FINAL REPORT TO THE
WATER RESEARCH COMMISSION
ON THE RESEARCH PROJECT

EVALUATION OF THE APPLICATION OF BACTERIOPHAGES AS INDICATORS OF WATER QUALITY

BY

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GLOSSARY

Bacteriophages (Phages)

Viruses which infect bacteria. These host bacteria support the replication of the phages. Phages can only infect specific host bacteria. The specificity is determined by phage receptor sites on certain surface parts of the host bacteria. In terms of size, morphology, structure, composition and mode of replication, phages resemble human viruses. As in the case of human viruses, nucleic acid content of different phages is solely single- or double-strand DNA or RNA. Many phages are detectable by simple and inexpensive techniques which yield results in less than 24 h.

Coliphages

Phages which infect *Escherichia coli* and certain closely related bacteria.

Somatic coliphages

The receptor site for these coliphages is at all times located on the cell wall of host bacteria. The hosts are, therefore, susceptible to infection at any time and under a wide range of conditions. For instance, infection can take place in natural water environments, and since some of the hosts can grow and metabolise in these environments, they can also support the replication of somatic coliphages in these environments. Somatic coliphages are, therefore, not specific for faecal pollution and their numbers can increase in environments such as wastewater, sand filters and activated carbon filters. Somatic coliphages include a wide variety of DNA and RNA phages which differ in morphology and structure. Since their hosts are *E coli* and closely related bacteria, somatic coliphages typically occur in large numbers in wastewater which contains human and animal excreta. Their numbers are often similar to those of faecal bacteria. Somatic coliphages are detectable by the simplest and least expensive of phage techniques. Somatic coliphages do, therefore, give an indication of sewage pollution, and their incidence and behaviour in water environments more closely resembles that of human viruses than faecal bacteria such as coliforms, enterococci and clostridia.

Male-specific coliphages

The receptor site for these phages is located on fertility (F) fimbriae of *E coli*. F fimbriae are short tube-like protrusions produced by certain bacteria for the transfer of nucleic acid (RNA or DNA) to other bacteria of the same or closely related species. It is, therefore, literally a sexual process and the fimbriae are also referred to as sex fimbriae. F fimbriae are produced only at

optimal growth conditions including temperatures of about 32-40°C. This implies that male-specific coliphages cannot multiply in natural water environments, and they are highly specific indicators of faecal pollution. They generally occur in sewage polluted water environments in numbers much lower than those of somatic coliphages. The genes which code for the production of F fimbriae are known as the fertility (F) factor. These genes have by genetic engineering techniques been incorporated in the nucleic acid (DNA) of a *Salmonella typhimurium* bacterium with the result that this bacterium now produces *E coli* F fimbriae and is susceptible to male-specific coliphages. Since *S typhimurium* bacteria are rare in most water environments, this host can be used for the selective detection of male-specific coliphages without interference by other coliphages. The *S typhimurium* host commonly used for this purpose is know as the WG49 or Havelaar host. Dr Arie H Havelaar has established the technology and expertise concerned. The technology for detecting male-specific phages is not as simple as for somatic coliphages.

F-RNA coliphages

Restricted group of male-specific coliphages. They closely resemble human enteroviruses with regard to features such as size, morphology, structure, nucleic acid consisting solely of single-stranded RNA, and failure to replicate in water environments. F-RNA coliphages are, therefore, valuable models or indicators for human enteric viruses in water environments. The receptor sites for F-RNA phages are located along the shaft of F fimbriae.

F-DNA coliphages

Restricted group of male-specific coliphages clearly distinguishable from F-RNA phages. They are typically filamentous phages which contain double-stranded DNA, and their receptor site is located at the tip of F fimbriae.

***Bacteroides fragilis* HSP40 phages**

Bacteroides fragilis bacteria are members of the normal flora of the gut of man and warm-blooded animals. Prof Juan Jofre and co-workers have found that phages which specifically infect *B fragilis* strain HSP40 are excreted only by humans and no animals. Reasons are not yet clear, but it may largely be due to this specific strain being highly specific for the human intestinal tract. Various bacteria are known to be specific for humans or animals. These phages can, therefore, be used to distinguish between faecal pollution of human and animal origin. The numbers of these phages in water environments are generally lower than those of male-specific phages, primarily

because they are excreted in relatively low numbers by only about 15% of humans. Since the host bacteria are strict anaerobes and plaque assays exceptionally vulnerable to interference by contaminant bacteria, the detection and enumeration of these phages is relatively complicated.

Plaques

Circular clearance zones in a lawn of host bacteria on petri dishes. Obtained by mixing a culture of host bacteria with phages (as in a test sample) in sloppy agar which is poured into a petri dish (usually on top of an agar layer rich in host nutrients) and incubated. Each phage will infect a host bacterium. The host bacterium replicates the phage and releases large numbers of the phage into the environment. These phages infect neighbouring host bacteria and the process is repeated with new phages spreading outward from the original point of infection. The process carries on as long as the host bacteria are in the stage of active growth and metabolism. When this stage comes to an end due to depletion of nutrients and the maximum density of bacteria has been reached, the host bacteria can no longer support the replication of phages and the plaques do not increase in size any further. The size and appearance of plaques produced by various phages differs and is distinctive to some extent. Phages such as somatic coliphages completely destroy their hosts and produce easily visible clear plaques. Male-specific phages produce faint plaques which are not always easily visible largely because many host bacteria in the lawn do not have F fimbriae and are not susceptible to the phages. Obtaining visible plaques does, therefore, require carefully controlled experimental conditions in which optimum numbers of host bacteria contain F fimbriae. The size of plaques produced by different phages may differ largely due to differences in the rate of phage replication and spread through the agar medium. In principle each phage in a test sample produces one plaque, which implies that the number of plaques gives an indication of the number of phages in the test sample. However, phages tend to clump together and such a clump will produce only one plaque.

Rnase

Enzyme which specifically degrades RNA nucleic acid. RNA phages are, therefore, inactivated by the enzyme but not DNA phages. The enzyme is, therefore, being used to distinguish between RNA and DNA phages, such as F-RNA and F-DNA male-specific coliphages.

Probe

Short piece of single-stranded RNA or DNA with nucleotide sequence homologous to that of a specific part of the nucleic acid of a phage. These probes can, therefore, hybridise with the nucleic acid of phages of choice. Hybridization of probes with phage nucleic acid immobilised on for instance membranes, can be detected by using probes labelled with radio-active markers or enzymes. This technique is used for the sensitive and highly specific detection of phages.

EXECUTIVE SUMMARY

Key concepts

Water quality; health aspects of water; safety of water for human consumption; efficiency of water treatment processes; cost effective assessment of water quality; guidelines for practical routine monitoring of water quality; reliable indicators for human viruses; waterborne diseases; distinguish between faecal pollution of human and animal origin; training of manpower; technology transfer.

OBJECTIVES :

The main objective was to evaluate and optimise the application of phages as indicators of water quality in practice. This objective was based on information which suggests that phages have attractive features for application as cost effective and practical indicators of water quality, particularly since they closely resemble human viruses in a number of relevant respects.

The intention was to accomplish the objective by investigating the behaviour of selected phages and groups of phages relative to that of other indicators and human viruses in water environments and water treatment processes. The results were used to identify and solve shortcomings in current procedures for using phages as indicators. Special attention was given to the numbers of phages in waste water and polluted water sources, and their survival in these environments. Available information left no doubt that the meaningful application of phages as indicators, and research on their incidence and behaviour in water environments, would require the development of more sensitive techniques for the qualitative detection and enumeration of phages.

The results were due to be applicable in practice for the following purposes:

- Assessment of water quality, particularly with regard to viruses
- Evaluation of the efficiency of water treatment and disinfection processes
- Formulation of water quality guidelines
- Design of cost-effective procedures for routine monitoring of the safety of drinking water

The project was intended to contribute to the training and education of manpower required for quality monitoring in the water industry and related health disciplines, and to the transfer of technology and expertise to relevant laboratories and authorities.

MOTIVATION :

The project logically followed on achievements accomplished in the 1990-92 WRC Project, 321/1/93, "Research on the Use of Bacteriophages as Indicator Organisms" by W O K Grabow and co-workers which may be summarised as:

* Establishment of technology, expertise, facilities and materials for research on the application of various phages and groups of phages as indicators of water quality.
* First application in South Africa of *Bacteroides fragilis* phages as indicators.
* Evidence that *B fragilis* HSP40 phages occur exclusively in human stools, which implies that a reliable indicator for distinction between human and animal faecal pollution may be available.
* Evidence that numbers of certain phages and groups of phages relative to those of other indicators and viruses in a variety of water environments meet the requirements of reliable, rapid, practical and economic indicators of water quality.
* Technology and materials for the application of phages as indicators has been transferred to a number of laboratories.
* Quality guidelines for acceptable levels of phages in drinking water supplies and water intended for recreation and other purposes have been proposed.
* A substantial contribution has been made to the training of manpower essential for quality monitoring in the water industry, as well as related health disciplines.

The objective of this project was to build on the above foundation in terms of additional support for the value of phages as water quality indicators, and to optimise technology for the application of phages as quality indicators in practice.

The merit of research along these lines was supported by:

* The Department of Water Affairs and Forestry has appointed a Task Force of experts to formulate guidelines for the quality of water intended for various purposes. This Task Force has included phages in guidelines for the quality of raw and treated drinking water supplies, as well as water used for recreational purposes. The Task Force identified further research on the application of phages as water quality indicators as priority.
* At international level interest in phage indicators is escalating. For instance, the International Organization for Standardization (ISO) is in the event of standardising techniques for world wide use of phages as indicators for a variety of purposes (ISO Committee Draft, 1991). The

Commission on Water Quality of the European Union is considering the inclusion of phages in quality specifications for drinking water and recreational waters. Growing world-wide interest in phage indicators has been outlined in more detail (IAWPRC Study Group on Health Related Water Microbiology, 1991).

TECHNICAL DETAILS OF PROJECT :

The agreement specified that the research would primarily be carried out in the laboratories of the Department of Medical Virology, University of Pretoria. This laboratory has the necessary facilities, expertise and materials for the study. The required infrastructure and arrangements for obtaining test samples from sites in and around Pretoria, as well as sites at Vereeniging, Bloemfontein, Pietermaritzburg, Cape Town and Windhoek, were in place.

The agreement furthermore specified that research would primarily focus on the following:

* Somatic coliphages, male-specific coliphages, and *B fragilis* HSP40 phages.
* Quantitative recovery of phages using adsorption-elution methods.
* Direct plaque assays on large volumes of water using single agar layers in large petri dishes.
* Qualitative presence-absence (P-A) tests on large volumes of water.
* Application of the new techniques in research on the incidence and behaviour of phages in selected water environments.
* Statistical evaluation of results was carried out according to procedures established with the Division on Biostatistics of the Medical Research Council.
* The study was carried out in close collaboration with international bodies such as ISO in order to ensure that technology and expertise are in line with the latest in the world.

RESEARCH OUTPUTS :

Details on some research outputs of the project have been recorded in the following publications, conference papers and reports:

Publications

Grabow W O K (1993a) Pathogenic organisms and indicators of faecal pollution. Proceedings: International Seminar on the Disinfection of Water and Wastewater in Developing Countries, Bello Horizonte, Brazil, 26-28 April. S471a Anais: Associacao Brasileira de Engenharia Sanitaria e Ambiental da Escola de Engenharia da UFMG, Bello Horizonte, Brasil. pp 17-31.

Grabow W O K (1993b) In: Specifications for enteric viruses and faecal coliforms in: South African Water Quality Guidelines, Volume 1 (Domestic Use) and Volume 2 (Recreational Use), Department of Water Affairs and Forestry, Pretoria.

Grabow W O K (1994a) Evaluation of the efficiency of "Stericlean". Hands-On (Newsletter of the Dental and Dental Technology Forum) 6/2, 7-9.

Grabow W O K (1994b) Health protection by disinfection of treated wastewater. In: Documentation Proceedings, International Conference on Integrated Wastewater Management - Collection, Treatment and Reuse, 203-214. European Water Pollution Control Association, Gesellschaft zur Förderung der Abwassertechnik, Hennef. 382 pp.

Grabow W O K, Wyn-Jones A P, Schildhauer C and Jofre J (1995a) Efficiency of the *Euroguard* domestic water treatment unit with regard to viruses, phages and bacteria. Water SA 21, 71-74.

Grabow W O K, Neubrech T E, Holtzhausen C S and Jofre J (1995b) *Bacteroides fragilis* and *Escherichia coli* bacteriophages: excretion by humans and animals. Water Science and Technology 31, 223-230.

Jagals P, Grabow W O K and De Villiers J C (1995) Evaluation of indicators for assessment of human and animal faecal pollution of surface run-off. Water Science and Technology 31, 235-241.

Grabow W O K (1995a) Progress in research on human viruses in water. In: Proceedings of the Fourth International Symposium on Contamination of the Environment by Viruses and Methods of Control held in Vienna on 2-4 September 1993, eds W Biffl and R Walter, University of Agriculture, Forestry and Renewable Resources, Vienna, Austria. Wiener Mitteilungen 128/2, 1-14.

Grabow W O K (1995b) Member of Working Group for ISO Standard: Water quality - Detection and enumeration of bacteriophages, Part 1: Enumeration of F-specific RNA bacteriophages. ISO 10705-1:1995. International Organization for Standardization, Geneva.

Grabow W O K (1995c) Member of Working Group for ISO Standard: Water quality - Detection and enumeration of bacteriophages, Part 2: Enumeration of somatic coliphages. ISO/CD 10705-2:1995. International Organization for Standardization, Geneva.

Grabow W O K (1995d) Member of Working Group for ISO Standard: Water quality - Detection and enumeration of bacteriophages, Part 3: Concentration of bacteriophages from water. International Organization for Standardization, Geneva.

Grabow W O K (1996a) Waterborne diseases: Update on water quality assessment and control. Water SA 22, 193-202.

Jagals P, Grabow W O K and Williams E (1996) The effects of supplied water quality on human health in an urban development with limited basic subsistence facilities. Water SA (in press).

Jagals P and Grabow W O K (1996) An evaluation of sorbitol-fermenting bifidobacteria as specific indicators of human faecal pollution of environmental water. Water SA 22, 235-238.

Grabow W O K, De Villiers J C, Erasmus B, Erasmus D, Engelbrecht L (1996a) Viruses in wastewater from an informal settlement. Proceedings of the Biennial Conference of the Water Institute of Southern Africa, 20-23 May. Water Institute of Southern Africa, Johannesburg.

Grabow W O K (1996b) New challenges in monitoring water for pathogens. Water, Sewage & Effluent 16, 51-54.

Grabow W O K (1996c) In: South African Water Quality Guidelines, Second Edition. Department of Water Affairs and Forestry, Pretoria. Volume 1: Domestic Use. Volume 2 : Recreational Use.

Grabow W O K (1996d) In: South African Water Quality Guidelines. Department of Water Affairs and Forestry, Pretoria. Volume 6: Coastal and Marine Environment.

Reports

Grabow W O K, Holtzhausen C S and de Villiers C J (1993c) Research on Bacteriophages as Indicators of Water Quality. WRC Report No 321/1/93. Water Research Commission, Pretoria. 147 pp.

Grabow W O K (1994c) Rand Water: Virological Quality of Water, April 1993 to March 1994. Department of Medical Virology, University of Pretoria.

Grabow W O K (1995e) Rand Water: Virological Quality of Water, April 1994 to March 1995. Department of Medical Virology, University of Pretoria.

Grabow W O K, Van der Veen A and De Villiers, J C (1995e) Marine Pollution: Pathogenic micro-organisms in shellfish. Report on Joint Venture Research Project: Foundation for Research Development and Water Research Commission, Pretoria. 179 pp.

Grabow W O K (1996e) Rand Water: Virological Quality of Water, April 1995 to March 1996. Department of Medical Virology, University of Pretoria.

Conference papers

Van der Veen A and Grabow W O K (1993) Accumulation of human viruses by oysters. Oral paper: Southern African Marine Science Symposium on Marine Science for a Sustainable Future, Club Mykonos, Saldanha Bay, 17-22 October.

Grabow W O K (1994d) Health protection by waste water treatment. Invited oral paper: Seminar on Waste Water Treatment, NCP Chlor Alkali, Johannesburg, 11 May.

Grabow W O K, Neubrech T E, Holtzhausen C S and Jofre J (1994d) *Bacteroides fragilis* and *Escherichia coli* bacteriophages: excretion by humans and animals. Oral paper: International Symposium on Health-Related Water Microbiology, Budapest, Hungary, 25-30 July.

Jagals P, Grabow W O K and De Villiers J C (1994) Evaluation of indicators for assessment of human and animal faecal pollution of surface run-off. Oral paper: International Symposium on Health-Related Water Microbiology, Budapest, Hungary, 25-30 July.

Grabow W O K und Botzenhart K (1994) Trends in der virologischen Wasserüberwachung. Vortrag: 46. Kongress der Deutschen Gesellschaft für Hygiene und Mikrobiologie, Kiel, 26. - 29. September. Zusammenfassung: Christiansen B, Gundermann K-O, Höller C, Rautenberg P, Schubert S, Ullmann U (Herausgeber)(1994) Fortschritte in der Hygiene und Mikrobiologie. Ergebnisse des 46. Kongresses der Deutschen Gesellschaft für Hygiene und Mikrobiologie in Kiel 1994, Seite 262. Pechstein Verlag, Dobersdorf. pp 335.

Grabow W O K (1994e) Health protection by disinfection of treated wastewater. Invited oral paper: International Conference on Integrated Wastewater Management - Collection, Treatment and Reuse. Conference Centre, National Laboratory of Civil Engineering, Lisbon, Portugal, 10-12 October.

Grabow W O K (1994f) WHO Guidelines for drinking-water quality: microbiological aspects. Invited oral paper: Joint WHO/UNEP/USEPA Regional Seminar on Drinking-Water Quality, Nairobi, Kenya, 28 November - 1 December 1994.

Grabow W O K, De Villiers J C, Erasmus B, Erasmus D and Engelbrecht L (1995f) Isolation and typing of cytopathogenic viruses in wastewater effluents from an informal settlement. Oral paper: International Congress on the Impact of Viral Infections in the Developing World, Johannesburg, South Africa, 9-14 July (see Congress Book of Abstracts).

Grabow W O K (1996g) Control of waterborne viral diseases. Invited oral paper: International Congress on Waterborne Pathogens, jointly organised by the German Association for Hygiene and Microbiology, and the World Health Organization, Bonn, Germany, 22-24 May (Proceedings in press).

Grabow WOK (1996h) Why monitor viruses in water and food. Invited paper: Symposium on Ecotoxicology, City University, Hong Kong, 4 July.

SUMMARY OF ACHIEVEMENTS

Methods for the quantitative enumeration of phages

Somatic coliphages

1. The double agar layer plaque technique specified in ISO (1995b) has been evaluated. This technique is intended for testing volumes of water up to about 10 ml. No evidence for improvement on the principles outlined in this technique has been found. There is no indication that a better host strain may be available. However, the project team believed that the ISO technique can be substantially simplified to render it more practical and user-friendly, and less expensive and labour intensive. These suggestions have been submitted to the ISO Working Group (WG) concerned. Some of the modifications have already been accepted, and others are under investigation. For instance, the proposal of accepting standard grade chemicals, which are much less expensive than analytical grade chemicals, provided they yield similar results, has now been accepted as general ISO policy. The proposal of the project to simplify the complicated, labour intensive and expensive procedure for the preparation of host inocula is being investigated by the ISO WG. The final report contains details on the technique recommended by the project team.

2. Possibilities for reducing the time required for the detection of phages have been investigated. Results revealed that using the above standard technique some plaques were visible after about 8 h incubation, but maximum plaques were visible only after some 20 h.

3. The value of 2,3,5-triphenyl-tetrazolium hydrochloride (tetrazolium) in plaque assays has been investigated. The results indicated that tetrazolium may enhance the early visibility of plaques. However, tetrazolium failed to reduce the time required for plaque assays to any meaningful extent.

4. Two rapid tests for the detection of phages described in the literature have been evaluated. Both techniques were rejected as impractical and failing to reduce the time required by the above plaque assay to meaningful extent. The results indicated that the development of meaningful rapid methods requires more intensive research, possibly using different approaches, which may be based on molecular techniques.

Male-specific coliphages

1. The double agar layer plaque assay specified in ISO (1995a) has been evaluated. This technique is intended for testing volumes of water up to about 10 ml. No evidence for improvement on the principles outlined in this technique has been found. In detailed studies on host strains the Havelaar strain specified in the ISO standard proved superior to the Cabelli strain. There is no indication that a better host strain may be available or readily developed. Unfortunately, however, the results and experience confirm that the time consuming, cumbersome and labour intensive procedure for the preparation of host cultures described in the ISO specification has to be followed closely. In the case of somatic coliphages, the ISO technique can be substantially simplified. The modified technique recommended is described in detail in the final report.

2. Research on rapid techniques and the use of tetrazolium yielded results similar to those for somatic coliphages.

3. Techniques for the confirmation of male-specific coliphages have been established. These are based on electron microscopy and RNase tests. Unfortunately the former requires sophisticated facilities and expertise, and the latter proved not easy to perform.

4. Typing of male-specific coliphages by gene probe hybridisation proved feasible. However, optimisation of technical details of the concept, assessment of the application of phage types for distinguishing between animal and faecal pollution, and application of the concept in practice, proved to require research beyond the objectives of this project.

***Bacteroides fragilis* HSP40 phages**

1. The double agar layer plaque assay described in the preceding WRC project has been evaluated. This technique is intended for testing volumes of water up to about 10 ml. Apart from minor modifications to ingredients of growth media, no evidence for improvement on the principles of the technique has been found. For instance, there is no indication of a host strain superior to HSP40 for the detection of phages excreted exclusively by humans.

2. As in the case of coliphages, tetrazolium proved to have certain benefits, and efforts to reduce the time required for the test were not successful.

Decontamination of water samples

Decontamination of water samples by means of chloroform treatment proved to slightly reduce counts of somatic coliphages and *B fragilis* HSP40 phages, and slightly increase counts of male-specific coliphages. Reasons for these observations, as well as alternative methods for decontamination such a membrane filtration, were not investigated in further detail because problems with contamination were successfully solved using aseptic working conditions and antibiotics for suppressing growth of contaminants.

Methods for the quantitative recovery of small numbers of phages from water

1. Amicon stirred cell units using membranes with molecular cut-off levels of 50 000 yielded an efficiency of recovery (EOR) in excess of 90% for all groups of phages in drinking water and environmental waters with limited pollution. The technique proved feasible for volumes of drinking water up to 1000 litres. Disadvantages included rapid clogging by turbid waters, and the cost of membranes.

2. The glass wool adsorption-elution procedure established in the laboratory has an EOR of 60-90 % for human viruses, but on average only about 1 % for a variety of phages tested. The results indicated that compared to human viruses, the adsorption of phages was much less efficient and so was the release of those phages that did adsorb. Efforts to improve the EOR by modifying the electrostatic charge on the glass wool using polyethylene-imine, and by increasing the pH of the elution buffer, made no meaningful difference.

3. The recovery of phages by adsorption-elution using conventional cellulose nitrate membrane filters with diameter 47 mm and pore size 0,45 μm as described by Sobsey, has been investigated in detail. Tests were carried out on 100 ml samples of a variety of environmental waters. Phage counts obtained were compared to those obtained by direct plaque assays. The latter yielded higher counts for waters which contained relatively high numbers of phages, but the membrane filtration procedure yielded positive results more often and higher counts for

waters which contained very low numbers of phages. This relatively inexpensive procedure would, therefore, seem to have merit for the recovery of phages from waters with low counts of phages such as drinking water.

Direct plaque assays on large volumes of water using large petri dishes

The merits of a single agar layer procedure using 140 mm diameter petri dishes described previously have been confirmed for groups of phages concerned. This procedure is intended for the detection of phages in 100 ml samples of water containing low numbers of phages such as drinking water supplies. Modification of the procedure by converting it into a double agar layer procedure substantially increased counts of phages obtained. This was confirmed for all three groups of phages concerned in comparative tests on a variety of water samples. The double agar layer procedure proved superior to the membrane filter adsorption-elution technique in terms of sensitivity and accuracy. Unfortunately, however, the technique is relatively expensive since the total cost of the ten disposable plastic petri dishes used amounts to some R 60. The recommended procedure for the new double agar layer plaque assay is described in detail in the final report.

Qualitative detection of phages in water using a presence-absence (P-A) procedure

These tests are based on adding nutrients and a culture of the appropriate host to a large volume of test water and overnight incubation. Phages present in the water, theoretically as few as a single viable particle, then infect the growing host and multiply. After overnight incubation these phages are present in large numbers which are readily detectable by conventional plaque assays. P-A procedures for all three groups of phages concerned were evaluated in tests on more than 50 samples of river water and more than 600 samples of treated drinking water. The results indicated that 500 ml was the optimum volume for samples of treated drinking water. Results for tests on 1000 ml did not differ significantly from those for 500 ml. The P-A tests proved more sensitive than direct plaque assays using large petri dishes or recovery of phages by membrane filter adsorption-elution. P-A tests are also relatively inexpensive and simple. However, since a single viable phage particle yields a positive result, the procedure is extremely sensitive to contamination, and the tests have to be conducted under absolutely aseptic conditions. P-A tests proved the method of choice for routine monitoring of the quality of treated drinking water supplies. The tests are suitable for monitoring the compliance of water supplies to a quality limit

based on the absence of phages from a particular volume of water. Details on numbers of phages present would require supplementary direct plaque assays. Recommended procedures for P-A tests are described in the final report.

Incidence of selected indicator phages in human and animal excreta

In an analysis of some 200 stool specimens from humans and a variety of domestic and wild animals, *B fragilis* HSP40 phages were detected only in 13 % of human stool specimens. Somatic and male-specific coliphages were excreted by considerably higher percentages of both humans and a variety of animals. These findings confirmed earlier observations that *B fragilis* HSP40 phages are excreted exclusively by humans and can be used to distinguish between faecal pollution of human and animal origin.

Incidence of phages, bacterial indicators and human viruses in waste and river water

Comparative analyses have been carried out on the following waters:

1. A total of 209 samples collected over a period of one year from a stream receiving diffuse effluents from an informal settlement in Mamelodi has been analysed. In the great majority of samples phages outnumbered human viruses. However, in small but meaningful number of samples human viruses were detected by conventional cell culture techniques in the absence of any of the groups of phages concerned. These results suggested that generally the absence of phages is a reliable indication of the absence of human viruses, but in exceptional cases phages may fail to reveal the presence of human viruses.

2. Basically similar results were obtained in studies on 150 samples of Klip River water and more than 50 samples of Vaal Dam sluice gate water. In this case, however, viruses were detected only in five samples which yielded negative results for *B fragilis* HSP40 phages in P-A tests on 500 ml volumes. Somatic and male-specific coliphages consistently outnumbered human viruses.

3. The above findings on natural water sources were confirmed in studies on waste water effluents from informal settlements at Botshabelo. The results highlighted the value of *B fragilis* HSP40 phages as indicators of human faecal pollution. The phages were detected only

in waste waters known to contain predominantly human wastes, and not in environmental waters known to contain predominantly animal wastes.

Incidence of phages, bacterial indicators and viruses in treated drinking water supplies

More than 600 samples of treated drinking water from various points in the Rand Water treatment system were analysed for human viruses, phages and bacterial indicators of faecal pollution, using the best available techniques. Human viruses were never detected, and phages rarely. The results showed that phages are valuable indicators of the absence of viruses, and of the efficiency of water treatment processes. The results indicated that P-A tests for somatic coliphages on 500 ml samples are practical, inexpensive and valuable components of systems for routine quality monitoring of treated drinking water supplies.

Survival of selected phages, bacterial indicators and viruses in water environments

The survival of phages, human viruses and bacterial indicators in various water environments has been compared. Samples of sea water, river water and buffer were seeded with human and animal stool specimens containing naturally occurring organisms. Reaction mixtures were seeded with laboratory cultures of polio viruses and selected phages which were not present in stool specimens. The survival of these organisms was determined under controlled laboratory conditions and in the open on roof top with or without exposure to sunlight. The results indicated that under most conditions resembling those in natural water environments at least some phages survived longer than laboratory strains of polio virus. Since polio virus is known as a relatively resistant member of enteric viruses, and phages have been shown to generally outnumber human viruses in most water environments, it would appear reasonable to conclude that the absence of phages is under most conditions a reliable indication of the absence of human viruses.

Survival of phages in water treatment processes

In routine analysis of water reclaimed from abattoir effluent by means of multiple-barrier system, viruses were detected only once in the absence of phages. These results indicated that enteric viruses are rarely detected in water treated by the processes concerned.

Practical aspects of phage indicators

Evidence has been presented that phages are detectable by relatively simple and inexpensive techniques. The following techniques which have been described and evaluated in detail for application in practice, proved reliable and sensitive:

* Double agar layer plaque assays using small petri dishes for samples up to 10 ml:
  Quantitative direct plaque assays for water which contains relatively high numbers of phages
* Double agar layer plaque assays using large petri dishes for samples up to 100 ml:
  Quantitative direct plaque assays for water which contains relatively small numbers of phages
* Presence-Absence (P-A) test for samples up to 500 ml or more:
  P-A test for water which contains very low numbers of phages

These techniques proved suitable for the quantitative enumeration of phages in waste water, river water, sea water and dam water, as well as the sensitive , routine qualitative monitoring of treated drinking water supplies.

