviruses (lower plot) in river water at 10°C and 20°C.
Figure 13. Long term survival of polioviruses (upper plot) and rotaviruses (lower plot) in sea water at 10°C and 20°C.
Figure 14. Inactivation of coliphages and thermotolerant coliform organisms (TTC) in sea water by solar radiation.

Figure 15. Inactivation of coliphages and thermotolerant coliform organisms (TTC) in sea water by solar radiation.
Figure 16. Inactivation of coliphages in river water by solar radiation.

Figure 17. Inactivation of thermotolerant coliform organisms in river water by solar radiation.
Figure 18. Inactivation of polioviruses in river and sea water, by solar radiation.

Figure 19. Inactivation of polioviruses in river and sea water, by solar radiation.