International collaboration on phage technology and water quality assessment

Work on this project has been carried out in close collaboration with the International Organization for Standardization (ISO), and leaders in the field in a number of laboratories abroad. Techniques recommended are based on those standardised by ISO, with modifications for improvement according to results obtained in this study.

Recommended water quality guidelines

Data obtained in this study were used to formulate recommendations for drinking water (absence of phages from 500 ml samples) and environmental waters used for direct contact recreation (less than 10 phages per 10 ml). Recommended test methods for these guideline levels, application of the tests and the interpretation of results are discussed in the report.

Recommended procedures for routine water quality monitoring

Techniques recommended for the routine enumeration of all three groups of phages and monitoring of water supplies have been described in detail in the report.

Technology transfer and capacity building

Details on technology and expertise developed in this project have been forwarded to a number of laboratories for application in practice. These laboratories include Rand Water, Umgeni Water, University of the North West at Mmabathu, Johannesburg Municipal Laboratory and the Windhoek Municipal Laboratory. Results obtained in this study have been used by the Department of Water Affairs and Forestry in the formulation of water quality guidelines.

FUTURE RESEARCH NEEDS AND PRIORITIES

The project has revealed attractive possibilities for further development of technology and expertise to utilise phages as indicators in water qualitative assessment and monitoring. Challenges such as the following warrant further investigation:

* Development of practical techniques for the identification of serogroups of male-specific coliphages. Information presently available suggests that genetic hybridisation using appropriate gene probes may prove the most practical approach, but would require evaluation against neutralisation assays using specific antibodies.
* Determination of the specificity of serogroups of male-specific coliphages for excretion by humans and animals. This would include screening stools from humans and a variety of animals for the presence of the five serogroups of male-specific coliphages presently known, and possibly additional serogroups.
* Evaluation of the application in practice of serogroups of male-specific coliphages for distinction between faecal pollution of human and animal origin.
* Improvement of the sensitivity of techniques for the qualitative and quantitative detection of *B fragilis* HSP40 phages.
* Development of rapid techniques for the detection and enumeration of phages. Molecular techniques may offer feasible possibilities for this purpose.
* Development of practical techniques for the detection of specific members of the groups of somatic coliphages, male-specific coliphages and *B fragilis* HSP40 phages which may have indicator features superior to those of the groups of phages presently used.
* Research on shortcomings of phages and approaches to overcome these shortcomings. This would include an investigation of circumstances, such as outbreaks of viral diseases, which may result in situations where phages are outnumbered by human viruses.
* Improvement in practical details of phage technology including detection methods, host strains, the handling of host strains, and the characterisation of phage isolates.
* Correlation of the incidence of phages in water and the incidence of enteric infections in consumers of the water.

SUMMARY AND CONCLUSIONS

1. Practical techniques for the quantitative and qualitative detection of three groups of phages (somatic coliphages, male-specific coliphages and *B fragilis* HSP40 phages) have been optimised and evaluated for application in practice.

2. Practical procedures for routine water quality monitoring have been established and evaluated.

3. Evidence has been presented that phages generally outnumber human viruses in water environments, and that the absence of phages offers a valuable indication of the absence of human viruses as well as the efficiency of water treatment processes. However, since there is no direct correlation between numbers of any phages and the wide variety of human viruses that may be present in water, the absence of phages is no absolute guarantee of the absence of human viruses. Phages would, therefore, best be applied as an important component of an appropriately battery of tests for assessment of water quality.

4. Details have been obtained on the incidence and behaviour of phages in various water environments on which reliable quality guidelines can be based.

5. A contribution has been made to the establishment of technology and expertise which has valuable benefits for the water industry and related health disciplines. The technology and expertise has been transferred to other laboratories, and a contribution has been made to the training of manpower. An infrastructure of collaboration with laboratories locally and abroad has been established.

2. PROJECT RESEARCH

2.1. Introduction

Awareness of the value of phages as indicators of water quality is rapidly gaining ground (IAWPRC Study Group on Health Related Water Microbiology, 1991; Grabow, 1996; Müller, 1997). This is reflected by the formulation of standard phage detection techniques by the International Organization for Standardization (ISO, 1995, 1997a,b,c), and the inclusion of phages in water quality guidelines by bodies such as the World Health Organization (World Health Organization, 1993, 1996), American Public Health Association (Standard Methods, 1995), and the Department of Water Affairs and Forestry (Department of Water Affairs, 1993). The Commission of the European Communities is considering the replacement of specifications for human viruses by guidelines for phages (A H Havelaar, personal communication).

Since this is a new indicator concept, the development of detection techniques for various phages, and the application of the techniques in practice, are still to large extent in an infant stage. This is illustrated by substantial differences in techniques recommended by, for instance, ISO and the American Public Health Association. Many questions about the techniques and their application remain unanswered. This includes the preparation of host cultures, the composition of growth media and procedures for the detection of small numbers of phages in large volumes of water.

The objectives of this study include research on the optimisation of phage detection methods, assessment of the efficiency and reliability of these methods, and evaluation of the application of these methods in practice.

2.2. Methods for the Quantitative Enumeration of Phages

2.2.1. Somatic coliphages

2.2.1.1. Double agar layer plaque assay

A technique based on principles specified in ISO (1995) has been developed and optimised. Details of the technique are recorded in Appendix 1. The technique is less expensive, time consuming and labour intensive than the ISO technique. Although

the techniques have not been compared directly, there is no evidence of meaningful difference in sensitivity or accuracy. The ISO Working Group concerned is presently investigating the possibility of adapting the ISO technique accordingly. At this stage there is no indication of superior techniques or possibilities for improvement of the method. The developed and optimised technique differs from the ISO technique in terms of certain ingredients of growth media, including cations and sources of carbohydrates. Evaluation and optimisation of these differences would involve a substantial amount of work well beyond the scope of this project. Although there is no reason to believe that these variations may cause meaningful differences in results, detailed evaluation and optimisation is fully justified for the elimination of uncertainties, formulation of standardised techniques, simplification of techniques, and possibly also reducing the cost of analyses.

2.2.1.1.1. Time required for plaques to appear and effect of tetrazolium

Parallel plaque assays were carried out using plates with (300 μg/ml in top layer) and without 2,3,5-triphenyl- tetrazolium -hydrochloride (tetrazolium) in the top layer of double agar layer plaque assays as described in Standard Methods (1995). Comparative assays were carried out on dechlorinated tap water seeded with a laboratory culture of the somatic coliphage V1 (double-stranded DNA with short tail), and on samples of river water containing naturally occurring phages. Each assay consisted of three plates per dilution, and each test was repeated at least three times. Plates were incubated at 37°C and plaques were counted at time intervals with least possible exposure of plates to temperatures below 35°C. No effort was made to count plaques between 8 and 20 h because obtaining results in this period is impractical for a normal working day of 8 h.

The results of typical experiments in Table 2.1 indicate that:

a) Maximum plaque counts were obtained only after 20 h incubation, and these were more than twice as high as counts recorded after 8 h.
b) Counts tended to be higher on plates which contained tetrazolium. It would appear that the difference was primarily due to better visibility of plaques under certain circumstances. The reason for this phenomenon is not clear. In subsequent comparative tests on river water containing naturally occurring phages, differences between counts on plates with and without tetrazolium were not meaningful, and

plaques actually tended to be more clearly visible on plates without tetrazolium. The inclusion of tetrazolium in double agar layer plaque assays as previously suggested (Standard Methods, 1995) would, therefore not seem to be justified, particularly in view of the additional cost and labour. Similar results were recorded for phage V1 seeded into dechlorinated tap water and naturally occurring somatic phages in river water.

According to Hurst *et al* (1994) plaque formation by phages belonging to the families *Podoviridae* and *Siphoviridae* (both double-stranded DNA and long non-contractile tails) was inhibited by 300 μg/ml of tetrazolium in top layers. The initial findings fail to confirm these observations.

2.2.1.2. **Rapid detection of somatic coliphages**

Two techniques described for the rapid detection of phages (Armon and Kott, 1993; Ijzerman *et al*, 1994) were evaluated. Both proved complicated, labour intensive and expensive, while the extent to which optimum results were available sooner than conventional plaque assays was not meaningful. In addition, both are qualitative presence/absence (P-A) tests with no meaningful advantages over the P-A tests routinely used in our laboratory. The techniques were, therefore, not studied in further detail and rejected as impractical.

Table 2.1 Time required for detection of plaques and effect of tetrazolium on detection of plaques in double agar layer plaque assays for somatic coliphages

Test samples	Incubation time (h)	Average plaque count	
		Without tetrazolium	With tetrazolium
Suspension of V1 coliphage in PBS buffer	3,5	0	0
	4,0	0,3	2
	4,5	1	3
	5,5	2	5
	6,0	5	10
	6,5	6	11
	7,0	15	30
	7,5	22	49
	8,0	22	51
	20	73	119
	24	73	119
K19 Klip River water	3,5	0	0.3
	4,0	0	0,3
	4,5	0	0,3
	5,0	0,3	1
	5,5	0,3	1
	6,0	0,3	2
	6,5	1	3
	20	3	5
	24	3	5

2.2.2. Male-specific coliphages

The double agar layer plaque assay described in Appendix 1 is based on the standard method defined in ISO (1995). A detailed assessment of the ISO technique unfortunately confirmed that the cumbersome, time consuming and expensive procedure for the preparation of host inoculum cultures was essential. There is no indication of a feasible alternative for obtaining host cells at optimum levels of susceptibility to male-specific coliphages.

2.2.2.1. Selection of the host of choice for male-specific coliphages

Presently available results from research carried out world-wide indicate that the only two host strains worth considering are:

Havelaar host : *Salmonella typhimurium* strain WG49 phage type 3 Nal^r (F'lac: :Tn5) NCTC 12484

Cabelli host : *Escherichia coli* HS(pFamp)R

Further details on these hosts have been recorded elsewhere (ISO, 1995).

After optimisation of procedures for application of the Havelaar host, the Havelaar and Cabelli hosts have been compared in tests on naturally occurring male-specific phages in selected water environments.

In comparative tests on 34 water samples collected from the Klip River at sampling point K19 (Fig 1), the Havelaar host yielded higher average and median counts for male-specific coliphages than the Cabelli host (Table 2.2). The same trend was evident from similar tests on another 12 samples of K19 water, and 12 samples of Vaal Dam sluice gate (A18) water (Tables 2.3 and 2.4). The latter Table shows that the Havelaar host yielded positive results more often than the Cabelli host for water which contained low numbers of phages. At this stage there is no indication of host strains that may prove superior to the Havelaar host for the detection of male-specific coliphages.

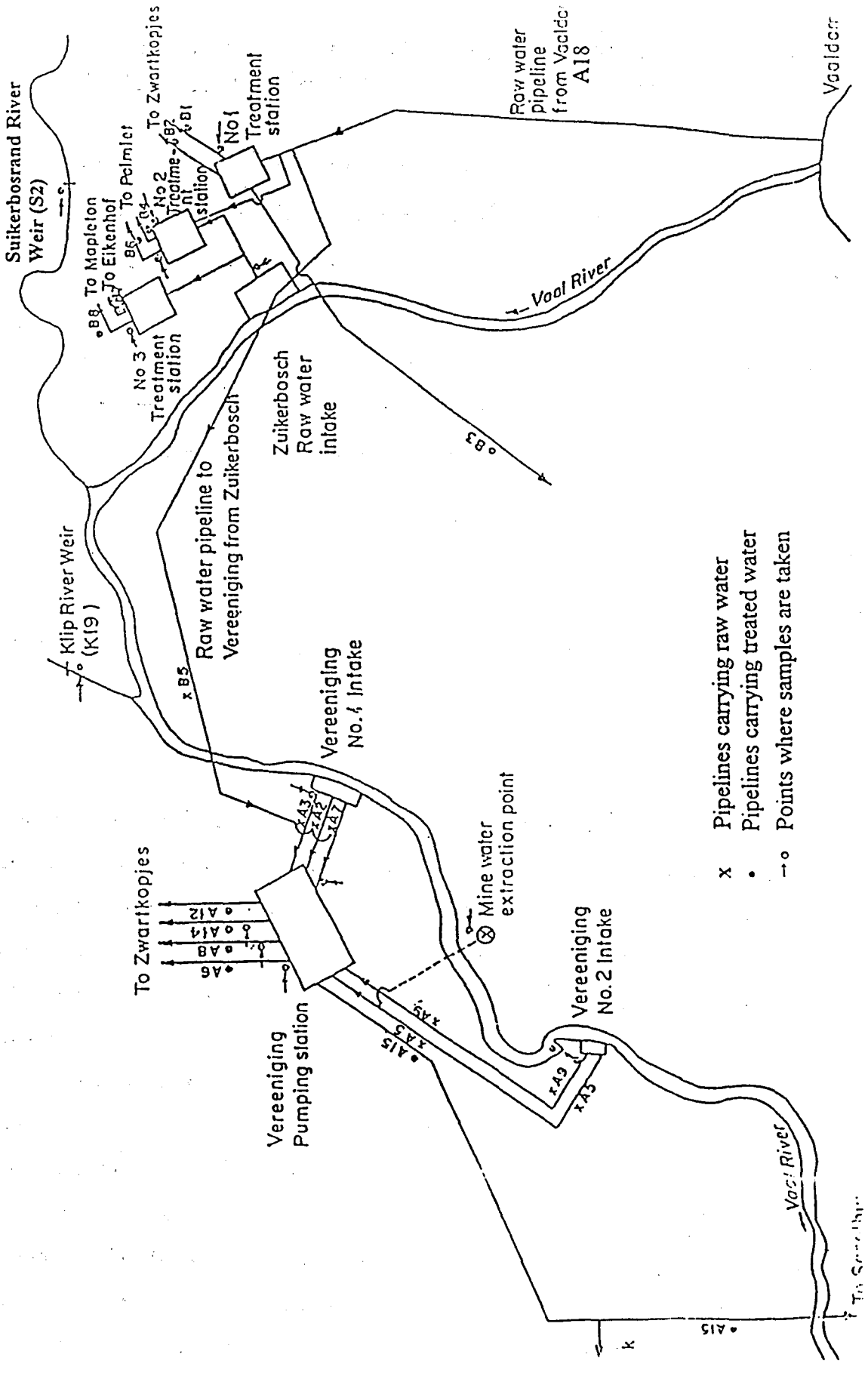

Figure 1. Map showing the location of sampling point K19 on the Klip River which feeds the Vaal River

Table 2.2 Comparison of the Cabelli and Havelaar hosts for the enumeration of male-specific coliphages in Klip River water at sampling point K19 using the direct double agar layer plaque assay

	Plaque forming units per 1 ml	
	Cabelli host	Havelaar host
No of tests	34	34
Range	0-24	0-28
Average	3,76	5,02
Median	1,0	2,0

2.2.2.2. Confirmation of male-specific coliphages

2.2.2.2.1. Electron microscopy

The distinctive morphology of male-specific coliphages was clearly visible by electron microscopic procedures routinely used in the laboratory.

2.2.2.2.2. RNase test

Test procedures based on the selective inactivation of male-specific coliphages by the RNase enzyme have been optimised and successfully established. The test has been used to characterise phages isolated on the Havelaar and Cabelli hosts. Phages from 116 plaques obtained in four tests on K19 river water using the Havelaar host, and the same number of plaques obtained in tests on the same water samples using the Cabelli host, were purified and cultivated for characterisation. Plaques were picked at random from both hosts. All plaques on the Havelaar host were faint plaques typical of male-specific coliphages. Many plaques on the Cabelli host were clear plaques typical of somatic phages. In total 93% of phages on the Havelaar host were RNase sensitive, ie RNA phages. The percentage of RNase sensitive plaques on the Cabelli host was much lower. These findings show that many plaques on the Cabelli host are not male-specific RNA phages, which includes the clear plaques. All RNase sensitive phages isolated from the Cabelli host, produced plaques on the Havelaar host. However, some RNase sensitive phages isolated from the Havelaar host failed to produce plaques on the Cabelli host, which suggests that the Cabelli host may not be susceptible to all male-specific RNA coliphages. RNase sensitive phages were confirmed as male-specific RNA (F-RNA) phages by electron microscopy. Exact

details of results are not presented because the results of RNase tests tended to prove difficult to interpret and were not particularly reliable. The findings were not investigated in further detail because the results left no doubt that the Havelaar host was more specific for F-RNA phages than the Cabelli host. These observations, together with results in Tables 2.2 to 2.4 according to which the Havelaar host yields higher plaque counts than the Cabelli host for phages in a variety of water environments, strongly suggest that the Havelaar host is superior to the Cabelli host for general purposes of the detection of F-RNA phages in water environments.

2.2.2.3. Typing of male-specific coliphages by gene probe hybridisation

Prof Sobsey kindly supplied materials and details on techniques for the typing serogroups of male-specific RNA coliphages by means of hybridisation using digoxigenin labelled oligonucleotide probes as described by Hsu *et al* (1995). Indications are that this procedure is more practical for the confirmation of male-specific RNA coliphages than conventionally used RNase inactivation, electron microscopy and serotyping. This procedure is expected to prove of major benefit in assessment of the value of male-specific RNA coliphages for water quality assessment. A particularly important advantage of the gene probe hybridisation procedure is that it offers a practical method for typing the five serotypes of male-specific RNA coliphages. Indications are that certain serogroups of these phages are specific for faecal pollution of human origin and others for faecal pollution of animal origin (Hsu *et al*, 1995). This implies that typing of male-specific coliphages may prove a useful tool for distinguishing between faecal pollution of human and animal origin. Details on serogroups and probes are summarised in the diagram below. The oligoprobes (base length 25-30) are end-labelled with digoxigenin for colorimetric immuno-enzymatic detection. Optimisation of techniques for the typing of the serogroups of male-specific coliphages requires inputs beyond the scope of this project.

Diagram: 2.1 Details on serogroups of male-specific (F-RNA) (F^+ RNA) coliphages and gene probes for their typing (Hsu *et al*, 1995)

Serogroup I	Serogroup II	Serogroup III	Serogroup IV
Phage MS2	Phage GA	Phage Qß	Phages FI & SP
Animals	Humans	Humans	Animals
Probe 1	Probe II	Probe III	Probe IV
Probe A		Probe B	

2.2.3. *Bacteroides fragilis* HSP40 phages

The double agar layer plaque assay described previously (Grabow *et al*, 1993, 1995) has been re-evaluated, modified and optimised . The latest version is described in Appendix 1. The P-A test procedure for the phages is also described in this Appendix, and is based on the same principles as those described for somatic and male-specific coliphages. Although it has been confirmed that these phages are highly specific for human faecal pollution, data obtained in this study on their incidence in various water environments show that their numbers tend to be rather low, in many cases lower than those of human viruses. This implies that although they specifically indicate faecal pollution of human origin, their absence from water environments does not necessarily imply the absence of human viruses, at least in terms of technology for the detection of these phages presently available. According to Prof J Jofre (personal communication) there is reason to believe that numbers of *B fragilis* HSP40 in sewage and polluted water environments are considerably higher than indicated by presently available techniques. There is, therefore, a need to upgrade techniques for the detection of *B fragilis* HSP40 phages because they serve a most valuable role in water quality assessment. One disadvantage of presently available techniques is that they are time consuming, cumbersome, labour intensive and relatively expensive, primarily because strict anaerobic incubation is required. Prof Jofre and his team are presently working on the possibility of cloning the gene that codes for the receptor site of *B fragilis* HSP40 phages into an aerobic bacterium such as *E coli* which may support the replication of the phages and can be used as host in convenient assays similar to those for coliphages.

2.2.4. Decontamination of water samples for phage testing

Chloroform decontamination was investigated in three comparative tests on hospital waste water. These samples were used to investigate the effect of chloroform on naturally occurring phages in waste water samples. Homogenised samples were divided into two, one was exposed to chloroform according to common procedure, and phage titrations were carried out on both. On average counts of somatic coliphages and *B fragilis* HSP-40 phages tended to be higher in chloroform treated samples, and counts of male-specific coliphages higher in untreated samples. Higher counts were probably due to the elimination of contaminant bacteria which overgrow the host organisms, which may apply in particular to the *B fragilis* phages. Reasons for the lower counts of male-specific coliphages in chloroform-treated samples were not clarified.

No further time was spent on questions regarding the decontamination of samples because problems with contamination were solved by rigorous application of aseptic laboratory procedures and antibiotic treatment. However, the observations that chloroform treatment may increase phage counts, at least under some circumstances, warrants further investigation. Decontamination of samples by membrane filtration would also seem to warrant consideration (Jofre, personal communication).

2.3. Methods for the Recovery of Small Numbers of Phages

2.3.1. Ultrafiltration

The efficiency of ultrafiltration for the recovery of phages has been evaluated. Tests were carried out on samples of phosphate buffered saline (PBS) and dechlorinated tap water seeded with known numbers of type-specific representatives of somatic and male-specific coliphages, and *B fragilis* HSP-40 phages. Tests were carried out on 200 ml, 300 ml and 1000 ml seeded test samples. Titres of phages in the seeded test samples ranged from 1 x 10^3 to 5 x 10^4 per ml. An Amicon stirred cell unit with 47 mm diameter membranes of 50 000 molecular cut-off level, was used. Test samples were filtered to a final volume of 20-35 ml. This concentrate was thoroughly stirred to suspend all phages which may have adsorbed to the membrane, and then transferred

to a measuring cylinder. Another 5 ml of PBS was added to the filter chamber and thoroughly stirred to remove remaining phages. This was then added to the concentrate, and the volume of the concentrate made up with PBS to a fixed volume for titration of the phages. In tests on seven 1000 ml samples seeded with MS2 male-specific coliphages, the average EOR was 94% (range 61-124%). Similar results were obtained for smaller volumes of test samples. Comparable results were obtained for the other two phages.

The results show that ultrafiltration can be used for the highly efficient recovery of phages from volumes of water of up to 1000 ml and possibly even more. The most important disadvantage of the method is that meaningful application is limited to water with low turbidity because the membranes clog readily and the presence of concentrated organic material in the final test sample may interfere with plaque assays. Contamination was a typical problem of ultrafiltration concentrates of river water. The technique proved well suited for drinking water. However, the procedure is relatively expensive. The membranes cost about R 500 a piece and are not reusable.

2.3.2. Glass wool adsorption-elution

The efficiency of recovery (EOR) of glass wool adsorption-elution for human enteric viruses is in the order of 60-95% according to reports from various laboratories (Grabow, 1996). The efficiency of this procedure for the recovery of phages has, therefore, been investigated. The MS2 male-specific coliphage was used as model in these experiments. In one series of experiments five 5-litre samples of dechlorinated tap water were seeded with MS2 phage. The range of phage counts in the water samples was $5,0 \times 10^4$ to $1,2 \times 10^6$ per ml. The preparation of filters containing 10 g of glass wool, the filtration, and the recovery by means of pH 9,5 beef extract-glycine buffer, were carried out as described for human viruses. The average EOR was 1,1% (range 0,3-1,8%). Titration of phages in the filtrates indicated that on average 72% (range 64-83%) of phages failed to adsorb, which implies that on average only 28% of phages adsorbed. These results suggest that adsorption of phages to the glass wool was poor, and that the release of adsorbed phages was also inefficient. Inactivation of phages by the pH 9,5 elution buffer was not tested but

appears unlikely in view of details on the resistance of human viruses to this buffer.

In an attempt to increase the efficiency of adsorption of phages, the glass wool was treated in packed columns with polyethylene-imine (PEI), according to procedures described for human viruses. PEI is used to change the electrostatic charge on conventional glass wool from negative to positive for recovery of negatively charged human viruses at neutral pH levels. Basically PEI was passed through a column to completely saturate the glass wool, the column stopcock was then closed with sufficient PEI left to cover the glass wool, and left like this for 24 h on the laboratory bench. The PEI was then drained and the column rinsed with 100 ml of distilled water. Tests were carried out using five 5-litre samples of dechlorinated tap water seeded with MS2 phage to counts of $5,0 \times 10^4$ to $4,6 \times 10^5$ per ml. Phages were eluted with the conventional pH 9,5 buffer. In this case the average percentage of phages in the filtrate was only 9% (range 0-21%), suggesting that on average 91% of phages had adsorbed. However, the average EOR was still only 6,4% (range 5,2-7,1%). These results show that PEI increases the adsorption of MS2 phage to the glass wool, but that the phages are not readily released by the elution buffer and procedure used. The eventual EOR is not much higher than without PEI treatment.

In an attempt to increase the release of phages from PEI-treated glass wool columns, a number of experiments were carried out in which the elution process was modified by increasing the volume of elution buffer, changing the pH of the elution buffer to pH 10,0 or 9,0, and increasing or decreasing the flow rate of the elution buffer. None of these modifications made any significant difference to the EOR. Since other possibilities for the detection of small numbers of phages in large volumes of water are available, further attempts to upgrade the recovery of phages by glass wool adsorption-elution were abandoned.

The results are interesting in terms of the following: Since the recovery process depends on the adsorption of viruses and phages to a particular surface, and subsequent release from that surface, the difference in EOR indicates differences in adsorption and elution properties between phages and human viruses. Since

adsorption to surfaces plays an important role in the behaviour and survival of human viruses in the environment, these differences in adsorption and elution properties may imply differences in the behaviour of viruses and phages in the environment, which would have implications for the indicator value of phages.

2.3.3. Membrane filter adsorption-elution

The method described by Sobsey *et al* (1990) has been evaluated. The method is based on adsorption-elution using conventional membrane filtration (Gelman GN-6 Metricel; 47 mm; 0,45 μm) and elution with 3% beef extract. Any volume of water that will pass through a membrane (100 ml or more) can be filtered and phages can afterwards be recovered by appropriate procedure using 5 ml beef extract.

In comparative tests on 22 samples of Klip River water at sampling point K19, direct double agar layer plaque assays using small dishes yielded higher average and median counts of somatic and male-specific coliphages than similar plaque assays on membrane filter concentrates of 100 ml samples. In the case of direct plaque assays the count per 100 ml was calculated from counts obtained in tests on 1 ml samples (Table 2.3 and 2.4). In similar comparative tests on 12 samples of Vaal Dam A18 water which contained considerably lower numbers of phages, the membrane filter concentrates yielded positive results more often for male-specific coliphages, and average and median counts were higher (Table 2.5). However, counts of somatic coliphages in Vaal Dam A18 water were higher by direct plaque assays (Table 2.6).

The higher counts obtained by direct plaque assays compared with plaque assays on 100 ml filter concentrates of K19 water, may be due to failure of the filters to recover all phages, which is reasonable to expect. However, the higher counts obtained by direct plaque assays may also at least to some extent be due to error introduced by multiplication of counts for 1 ml test samples by 100 to calculate counts per 100 ml for comparison to results obtained by membrane filter tests. Despite potential shortcomings in tests on water containing numbers of phages large enough for direct titration, the value of membrane filter recovery was clearly illustrated in test on A18 water which contained low numbers of male-specific coliphages (Table 2.5). These

findings indicate that the membrane filter method has merit for the detection of small numbers of phages in large volumes of water. Counts of somatic coliphages in A18 water were probably high enough for direct plaque assays (Table 2.6) eliminating the benefits of membrane filter recovery. This possibility is supported by results of tests on alternative methods for the detection of low numbers of phages described in the next section.

2.4. Direct Plaque Assay for Small Numbers of Phages in Water

Direct plaque assays on 100 ml samples of water using single agar layers in 10 large petri dishes according to the principles described earlier (Grabow *et al*, 1993) have been re-evaluated and optimised.

In comparative tests on naturally occurring phages in waters from various environments phages were enumerated by conventional double agar layer plaque assays using small petri dishes (90 mm diameter) in threefold on 1 ml samples (SP-DL), and single agar layer assays on 100 ml samples using 10 large petri dishes (140 mm diameter) (LP-SL). Counts per 100 ml for assays using small petri dishes were calculated by multiplying average counts for 1 ml by 100. Results show that LP-SL yielded higher counts and positive results more often than SP-DL for waters which contained very low numbers of somatic coliphages but not for waters which contained relatively high counts of phages (Tables 2.6 to 2.9).

NOTE:

SP-DL = Small Plates with Double agar Layer
LP-SL = Large Plates with Single agar Layer
LP-DL = Large Plates with Double agar Layer

Comparative tests similar to those recorded in Tables 2.6 to 2.9 were carried out in tests on dechlorinated tap water seeded with somatic coliphage V1, male-specific coliphage MS2 and *B fragilis* phage B40-8. Results were similar to those recorded for naturally occurring phages in Tables 2.6 to 2.9. Reasons for the lower numbers of plaques on the large petri dishes remain to be explained, but may be due to less

ideal host infection and replication conditions on the large petri dishes. The difference may also at least in part be due to error introduced by multiplying counts for 1 ml by 100 to obtain a count per 100 ml for SP-DL. However, the results still imply that the procedure offers a useful means for the direct quantitative enumeration of very low numbers of all three groups of phages in 100 ml volumes of water. The volume of water to be analysed can obviously be increased by increasing the number of plates. The procedure would remain relatively simple. In the case of tests for *B fragilis* HSP-40 phages in large volumes of water, this approach may even be superior to P-A tests, taking into account the shortcomings of P-A tests for these phages outlined in the next section.

Unfortunately cost is a factor in using large petri dishes for direct plaque assays on large volumes of water. The cost of a single disposable large petri dish (140 mm) is about R 5,00, which implies that the total cost for a test using 10 plates amounts to R 50,00 for petri dishes alone. The cost of small petri dishes (90 mm) is about R 0,50, which implies that the cost of petri dishes for a three-fold test using three dilutions amounts to R 4,50. Cost would, therefore, be another reason for using large plates only for the quantitative enumeration of very small numbers of phages, as may be expected in drinking water supplies and environmental waters with limited pollution. The cost of using large petri dishes could, of course, be reduced by using reusable glass petri dishes.

Reasons for the differences in counts obtained by small and large plates were investigated, and attempts were made to increase the sensitivity and accuracy of counts obtained by large plates. One difference between plaque assays using large and small petri dishes is the double agar layer used with the small plates. The possibility of the double layer playing a role in plaque formation was investigated by comparing SP-DL, LP-SL and large plates with double agar layers (LP-DL). Comparative tests on various waters showed that LP-DL yielded higher counts and positive results more often than LP-SL (Tables 2.7 to 2.11). The LP-DL approach has, therefore, been optimised and investigated in further detail. A recommended procedure for LP-DL is described in Appendix 1. Although LP-DL yielded higher counts than LP-SL,

counts for waters which contained relatively high numbers of phages were still lower than those obtained by SP-DL. The agar medium used for the bottom layer in LP-DL plates represents a minor factor in the cost of the assays, which implies that the difference in the cost of LP-DL and LP-SL is minimal. The additional labour required for preparing the large plates with bottom agar is likewise minimal.

Reasons for higher counts obtained by SP-DL than LP-DL are not clear and were not investigated in further detail. Theoretically both may be expected to detect all plaque forming units because the only difference is the diameter of the plates. The most likely reason for the differences would, therefore, appear to be associated with error introduced by the calculation of counts per 100 ml for SP-DL test carried out on 1 ml test samples.

Whatever the reason for differences in counts per 100 ml obtained by SP-DL and LP-DL, the results clearly illustrate the value of LP-DL for the direct enumeration of very low numbers of phages in water. In order to determine levels of phage numbers best suited for SP-DL and LP-DL, comparative plaque assays were carried out on dilutions of Klip River K19 water. The results in Tables 2.12 and 2.13 indicate a cut-off level at counts of about 1000 plaques per 100 ml, with SP-DL yielding higher counts above this level and LP-DL below this level.

Table 2.3 Counts of somatic coliphages in Klip River K19 water and Vaal Dam sluice gate A18 water obtained by plaque assays directly on water samples and on 100 ml membrane filter concentrates

	Plaque forming units per 100 ml			
	Klip River K19		Vaal Dam A18	
	Direct	Concentrate	Direct	Concentrate
Test series	95-07-10 to 96-07-22		96-01-08 to 96-08-19	
No of tests	22	22	19	19
No positive	22	20	14	7
Range	200-6030	0-2170	0-700	0-210
Average	2182	598	107	15
Median	1180	150	30	0
Standard deviation	2104	710	180	48

Direct plaque assays : Count per 100 ml calculated from results of tests on 1 ml samples
Membrane filters : Plaque assays on phages recovered from 100 ml samples

Table 2.4 Counts of male-specific coliphages in Klip River K19 water obtained by plaque assays directly on water samples and on 100 ml membrane filter concentrates

	Plaque forming units per 100 ml			
	Cabelli host		Havelaar host	
	Direct	Concentrate	Direct	Concentrate
Test series 1:	95-07-10 to 96-04-01			
No of tests	16	16	16	16
No positive	13	14	16	16
Range	0-2400	0-1500	60-2800	45-500
Average	371	255	553	234
Median	80	70	250	150
Standard deviation	696	429	804	187
Test series 2:			95-07-10 to 96-07-22	
No of tests	-	-	27	27
No positive	-	-	27	27
Range	-	-	60-4800	45-700
Average	-	-	761	262
Median	-	-	300	200
Standard deviation	-	-	1068	205

Direct plaque assays : Count per 100 ml calculated from results of tests on 1 ml samples
Membrane filters : Plaque assays on phages recovered from 100 ml samples
- = Not done

Table 2.5 Counts of male-specific coliphages in Vaal Dam sluice gate (A18) water determined by plaque assays directly on water samples and on 100 ml membrane filter concentrates

	Plaque forming units per 100 ml			
	Cabelli host		Havelaar host	
	Direct	Concentrate	Direct	Concentrate
Test series 1:	96-01-08 to 96-04-01			
No of tests	12	12	12	12
No positive	0	3	1	4
Range	0	0-25	0-60	0-45
Average	0	3	5	8
Median	0	0	0	0
Standard deviation	0	8	17	15
Test series 2:			96-01-08 to 96-08-19	
No of tests	-	-	24	24
No positive	-	-	1	7
Range	-	-	0-60	0-45
Average	-	-	3	7
Median	-	-	0	0
Standard deviation			12	14

Direct plaque assays : Count per 100 ml calculated from results of tests on 1 ml samples
Membrane filters : Plaque assays on phages recovered from 100 ml samples
- = Not done

Table 2.6 Counts of somatic coliphages in Klip River K19 water and Vaal Dam sluice gate A18 water obtained by using double agar layer small plates, membrane filter concentrates and single agar layer large plates

	Plaque forming units per 100 ml		
	Small plates	Concentrates	Large plates
Klip River K19	96-01-08 to 96-07-08		
No of tests	15	15	15
No positive	15	13	15
Range	200-5900	0-1835	50-3760
Average	2030	416	1117
Median	12	100	600
Standard deviation	2048	646	1209
Vaal Dam A18	96-01-08 to 96-07-22		
No of tests	16	16	16
No positive	11	5	13
Range	0-700	0-210	0-300
Average	125	16	63
Median	47	0	47
Standard deviation	192	52	80

Small plates : 90 mm diameter disposable plastic petri dishes:
Count per 100 ml calculated
Large plates : 140 mm diameter disposable plastic petri dishes : 100 ml tested

Table 2.7 Counts of somatic coliphages in Klip River K19 water obtained by plaque assays using double agar layer small plates, and single or double agar layer large plates

	Plaque forming units per 100 ml		
	Small plates	Large plates	
	Double layer	Single layer	Double layer
Test series 1	95-10-09 to 95-12-04		
No of tests	7	7	-
No positive	7	7	-
Range	200-1700	100-500	-
Average	1100	271	-
Median	1300	300	-
Standard deviation	597	180	
Test series 2	95-12-11 to 96-05-20		
No of tests	21	21	21
No positive	21	21	21
Range	80-4700	18-1700	50-1900
Average	1256	565	651
Median	900	400	500
Standard deviation	1099	461	508
Test series 3	96-06-10 to 96-10-14		
No of tests	10	-	10
No positive	10	-	10
Range	467-5900	-	740-3760
Average	3582	-	1970
Median	4450	-	1890
Standard deviation	2013	-	1000

Small plates : 90 mm diameter disposable plastic petri dishes
Count per 100 ml calculated

Large plates : 140 mm diameter disposable plastic petri dishes : 100 ml tested - =Not done

Table 2.8 Counts of somatic coliphages in Vaal Dam sluice gate A18 water obtained by plaque assays using double agar layer small plates, and single or double agar layer large plates

	Plaque forming units per 100 ml		
	Small plates	Large plates	
	Double layer	Single layer	Double layer
Test series 1	95-10-09 to 95-12-04		
No of tests	7	7	-
No positive	2	7	-
Range	0-60	3-13	-
Average	17	7	-
Median	0	6	-
Standard deviation	29	4	-
Test series 2	95-12-11 to 96-05-20		
No of tests	21	21	21
No positive	15	11	15
Range	0-700	0-300	0-400
Average	143	51	61
Median	70	10	13
Standard deviation	202	90	108
Test series 3	96-06-10 to 96-08-07		
No of tests	8	-	8
No positive	6	-	8
Range	0-170	-	8-93
Average	49	-	47
Median	33	-	47
Standard deviation	55	-	25

Small plates : 90 mm diameter disposable plastic petri dishes
Count per 100 ml calculated

Large plates : 140 mm diameter disposable plastic petri dishes : 100 ml tested
- =. Not done

Table 2.9 Counts of somatic coliphages in Apies River water obtained by using double agar layer small plates, and single or double agar layer large plates

	Plaque forming units per 100 ml		
	Small plates	Large plates	
	Double layer	Single layer	Double layer
No of tests	10	10	10
No positive	10	10	10
Range	1000-1600	80-1000	200-400
Average	1250	228	260
Median	1150	105	200
Standard deviation	207	276	84

Small plates : 90 mm diameter disposable plastic petri dishes:
Count per 100 ml calculated
Large plates : 140 mm diameter disposable plastic petri dishes : 100 ml tested

Table 2.10 Counts of somatic coliphages in treated waste water obtained by plaque assays using double agar layer small plates and double agar layer large plates

	Plaque forming units per 100 ml	
	Small plates	Large plates
Secondary treated effluent	96-07-09 to 96-08-27	
No of tests	5	5
No positive	5	5
Range	200-3830	532-2600
Average	1460	1510
Median	870	1506
Standard deviation	1467	932
Chlorinated sand filter effluent	96-07-09 to 96-08-27	
No of tests	5	5
No positive	5	5
Range	533-3000	312-2510
Average	1167	1299
Median	667	755
Standard deviation	1051	1113

Small plates : 90 mm diameter disposable plastic petri dishes:
Count per 100 ml calculated
Large plates : 140 mm diameter disposable plastic petri dishes : 100 ml tested

Samples collected at the Daspoort sewage treatment works in Pretoria

Table 2.11 Counts of somatic coliphages in diffuse effluents from an informal settlement obtained by plaque assays using double agar layer small plates and double agar layer large plates

	Plaque forming units per 100 ml	
	Small plates	Large plates
Secondary treated effluent	96-07-15 to 96-08-07	
No of tests	4	4
No positive	4	4
Range	1100-4400	222-3000
Average	2200	1022
Median	1650	432
Standard deviation	1500	1327
Chlorinated sand filter effluent	96-07-15 to 96-08-07	
No of tests	5	5
No positive	3	5
Range	0-60	15-87
Average	20	44
Median	10	39
Standard deviation	25	26

Small plates : 90 mm diameter disposable plastic petri dishes:
Count per 100 ml calculated
Large plates : 140 mm diameter disposable plastic petri dishes : 100 ml tested

Samples collected at two sites from diffuse effluents from an informal settlement in Atteridgeville

Table 2.12 Counts of somatic coliphages in dilutions of Klip River K19 water obtained by plaque assays using double agar layer small plates, and double agar layer large plates

Date	Plaque forming units per 100 ml	
	Small plates	Large plates
96-08-19		
200:0	2 000	1 740
180:20	1 800	1 680
160:40	1 800	1 670
140:60	1 900	1 637
120:80	1 400	1 265
100:100	1 000	1 087
80:120	900	864
60:140	533	715
40:160	560	557
20:180	300	456
96-08-26		
200:0	467	740
180:20	400	697
160:40	100	778
140:60	200	613
120:80	300	596
100:100	167	510
80:120	167	466
60:140	100	352
40:160	100	202
20:180	67	147

Small plates : 90 mm diameter disposable plastic petri dishes:
Count per 100 ml calculated

Large plates : 140 mm diameter disposable plastic petri dishes : 100 ml tested

Dilutions of Klip River K19 water in sterile phosphate buffered saline (PBS): 200:0 = undiluted K19 water

Table 2.13 Counts of somatic coliphages in dilutions of Klip River K19 water obtained by plaque assays using double agar layer small plates, and double agar layer large plates

Date	Plaque forming units per 100 ml	
	Small plates	Large plates
96-09-16		
200:0	4 700	2 149
180:20	4 100	1 834
160:40	4 200	1 783
140:60	4 100	1 673
120:80	3 400	1 446
100:100	2 800	1 237
80:120	2 300	975
60:140	1 400	954
40:160	1 400	712
20:180	500	400
96-10-14		
200:0	800	940
180:20	567	925
160:40	567	934
140:60	367	766
120:80	500	612
100:100	267	540
80:120	200	436
60:140	233	372
40:160	133	190
20:180	100	128

Small plates : 90 mm diameter disposable plastic petri dishes:
Count per 100 ml calculated
Large plates : 140 mm diameter disposable plastic petri dishes : 100 ml tested
Dilutions of Klip River K19 water in sterile phosphate buffered saline (PBS) : 200:0 = undiluted K19 water

2.5. Qualitative Detection of Small Numbers of Phages

2.5.1. Qualitative presence/absence (P-A) tests

P-A test procedures for somatic and male-specific coliphages, and *Bacteroides fragilis* phages, have been standardised for routine application in water quality monitoring. Details on the recommended test procedures are recorded in Appendix 1. An evaluation of various procedures indicated that the optimum volume of water to be tested in the quality assessment of drinking water supplies was 500 ml. Results of tests on 1000 ml samples did not differ significantly from those on 500 ml samples. Testing of larger volumes is possible, but becomes impractical and expensive, and the need for that level of sensitivity in routine monitoring of the quality of drinking water supplies seems unlikely.

The standardised P-A test procedures for all three groups of phages were applied in tests on more than 50 samples of river water (mainly Klip River at site K19), and more than 600 samples of treated drinking water, most of which supplied by Rand Water. In a substantial number of tests positive results were obtained by P-A tests but not by conventional direct plaque assays (SP-DL). These results confirm that P-A tests were much more sensitive than direct plaque assays for all three groups of phages. Details of results are presented in 2.7.

The findings confirm that P-A tests offer a simple and inexpensive means for the qualitative detection and screening of small numbers of phages in large volumes of water. Since many water quality guidelines and specifications are based on the presence, and not quantitative numbers, of indicators or pathogens in a specified volume of test water, these P-A tests for phages fulfil a useful role in water quality assessment.

The team experienced that these highly sensitive P-A tests have to be applied with caution, particularly in routine quality monitoring of treated drinking water supplies. Positive results tend to be inter-preted as an indication of unacceptable quality. This may, however, not be the case because the health risks reflected by the presence of

phages at that level have not yet been established. In most situations these tests should be applied as a highly sensitive screening component of a battery of tests including additional tests for appropriately selected indicators and pathogens. The test procedure serves a most valuable purpose as highly sensitive monitoring procedure for the efficiency of drinking water treatment processes. The tests are, of course, useful tools for various research purposes.

In view of their basic sensitivity the P-A tests are obviously vulnerable to contamination. The tests do, therefore, have to be carried out under carefully controlled laboratory conditions, and samples have to be collected aseptically, because contamination with a single viable phage particle will lead to a positive result.

Some questions about the reliability of P-A tests for *B fragilis* phages remain to be clarified. Reason is that results of tests in which known numbers of phages were seeded into samples of water, indicate that P-A tests failed to detect all viable phages. Theoretically this may be due to problems with the inoculation of viable host bacteria into the test suspension (Jofre, personal communication). With the facilities presently available in our laboratory, anaerobic host cultures are inoculated into test water samples which contain oxygen. This exposure to oxygen probably inactivates a large number of the host bacteria. This implies that many phages in the test sample may adsorb to inactive host bacteria or receptor material of lysed host bacteria. These phages would fail to replicate. The test water becomes anaerobic only during anaerobic incubation after inoculation of the host culture. During these fully anaerobic conditions host bacteria which have survived the oxygen shock become metabolically active and phages which have adsorbed to these bacteria are replicated. The problem may be solved by prior removal of oxygen from the test sample of water and inoculation of the host culture under anaerobic conditions. This could possibly be carried out in an anaerobic inoculation cabinet which is expensive and presently not available to us. Alternative solutions should be investigated.

Theoretically it is possible to obtain quantitative estimates from P-A tests on large volume dilution series of test samples. However, this has not been investigated

because the procedure is cumbersome, labour intensive, expensive and subject to the statistical shortcomings of all MPN assays. In addition, more practical alternatives for the quantitative enumeration of small numbers of phages in large volumes of water are available.

2.6. Incidence of Indicator Phages in Human and Animal Wastes

Details on studies along these lines have been published (Grabow *et al*, 1995) (Appendix 2). The results indicate that *B fragilis* HSP40 phages are highly specific for humans and excreted by about 14% of individuals. Somatic and male-specific coliphages were excreted by considerably higher percentages of both humans and a variety of animals. Since that time another 100 human stool specimens have been analysed. *Bacteroides fragilis* HSP40 phages were detected in 13% of these specimens by P-A tests on suspensions containing 1,0 g wet mass stool. In no case were phages detected by direct plaque assays which indicates that the phages were present in low numbers in all positive specimens, at least by the plaque assays used.

2.7. Phages, Bacteria and Viruses in Waste and Environmental Waters

The results of a study on waste water from an informal settlement have been published (Grabow *et al*, 1996) (Appendix 3). In an Analysis of 209 samples collected over a period of one year, human enteric viruses were recovered from a number samples which yielded negative results in conventional tests for somatic and male-specific coliphages, *B fragilis* HSP40 phages, and even faecal bacteria such as faecal coliforms and enterococci. The results reveal shortcomings in the indicator value of these commonly used indicators when using conventional methods.

Some 150 samples of water from the Klip River collected at weekly intervals at sampling point K19, and more than 50 samples of Vaal Dam sluice gate water collected at sampling point A18, have been analysed for phages, bacterial indicators of faecal pollution and human viruses. Typical results are presented in Appendix 4. The consistent presence of somatic and F-RNA coliphages confirms faecal and sewage pollution. The detection of *B fragilis* HSP40 phages in some of the samples confirms faecal pollution specifically of human origin. However, these phages were rarely detected and only by presence-absence tests on 500 ml samples. This suggests the phages were present in low numbers or the detection methods have shortcomings.

The isolation of viruses from five samples which yielded negative results for *B fragilis* HSP40 phages in presence-absence tests, implies that these phages are not reliable indicators for the presence of viruses in water environments. Cytopathogenic viruses were never detected in the absence of somatic coliphages, F-RNA coliphages, faecal coliforms or enterococci. However, viruses were occasionally isolated from water in which *B fragilis* HSP40 phages were not even detected by P-A tests on 500 ml samples. Although counts of all phages and bacterial indicators were considerable lower in Vaal Dam sluice gate (A18) water than in Klip River (K19) water, evidence of faecal pollution was consistently present. Appendix 3 also shows a graphical presentation of somatic coliphage counts recorded by the team's and Rand Water's laboratories. Despite all efforts to standardise laboratory procedures as far as possible, variation in results is evident. This variation may at least in part be due to inherent variation in technology for enumerating micro-organisms, the fact that the samples analysed were not identical even though they were collected from the same water at the same site and time, and the longer transit period for samples analysed in our laboratory.

Details on phages in diffuse effluents from the Botshabelo township have been published (Jagals *et al*, 1995). Among other things, this study confirms the value of *B fragilis* HSP40 phages as indicators of human faecal pollution.

2.8. Phages, Bacteria and Viruses in Drinking Water Supplies

More than 600 samples of treated drinking water from various points in the Rand Water treatment system were analysed for human viruses, phages and bacterial indicators of faecal pollution. Samples were collected each week over a period of three years. Tests included recovery of viruses by glass wool adsorption-elution, P-A tests on 500 ml samples for somatic coliphages, male-specific coliphages and *B fragilis* HSP40 phages, and conventional membrane filter tests for coliform bacteria. Details of the study have been reported (Grabow, 1997). In summary, human viruses were never detected in the treated drinking water, and phages rarely if ever. This implies that P-A test on 500 ml samples are feasible and a valuable component of indicator systems for routine monitoring of the quality of treated drinking water

supplies. These phage results indicate the absence of human viruses as well as efficient treatment of the water.

2.9. Survival of Phages, Bacteria and Viruses in Water Environments

2.9.1. Laboratory experiments on the survival of phages and other indicators in sea water

Experiments were carried out on sea water seeded with human stool and marine guano to investigate the survival of naturally occurring organisms under controlled laboratory conditions. Seeded suspensions were continuously stirred by magnetic stirrer on the laboratory bench, at temperatures of 22-28°C, and pH levels of 7,4-8,0. A laboratory strain of poliovirus was seeded into the suspensions. Laboratory experiments were carried out on one litre of sea water samples each seeded with 1,0 g of fresh human stool or marine bird droppings. The results of typical experiments are presented graphically in Appendix 5. In summary, these results indicate that somatic coliphages survived longest, and were still detectable after 22 days. Enterococci in human stool seemed to survive longer than faecal coliforms, and in guano it was the other way round. Survival of male-specific coliphages was relatively short. All organisms except male-specific coliphages survived for longer than poliovirus. Neither the human stool nor the guano contained *B fragilis* HSP40 phages, and the human stool contained no somatic or male-specific coliphages. Differences in numbers of organisms in the suspensions may partly explain differences in survival times.

2.9.2. Survival of phages and indicators in river water exposed to sunlight

Experiments have been carried out using water from the Apies River. Seeded water was stirred in shallow glass bowls on roof-top. Controls were run under identical conditions except that the beakers were protected from sunlight. Results of a typical experiment in Appendix 5 show that exposure to sunlight has a major effect on the survival of all organisms tested. The tests on survival in river water were supplemented by similar tests using PBS buffer and sea water. the results indicate a general trend for phages to survive at least as long as human viruses under conditions resembling those in natural water environments.

2.9.3. Survival of phages in water treatment processes

The survival of phages, bacterial indicators of faecal pollution and human/animal viruses in a multiple barrier system for the reclamation of water from abattoir effluent has been investigated. No *B fragilis* HSP40 phages were detected in the abattoir effluent which confirms the specificity of the phages for human excreta. Enteric viruses were isolated from occasional samples which yielded negative results for somatic coliphages even in P-A tests on 500 ml samples.

2.10. Indicator Value of Phages in Terms of Numbers and Survival

Data obtained in this study show that:

* Under most circumstances the phages covered by this study outnumbered at least those human viruses which are detectable by conventional techniques.
* The phages concerned tended to survive at least as long as human viruses in a variety of natural water environments and laboratory conditions resembling natural environments.
* The phages were at least as resistant as some enteric viruses to water treatment and disinfection processes.

These features meet fundamental requirements of indicators for routine monitoring of water quality and assessment of the efficiency of treatment processes (Grabow, 1996). However, human viruses were occasionally detected in the absence of at least some of the phages concerned. This is not altogether surprising since viruses may be excreted by infected individuals in numbers of 10^{10} or more per gram of faeces. In situations such as disease outbreaks or peaks of seasonal incidence viruses may outnumber many phages just like they may outnumber commonly used bacterial indicators of faecal pollution. These results and considerations underline the value and importance of using phages as an important component of appropriately selected combinations of indicators for water quality monitoring.

2.11. Practical Aspects of Phage Indicators

Evidence has been presented that:

* Phages are detectable by relatively simple and inexpensive techniques.
* Practical techniques have been established for the sensitive and accurate qualitative

and quantitative detection of phages in a water containing phages in numbers ranging from high to very low.

* Phage tests do not require sophisticated and costly laboratory facilities or equipment, and can be applied by most water quality laboratories.
* Results of many phage tests are available within 24 h.
* Phages are not pathogenic and constitute no health hazard to laboratory workers.

These features meet fundamental requirements of indicators for water quality assessment (Grabow, 1996).

2.12. International Collaboration

An infrastructure for collaboration with international experts and research bodies has been established including the International Organization for Standardization, Universities of Barcelona and North Carolina, and the Netherlands National Institute for Environmental Hygiene (Grabow, 1996).

2.13. Recommended Water Quality Guidelines

Data on the following features of phages indicators obtained in this study have been used to formulate recommendations for water quality guidelines:

* Relative incidence of the phages concerned, human viruses and commonly used bacterial indicators of faecal pollution, in water environments.
* Relative resistance of phages to water treatment and disinfection processes.
* Feasibility of phage detection methods. This includes cost, time, labour, expertise and facilities required for carrying out the tests.

The following quality guidelines are based on the above considerations:

Drinking water : Absence of any phages from 500 ml samples.

Test method : Presence-Absence test on 500 ml samples.
Positive results to be followed up by quantitative tests on at least 100 ml samples using double agar layer plaque assays on large petri dishes (LP-DL), or conventional plaque assays (SP-DL) on membrane filter concentrates of 500 ml samples.

Environmental waters (ie rivers, dams, sea water) intended for direct contact recreation (ie swimming) : Less than 10/10 ml of any phages.

Test method : Direct quantitative plaque assay on 10 ml samples using conventional small plates with double agar layer (SP-DL).

Interpretation:

* The recommended guidelines are no absolute guarantee for the absence of pathogens such as human viruses and protozoan parasites.
* In view of potential shortcomings under certain conditions, the phage tests should be used as one component of a combination of tests. The combination of tests for indicators and pathogens should be appropriately selected for the purpose concerned.
* The recommended guidelines are not based on data directly related to health risks. The guide-lines do, therefore, not give any direct indication of risks of infection by any pathogens.
* Data recorded in this study could also be used to formulate guidelines for treated waste water to be discharged into environmental water sources such as rivers, dams and the sea.
* Techniques defined in this study could be used to distinguish between faecal pollution of human and animal origin. Under circumstances this would cast valuable light on risks of infection.

2.14. Recommended Procedure for Routine Water Quality Monitoring

Techniques recommended for general and routine use in water quality monitoring and research are described in Appendix 1. These techniques are based on technology development and research carried out in this project. The techniques are representative of the best technology internationally recommended at the time of printing.

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Appendix 1

Methods for the detection of phages

Plaque Assay for Somatic Coliphages
using
Small Petri Dishes with Double Agar Layer (SP-DL)

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Principle

1. Conventional plaque assay for somatic coliphages in small volumes of water (generally 1,0 ml) using small petri dishes (90 mm diam) based on principles originally formulated by Adams.
2. *Escherichia coli* strain C is exceptionally susceptible to a wide range of somatic coliphages largely because it has an impaired nucleic acid restriction enzyme.
3. The media described here differ somewhat from those specified in ISO/DIS 10705-2:1997. There is no evidence that one may be superior to the other. In terms of basic theoretical considerations there is no reason to expect meaningful differences in results.

Test procedure

1. Steam the required number of test tubes with top agar to liquefy agar and adjust to 48°C in a heating block.
2. Add 0,5 ml of the host culture to the top agar.
3. Add 1,0 ml of the test sample, or an appropriate dilution of the test sample, to the top agar in each test tube.
4. Mix gently and pour the top agar mixture with minimum delay onto the bottom agar layer in a 90 mm phage agar plate.
5. Repeat the above in tenfold to obtain counts per 10 ml. If tenfold dilutions are required, three plates should preferably be used for each dilution to obtain meaningful results.
6. Incubate inverted plates overnight at 35-37°C and count plaques of somatic coliphages.

Materials

Growth medium (Nutrient broth)
Prepare ordinary nutrient broth (Difco or equivalent) according to the manufacturer's instructions, heat to dissolve, dispense in convenient containers, ie, 100 ml quantities in 200 ml medical flats, autoclave, store at about 4°C for not longer than 30 days.

Phage bottom agar

Bacto agar	14,0 g
Tryptone	13,0 g
NaCl	8,0 g
Na_2CO_3 solution	5,0 ml
Glucose	1,5 g
Dist water	1000 ml

Heat to dissolve agar, and autoclave.
Pour about 20 ml in 90 mm diameter petri dishes.
Store at 4±2 °C: maximum 10 days.

Phage top agar

Bacto agar	8,0 g
Tryptone	10,0 g
NaCl	8,0 g
Glucose	3,0 g
Na_2CO_3 solution	5,0 ml
$MgCl_2$ solution	1,0 ml
Dist water	1000 ml

Autoclave and cool to 55-60 °C.
Add 6,0 ml $CaCl_2$ solution.
Add nalidixic acid solution if considered necessary (1,0 ml/100 ml).
Distribute 2,5 ml aliquots into test tubes with caps.
Store at 4±2 °C: maximum 30 days.

Host culture

Escherichia coli strain C (ATCC 13706) = WG4
Nalidixic acid resistant mutant of WG4 = WG5

Preparation of host culture

Inoculate typical colonies from a stock agar plate, or a loopful of growth from a stock agar slant, into 50 ml of prewarmed growth medium and incubate for 3±1 h at 36±2 °C with gentle shaking. Use immediately or take the inoculum culture from the incubator and quickly cool to 5-10 °C, preferably by placing onto melting ice. Use this inoculum culture the same working day. The inoculum culture should ideally have a count of 1-10 x 10^8 per ml.

Test sample

Water (eg drinking water, wastewater, river water, seawater) or liquid suspension (eg suspension of shellfish meat). Make tenfold dilution in peptone saline solution as necessary.

Nalidixic acid solution

Dissolve 0,5 g of nalidixic acid in 4 ml of 1 M NaOH. Add 16 ml of sterile water, mix well. Decontaminate by membrane filtration, eg syringe filter, 0,22 μm membrane.
Store at 4±2 °C : maximum 4 weeks.

$MgCl_2$ solution

Prepare 4 M stock solution by dissolving 820 g of $MgCl_2.6H_2O$ crystals in 1000 ml of water; sterilise by autoclaving; store at room temp in the dark.

Na_2CO_3 solution

Dissolve 150 g of Na_2CO_3 in 1000 ml of water by gentle heating. Store at 4±2 °C : maximum 6 months.

$CaCl_2$ solution

Prepare 1 M stock solution by dissolving 147 g of $CaCl_2.2H_2O$ in 1000 ml water by gentle heating. Decontaminate by membrane filtration, eg syringe filter, 0,22 μm membrane.
Store at 4±2 °C : maximum 6 months.

Peptone saline solution

Dissolve 1,0 g peptone and 8,5 g sodium chloride in 950 ml water by boiling. Adjust pH to 7,0 ± 0,1 using 1 M NaOH or HCl. Make up to 1000 ml with water, and dispense in convenient volumes.

Autoclave. Store at 4±2 °C : maximum 6 months.

Notes

1. Tests to be carried out according to basic principles outlined in:
   1.1. Grabow W O K, Holtzhausen C S and de Villiers C J (1993) Research on Bacteriophages as Indicators of Water Quality. WRC Report No 321/1/93. Water Research Commission, Pretoria. 147 pp.
   1.2. ISO 10705-1:1995. Water Quality - Detection and Enumeration of Bacteriophages. Part 1: Enumeration of F-specific RNA bacteriophages. International Organization for Standardization, Geneva. 15 pp.
   1.3. ISO/DIS 10705-2:1997. Water Quality - Detection and Enumeration of Bacteriophages. Part 2: Enumeration of somatic coliphages. International Organization for Standardization, Geneva. 17 pp.
2. In the case of heavily contaminated test samples (eg waste water), interfering microbial growth may be suppressed by the addition of 1,0 ml of the nalidixic acid solution to 100 ml of molten phage top agar.
   Final concentration of nalidixic acid in phage top agar = 250 μg/ml.
   The resistant mutant WG5 must be used as host in these assays.
   Nalidixic acid is generally not necessary for testing treated drinking water.
3. A heating block should be used for tubes with top agar instead of a water bath if possible in order to avoid contamination by phages in water bath water.
4. Always use distilled water for the preparation of media and reagents.
5. Autoclaving = 121°C/15 min.

Commercial media and reagents

The following or equivalent are recommended:

Difco: Bacto Agar, Nutrient Broth, Tryptone, Peptone

Sigma: Glucose, nalidixic acid, NaCl, $CaCl_2.2H_2O$, $MgCl_2.6H_2O$

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Plaque Assay for F-RNA (male-specific) Coliphages
using
Small Petri Dishes with Double Agar Layer (SP-DL)

W O K Grabow, J C de Villiers, M Uys, M A Vrey
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Principle

1. Conventional plaque assay for F-RNA coliphages in small volumes of water (generally 1,0 ml) using small petri dishes (90 mm diam).
2. *Salmonella typhimurium* strain WG49 (Havelaar) has been selected in detailed evaluations by laboratories world-wide as the host of choice.
3. F-RNA coliphages share a number of fundamentally important features with enteric viruses and meet important requirements of indicators for water quality assessment and routine monitoring.

Test procedure

1. Steam the required number of test tubes with top agar (ssTYGA) to liquefy agar and adjust to 48°C in a heating block.
2. Add 1,0 ml of the test sample, or an appropriate dilution of the test sample, to the top agar in each test tube.
3. Add 1,0 ml of the host culture to the top agar and mix gently.
4. Pour the top agar mixture with minimum delay onto the bottom agar layer (TYGA) in a 90 mm phage agar plate.
5. Repeat the above in tenfold to obtain counts per 10 ml. If tenfold dilutions are required, three plates should preferably be used for each dilution to obtain meaningful results. Use peptone-saline solution for dilutions.
6. Incubate inverted plates overnight (18±2 h) at 35-37°C and count plaques of F-RNA coliphages (faint lysogenic plaques).

When high bacterial background flora may interfere with growth of the host and replication of phages, the addition of nalidixic acid is recommended to suppress contaminant growth.
Add 0,2 ml of stock nalidixic acid solution to 50 ml top agar (ssTYGA): final conc 100μg/ml.
Nalidixic acid is heat resistant and can be added to the top agar prior to autoclaving.

Confirmatory test

When there is reason to suspect that F-RNA phages may constitute less than 90 % of plaques obtained by the above procedure, or absolute identity is essential, the identity of F-RNA phages should be confirmed as follows:
Prepare plates in parallel to those described above but add 5,0 ml RNase solution to 50 ml top agar just after the addition of the calcium-glucose solution, to obtain a final RNase concentration of 100 μg/ml. In some cases it may be necessary to increase the RNase concentration to 400 μg/ml. F-RNA phages may also be confirmed by electron microscopy, and serological and molecular techniques.

Materials

Host for F-RNA coliphages:
Salmonella typhimurium WG49 (Havelaar):
Salmonella typhimurium strain WG49 phage type 3 Nal^r (F'lac: :Tn5)
Reference: NCTC 12484

Inoculum culture of WG49 for F-RNA phages

1. **Working culture**

Thaw one vial of stock culture and streak for single colonies on selective lactose-containing agar medium such as McConkey.
Incubate at 37±1 °C for 18±2 h.
Pick 3-5 typical lactose-positive colonies and add to 50 ml TYGB in a conical flask (about 300 ml).
Incubate at 37±1 °C for 5±1 h while shaking at 100±10/min.
Add 10 ml of glycerol (870 g/l) and mix well.
Distribute 1,2 ml aliquots into plastic vials (Eppendorf).
Store at -70±10 °C: maximum 2 years.

2. **Inoculum culture**

Add 0,5 ml of working culture thawed at room temp to 50 ml of TYGB in a conical flask (about 300 ml) or nephelometric conical flask with side arm for optical density reading.
Incubate at 37±1 °C while shaking at 100±10/min.
Measure turbidity every 30 min using a spectrophotometer.
When a turbidity equivalent to approximately 10^8 colony forming units (cfu) per ml has been reached*, cool culture quickly on melting ice and use within 2 h.

* The required density of host cells is generally reached at an optical density (OD) reading of 0,8-1,0 after 2-4 h incubation. However, this is variable and a growth curve has to be prepared for each batch of working cultures using procedures described in detail (ISO, 1995).

Tryptone - Yeast extract - Glucose - Broth (TYGB)

1. **Basal medium**

Trypticase peptone (BBL)	10,0 g
Yeast extract (Difco)	1,0 g
NaCl (Sigma)	8,0 g
Dist water	1000 ml

Dissolve with gentle heating if necessary.
Adjust pH to be 7,2±0,1 at 25 °C after autoclaving.
Distribute 200 ml volumes into bottles and autoclave (121±1 °C for 15 min).
Store at 4±2 °C in dark: maximum 6 months.

2. **Complete medium**

Basal medium	200 ml
Calcium-glucose solution	2,0 ml

Add and mix aseptically
Store at 4±2 °C in dark: maximum 6 months.

Tryptone - Yeast extract - Glucose - Agar (TYGA)
Prepare basal medium as for TYGB but add 15 g of agar (depending on the gel strength of the agar used the concentration may have to be adjusted to 12-20 g).
After autoclaving cool to 50-55 °C, add prewarmed calcium-glucose solution as for TYGB, mix gently, and pour 20 ml quantities into 90 mm diameter petri dishes.
Store plates at 4±2 °C: maximum 6 months.

Top agar (ssTYGA)
Prepare basal medium as for TYGA but use 8 g of agar instead of 15 g (depending on the gel strength of the agar used, the concentration may have to be adjusted in the range 6-10 g).
Autoclave and cool to 55-60 °C.
Add calcium-glucose solution: 0,5 ml/50 ml.
Distribute 2,5 ml aliquots into test tubes with caps.
Store tubes at 4±2 °C: maximum 6 months.

Calcium-glucose solution

$CaCl_2 \cdot 2H_2O$ (Sigma)	3,0 g
Glucose (Sigma)	10,0 g
Dist water	100 ml

Dissolve with gentle heating if necessary
Decontaminate by filtration: 0,22 µm membrane
Store at 4 °C: maximum 6 months

Nalidixic acid stock solution

Nalidixic acid (Sigma)	2500 mg
NaOH (1M)	2,0 ml
Dist water	98,0 ml

Dissolve nalidixic acid in NaOH
Add water
Decontaminate by filtration: 0,22 µm membrane
Store at 4 °C: maximum 2 weeks

Peptone saline solution
Dissolve 1,0 g peptone and 8,5 g sodium chloride in 950 ml water by boiling. Adjust pH to 7,0 ± 0,1 using 1 M NaOH or HCl. Make up to 1000 ml with water, and dispense in convenient volumes.
Autoclave.
Store at 4±2 °C : Maximum 6 months.

RNase solution

RNase	100 mg
Dist water	100 ml

Dissolve RNase by heating for not more than 10 min at 100 °C.
Distribute 0,5 ml aliquots into plastic vials (Eppendorf).
Store at -2 °C: maximum 12 months.
Recommended RNase: RNase A from bovine pancreas (Boehringer Mannheim).

Notes

1. Tests to be carried out according to basic principles outlined in:

1.1. Grabow W O K, Holtzhausen C S and de Villiers C J (1993) Research on Bacteriophages as Indicators of Water Quality. WRC Report No 321/1/93. Water Research Commission, Pretoria. 147 pp.

1.2. ISO 10705-1:1995. Water Quality - Detection and Enumeration of Bacteriophages. Part 1: Enumeration of F-specific RNA bacteriophages. International Organization for Standardization, Geneva. 15 pp.

3. Water baths are a common source of phage contamination unless carefully controlled. A heating block is, therefore, recommended for tubes with top agar in order to avoid risk of contamination.
4. Always use distilled water for the preparation of media and reagents.
5. Autoclaving = 121°C/15 min.
6. The names of commercial media and reagents are indicated merely as a guide. A variety of alternative commercial equivalents may yield similar and acceptable results. Analytical grade products are required only when proven to yield results superior to those obtained by means of standard grade reagents.

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Plaque Assay for *Bacteroides fragilis* HSP40 Phages
using
Small Petri Dishes with Double Agar Layer (SP-DL)

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Principle

1. Conventional plaque assay for *B fragilis* HSP-40 phages in small volumes of water (generally 1,0 ml) using small petri dishes (90 mm diam) based on principles originally formulated by Adams.
2. As far as is known, these phages are excreted only by about 14 % of humans and not by any animals. The phages can, therefore, be used to distinguish between human and animal faecal pollution.
3. *Bacteroides fragilis* is a strictly anaerobic bacterium, which requires incubation under absolutely anaerobic conditions. Implications are that these plaque assays are more complicated, labour intensive and expensive in terms of facilities and materials than corresponding tests for phages of aerobic bacteria such as *Escherichia coli*. Also, production of visible plaques is slower, and generally requires 2 days of incubation instead of 1 day as with coliphages.

Test procedure

1. Steam the required number of test tubes with top agar to liquefy agar and adjust to 48 °C in a heating block.
2. Add 1,0 ml of the host culture to the top agar in each tube.
3. Add 1,0 ml of the test sample, or an appropriate dilution of the test sample, to the top agar in each tube.
4. Pour the top agar mixture with minimum delay onto the bottom agar layer in a 90 mm phage agar plate.
5. Repeat the above in tenfold to obtain counts per 10 ml. If tenfold dilutions are required, three plates should preferably be used for each dilution to obtain meaningful results.
6. Incubate inverted plates anaerobically at 35-37°C for 2 days and count plaques.

Materials

Growth medium (BPRM)

Tryptone	(Difco)	10,0 g
Peptone	(Difco)	10,0 g
Yeast extract	(Difco)	2,0 g
NaCl	(Sigma)	5,0 g
$CaCl_2 \cdot 2H_2O$ (0,5% in H_2O)	(Merck)	10,0 ml
$MgSO_4 \cdot 7H_2O$ (1,2% in H_2O)	(Merck)	10,0 ml
L-Cysteine monohydrochloride	(Sigma)	0,5 g
Dist water		955 ml

Dissolve ingredients and autoclave (121°C for 15 min).
Keep autoclaved medium at 60-80°C for further processing.
Add the following:

Glucose (1M in distilled H_2O)	(Sigma)	10,0 ml
Haemin (0,1% in 0,02% NaOH)	(Sigma)	10,0 ml
Na_2CO_3 (1M)		25,0 ml

Adjust pH to 7,0 using concentrated HCl, and add:

Kanamycin sulphate	(Sigma)	final concentration	100 μg/ml
Vancomycin	(Sigma)	final concentration	7,5 μg/ml

Bottom agar

Prepare like growth medium but add 17,0 g of Bacto agar (Difco) and autoclave basic ingredients.
Cool to about 60-80 °C, and add glucose, haemin, Na_2CO_3 and antibiotics as for BPRM.
Pour 20 ml volumes into 90mm diameter petri dishes.
Store plates at 4±2 °C: Maximum 10 days.

Top agar

Same as bottom agar, except for 7,0 g instead of 17,0 g agar (depending on the gel strength of the agar used, the concentration of the agar may have to be adjusted to obtain a suitable top layer).
Distribute 2,5 ml aliquots into test tubes with caps.
Store at 4±2 °C: Maximum 10 days.

Host culture

Bacteroides fragilis strain HSP40.
Reference: ATCC 51477.
Streak reference ATCC or stock culture on bottom agar for single colonies.
Incubate anaerobically at 35-37 °C for 2 days.

Stock cultures:

Inoculate 50 ml of BPRM medium with 3 typical colonies.
Incubate anaerobically at 35-37 °C for 2 days.
Add 10 ml of glycerol (870 g/l) and mix well.
Distribute to 1,5 or 2,0 ml plastic vials (Eppendorf).
Fill vials to top and seal tightly to promote anaerobic conditions.
Store at -70±10 °C: maximum 2 years.
Regularly check the host for the following properties:

Pale colonies, pleomorphic or uniform Gram-negative rods with irregular staining.
Susceptible to type specific phage B40-8.
Growth stricly anaerobic.

Working culture:
Thaw one vial of stock culture and streak for single colonies on bottom agar.
Incubate anaerobically at 37±1 °C for 2 days.
Store at 4±2 °C: maximum 7 days.
Inoculum culture:
Pick 3 typical colonies of *B fragilis* HSP40 from working culture plate and inoculate required volume of BPRM broth, ie 50 ml.
Incubate anaerobically at 37±1 °C for 2 days.

Notes

1. Tests to be carried out according to basic principles outlined in:
1.1. Grabow W O K, Holtzhausen C S and de Villiers C J (1993) Research on Bacteriophages as Indicators of Water Quality. WRC Report No 321/1/93. Water Research Commission, Pretoria. 147 pp.
1.2. Tartera C, Araujo R, Michel T and Jofre J (1992) Culture and decontamination methods affecting enumeration of phages infecting *Bacteroides fragilis* in sewage. Appl Environ Microbiol 58, 2670-2673.
2. Success of the method heavily depends upon the extent to which strict anaerobic conditions are maintained during incubation, as well as the extent to which exposure of host cultures, growth media and test samples to oxygen is restricted. Application of the commercial BBL Gaspak Anaerobic System is recommended. However, similar or superior results may be obtained by alternative systems. Ideally, the plaque assays should be carried out in anaerobic inoculation cabinets, and oxygen should be removed from test samples.
3. A heating block should be used for tubes with top agar instead of a waterbath if possible in order to avoid contamination by phages in waterbath water.
4. Always use distilled water for the preparation of media and reagents.
5. Autoclaving = 121°C/15 min.
6. Names of commercial media and reagents are mentioned merely as a guide. Alternative products may yield similar results. Analytical grade reagents are required only when proven to yield results superior to those obtained by standard grade reagents.

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Direct Plaque Assay for Somatic Coliphages in 100 ml Water Samples
using
Large Petri Dishes with Double Agar Layer (LP-DL)

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Principle

1. Intended for the enumeration of small numbers of somatic coliphages in 100 ml water samples.
2. Intended for water with low numbers of phages such as treated drinking water or environmental water with limited pollution.
3. Based on the addition of cations, host nutrients, nalidixic acid (if necessary), and host inoculum to 100 ml of water, mixing and pouring in equal volumes into 140 mm petri dishes with bottom agar, and counting of plaques after overnight incubation.
4. The procedure could be adapted to accommodate other volumes of water, but tends to become impractical for volumes of more than 100 ml in terms of labour and cost. There would, however, not seem to be meaningful need for direct plaque assays for somatic coliphages in volumes of water in access of 100 ml.

Test procedure

1. Steam a bottle of PAC to liquefy agar and adjust temperature to 48 °C (ie water bath).
2. Pour 100 ml of water to be tested into a convenient sterile container, eg 200 ml screw cap medical flat.
3. Add 1,0 ml Ca solution.
4. If interference by microbial growth is expected, add 1,0 ml of NA solution.
5. Adjust temperature of water sample to 35-37 °C (ie water bath or incubator).
6. Add 5,0 ml of host culture to water sample, using WG4 in the absence of NA, and WG5 in the presence of NA.
7. Keep inoculated water sample for about 3 min at 35-37 °C.
8. Add inoculated water sample gently to the bottle of PAC; mix gently to avoid the formation of bubbles by turning bottle end over end once.
9. Pour mixture swiftly but gently in equal volumes into ten 140 mm diameter plastic petri dishes with bottom layer and let solidify with lids partly open.
10. Incubate inverted plates overnight at 35-37 °C and count plaques.

Materials

PAC (Phage Agar Concentrate)

Meat extract powder (Lab Lemco, Oxoid)	14,0 g
Yeast Extract Powder (Oxoid)	4,0 g
NaCl	4,0 g
Peptone (Difco)	12,0 g
Na_2CO_3	1,0 g
$MgCl_2.6H_2O$	1,0 g
Agar (Bacto)	14,0 g
Dist water	1000 ml

Heat to dissolve agar.
Adjust pH to 7,2.
Dispense 100 ml quantities into suitable bottles, eg, 250 ml screw cap medical flats.
Autoclave.
Store at 4±2 °C: maximum 4 months.

Bottom agar

Agar (Bacto, Difco)	14,0 g
Tryptone (Bacto, Difco)	13,0 g
NaCl	8,0 g
Glucose	1,5 g
Na_2CO_3	1,0 g
Dist water	1000 ml

Autoclave.
Cool to 45-50 °C and pour 30 ml into each of 140 mm diameter petri dishes. Allow to solidify.
Store at 4±2 °C: maximum 2 months.

Ca solution

$CaCl_2.2H_2O$	13,0 g
Dist water	100 ml

Autoclave.
Store at 4 °C or room temperature: maximum 4 months.

NA solution

Nalidixic acid (Sigma)	0,7 g
Sterile dist water	20 ml

If the nalidixic acid does not dissolve directly in water, it can first be dissolved in 5 ml 1M NaOH and then added to 15 ml water.
Decontaminate by filtration using a 0,22 μm pore size membrane.
Store at 4±2 °C: maximum 2 weeks.

Host culture

Escherichia coli strain C (ATCC 13706), nalidixic acid sensitive = WG4
Nalidixic acid resistant mutant of WG4 = WG5
Inoculum:
Inoculate colony from stock agar plate or slant into 50 ml nutrient broth (Difco).
Incubate at 35-37 °C with shaking for 4-5 h to obtain cultures in the logarithmic growth phase.

Notes

1. Tests to be carried out according to basic principles outlined in:

1.1. Grabow W O K, Holtzhausen C S and de Villiers C J (1993) Research on Bacteriophages as Indicators of Water Quality. WRC Report No 321/1/93. Water Research Commission, Pretoria. 147 pp.

1.2. ISO 10705-1:1995. Water Quality - Detection and Enumeration of Bacteriophages. Part 1: Enumeration of F-specific RNA bacteriophages. International Organization for Standardization, Geneva. 15 pp.

1.3. ISO/DIS 10705-2:1997. Water Quality - Detection and Enumeration of Bacteriophages. Part 2: Enumeration of somatic coliphages. International Organization for Standardization, Geneva. 17 pp.

2. This double agar layer plaque assay using 140 mm petri dishes (LP-DL), tends to yield slightly lower counts than conventional double agar layer plaque assays using 90 mm petri dishes (SP-DL) in assays on waters which contain relatively high numbers of somatic coliphages. Therefore, LP-DL is recommended only for assays on waters which contain numbers of phages below the reliable detection limit of SP-DL. This implies that LP-DL would primarily be used for treated drinking water and environmental waters with low levels of sewage pollution. SP-DL would primarily be used for waste water and environmental waters with relatively heavy faecal pollution.

3. Commercial products other than those mentioned may yield similar results. Analytical grade reagents are recommended only if evidence is available that they yield results superior to those obtained by using standard grade reagents from reliable manufacturers.

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Somatic Coliphages, F-RNA Coliphages and *Bacteroides fragilis* HSP40 Phages

Detection by

Qualitative Presence-Absence Tests on 500 ml Samples of Water

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Principle

These qualitative tests are intended for the detection of low numbers of phages in 500 ml samples of water. This would include routine monitoring of treated drinking water. The procedure is based on:

1. Neutralise chlorine residuals in 500 ml samples of test water.
2. Add nutrients for cultivation of the appropriate hosts to the water samples.
3. Add cations to promote adsorption of phages to host bacteria.
4. Add antibiotics to select for bacterial host concerned.
5. Add inoculum of host bacteria in broth culture.
6. Incubate overnight at 35-37°C.
7. Test for presence of phages by conventional plaque assay or qualitative spot test.

Test procedures

The following procedures are for testing 500 ml samples. Theoretically any volume of water can be tested by corresponding adjustment of nutrients and inocula.

Chlorine residuals in test samples of treated drinking water may be neutralised by means of sodium thiosulphate. The addition of 1 ml of a 1 % solution of thiosulphate in water, to 500 ml samples of conventional treated drinking water supplies, is generally sufficient. However, this should be confirmed by testing samples for chlorine residuals.

1. Somatic coliphages and F-RNA coliphages

1.1. Weigh the following nutrients into an appropriate container, ie 500-1000 ml glass flask:

Trypticase peptone (BBL)	6,0 g
Yeast extract (Difco)	0,5 g
NaCl	4,0 g

1.2. Add test water 500 ml

1.3. Add Calcium-glucose solution 1,0 ml

1.4 Add nalidixic acid stock solution:

Somatic coliphages	(Final concentration 250 μg/ml)	5,0 ml
F-RNA coliphages	(Final concentration 100 μg/ml)	2,0 ml

1.5. Add inoculum culture of host 3,0 ml

1.6. Incubate overnight (20±2 h) at 35-37°C

1.7. Test for presence of somatic or F-RNA coliphages using plaque assay (tenfold dilutions: 0 to at least -8) or spot test.

2. *Bacteroides fragilis* HSP40 phages

Add the following to a suitable container, ie 500 ml glass bottle with screw cap:

Tryptone	(Difco)	5,0 g
Peptone	(Difco)	5,0 g
Yeast extract	(Difco)	1,0 g
NaCl	(Sigma)	2,5 g
$CaCl_2 \cdot 2H_2O$ (0,5% in H_2O)	(Merck)	5,0 ml
$MgSO_4 \cdot 7H_2O$ (1,2% in H_2O)	(Merck)	5,0 ml
L-Cysteine monohydrochloride	(Sigma)	0,3 g
Glucose (1M in distilled H_2O)	(Sigma)	5,0 ml
Haemin (0,1% in 0,02% NaOH)	(Sigma)	5,0 ml
Na_2CO_3 (1M)		13,0 ml
Test water sample		400 ml

Mix thoroughly and adjust pH to 7,0 using concentrated HCl.

Add the following:

Kanamycin sulphate	(Sigma)	final concentration	100 μg/ml
Vancomycin	(Sigma)	final concentration	7,5 μg/ml
Inoculum culture of host			60 ml

Mix thoroughly, fill bottle to top with test water.

Put parafilm over mouth of bottle and screw cap on tightly to promote anaerobic condition.

Incubate overnight (20±2 h) at 35-37°C.

Test for presence of *B fragilis* HSP40 phages using plaque assays on 0 to at least -8 tenfold dilutions.

Materials

Host cultures:

Somatic coliphages:

Escherichia coli strain C (WG4, Havelaar): Nalidixic acid sensitive.

Reference: ATCC 13706.

Escherichia coli WG5 (Havelaar): Nalidixic acid resistant mutant of *E coli* strain C

F-RNA coliphages:

Salmonella typhimurium WG49 (Havelaar):

Salmonella typhimurium strain WG49 phage type 3 Nal^r (F'lac: :Tn5)

Reference: NCTC 12484

Inoculum cultures of WG4 and WG5 for somatic coliphages

Inoculate 3-5 typical colonies from a stock agar plate, or a loopful of growth from a stock agar slant, into nutrient broth (Difco) or TYGB and incubate at 35-37 °C for 4-5 h with shaking.

Inoculum culture of WG49 for F-RNA phages

1. **Working culture**

   Thaw one vial of stock culture and streak for single colonies on selective lactose-containing agar medium such as McConkey.
   Incubate at 37±1 °C for 18±2 h.
   Pick 3-5 typical lactose-positive colonies and add to 50 ml TYGB in a conical flask (about 300 ml).
   Incubate at 37±1 °C for 5±1 h while shaking at 100±10/min.
   Add 10 ml of glycerol (870 g/l) and mix well.
   Distribute 1,2 ml aliquots into plastic vials (Eppendorf).
   Store at -70±10 °C: maximum 2 years.

2. **Inoculum culture**

   Add 0,5 ml of working culture thawed at room temp to 50 ml of TYGB in a conical flask (about 300 ml) or nephelometric conical flask with side arm for optical density reading.
   Incubate at 37±1 °C while shaking at 100±10/min.
   Measure turbidity every 30 min using a spectrophotometer.
   When a turbidity equivalent to approximately 10^8 colony forming units (cfu) per ml has been reached*, cool culture quickly on melting ice and use within 2 h.

   * The required density of host cells is generally reached at an optical density (OD) reading of 0,8-1,0 after 2-4 h incubation. However, this is variable and a growth curve has to be prepared for each batch of working cultures using procedures described in detail (ISO, 1995).

Inoculum culture of *Bacteroides fragilis* HSP40 host

Host culture

Bacteroides fragilis strain HSP40.
Reference: ATCC 51477.
Streak reference ATCC or stock culture on bottom agar for single colonies.
Incubate anaerobically at 35-37 °C for 2 days.

Stock cultures:

Inoculate 50 ml of BPRM medium with 3 typical colonies.
Incubate anaerobically at 35-37 °C for 2 days.
Add 10 ml of glycerol (870 g/l) and mix well.
Distribute to 1,5 or 2,0 ml plastic vials (Eppendorf).
Fill vials to top and seal tightly to promote anaerobic conditions.
Store at -70±10 °C: maximum 2 years.
Regularly check the host for the following properties:

   Pale colonies, pleomorphic or uniform Gram-negative rods with irregular staining.
   Susceptible to type specific phage B40-8.
   Growth strictly anaerobic.

Working culture:

Thaw one vial of stock culture and streak for single colonies on bottom agar.
Incubate anaerobically at 37±1 °C for 2 days.
Store at 4±2 °C: maximum 7 days.

Inoculum culture:

Pick 3 typical colonies of *B fragilis* HSP40 from working culture plate and inoculate required volume of BPRM broth, ie 100 ml.
Incubate anaerobically at 37±1 °C for 2 days.

Calcium-glucose solution

$CaCl_2 \cdot 2H_2O$	(Sigma)	3,0 g
Glucose	(Sigma)	10,0 g
Dist water		100 ml

Dissolve with gentle heating if necessary
Decontaminate by filtration: 0,22 μm membrane
Store at 4 °C: maximum 6 months

Nalidixic acid stock solution

Nalidixic acid	(Sigma)	2500 mg
NaOH (1M)		2,0 ml
Dist water		98,0 ml

Dissolve nalidixic acid in NaOH
Add water
Decontaminate by filtration: 0,22 μm membrane
Store at 4 °C: maximum 2 weeks

Tryptone - Yeast extract - Glucose - Broth (TYGB)

1. **Basal medium**

Trypticase peptone	(BBL)	10,0 g
Yeast extract	(Difco)	1,0 g
NaCl	(Sigma)	8,0 g
Dist water		1000 ml

Dissolve with gentle heating if necessary.
Adjust pH to be 7,2$\pm$0,1 at 25 °C after autoclaving.
Distribute 200 ml volumes into bottles and autoclave (121$\pm$1 °C for 15 min).
Store at 4$\pm$2 °C in dark: maximum 6 months.

2. **Complete medium**

Basal medium	200 ml
Calcium-glucose solution	2,0 ml

Add and mix aseptically
Store at 4$\pm$2 °C in dark: maximum 6 months.

Growth medium (BPRM)

Tryptone	(Difco)	10,0 g
Peptone	(Difco)	10,0 g
Yeast extract	(Difco)	2,0 g
NaCl	(Sigma)	5,0 g
$CaCl_2 \cdot 2H_2O$ (0,5% in H_2O)	(Merck)	10,0 ml
$MgSO_4 \cdot 7H_2O$ (1,2% in H_2O)	(Merck)	10,0 ml
L-Cysteine monohydrochloride	(Sigma)	0,5 g
Dist water		955 ml

Dissolve ingredients and autoclave (121°C for 15 min).
Keep autoclaved medium at 60-80°C for further processing.
Add the following:

Glucose (1M in distilled H_2O)	(Sigma)	10,0 ml
Haemin (0,1% in 0,02% NaOH)	(Sigma)	10,0 ml
Na_2CO_3 (1M)		25,0 ml

Adjust pH to 7,0 using concentrated HCl, and add:

Kanamycin sulphate	(Sigma)	final concentration	100 μg/ml
Vancomycin	(Sigma)	final concentration	7,5 μg/ml

Notes

1. Tests to be carried out according to basic principles outlined in:

1.1. Grabow W O K, Holtzhausen C S and de Villiers C J (1993) Research on Bacteriophages as Indicators of Water Quality. WRC Report No 321/1/93. Water Research Commission, Pretoria. 147 pp.

1.2. ISO (1995) ISO 10705-1:1995, Water quality - Detection and enumeration of bacteriophages - Part 1: Enumeration of F-specific RNA bacteriophages. International Organization for Standardization, Geneva. 11 pp.

1.3. ISO (1997) ISO/DIS 10705-2, Water quality - Detection and enumeration of bacteriophages. Part 2: Enumeration of somatic coliphages. International Organization for Standardization, Geneva. 17 pp.

1.4. Tartera C, Araujo R, Michel T and Jofre J (1992) Culture and decontamination methods affecting enumeration of phages infecting *Bacteroides fragilis* in sewage. Appl Environ Microbiol 58, 2670-2673.

2. The P/A test is extremely sensitive to contamination. A single phage contaminant will yield a false positive result. Therefore, the utmost care must be taken to avoid contamination. Water baths should be used with special caution because these are often sources of phage contamination.

3. Commercial names of media and chemicals are mentioned merely as guide. Any alternative media or chemicals which yield equivalent results are acceptable. Analytical grade reagents are required only when standard grade reagents have been proved inferior.

4. The addition of nalidixic acid is required only when the bacterial flora of the water sample may interfere with replication of the host inoculum, multiplication of phages, or subsequent detection of phages.

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Appendix 2

Phages excreted by humans and animals

Pergamon

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BACTEROIDES FRAGILIS AND *ESCHERICHIA COLI* BACTERIOPHAGES: EXCRETION BY HUMANS AND ANIMALS

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ABSTRACT

The faecal excretion of somatic and male-specific coliphages, and phages of *Bacteroides fragilis* strain HSP40, by humans and a variety of animals has been investigated as part of research on indicator features of phages. Ninety human stool specimens were obtained from black and white male and female individuals varying in age from 6 months to 85 years. Sixty-five faecal samples from domestic animals including cattle, sheep, pigs, horses, dogs, cats, geese and rabbits, were obtained from various agricultural and domestic sources. The National Zoological Gardens in Pretoria kindly supplied 38 stool specimens from higher primates (gorilla, orang-outang and chimpanzee). Thirty-seven stool specimens from chacma baboons and vervet monkeys were obtained from the Zoological Gardens and our animal research centre. Five specimens of seabird droppings were obtained from the west coast of South Africa. The qualitative presence of phages was determined by an enrichment procedure followed by a plaque spot test. Double agar layer plaque assays were used to titrate phages. *Bacteroides fragilis* phages were detected in 13% of human stool samples, but not in any animal faeces. Somatic coliphages were detected in 54% of human, 56% of domestic animal, 57% of monkey and baboon, 53% of higher primate, and 60% of seabird specimens. Male-specific coliphages were detected in 26% of human, 90% of domestic animal, 76% of monkey and baboon, 63% of higher primate, and 20% of seagull faecal samples. Titres of phages in selected samples varied from undetectable by direct plaque assay to 4.5×10^6 somatic and 3.2×10^4 male-specific coliphages per gram of seabird droppings. Faecally polluted environments may, therefore, contain substantial numbers of somatic and male-specific coliphages of human and animal origin. The results confirm earlier observations that *B fragilis* phages can be used to distinguish between faecal pollution of human and animal origin.

KEYWORDS

Bacteriophages, *Bacteroides fragilis*, *Escherichia coli*, humans, animals, indicators

INTRODUCTION

Bacteriophages (phages) are viruses which infect bacteria. These bacterial viruses share properties with human viruses in terms of structure, composition, size and morphology. In view of this resemblance some phages have attractive features for application as indicators or models of human viruses (IAWPRC Study Group on Health Related Water Microbiology, 1991; Grabow *et al*, 1993). An important indicator feature of phages is that many of them are detectable by simple, rapid and inexpensive techniques. In addition, phages constitute no human health risk. Since phages can, like human viruses, only multiply in specific metabolically active host cells, the incidence in the environment of phages which infect bacteria such as *Escherichia coli* (coliphages), is as specific for faecal pollution as the host cells. A group of coliphages known as somatic coliphages is, therefore, widely being used as indicator of water quality and the potential presence of human viruses. These phages occur in sewage-polluted environments in numbers which usually exceed those of cytopathogenic human enteric viruses by several orders of magnitude. In addition, coliphages tend to be at least as resistant to unfavourable environmental conditions as human viruses.

One indicator shortcoming of somatic coliphages is that at least some of them can also infect bacteria related to *E coli*. Some of these hosts are metabolically active in certain water environments to the extent that they can multiply and support the replication of somatic coliphages. This implies that the presence and numbers of somatic coliphages in water environments are neither qualitatively nor quantitatively related to faecal pollution. Another group of coliphages known as male-specific or F-specific RNA coliphages can only infect hosts with sex fimbriae (pili) coded for by the fertility (F) genetic factor. These fimbriae, which carry the receptor sites for male-specific phages, are produced only at temperatures above about 30°C. Since sewage or waters in the open environment rarely reach this temperature, possibilities for male-specific coliphages to multiply in the environment are negligible which implies that these phages are specific indicators of faecal pollution. Another important indicator feature of male-specific coliphages, such as the type-specific phage MS2, is that their size and structure very closely resemble that of typical enteroviruses like poliovirus, and they even have a single-stranded RNA genome like enteroviruses. In view of these features male-specific coliphages are rapidly gaining ground as water quality indicators, and the International Organization for Standardization has formulated a standard procedure for their detection and enumeration (ISO, 1993).

Phages which infect *Bacteroides fragilis* bacteria also have attractive indicator features. These bacteria are strictly anaerobic and only multiply in the gastrointestinal tract of warm-blooded animals (where they outnumber *E coli* by 100-fold or more), which implies that the phages are specific for faecal pollution. In an elaborate search for the best possible host strain for these phages, Tartera and Jofre (1987) discovered that one particular strain designated *B fragilis* HSP40, only detected phages in 10% of 40 human stool specimens, and not in faecal specimens from 40 cows, 50 pigs, 21 rabbits, 28 mice, 20 hens or 10 quail. This suggested that *B fragilis* HSP40 phages were highly specific indicators of human faecal pollution, which, among other things, implied that the phages could be used to distinguish between faecal pollution of human and animal origin.

The ability to distinguish between faecal pollution of human and animal origin has valuable benefits for water quality assessment and control (Jagals *et al*, 1995). The objective of this study was, therefore, to confirm the specificity of *B fragilis* HSP40 by testing excreta from humans and animals in another part of the world, and screening

animals not previously tested, including higher primates closely related to humans. The stool specimens were also screened for somatic coliphages, and male-specific coliphages using two host strains, in order to compare their incidence to that of *B fragilis* HSP40 phages, to study their incidence in excreta in a part of the world not previously investigated, and to investigate their incidence in the excreta of animals not previously studied. This information is essential for assessing the value of all these phages as indicators of faecal pollution.

MATERIALS AND METHODS

Stool specimens

Human stool specimens were obtained at our two teaching hospitals and a private pathology firm, all in Pretoria, from black and white male and female individuals. Stool specimens from domestic animals (cattle, sheep, pigs, horses, dogs, cats, geese and rabbits) were obtained from various farms, private homes and the National Zoological Gardens in Pretoria. Stool specimens from chacma baboons (*Papio ursinus*) and vervet monkeys (*Cercopithecus aethiops*) were collected at our animal research centre and the National Zoological Gardens. Stool specimens from higher primates (gorilla, orang-outang and chimpanzee) were kindly supplied by the National Zoological Gardens. Specimens of seabird droppings were collected from mariculture shellfish rafts on the West coast of South Africa, and were predominantly from cape cormorant (*Phalacrocorax capensis*), southern blackbacked gull (*Larus dominicanus*), Hartlaub's gull (*Larus hartlaubii*) and related species of seabirds. Human stool specimens were from different individuals. Specimens from animals were also from different individuals, except for the higher primates of which only limited numbers were available. In the case of these animals, specimens were collected from the same individual not more than once a week, and half the batch was collected 10 months after the other, in order to cover natural changes in intestinal flora. Apart from the seabird droppings, all human and animal stool specimens were collected in and near Pretoria. Fresh specimens of faeces and droppings were collected in sterile MacCartney bottles. Specimens were tested within 5 h of collection or frozen at -20°C until examination.

Bacterial host strains

Somatic coliphages:
Escherichia coli strain C (ATCC 13706) (WG5)
kindly donated by AH Havelaar (Grabow *et al*, 1993)
Male-specific coliphages:
1. *Salmonella typhimurium* strain 3 Nal^r (Flac::Tn5) (WG49) (NCTC 12484)
   kindly donated by AH Havelaar (ISO, 1993)
2. *Escherichia coli* strain HS(pFamp)R
   kindly donated by VJ Cabelli (Debartolomeis and Cabelli, 1991)

***Bacteroides fragilis* HSP40 phages:**
Bacteroides fragilis strain HSP40
from our own culture collection (Tartera and Jofre, 1987)

Enrichment procedure

Stool specimens (1 g wet mass) were incubated in 22 ml volumes of growth medium inoculated with 3 ml quantities of host cultures in the exponential growth phase. Separate enrichments were carried out for each host, using the growth medium specified for the host (Debartolomeis and Cabelli, 1991; Grabow *et al*, 1993; ISO, 1993; Tartera and Jofre, 1987). Enrichment cultures were incubated at 37°C for 24 h, *B fragilis*

anaerobically and the rest aerobically. The qualitative presence of phages in the enrichment cultures was then tested by means of a spot test.

Spot test

Enrichment cultures (1 ml) were decontaminated by adding 0.4 ml chloroform, brief vortexing, leaving on the bench for 10 min, and centrifugation at 7000 rpm for 5 min. Plates for spot tests for each host were prepared as for double agar layer plaque assays except that no test suspension was added to the top layer. Plates were left on the bench with lids removed for about 60 min to allow excess surface moisture of the seeded overlay to dry. A sterile pasteur pipette was then used to spot one drop from each decontaminated enrichment culture onto the top agar of a plate seeded with the corresponding host culture. Not more than three evenly distributed spots were placed on each plate, and allowed to dry. Plates were then incubated at 37°C for 24 h, *B fragilis* anaerobically and the rest aerobically. The presence of phages in the spot was visible from lysis of the host bacteria which caused clearly visible plaques, generally confluent. Phages present in positive enrichment cultures were further investigated by plaque assays. Individual plaques were picked for purification and propagation. Phages which yielded plaques on one or more of the two hosts for male-specific coliphages, were tested for ability to produce plaques at both 25°C and 37°C. Phages which produced plaques at 25°C were not considered male-specific coliphages (Debartolomeis and Cabelli, 1991) and discarded.

Double agar layer plaque assays

These assays were basically carried out in triplicate on tenfold physiological saline dilutions of test samples as described by Grabow and Coubrough (1986) using logarithmic phase cultures of hosts. Growth and plating media used for each host were as previously described: *E coli* C (Grabow and Coubrough, 1986), *S typhimurium* WG49 (ISO, 1993), *E coli* HS(pFamp)R (Debartolomeis and Cabelli, 1991), *B fragilis* HSP40 (Tartera and Jofre, 1987).

RESULTS

The 90 human stool specimens were from 62 white and 28 black individuals, 48 males and 42 females, varying in age from 6 months to 85 years. Of these stool donors 48 suffered from gastroenteritis, diarrhoea, dysentery or related intestinal infections, while 42 were not known to have any intestinal disorders. The results summarised in Table 1 did not show any meaningful relationship between the incidence of phages and the variables of ethnic group, age, sex or intestinal illness.

The results summarised in Table 1 show that the incidence of somatic coliphages in human stools (54%) was similar to that of higher primates (53%) and primates (57%), but lower than that of domestic animals (70%) and higher than that of birds (48%). The incidence of male-specific coliphages as determined by means of the host strain *E coli* HS(pFamp)R, was lower in human stool specimens (26%) than in those of higher primates (63%), primates (76%), domestic animals (60%) and birds (36%). The incidence of phages in different animals varied. For instance, somatic coliphages were present in 100% of specimens from pigs and cats, but only in 38% of gorilla and rabbit specimens. The incidence of male-specific coliphages displayed similar variation.

An outstanding feature of the results is that *B fragilis* HSP40 phages were detected only in 13% of human stool specimens and not in excreta of any animals or birds.

Table 1. Qualitative detection of somatic coliphages, male-specific coliphages and *Bacteroides fragilis* phages in stool specimens of humans and animals

Specimens	n	Percent positive faecal specimens		
		Somatic	Male-specific	*B fragilis*
Human	90	54	26	13
Higher primates				
Gorilla	16	38	63	0
Chimpanzee	15	40	47	0
Orang-outang	12	92	83	0
Total	43	53	63	0
Primates				
Chacma baboon	18	61	67	0
Vervet monkey	19	53	84	0
Total	37	57	76	0
Domestic animals				
Pig	8	100	50	0
Sheep	10	40	60	0
Horse	7	86	29	0
Cattle	9	78	78	0
Rabbit	8	38	63	0
Dog	4	75	75	0
Cat	4	100	75	0
Total	50	70	60	0
Birds				
Goose	20	45	40	0
Seabird	5	60	20	0
Total	25	48	36	0

The *E coli* host strain HS(pFamp)R was used for the detection of male-specific phages.

Results of comparative tests summarised in Table 2 show that the *E coli* host strain HS(pFamp)R yielded positive results for male-specific coliphages in 62 (57%) and *S typhimurium* WG49 in 31 (29%) of 108 randomly selected human and animal faecal specimens tested. The *E coli* host yielded positive results for 39 specimens for which the *S typhimurium* host yielded negative results, and the *S typhimurium* host yielded positive results for 8 specimens for which the *E coli* host yielded negative results.

Table 2. Male-specific coliphages detected in human and animal faeces using host strains *Escherichia coli* HS(pFamp)R and *Salmonella typhimurium* WG49

Host	Percentage of specimens positive for male-specific coliphages				
	Human n = 2	Higher Primates n = 16	Primates n = 24	Domestic Animals n = 46	Birds n = 20
E coli	50	56	75	57	40
S typhimurium	50	19	58	20	20

Primates = chacma baboons and vervet monkeys; Birds = geese

Titres (plaques per gram wet mass) of phages recorded in typical faecal specimens were:

Somatic coliphages:	Human	= 290 000; 4 600; 4 300; 140
	Orang-outang	= 110 000; 180 000
	Seabird	= 4 500 000
Male-specific coliphages:	Gorilla	= 56
	Chimpanzee	= No plaques detected by direct titration
	Orang-outang	= 130; 46
	Chacma baboon	= 360
	Vervet monkey	= 36; 53
	Seabird	= 32 000

DISCUSSION

This study represents one of the most extensive investigations which has to date been carried out on the incidence of somatic coliphages, male-specific coliphages and *B fragilis* HSP40 phages in human and animal excreta. The incidence of these phages in the excreta of humans and animals in this part of the world, and in the excreta of some of the animals, notably primates (chacma baboon and vervet monkey), higher primates (gorilla, chimpanzee and orang-outang) and seabirds, has not previously been investigated. Although the number of samples tested is not sufficient for statistically significant conclusions on the excretion of each of the groups of phages by humans and individual species of animals, the results do give a valuable indication of tendencies of phage excretion, which casts valuable light on the value of these phages as indicators of faecal pollution.

An important finding is the confirmation of earlier observations (Tartera and Jofre, 1987) that *B fragilis* HSP40 phages are indeed highly specific for human faeces, and that these phages were not even detectable in the excreta of closely related higher primates (Table 1). The incidence of *B fragilis* HSP40 phages in 13% of stool specimens of 90

individuals in and near Pretoria, is close to the 10% reported by Tartera and Jofre (1987) for specimens collected in Barcelona. The marginal difference may partly be due to a more sensitive enrichment procedure used in the present study.

Reasons for the human specificity of *B fragilis* HSP40 phages remain to be elucidated. One possibility may be that the host or hosts of these phages are highly specific for the human intestinal tract. Specificity of certain species or strains of enteric bacteria for either humans or animals is known (Jagals *et al*, 1995). Research on differences between *B fragilis* strain HSP40 and other members of the *Bacteroides fragilis* species which occur in large numbers in the intestinal tract of both humans and animals, may cast light on this phenomenon.

Results on the incidence of phages in stool specimens from man and animals obtained in various studies are difficult to compare due to many variables such as differences in phage host strains and phage detection methods used, statistical fluctuations due to small numbers of samples tested, different populations studied, and differences in diet of study subjects. However, some tendencies would seem apparent. For instance, the incidence of somatic coliphages in the stool of domestic animals (Table 1) which ranged from 38% to 100%, is similar to that reported by Dhillon *et al* (1976), Osawa *et al* (1981) and Havelaar *et al* (1986). The incidence of somatic coliphages in the faeces of primates and birds would seem to fall into the same order (Table 1). The 54% incidence of somatic coliphages in human stool samples (Table 1) is similar to results of Dhillon *et al* (1976) and Havelaar *et al* (1986), but higher than the 24% of Osawa *et al* (1981), and the 1,6% to 14% of Furuse *et al* (1983).

The incidence of up to 75% of male-specific coliphages in domestic animal stool samples (Table 1) is considerably higher than the 0% and 0-33% reported by Dhillon *et al* (1976) and Osawa *et al* (1981), respectively, but would appear to be in agreement with the findings of Havelaar *et al* (1986). The incidence of the phages in primates and birds would seem to be similar to that in domestic animals (Table 1). The 26% incidence of male-specific coliphages in human stool samples is considerably higher than the 0% of Dhillon *et al* (1976) and the low incidence reported by Havelaar *et al* (1986), but similar to the 0-33% of Osawa et al (1981). The sensitive enrichment procedure used in this study for the detection of phages may at least in part account for some of these differences. The absence of a relation between the incidence of both somatic and male-specific coliphages and age, state of health, sex or ethnic groups with differences in diet and lifestyle, is interesting because Furuse *et al* (1983) detected coliphages in 1.6% of stools from healthy individuals and in 14% of those of hospitalised patients.

Reasons for the more frequent detection of male-specific coliphages by means of the *E coli* host HS(pFamp)R than by *S typhimurium* WG49 have not been investigated. The percentage of false positive isolations may be higher with the former host. This difference should be investigated because it has implications for the standardisation of detection methods, and because in our hands the *E coli* host proved more user-friendly (unpublished observations).

The variation in incidence and counts of phages in excreta of humans and animals may be related to the natural fluctuation in the bacterial flora of humans and animals. As the colonisation of a particular bacterial host in the gastrointestinal tract reaches a maximum, the production of its phages reaches a maximum, and as the host disappears, the phages disappear. The high counts of phages in some specimens, notably those of seabirds, would seem to be in agreement with counts of 10^6 to 10^8 per gram of faeces

reported by Dhillon *et al* (1976) and Havelaar *et al* (1986). This and earlier studies do, therefore, show that both somatic and male-specific coliphages are excreted in substantial numbers by many humans and animals, and that *B fragilis* HSP40 phages are excreted only by humans, which adds to evidence supporting their use as indicators.

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Appendix 3

Phages in waste water from an informal settlement

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VIRUSES IN WASTE WATER FROM AN INFORMAL SETTLEMENT

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SUMMARY

Viruses are a major cause of waterborne diseases. Details on viruses in waste water are essential for management strategies aimed at the protection of water sources and the control of waterborne diseases. Virological screening of waste water is also carried out for monitoring enteric viruses circulating in communities, and assessment of public health campaigns such as poliomyelitis vaccination. In this study information has been obtained on viruses in diffuse effluents from a typical low socio-economic informal settlement with restricted sanitation and water supply. Sewage from an adjacent community with a sewerage system has likewise been analysed. The study area was selected as representative of communities which tend to be exceptionally vulnerable to enteric infectious diseases, and waste water from the settlements may be expected to contain high levels of waterborne pathogens. A total of 209 samples collected over a period of 9 months in 1994 were analysed for cytopathogenic enteric viruses and related indicators. Conventional cell culture propagation using three cell types yielded 486 isolates of viruses which consisted of 263 coxsackie B viruses (54%), 109 polioviruses (22%), 101 untyped enteroviruses (21%), 9 adenoviruses (2%), 2 echoviruses (<1%), and 2 reoviruses (<1%). All six types of coxsackieviruses were isolated, but 48% were type B2. All three types of polioviruses were isolated, all of them vaccine strains. Counts of faecal indicators and the incidence of viruses tended to increase after rainfall, which indicates that stormwater run-off was heavily polluted. Even though viruses were not enumerated, the isolation of viruses from 72 % of stream samples and 96% of sewage samples, suggests that the numbers of viruses in the waters were exceptionally high. No viruses were detected in the stream upstream of the settlement. Viruses outnumbered faecal bacteria and phages commonly used as indicators in a substantial number of stream water samples. This confirms the exceptional high incidence of viruses in the waters concerned as well as shortcomings of faecal bacteria and phages commonly used as indicators of water quality. The high incidence of all three vaccine strains of polioviruses in the absence of wild-type strains, confirms the success of poliomyelitis vaccination in the communities concerned. The results show that diffuse effluents from the settlement heavily pollute the stream with viruses and other faecal organisms. This has major implications for risks of infection constituted by the stream. The findings underline the need for efficient sanitary services in communities of this kind in order to protect water sources and to control waterborne diseases.

INTRODUCTION

Viruses are excreted in large numbers by infected individuals and may remain infectious for days, weeks or months in water environments (Grabow, 1996). In addition, the minimum infectious dose of viruses may be as low as a single particle. Viruses are, therefore, a common cause of waterborne disease. Details on the incidence and behaviour of viruses in water environments are limited because their enumeration requires advanced technology, and commonly used faecal bacteria have shortcomings as indicators for viruses. Details on viruses in waste water are essential for health risk assessment and management strategies aimed at the protection of water sources and the control of waterborne diseases. Virological screening of wastewater is also used for other purposes such as the investigation of enteric viruses circulating in communities, and monitoring of public health campaigns such as poliomyelitis vaccination (Tambini *et al*, 1993; Van der Avoort *et al*, 1995). This study focuses on viruses in diffuse effluents from a typical low socio-economic informal settlement with restricted sanitation and water supply. These communities are exceptionally vulnerable to enteric infections (Von Schirnding *et al*, 1993), and waste water effluents are likely to contain high levels of faecal organisms.

MATERIALS AND METHODS

A total of 209 samples were collected over a period of 9 months in 1994 and analysed for enteric viruses and related indicators. These samples were collected at regular intervals from a stream which runs through the settlement and a sewerage pipeline which serves an adjacent community. The stream was sampled about 3 km upstream of the settlement, right in the settlement and about 1 km downstream of the settlement. Cytopathogenic viruses were isolated from unconcentrated samples by conventional cell culture propagation using the BGM monkey kidney and PLC/PRF/5 human liver cell lines, and primary vervet kidney cells (Grabow and Taylor, 1993). Faecal coliform bacteria, enterococci, somatic and male-specific coliphages, and phages infecting *Bacteroides fragilis* HSP40 were enumerated by conventional methods (Grabow, 1996). Viruses were typed by cytopathogenic effect (CPE) in cell cultures and stained cover slips, inoculation of newborn mice, neutralisation tests using the Lim Benyesh-Melnick antiserum pools for enteroviruses, and molecular techniques using routine procedures (Grabow *et al*, 1992). The National Institute for Virology, Johannesburg, kindly rendered assistance in the typing of certain isolates, notably the confirmation of vaccine strains of polioviruses. Adenovirus typing was limited to distinction between enteric and non-enteric types using the commercial adenoclone-type 40/41 immunoassay (Cambridge Biotech, Worcester, MA).

RESULTS

No viruses were detected in samples of water from the stream upstream of the settlement. In these samples counts of faecal coliforms were in the range of 100 to 2800 per 100 ml. Somatic and male-specific coliphages were detected only on rare occasions, and counts were in the range of 0 to 200 per 100 ml. A total of 486 viruses were isolated from 72% of 142 samples of stream water collected in and below the settlement, and from 96% of 53 sewage samples. The isolates consisted of 263 coxsackie B viruses (54%), 109 polioviruses (22%), 9 adenoviruses (2%), 2 echoviruses (0,3%) and 2 reoviruses (0,4%). The remaining isolates consisted of 42 viruses only typed as enteroviruses (9%), and 59 only typed as enteroviruses other than coxsackie- or polioviruses (12%). All six types of coxsackie B viruses were isolated, but 48% were coxsackie B2. All three types of polioviruses were isolated. All the poliovirus isolates were vaccine strains. All 9 adenovirus isolates were non-enteric types. Details on viruses isolated from the stream and sewage samples are jointly summarised in Table 1, because the relative proportions of various viruses isolated from the stream and sewage did not differ significantly. Table 2 compares the percentages of viruses isolated on each of the three cell culture types, which gives an indication of the efficiency of the cells for the isolation of viruses from environmental waters. Many of the viruses only yielded a CPE after two or three blind passages.

Maximum levels of faecal coliforms, enterococci, somatic coliphages and male-specific (F-RNA) coliphages are recorded in Table 3. These results show that maximum levels of enterococci and coliphages in the stream were close to those in sewage. Counts of indicator organisms and the incidence of viruses tended to increase after rainfall, which shows that stormwater run-off contained heavy loads of faecal excreta. The following numbers of samples had counts of faecal bacteria or phages lower than those of viruses: faecal coliforms (2 samples), enterococci (14 samples), somatic coliphages (4 samples), male-specific coliphages (25 samples) and *B fragilis* HSP40 phages (many samples). The latter number of samples is not clearly defined because low counts of phages could at times have been due to malfunctioning of the phage test. Counts of faecal bacteria and phages were lower in the stream at the sampling site 1 km downstream of the settlement. In this area the stream slowly passed through some reed-beds and stagnant patches of water which clearly reduced counts of the organisms. The percentage of samples from which viruses were isolated was, however, the same (72%) for samples collected from the stream inside and downstream of the settlement, suggesting no significant reduction in numbers of viruses.

Table 1. Viruses isolated from 142 samples of polluted stream water and 53 samples of sewage

Virus	Isolates on each cell culture			Total
	PLC	BGM	VK	
Coxsackie B1	9	9	1	19
Coxsackie B2	47	43	37	127
Coxsackie B3	16	10	14	40
Coxsackie B4	16	26	18	60
Coxsackie B5	2	0	1	3
Coxsackie B6	3	5	6	14
Total coxsackie B	93	93	77	263
Polio Sabin 1	39	26	15	80
Polio Sabin 2	6	10	2	18
Polio Sabin 3	6	2	3	11
Total polio	51	38	20	109
Echo 7	0	0	1	1
Echo 27	0	0	1	1
Entero non-cox/polio	22	24	13	59
Entero untyped	10	17	15	42
Adeno	9	0	0	9
Reo	0	0	2	2
Total isolates	185	172	129	486

Samples collected from stream inside settlement and about 1 km downstream.

Entero non-cox/polio = enteroviruses other than coxsackie or polio.

Entero untyped = enteroviruses not typable by available neutralising typing antisera.

Relative composition of viruses isolated from the stream polluted by diffuse effluents from the informal settlement did not differ significantly from that of sewage of an adjacent community.

Table 2. Comparison of three cell culture types for the isolation of cytopathogenic viruses from a polluted stream and sewage in developing communities

Virus	Percentage isolates on each cell culture			Percentage of total
	PLC	BGM	VK	
Coxsackie B1	47	47	6	4
Coxsackie B2	37	34	29	26
Coxsackie B3	40	25	35	9
Coxsackie B4	27	43	30	13
Coxsackie B5	67	0	33	1
Coxsackie B6	21	36	43	3
Total coxsackie B	35	35	30	54
Polio Sabin 1	49	33	18	17
Polio Sabin 2	33	56	11	1
Polio Sabin 3	55	18	27	2
Total polio	41	38	21	22
Echo 7	0	0	100	0,3
Echo 27	0	0	100	0,3
Entero non-cox/polio	37	41	22	12
Entero untyped	24	40	36	9
Adeno	100	0	0	2
Reo	0	0	100	0,4
Total isolates	38	35	27	100

Total number of isolates = 486.

Percentages calculated from numbers of isolates presented in Table 1.

PLC = PLC/PRF/5 human liver cell line.

BGM = Buffalo Green Monkey kidney cell line.

VK = Primary vervet kidney cells.

Table 3. Maximum levels of faecal bacteria and coliphages in the stream and sewage

Indicator	Count/ml	
	Stream	Sewage
Faecal coliforms	733 000	30 000 000
Enterococci	33 300	62 000
Somatic coliphages	20 000	22 500
F-RNA coliphages	1016	1200

DISCUSSION

This study represents the most comprehensive survey of viruses in water recorded to date in South Africa, and probably the most detailed investigation of viruses in diffuse effluents from an informal settlement anywhere in the world. Although viruses have not been enumerated, the high percentage of samples from which viruses were isolated without the application of recovery techniques suggests that the incidence of viruses in the stream downstream of the settlement as well as the sewage were exceptionally high compared with data on the general incidence of viruses in sewage and waste water, according to which numbers of cytopathogenic viruses are generally about 200 to 11 000 per litre, but may exceed 100 000 per litre (WHO Scientific Group, 1979; Dahling *et al*, 1989). The viruses isolated in this study are limited to viruses which cause a cytopathogenic effect in cell cultures. These viruses probably only represent the tip of the iceberg of the total number of viruses in the waters because the great majority of viruses concerned are not detectable by cell culture propagation (Bern and Glass, 1994; Grabow, 1996).

The finding that viruses outnumbered faecal coliforms, enterococci and all three phages in a substantial number of river water samples, confirms the exceptionally high numbers of viruses, as well as shortcomings of commonly used indicators for indicating the presence of viruses. The high proportion of coxsackie B viruses (54% of all isolates, Table 2) is in agreement with results reported for sewage in other parts of the world (Dahling *et al*, 1989). The finding that the three vaccine strains of polioviruses represented 22% of all viruses isolated (Table 2), in the absence of wild type strains, confirms the success of poliomyelitis vaccination campaigns in the communities concerned and indicates that the risk of poliomyelitis in the communities is low.

A total of 101 isolates (Table 1) have only been typed as far as enteroviruses, or enteroviruses other than coxsackie or polio. These isolates probably belong to the wide range of 33 different serological types of echoviruses. This has been confirmed by typing two of the isolates in this group, which turned out to be echovirus types 7 and 27. Typing of these viruses is extremely labour intensive and expensive, and contributes little to the objectives of this particular study. The nine isolates of adenoviruses (Table 1) were likewise not typed any further than confirming that they were not enteric adenoviruses, ie types 40 or 41. These adenoviruses do, therefore, belong to any of the remaining 44 serological types.

The PLC/PRF/5 cell line proved most useful for the isolation of viruses from environmental waters because 38% of viruses were isolated on these cells, compared with 35% on BGM cells and 27% on primary vervet kidney (VK) cells (Table 2). PLC/PRF/5 cells were particularly susceptible to polioviruses, and all 9 adenoviruses were isolated on these cells. The susceptibility of PLC/PRF/5 cells to adenoviruses would seem to be in agreement with their ability to support the replication of enteric adenoviruses (Grabow *et al*, 1992). However, primary vervet kidney cells would seem to be more susceptible to echoviruses and reoviruses than the other two cell types. In combination the data on viruses isolated (Table 2) suggest that ideally all three cell types should be applied in parallel for the isolation of viruses from environmental waters. In a previous comprehensive evaluation of a wide variety of cell culture systems, the three cell culture types used in this study have been selected as the most sensitive for the isolation of viruses from environmental waters (Potgieter and Grabow, 1995). The low number of reoviruses isolated in this study would appear surprising because relatively higher numbers of reoviruses were recorded in studies on other waters (Grabow and Nupen, 1981; Dahling *et al*, 1989). This may imply variation in the incidence of reoviruses because the cell cultures and conditions of viral propagation used in this study are ideally suited for the isolation of reoviruses.

This study shows that diffuse effluents from the settlement heavily polluted the stream with viruses and other faecal organisms. At peak levels of pollution, particularly when stormwater run-off from the settlement entered the stream, counts of enterococci and phages in the stream were close to those in raw sewage (Table 3). These observations have major implications for the downstream utilisation of the water in the stream, because the quality by far exceeded levels considered acceptable for the utilisation of water for domestic purposes, recreation and irrigation. Since the study site was selected as typical of similar situations elsewhere, the results underline the importance of appropriate sanitary services for informal settlements.

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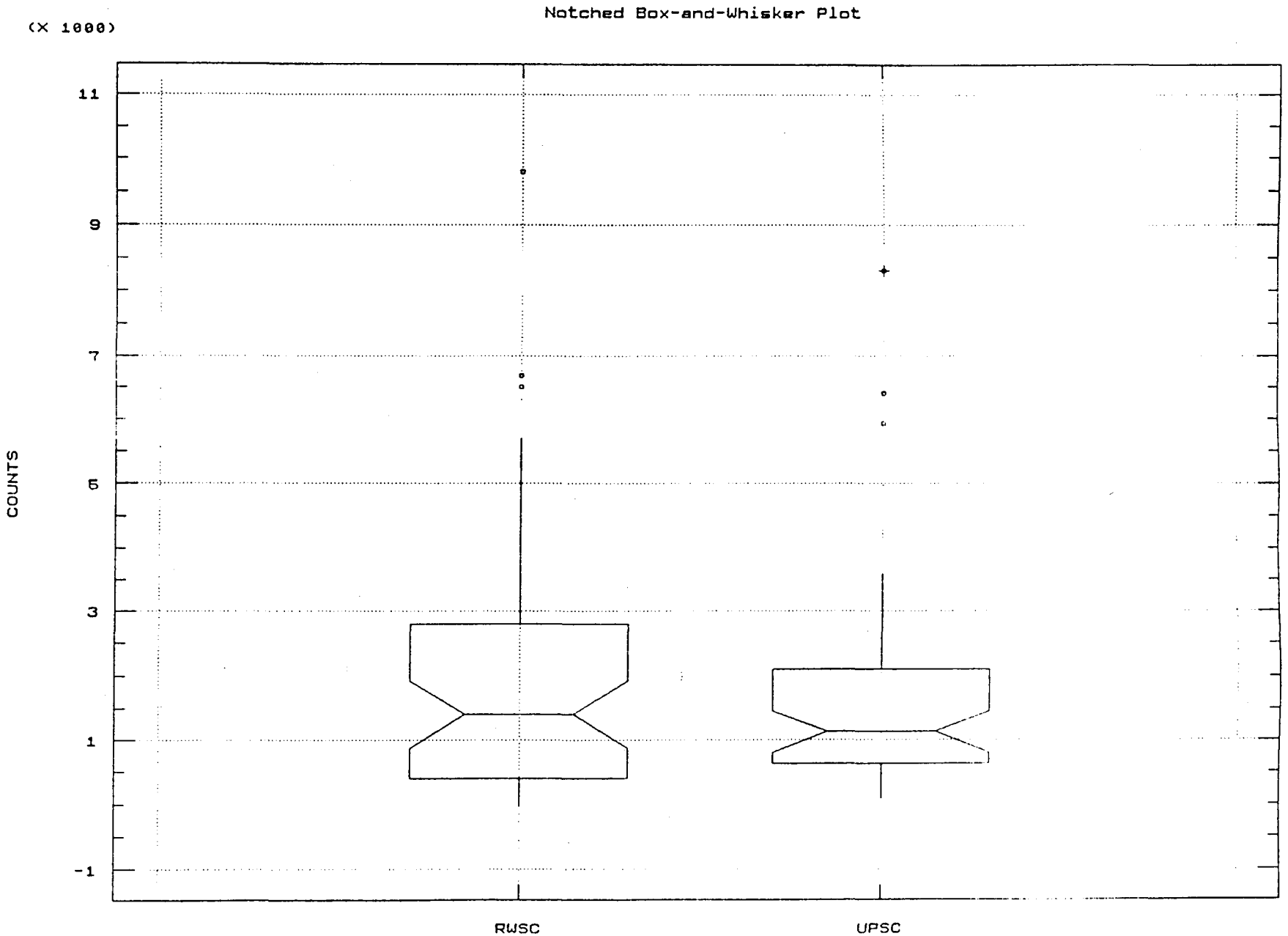
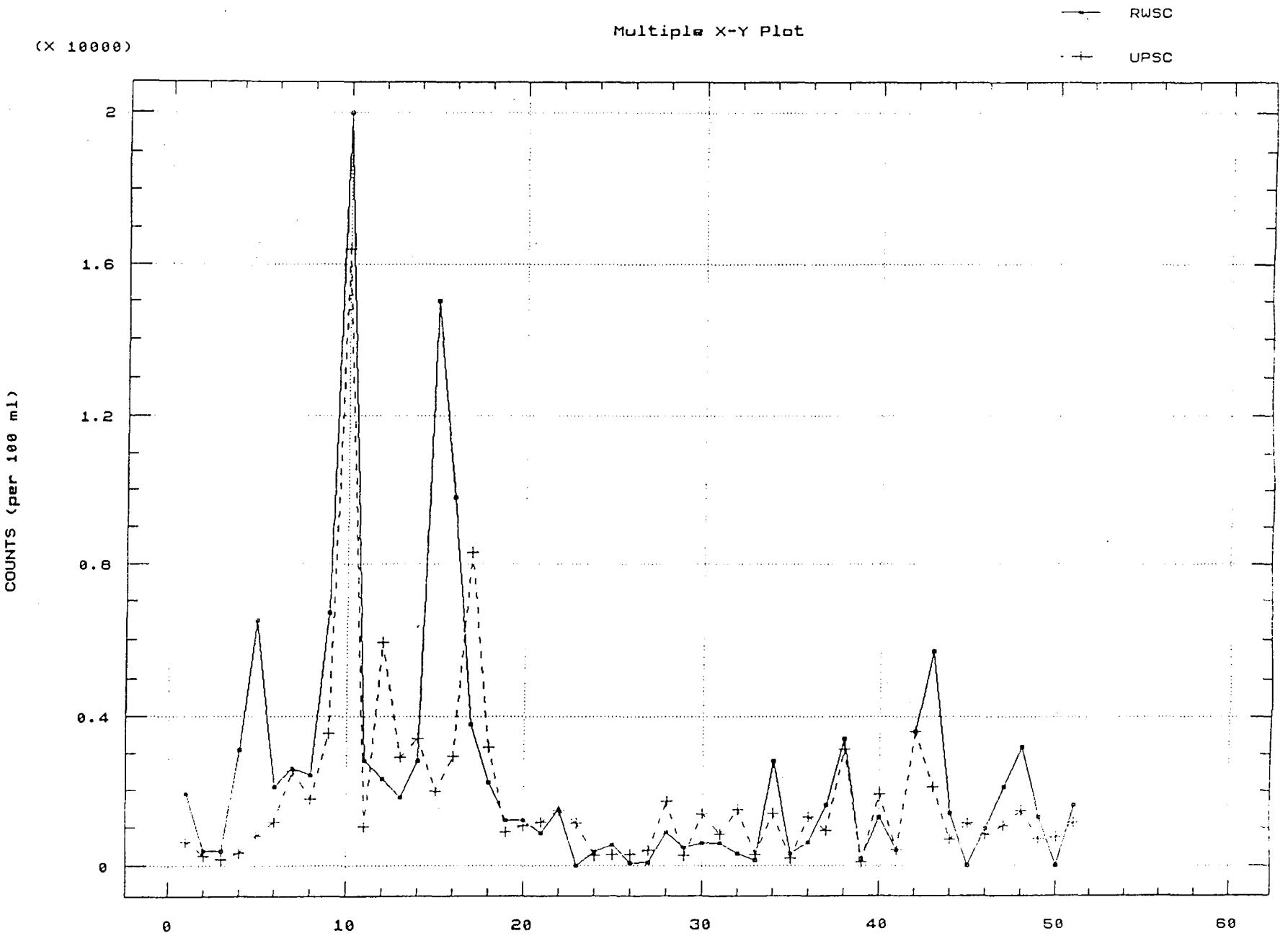


Counts of somatic coliphages (SC) recorded by Rand Water (RW) and the University of Pretoria (UP) laboratories for 51 samples of Klip River (K19) water collected from April 1995 to March 1996



Counts of somatic coliphages (SC) recorded by Rand Water (RW) and the University of Pretoria (UP) laboratories for 51 samples of Klip River (K19) water collected from April 1995 to March 1996

Appendix 4

Phages in Klip River and Vaal Dam water

Table 1. Klip River (K19) water:
Human viruses and related indicators

Date	Count per 100 ml											
	Faecal coliforms		Enterococci	Somatic coliphages			MS phages		*Bact* phages		Viruses	
	RW	UP	UP	RW	UP		UP		UP		UP	
95-04-03	19 000	1000	350	1900	-	633	-	100	A	-	0	
95-04-10	6500	463	230	380	-	267	-	67	A	-	0	
95-04-18	2700	210	70	380	-	167	-	100	A	-	0	
95-04-24	8100	657	120	3100	-	333	-	167	A	-	0	
95-05-02	6000	203	110	6500	-	800	-	233	A	-	Adeno	
95-05-08	42 000	353	180	2100	-	1160	-	630	A	-	0	
95-05-15	7700	1600	133	2600	-	2530	P	0	A	-	0	
95-05-22	1500	403	226	2400	-	1760	-	230	A	-	0	
95-05-29	4200	576	180	6700	-	3560	-	200	A	-	0	
95-06-05	8600	1560	300	20 000	-	16 400	-	5130	P	-	0	

- = not done or results not available
Counts = average of tests in threefold
Presence/Absence tests on 500 ml samples: P = Present, A = Absent
? = test in progress

continued

Date	Count per 100 ml													
	Faecal coliforms		Enterococci	Somatic coliphages			MS phages		*Bact* phages		Viruses			
	RW	UP	UP	RW		UP	UP		UP		UP			
95-06-12	6000	1030	136	2800	-	1030	-	260	A	-	0			
95-06-19	7400	560	50	2300	-	5930	-	960	A	-	0			
95-06-26	3000	446	203	1800	-	2900	-	930	A	-	0			
95-07-03	27 000	2730	166	2800	-	3400	-	2100	A	-	0			
95-07-10	2500	400	103	15 000	-	2960	-	960	A	-	0			
95-07-17	4600	600	203	9800	-	2930	-	2360	A	-	0			
95-07-24	2000	1300	166	3800	-	8300	-	1760	A	-	0			
95-07-31	750	450	150	2200	-	3160	-	1030	A	-	0			
95-08-07	1200	380	156	1200	-	900	-	260	A	-	0			
95-08-14	680	446	170	1200	-	1060	-	600	A	-	0			
95-08-21	4700	2760	636	870	-	1160	-	430	A	-	0			
95-08-28	6100	1200	63	1500	-	1460	-	230	P	-	0			
95-09-04	4900	1130	57	-	-	1130	-	30	A	-	0			
95-09-11	5200	1116	400	370	-	260	-	30	A	-	0			
95-09-18	3600	1830	256	550	-	300	-	60	A	-	0			

continued

Date	Count per 100 ml											
	Faecal coliforms		Enterococci	Somatic coliphages			MS phages		*Bact* phages		Viruses	
	RW	UP	UP	RW		UP	UP		UP		UP	
95-09-26	1000	646	120	80	-	300	-	33	A	-	0	
95-10-02	8300	2500	246	110	-	400	-	33	A	-	0	
95-10-09	3000	1433	427	900	-	1700	-	100	A	-	Reo	
95-10-16	3800	21 600	353	480	-	260	-	100	A	-	0	
95-10-23	3000	1866	1110	600	-	1360	-	430	A	-	0	
95-10-30	190	3660	512	600	-	850	-	67	A	-	0	
95-11-06	2500	1123	330	310	-	1500	-	530	A	-	0	
95-11-13	3200	3500	406	150	-	300	P	0	A	-	0	
95-11-20	7400	3130	1560	2800	-	1400	-	60	A	-	0	
95-11-27	5000	3000	670	320	-	200	-	30	P	-	0	
95-12-04	1300	7630	610	630	-	1300	-	60	A	-	0	
95-12-11	6100	2870	970	1600	-	930	-	100	A	-	0	
95-12-18	13 000	1070	720	3400	-	3130	-	100	A	-	Reo	

continued

Date	Count per 100 ml								
	Faecal coliforms		Streptococci	Somatic coliphages		MS phages		*Bact* phages	Viruses
	RW	UP	UP	RW	UP	UP		UP	UP
96-01-02	820	270	120	180	- 100	-	60	A -	0
96-01-08	7200	-	-	1300	- 1900	-	160	A -	0
96-01-15	2100	-	-	400	- 400	P	0	A -	0
96-01-22	7000	-	-	3600	- 3600	-	60	A -	0
96-01-29	7900	-	-	5700	- 2100	P	0	A -	0
96-02-05	1600	-	-	1400	- 700	P	0	A -	0
96-02-12	-	-	-	-	- 1130	P	0	P 0	Reo
96-02-19	4000	-	-	980	- 830	-	60	A -	Reo
96-02-26	66	-	-	2100	- 1060	P	0	A -	0
96-03-04	4800	-	-	3200	- 1460	-	300	P 0	0
96-03-11	8400	-	-	1300	- 730	-	60	A -	0
96-03-18	26 000	-	-	-	- 760	-	160	A -	Reo
96-03-25	6800	-	-	1600	- 1160	-	100	A -	0
96-04-01	24 000	-	-	6300	- 2630	-	300	A -	Reo
96-04-09	4100	-	-	2600	- 760	-	100	A -	0

continued

Date	Count per 100 ml								
	Faecal coliforms		Streptococci	Somatic coliphages		MS phages	*Bact* phages	Viruses	
	RW	UP	UP	RW	UP	UP	UP	UP	
96-04-15	44 000	-	-	1700	- 700	- 200	A	-	0
96-04-22	9000	-	-	5300	- 2860	- 830	A	-	Reo
96-04-29	8700	-	-	1300	- 1230	- 260	A	-	Reo
96-05-06	4100	-	-	580	- 1560	- 330	A	-	Reo
96-05-13	33 000	-	-	1470	- 930	- 260	A	-	0
96-05-20	10 000	-	-	4200	- 4700	- 1800	A	-	Reo
96-05-27	5800	-	-	7000	- 1530	- 1330	A	-	Reo
96-06-03	1300	-	-	2200	- 2500	- 200	A	-	0
96-06-10	12 000	-	-	1620	- 5260	- 1330	A	-	0
96-06-18	3900	-	-	5300	- 5900	- 830	A	-	Adeno
96-06-24	4200	-	-	1900	- 4060	- 1160	A	-	Reo
96-07-01	4700	-	-	3300	- 5530	- 4800	A	-	Reo
96-07-08	2200	-	-	3100	- 5530	- 800	A	-	0
96-07-15	2900	-	-	1800	- 4300	P 0	A	-	Reo
96-07-22	3600	-	-	3000	- 6030	- 1070	A	-	Reo

continued

Date	Count per 100 ml											
	Faecal coliforms		Streptococci	Somatic coliphages			MS phages		*Bact* phages		Viruses	
	RW	UP	UP	RW		UP	UP		UP		UP	
96-07-29	940	-	-	4600	-	13 400	-	11 800	A	-	Reo	
96-08-05	790	-	-	1700	-	7270	-	2100	A	-	Reo	
96-08-12	1500	-	-	1000	-	2300	-	900	A	-	0	
96-08-19	1400	-	-	2200	-	2000	-	470	A	-	0	
96-08-26	660	-	-	100	-	470	-	170	A	-	0	
96-09-02	1500	-	-	1750	-	2070	-	630	A	-	0	
96-09-09	1400	-	-	580	-	700	-	500	A	-	0	
96-09-16	1500	-	-	3100	-	3600	-	330	A	-	0	
96-09-23	3600	-	-	3600	-	2400	-	1400	A	-	0	
96-09-30	920	-	-	870	-	700	-	130	A	-	0	
96-10-07	6400	-	-	3200	-	1300	-	100	A	-	0	
96-10-14	3200	-	-	610	-	1600	-	900	A	-	0	
96-10-21	600	-	-	2500	-	1300	-	400	A	-	?	
96-10-28	130	-	-	1300	-	1300	-	600	A	-	0	
96-11-04	6200	-	-	1000	-	1100	-	500	A	-	0	

continued

Date	Count per 100 ml											
	Faecal coliforms		Streptococci	Somatic coliphages			MS phages		*Bact* phages		Viruses	
	RW	UP	UP	RW		UP	UP		UP		UP	
96-11-11	2500	-	-	840	-	1600	-	1200	A	-	0	
96-11-18	4200	-	-	2400	-	1300	-	800	A	-	?	
96-11-25	4600	-		1800	-	3500	-	700	A	-	?	
96-12-02	2500	-	-	770	-	800	-	100	A	-	?	
96-12-09	7800	-	-	2700	-	1800	-	1100	A	-	?	
96-12-17	2200	-	-	2690	-	1200	-	400	A	-	?	
97-01-06	-	-	-	-	-	500	-	100	A	-	?	

Table 2. **Vaal Dam sluice gate (A18) water: Human viruses and related indicators**

Date	Count per 100 ml											
	Faecal coliforms		Enterococci	Somatic coliphages			MS phages		*Bact* phages		Viruses	
	RW	UP	UP	RW	UP		UP		UP		UP	
95-07-31	-	51	84	-	P	0	P	0	A	-		0
95-08-07	-	9	30	-	P	0	P	0	A	-		0
95-08-14	-	33	70	-	P	0	P	0	A	-		0
95-08-21	-	32	70	-	P	0	P	0	A	-		0
95-08-28	-	35	72	-	P	0	P	0	A	-		0
95-09-04	-	62	85	-	P	0	P	0	A	-		0
95-09-11	-	12	34	-	P	0	P	0	A	-		0
95-09-18	-	43	129	-	P	0	P	0	A	-		0
95-09-26	-	49	82	-	P	0	P	0	A	-		0
95-10-02	-	40	75	-	P	0	P	0	A	-		0
95-10-09	-	55	59	-	P	0	P	0	A	-		0
95-10-16	-	95	140	-	P	0	P	0	A	-		0
95-10-26	-	153	178	-	P	0	P	0	A	-		0

- = not done or results not available; counts = average of tests in threefold; *Bact* = *Bacteroides fragilis* HSP40 phages;
Presence/Absence tests on 500 ml samples: P = Present, A = Absent, ? = test in progress

continued

Date	Count per 100 ml											
	Faecal coliforms		Enterococci	Somatic coliphages			MS phages		*Bact* phages		Viruses	
	RW	UP	UP	RW	UP		UP		UP		UP	
95-10-30	-	166	165	-	P	0	P	0	A	-		0
95-11-06	-	53	105	-	P	0	P	0	A	-		0
95-11-13	-	193	122	-	P	0	P	0	A	-		0
95-11-20	-	130	110	-	P	0	P	0	A	-		0
95-11-27	-	108	85	-	-	60	P	0	A	-		0
95-12-04	-	56	40	-	P	0	P	0	A	-		0
95-12-11	-	64	49	-	P	0	P	0	A	-		0
95-12-18	-	147	92	-	-	33	P	0	A	-		0
96-01-02	-	43	30	-	-	640	P	0	A	-		0
96-01-08	-	-	-	-	-	360	P	0	A	-		0
96-01-15	-	-	-	-	-	60	P	0	A	-		0
96-01-22	-	-	-	-	-	30	P	0	A	-		0
96-01-29	-	-	-	-	P	0	P	0	A	-		0

continued

Date	Count per 100 ml											
	Faecal coliforms		Enterococci	Somatic coliphages			MS phages		*Bact* phages		Viruses	
	RW	UP	UP	RW	UP		UP		UP		UP	
1996-02-05	-	-	-	-	P	0	P	0	A	-		0
1996-02-12	-	-	-	-	-	100	P	0	A	-		0
1996-02-19	-	-	-	-	-	730	P	0	A	-		0
1996-02-26	95	-	-	310	-	360	P	0	A	-		0
1996-03-04	610	-	-	-	-	130	P	0	A	-		0
1996-03-11	10	-	-	30	-	130	P	0	A	-		0
1996-03-18	18	-	-	0	P	0	P	0	A	-		0
1996-03-25	190	-	-	30	P	0	P	0	A	-		0
1996-04-01	30	-	-	20	P	0	P	0	A	-		0
1996-04-09	180	-	-	0	P	0	P	0	A	-		0
1996-04-15	57	-	-	60	P	0	P	0	A	-		0
1996-04-22	73	-	-	0	P	0	P	0	A	-		0

continued

Date	Count per 100 ml											
	Faecal coliforms		Enterococci	Somatic coliphages			MS phages		*Bact* phages		Viruses	
	RW	UP	UP	RW	UP		UP		UP		UP	
1996-04-29	130	-	-	60	-	100	P	0	A	-		0
1996-05-06	61	-	-	0	-	100	P	0	A	-		0
1996-05-13	0	-	-	0	P	0	P	0	A	-		0
1996-05-20	0	-	-	2500	-	30	P	0	A	-		Ent
1996-05-27	130	-	-	20	-	60	P	0	A	-		0
1996-06-03	62	-	-	10	-	100	P	0	A	-		0
1996-06-10	52	-	-	20	-	30	P	0	A	-		0
1996-06-18	76	-	-	0	P	0	P	0	A	-		0
1996-06-24	51	-	-	510	-	30	P	0	A	-		0
1996-07-01	28	-	-	10	P	0	P	0	A	-		0
1996-07-08	110	-	-	10	-	170	P	0	A	-		0
1996-07-15	61	-	-	10	-	30	P	0	A	-		0

continued

Date	Count per 100 ml											
	Faecal coliforms		Enterococci	Somatic coliphages			MS phages		*Bact* phages		Viruses	
	RW	UP	UP	RW	UP		UP		UP		UP	
1996-07-22	-	-	-	-	-	30	P	0	A	-		0
1996-07-29	24	-	-	10	-	70	P	0	A	-		0
1996-08-05	30	-	-	0	-	60	P	0	A	-		0
1996-08-12	35	-	-	0	P	0	P	0	A	-		0
1996-08-19	22	-	-	0	-	100	P	0	A	-		0
1996-08-26	1	-	-	0	P	0	P	0	A	-		0
1996-09-02	20	-	-	0	-	130	P	0	A	-		0
1996-09-09	32	-	-	10	P	0	P	0	A	-		0
1996-09-16	120	-	-	0	P	0	P	0	A	-		0
1996-09-23	48	-	-	0	P	0	P	0	A	-		0

continued

Date	Count per 100 ml											
	Faecal coliforms		Enterococci	Somatic coliphages			MS phages		*Bact* phages		Viruses	
	RW	UP	UP	RW	UP		UP		UP		UP	
1996-09-30	14	-	-	0	P	0	P	0	A	-	0	
1996-10-07	32	-	-	0	P	0	P	0	A	-	0	
1996-10-14	72	-	-	30	-	100	P	0	A	-	0	
1996-10-21	22	-	-	10	P	0	P	0	A	-	?	
1996-10-28	1500	-	-	30	P	0	P	0	A	-	0	
1996-11-04	67	-	-	-	-	-	-	-	-	-	-	
1996-11-11	92	-	-	0	P	0	P	0	A	-	0	
1996-11-18	26	-	-	20	P	0	P	0	A	-	0	
1996-11-25	-	-	-	-	P	0	P	0	A	-	?	
1996-12-02	19	-	-	0	P	0	P	0	A	-	0	
1996-12-09	110	-	-	10	P	0	P	0	A	-	?	
1996-12-17	150	-	-	0	P	0	P	0	A	-	?	

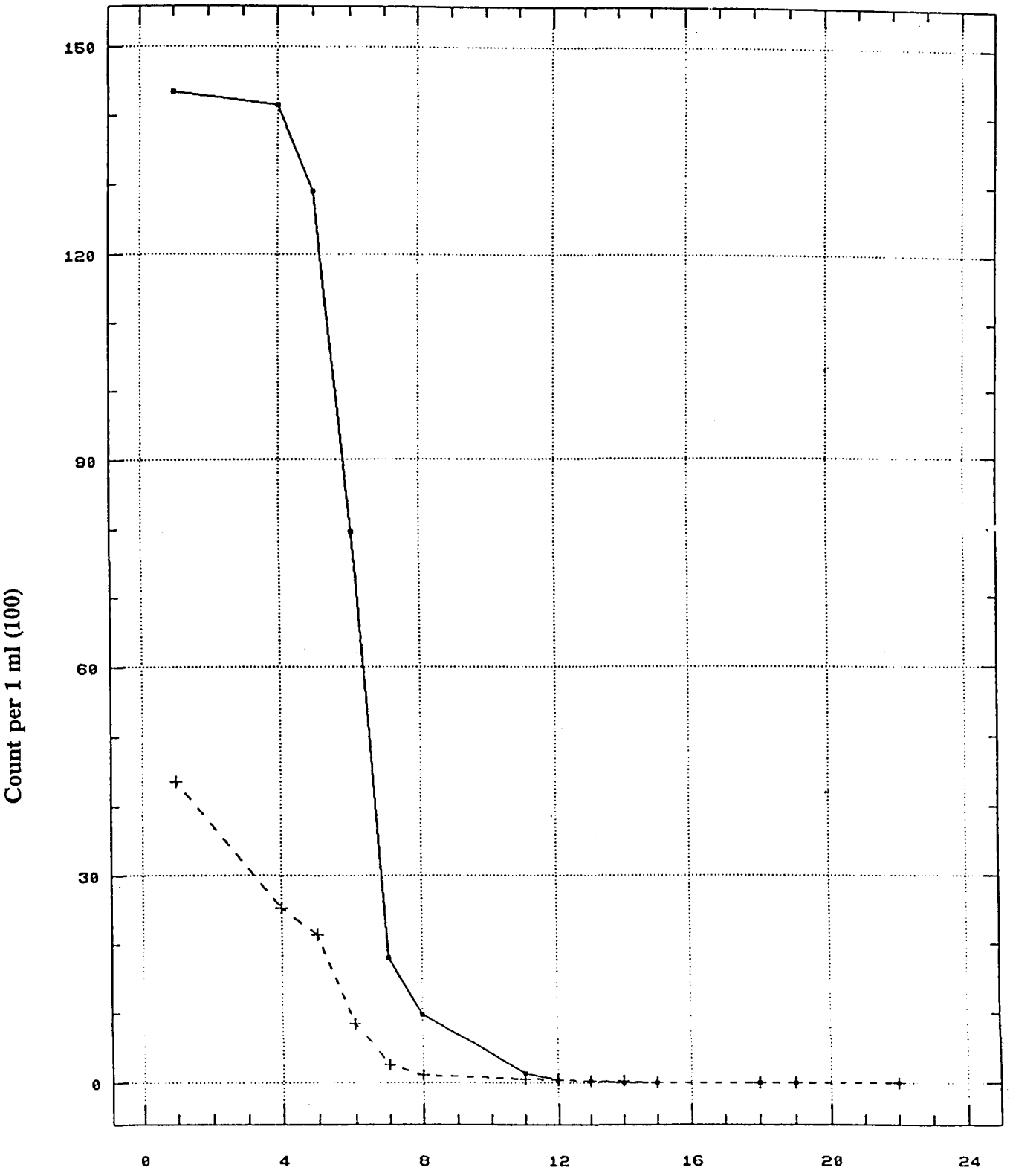
continued

Date	Count per 100 ml							
	Faecal coliforms		Enterococci	Somatic coliphages		MS phages	*Bact* phages	Viruses
	RW	UP	UP	RW	UP	UP	UP	UP
1997-01-06	-	-	-	0	P 0	P 0	A -	?

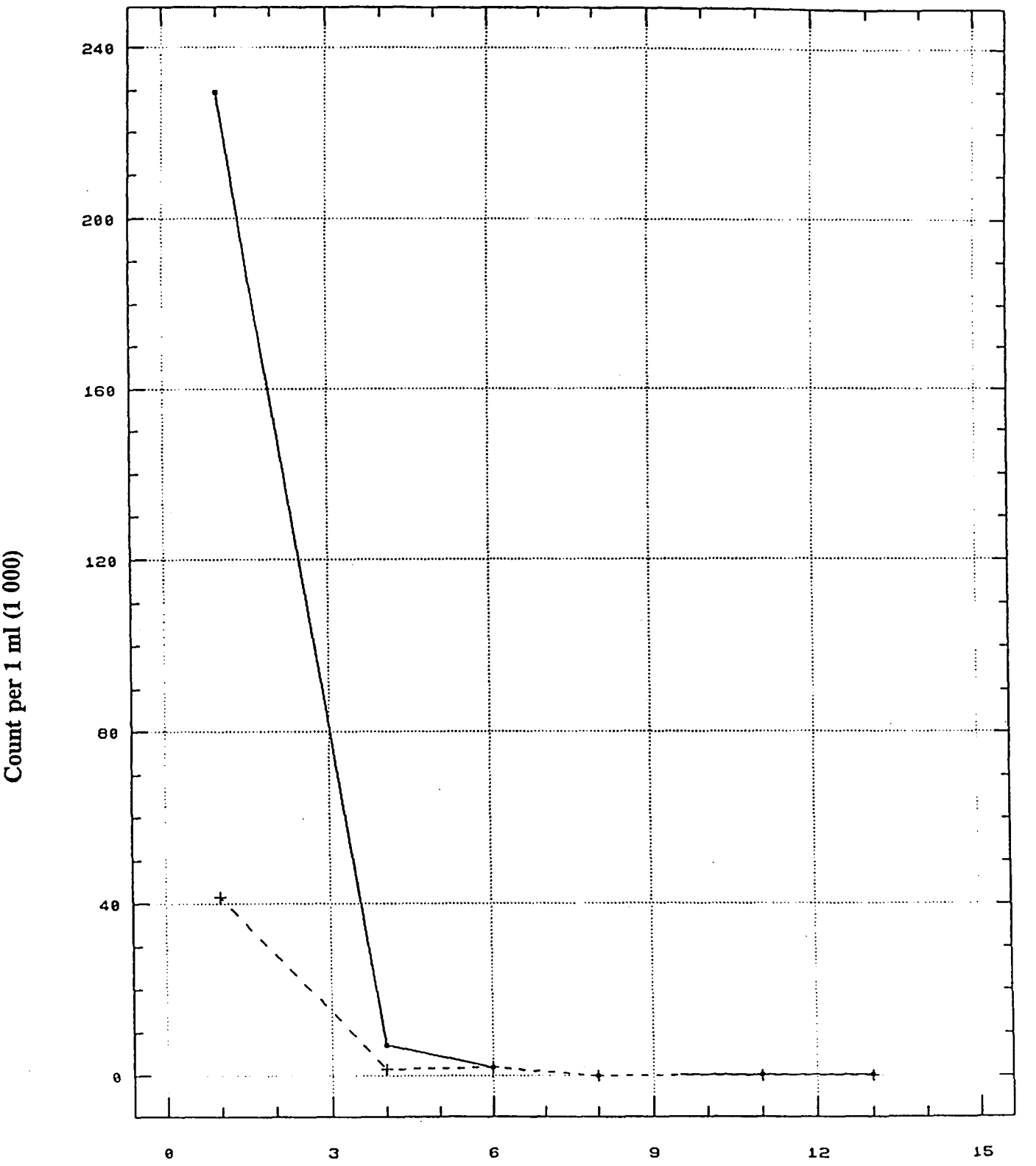
C:\wpd\vaal-18.97-01-24.

Appendix 5

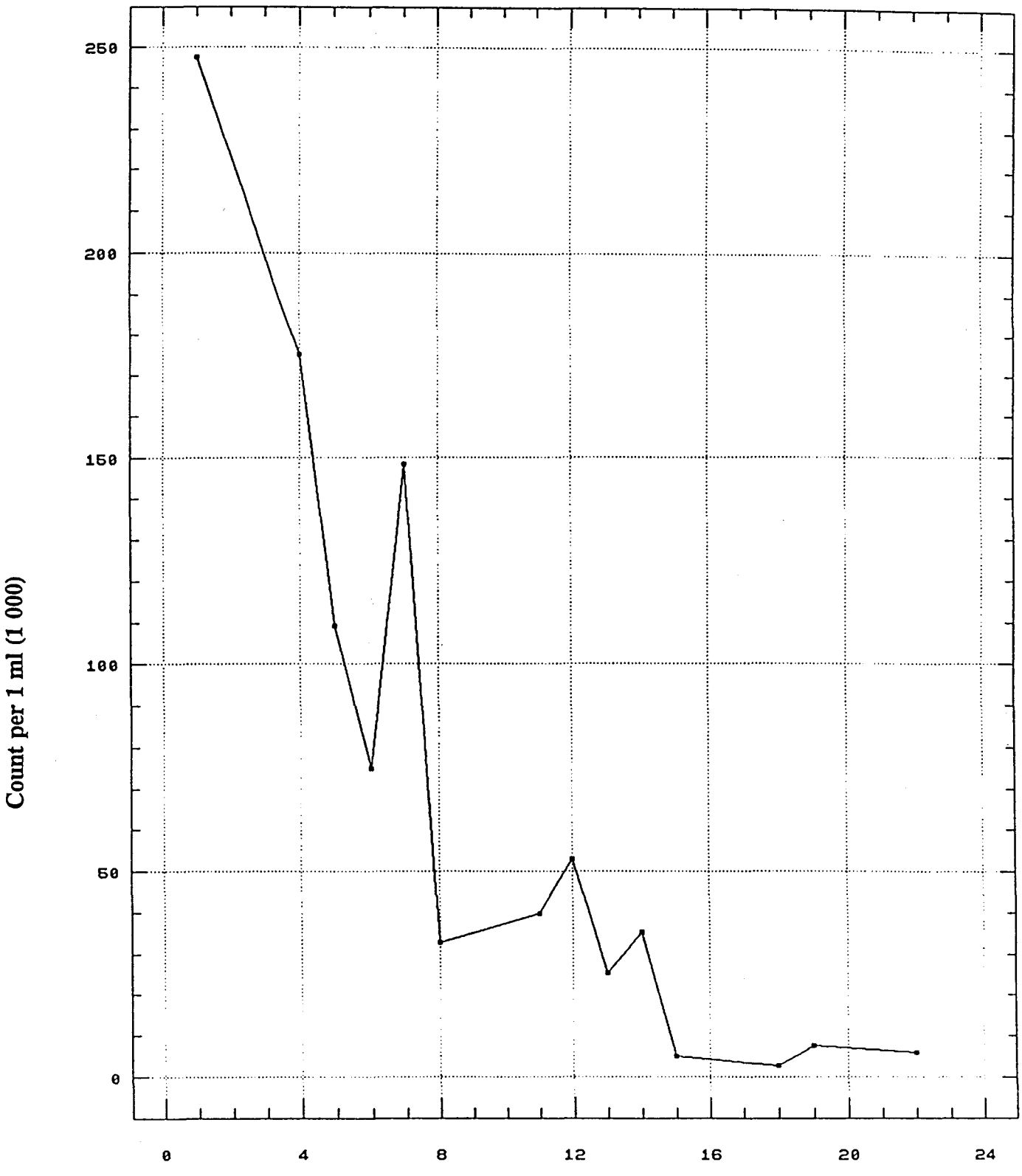
Survival of phages, viruses and bacteria in water



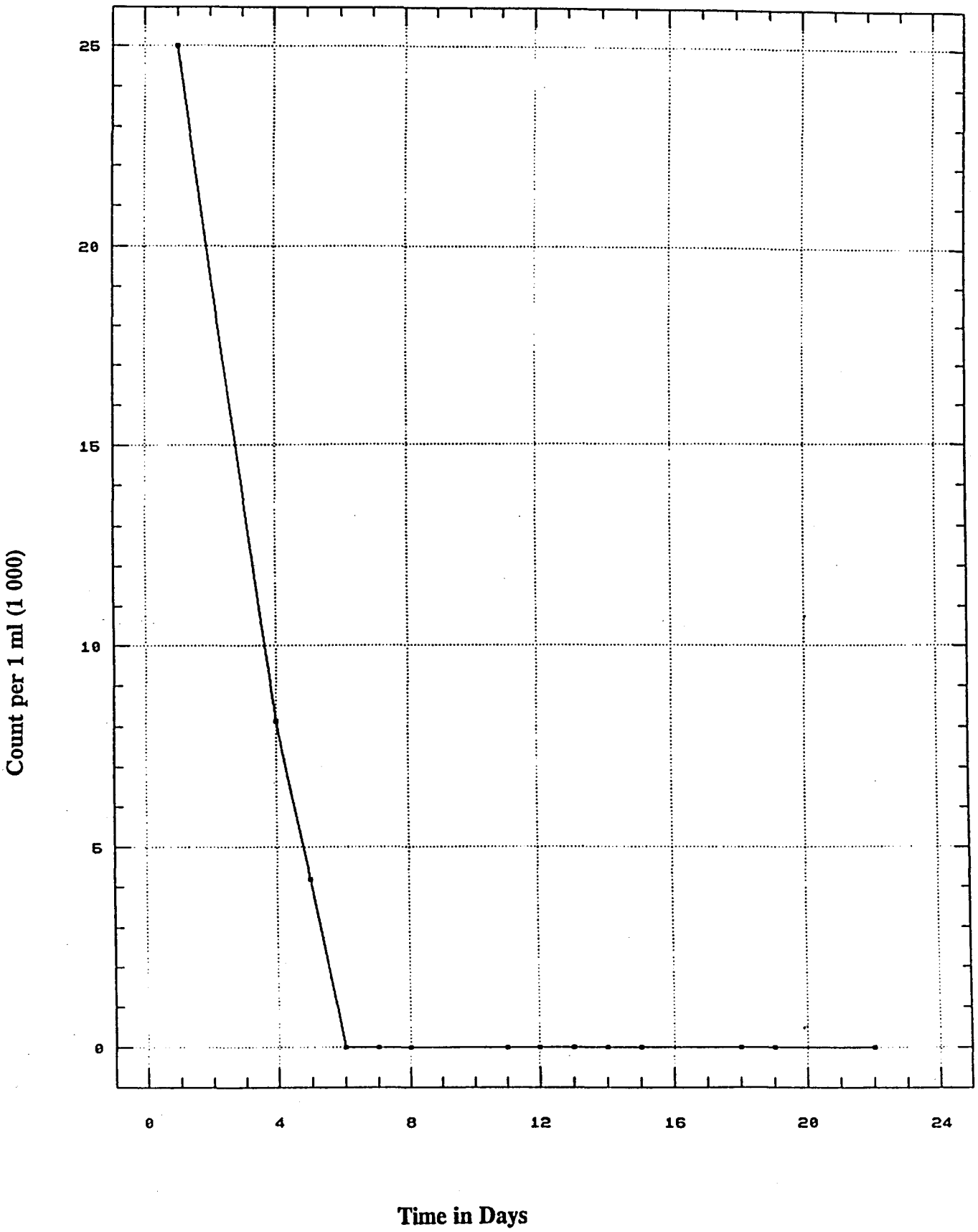
Survival on the laboratory bench in sea water seeded with bird droppings and human stool: Enterococci



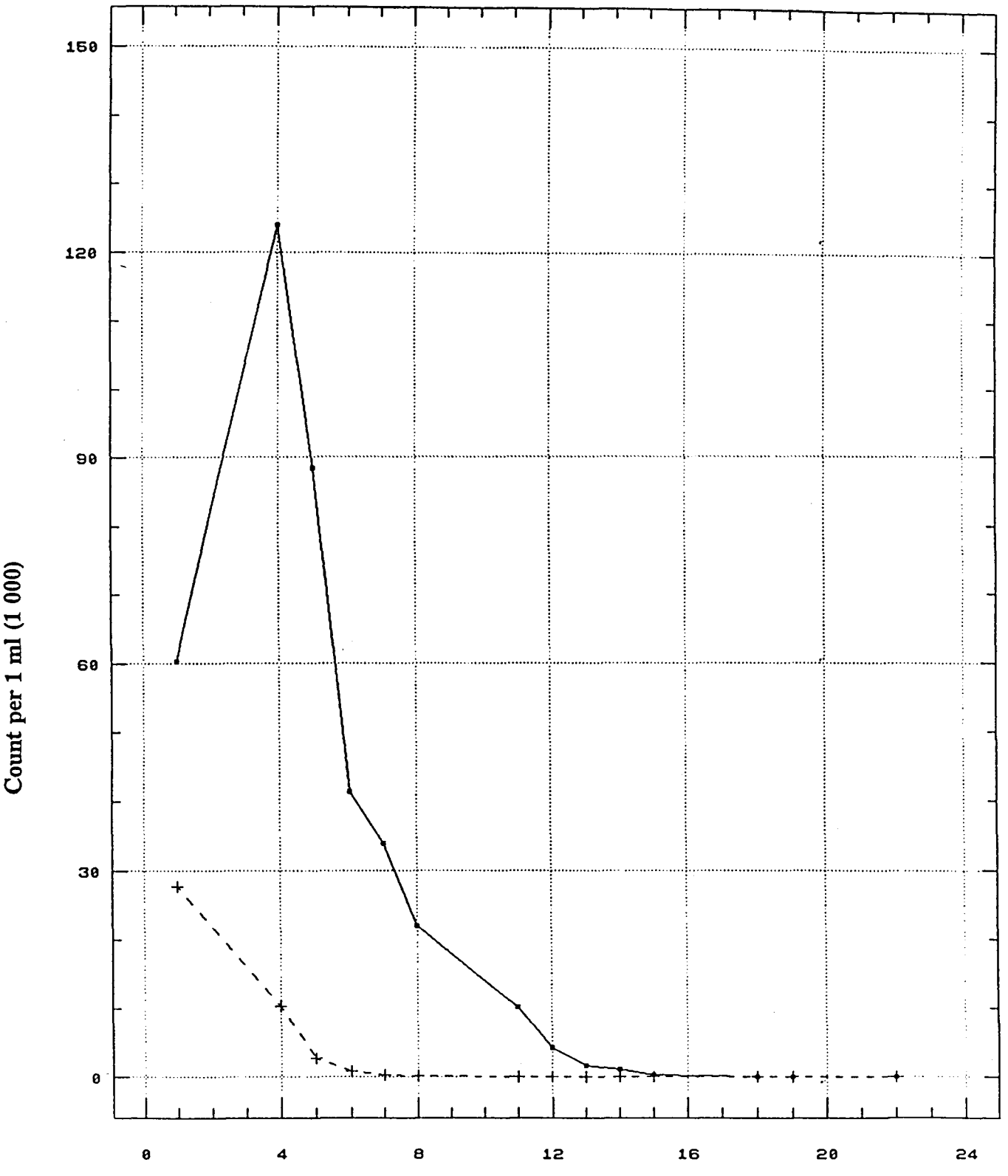
Survival on the laboratory bench in sea water seeded with bird droppings and human stool: Seeded poliovirus



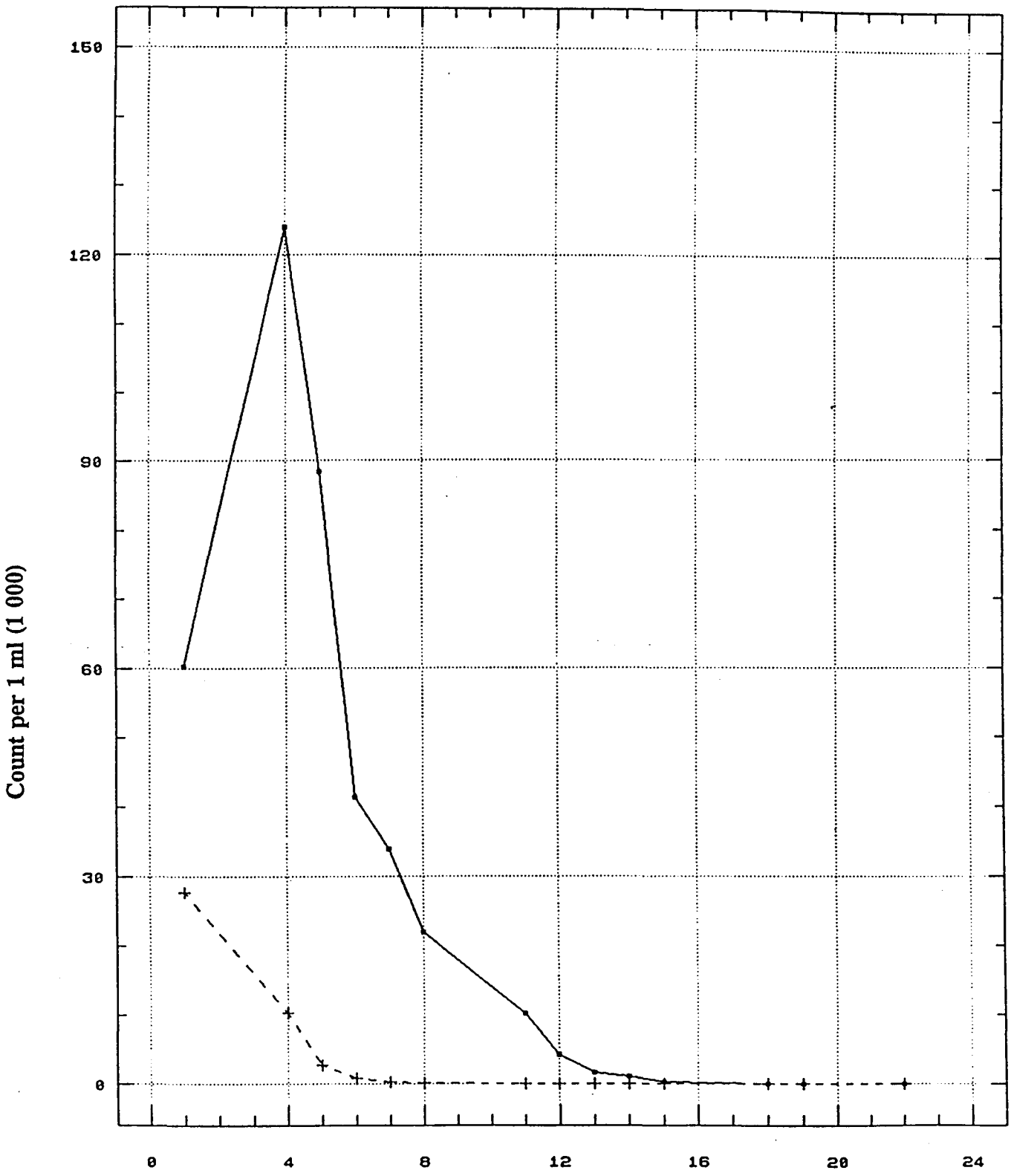
Survival on the laboratory bench in sea water seeded with bird droppings and human stool: Somatic coliphages



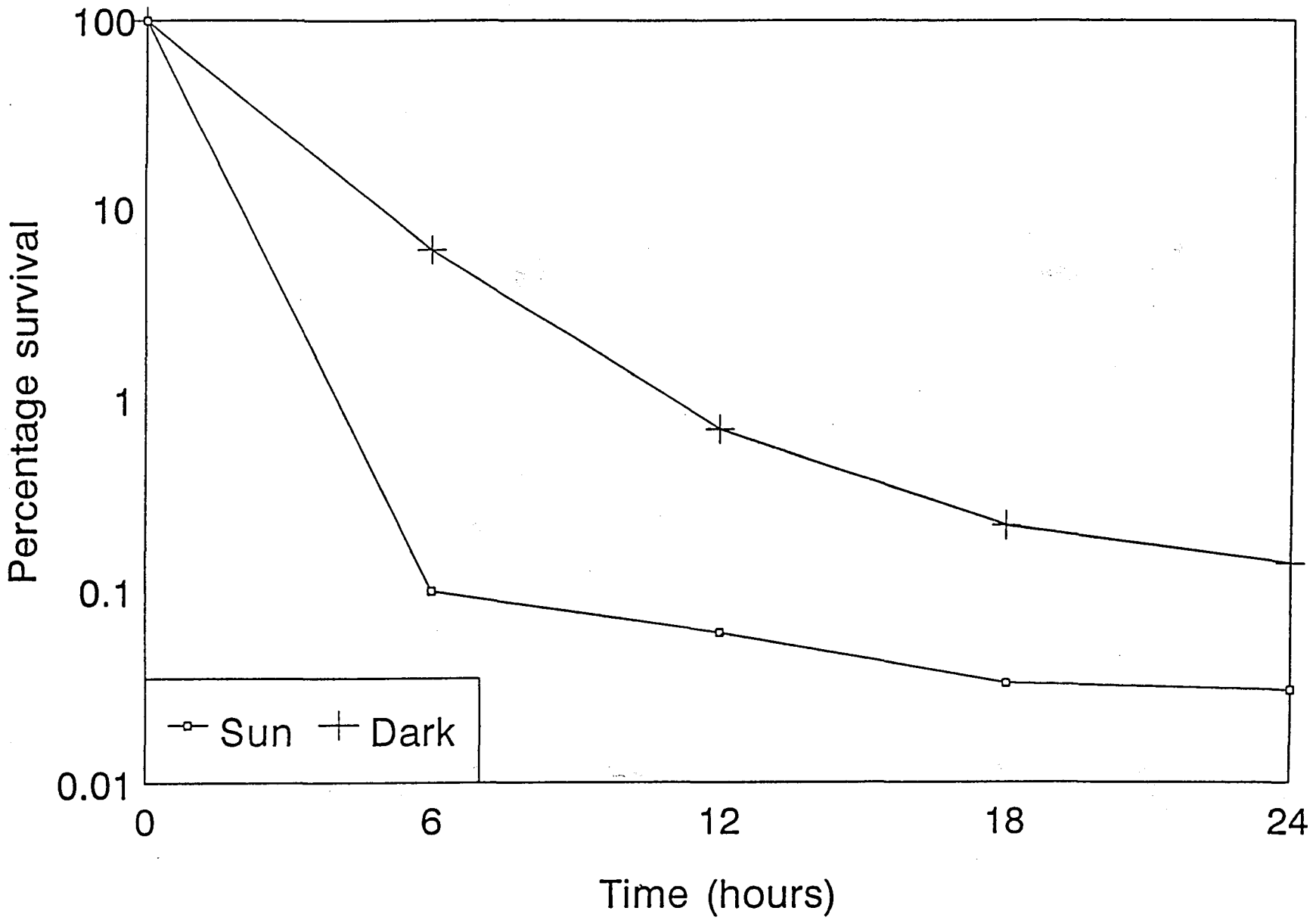
Survival on the laboratory bench in sea water seeded with bird droppings and human stool: Male-specific coliphages



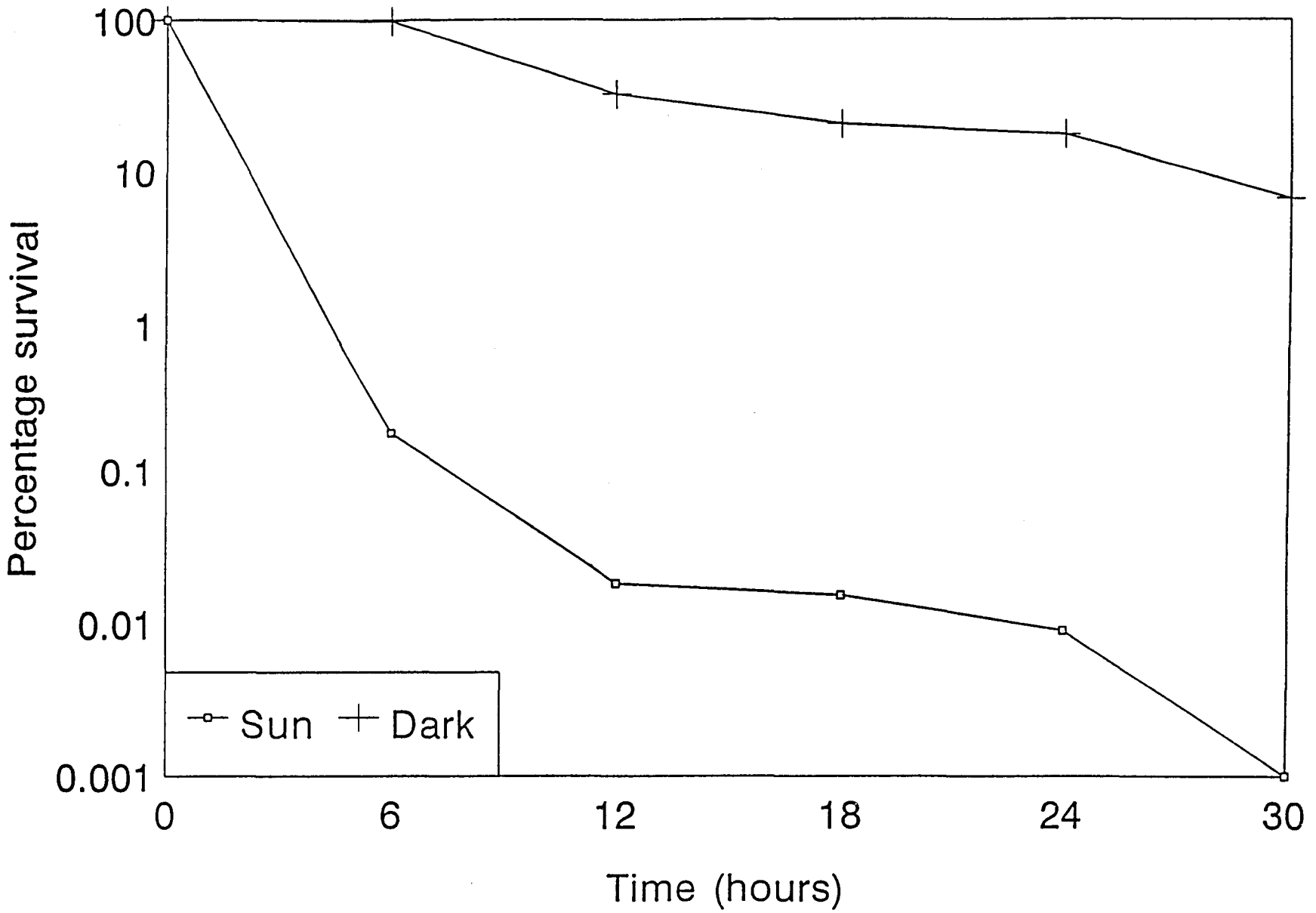
Survival on the laboratory bench in sea water seeded with bird droppings and human stool: Faecal coliforms



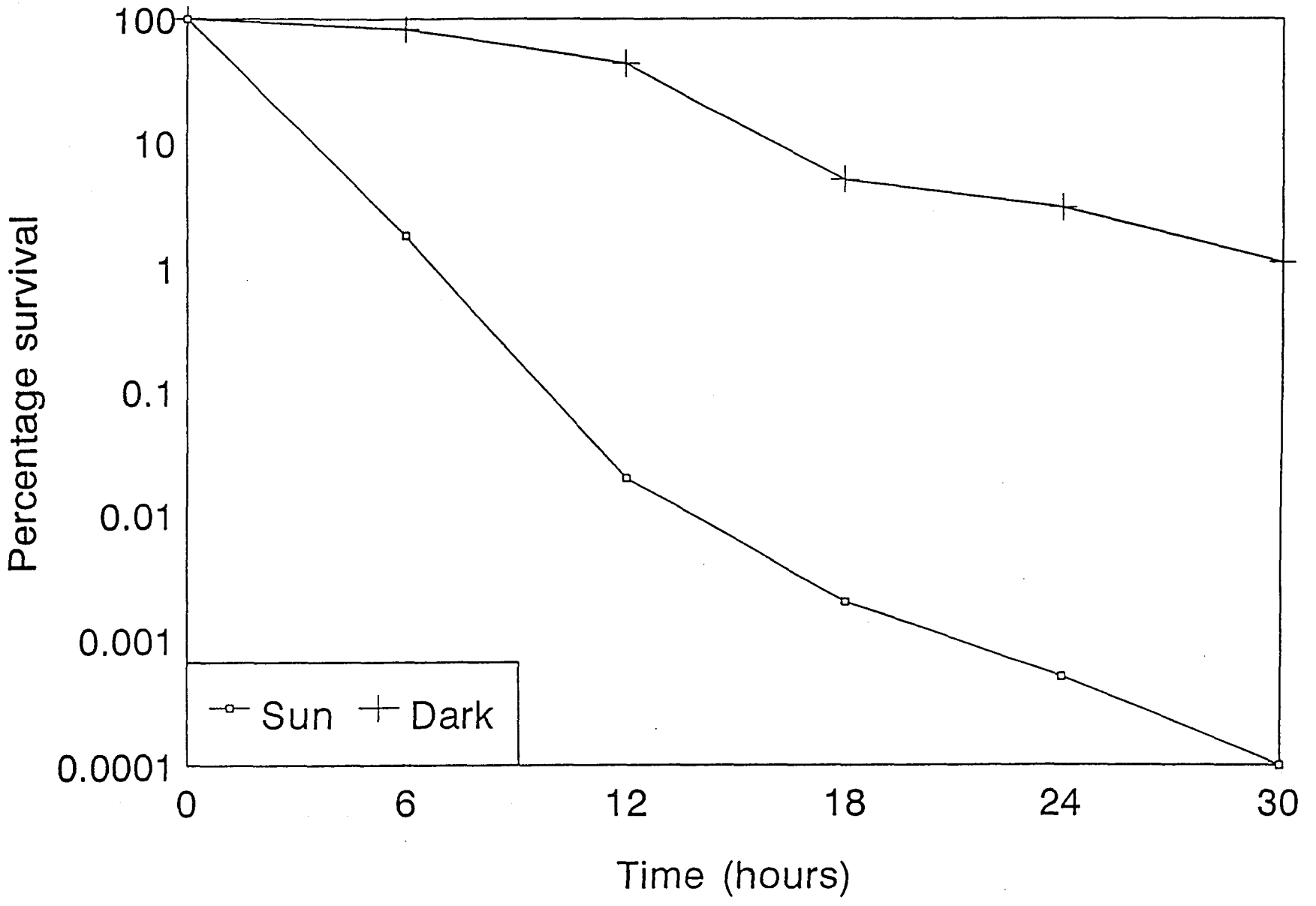
Survival on the laboratory bench in sea water seeded with bird droppings and human stool: Faecal coliforms



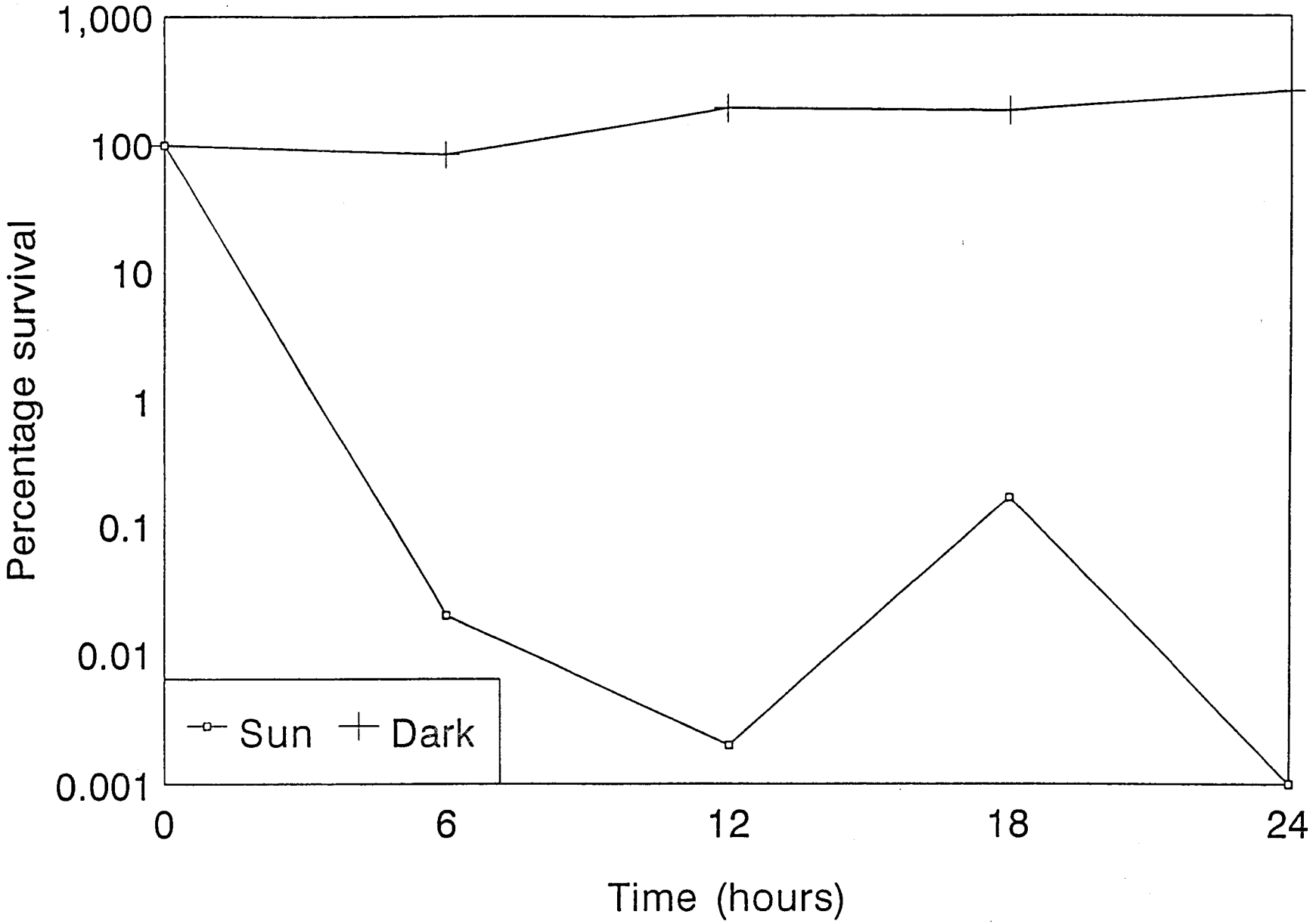
Survival in Seeded buffer in the presence and absence of sunlight:
Poliovirus type 1



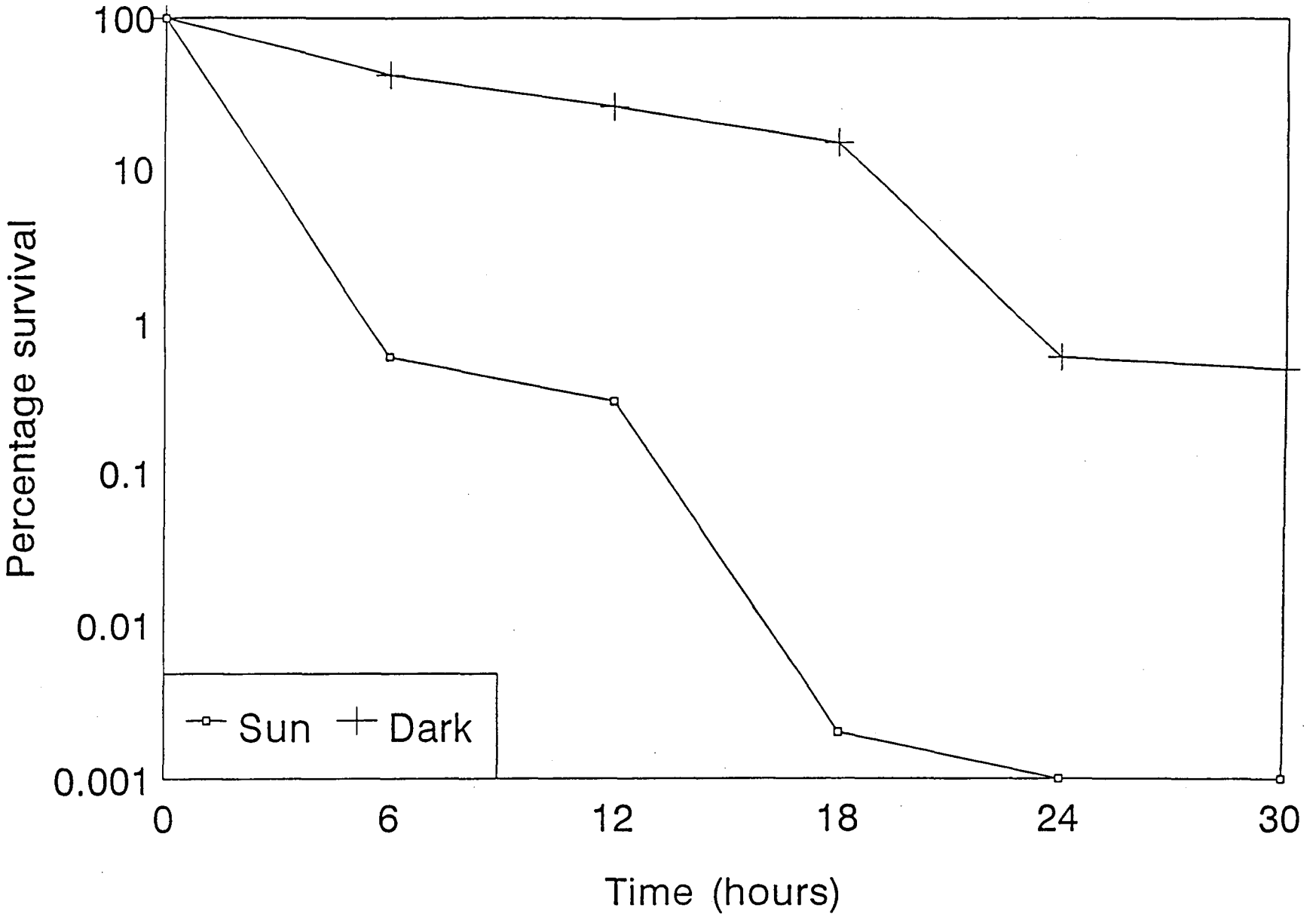
Survival in Seeded buffer in the presence and absence of sunlight:
Somatic coliphage V1



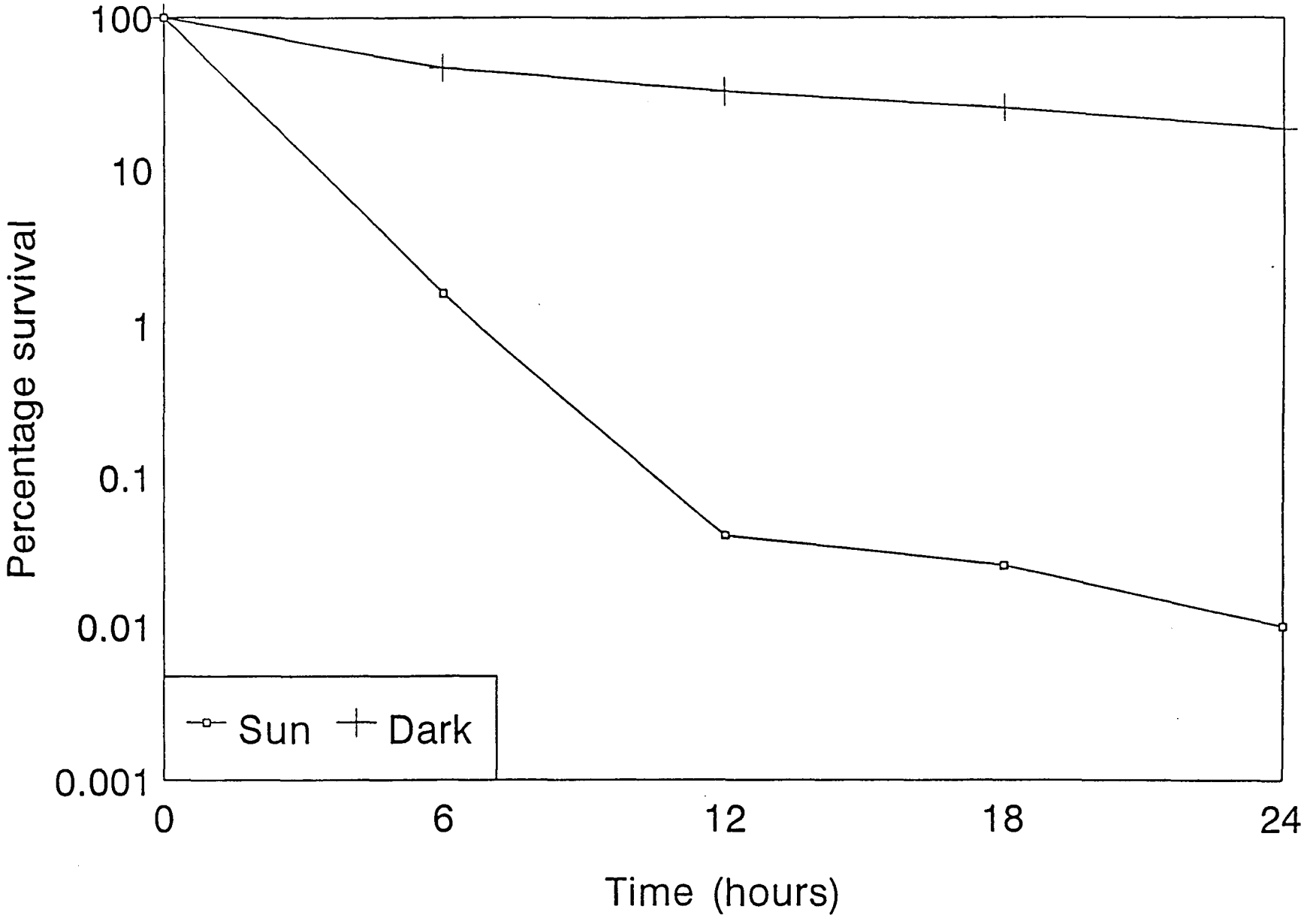
Survival in Seeded buffer in the presence and absence of sunlight:
Male-specific coliphage MS2



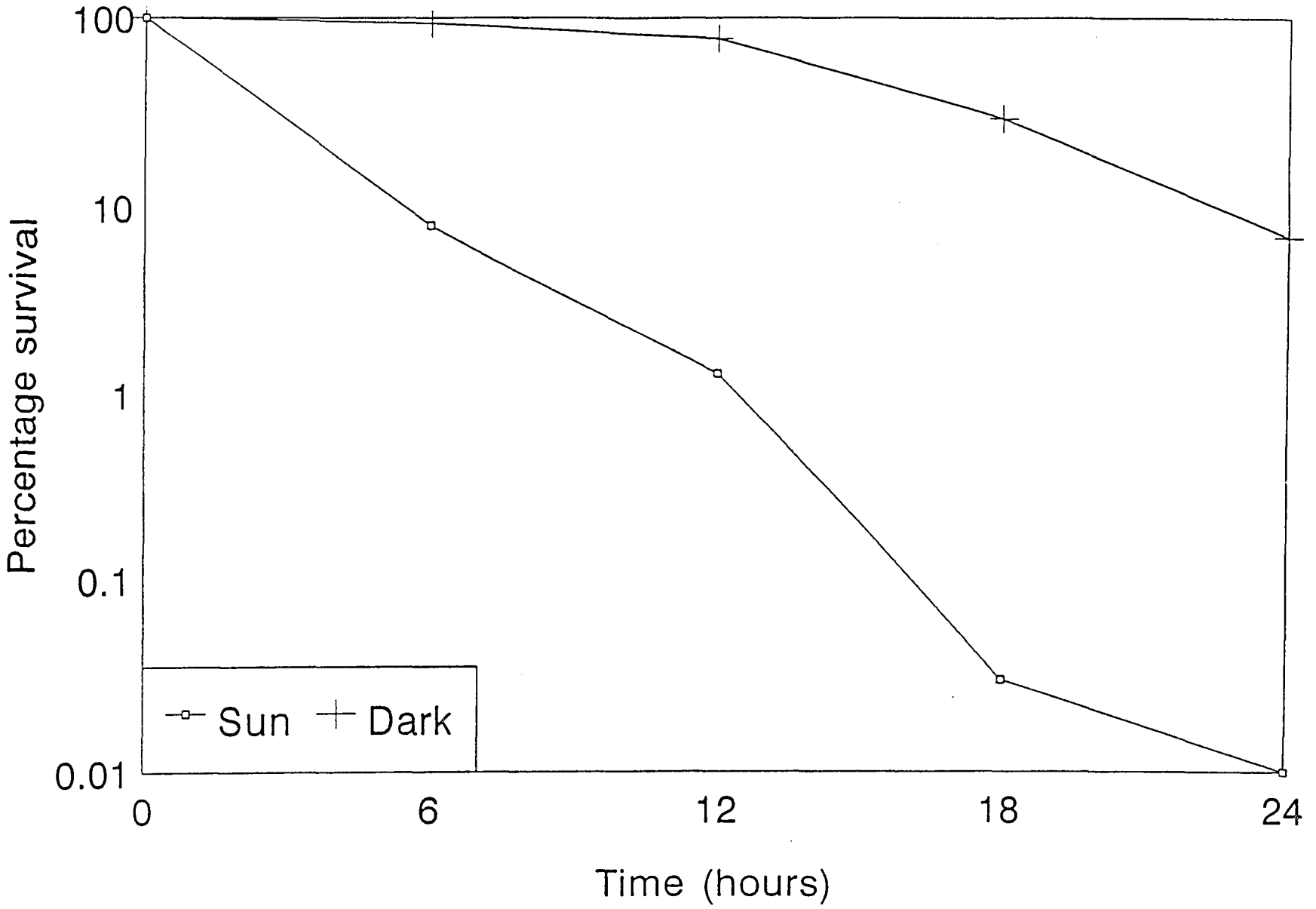
Survival in Seeded buffer in the presence and absence of sunlight:
Enterococcus faecalis



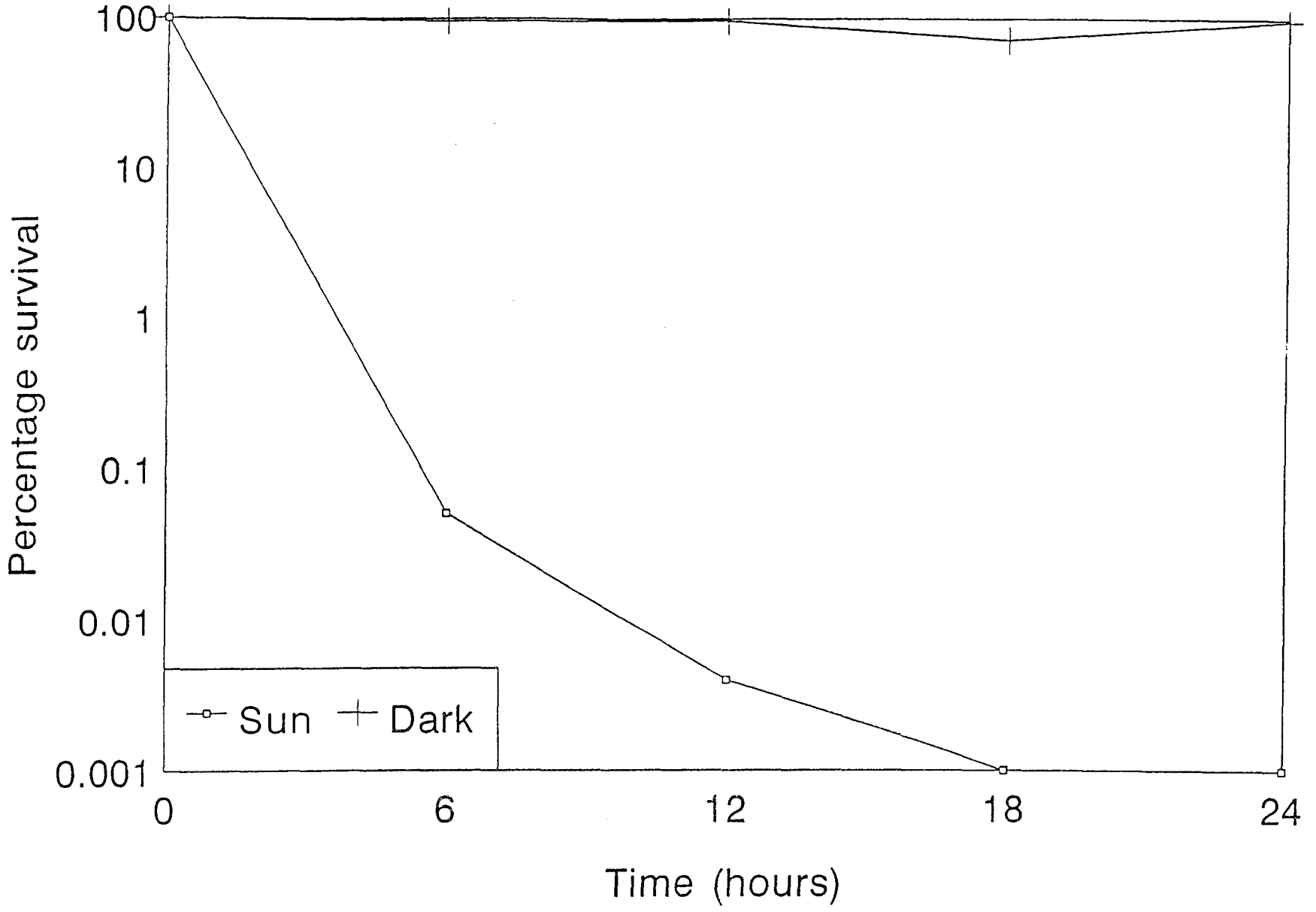
Survival in seeded Apies River water in the presence and absence of sunlight:
Enterococcus faecalis



Survival in seeded Apies River water in the presence and absence of sunlight: Poliovirus type 1



Survival in seeded Apies River water in the presence and absence of sunlight: Male-specific coliphage MS2



Survival in seeded Apies River water in the presence and absence of sunlight: Somatic coliphage V1

FINAL REPORT TO THE
WATER RESEARCH COMMISSION
ON THE RESEARCH PROJECT

EVALUATION OF THE APPLICATION OF BACTERIOPHAGES AS INDICATORS OF WATER QUALITY

BY

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CONTENTS

GLOSSARY

Bacteriophages (Phages)

Viruses which infect bacteria. These host bacteria support the replication of the phages. Phages can only infect specific host bacteria. The specificity is determined by phage receptor sites on certain surface parts of the host bacteria. In terms of size, morphology, structure, composition and mode of replication, phages resemble human viruses. As in the case of human viruses, nucleic acid content of different phages is solely single- or double-strand DNA or RNA. Many phages are detectable by simple and inexpensive techniques which yield results in less than 24 h.

Coliphages

Phages which infect *Escherichia coli* and certain closely related bacteria.

Somatic coliphages

The receptor site for these coliphages is at all times located on the cell wall of host bacteria. The hosts are, therefore, susceptible to infection at any time and under a wide range of conditions. For instance, infection can take place in natural water environments, and since some of the hosts can grow and metabolise in these environments, they can also support the replication of somatic coliphages in these environments. Somatic coliphages are, therefore, not specific for faecal pollution and their numbers can increase in environments such as wastewater, sand filters and activated carbon filters. Somatic coliphages include a wide variety of DNA and RNA phages which differ in morphology and structure. Since their hosts are *E coli* and closely related bacteria, somatic coliphages typically occur in large numbers in wastewater which contains human and animal excreta. Their numbers are often similar to those of faecal bacteria. Somatic coliphages are detectable by the simplest and least expensive of phage techniques. Somatic coliphages do, therefore, give an indication of sewage pollution, and their incidence and behaviour in water environments more closely resembles that of human viruses than faecal bacteria such as coliforms, enterococci and clostridia.

Male-specific coliphages

The receptor site for these phages is located on fertility (F) fimbriae of *E coli*. F fimbriae are short tube-like protrusions produced by certain bacteria for the transfer of nucleic acid (RNA or DNA) to other bacteria of the same or closely related species. It is, therefore, literally a sexual process and the fimbriae are also referred to as sex fimbriae. F fimbriae are produced only at

optimal growth conditions including temperatures of about 32-40°C. This implies that male-specific coliphages cannot multiply in natural water environments, and they are highly specific indicators of faecal pollution. They generally occur in sewage polluted water environments in numbers much lower than those of somatic coliphages. The genes which code for the production of F fimbriae are known as the fertility (F) factor. These genes have by genetic engineering techniques been incorporated in the nucleic acid (DNA) of a *Salmonella typhimurium* bacterium with the result that this bacterium now produces *E coli* F fimbriae and is susceptible to male-specific coliphages. Since *S typhimurium* bacteria are rare in most water environments, this host can be used for the selective detection of male-specific coliphages without interference by other coliphages. The *S typhimurium* host commonly used for this purpose is know as the WG49 or Havelaar host. Dr Arie H Havelaar has established the technology and expertise concerned. The technology for detecting male-specific phages is not as simple as for somatic coliphages.

F-RNA coliphages

Restricted group of male-specific coliphages. They closely resemble human enteroviruses with regard to features such as size, morphology, structure, nucleic acid consisting solely of single-stranded RNA, and failure to replicate in water environments. F-RNA coliphages are, therefore, valuable models or indicators for human enteric viruses in water environments. The receptor sites for F-RNA phages are located along the shaft of F fimbriae.

F-DNA coliphages

Restricted group of male-specific coliphages clearly distinguishable from F-RNA phages. They are typically filamentous phages which contain double-stranded DNA, and their receptor site is located at the tip of F fimbriae.

***Bacteroides fragilis* HSP40 phages**

Bacteroides fragilis bacteria are members of the normal flora of the gut of man and warm-blooded animals. Prof Juan Jofre and co-workers have found that phages which specifically infect *B fragilis* strain HSP40 are excreted only by humans and no animals. Reasons are not yet clear, but it may largely be due to this specific strain being highly specific for the human intestinal tract. Various bacteria are known to be specific for humans or animals. These phages can, therefore, be used to distinguish between faecal pollution of human and animal origin. The numbers of these phages in water environments are generally lower than those of male-specific phages, primarily

because they are excreted in relatively low numbers by only about 15% of humans. Since the host bacteria are strict anaerobes and plaque assays exceptionally vulnerable to interference by contaminant bacteria, the detection and enumeration of these phages is relatively complicated.

Plaques

Circular clearance zones in a lawn of host bacteria on petri dishes. Obtained by mixing a culture of host bacteria with phages (as in a test sample) in sloppy agar which is poured into a petri dish (usually on top of an agar layer rich in host nutrients) and incubated. Each phage will infect a host bacterium. The host bacterium replicates the phage and releases large numbers of the phage into the environment. These phages infect neighbouring host bacteria and the process is repeated with new phages spreading outward from the original point of infection. The process carries on as long as the host bacteria are in the stage of active growth and metabolism. When this stage comes to an end due to depletion of nutrients and the maximum density of bacteria has been reached, the host bacteria can no longer support the replication of phages and the plaques do not increase in size any further. The size and appearance of plaques produced by various phages differs and is distinctive to some extent. Phages such as somatic coliphages completely destroy their hosts and produce easily visible clear plaques. Male-specific phages produce faint plaques which are not always easily visible largely because many host bacteria in the lawn do not have F fimbriae and are not susceptible to the phages. Obtaining visible plaques does, therefore, require carefully controlled experimental conditions in which optimum numbers of host bacteria contain F fimbriae. The size of plaques produced by different phages may differ largely due to differences in the rate of phage replication and spread through the agar medium. In principle each phage in a test sample produces one plaque, which implies that the number of plaques gives an indication of the number of phages in the test sample. However, phages tend to clump together and such a clump will produce only one plaque.

Rnase

Enzyme which specifically degrades RNA nucleic acid. RNA phages are, therefore, inactivated by the enzyme but not DNA phages. The enzyme is, therefore, being used to distinguish between RNA and DNA phages, such as F-RNA and F-DNA male-specific coliphages.

Probe

Short piece of single-stranded RNA or DNA with nucleotide sequence homologous to that of a specific part of the nucleic acid of a phage. These probes can, therefore, hybridise with the nucleic acid of phages of choice. Hybridization of probes with phage nucleic acid immobilised on for instance membranes, can be detected by using probes labelled with radio-active markers or enzymes. This technique is used for the sensitive and highly specific detection of phages.

EXECUTIVE SUMMARY

Key concepts

Water quality; health aspects of water; safety of water for human consumption; efficiency of water treatment processes; cost effective assessment of water quality; guidelines for practical routine monitoring of water quality; reliable indicators for human viruses; waterborne diseases; distinguish between faecal pollution of human and animal origin; training of manpower; technology transfer.

OBJECTIVES :

The main objective was to evaluate and optimise the application of phages as indicators of water quality in practice. This objective was based on information which suggests that phages have attractive features for application as cost effective and practical indicators of water quality, particularly since they closely resemble human viruses in a number of relevant respects.

The intention was to accomplish the objective by investigating the behaviour of selected phages and groups of phages relative to that of other indicators and human viruses in water environments and water treatment processes. The results were used to identify and solve shortcomings in current procedures for using phages as indicators. Special attention was given to the numbers of phages in waste water and polluted water sources, and their survival in these environments. Available information left no doubt that the meaningful application of phages as indicators, and research on their incidence and behaviour in water environments, would require the development of more sensitive techniques for the qualitative detection and enumeration of phages.

The results were due to be applicable in practice for the following purposes:

- Assessment of water quality, particularly with regard to viruses
- Evaluation of the efficiency of water treatment and disinfection processes
- Formulation of water quality guidelines
- Design of cost-effective procedures for routine monitoring of the safety of drinking water

The project was intended to contribute to the training and education of manpower required for quality monitoring in the water industry and related health disciplines, and to the transfer of technology and expertise to relevant laboratories and authorities.

MOTIVATION :

The project logically followed on achievements accomplished in the 1990-92 WRC Project, 321/1/93, "Research on the Use of Bacteriophages as Indicator Organisms" by W O K Grabow and co-workers which may be summarised as:

* Establishment of technology, expertise, facilities and materials for research on the application of various phages and groups of phages as indicators of water quality.
* First application in South Africa of *Bacteroides fragilis* phages as indicators.
* Evidence that *B fragilis* HSP40 phages occur exclusively in human stools, which implies that a reliable indicator for distinction between human and animal faecal pollution may be available.
* Evidence that numbers of certain phages and groups of phages relative to those of other indicators and viruses in a variety of water environments meet the requirements of reliable, rapid, practical and economic indicators of water quality.
* Technology and materials for the application of phages as indicators has been transferred to a number of laboratories.
* Quality guidelines for acceptable levels of phages in drinking water supplies and water intended for recreation and other purposes have been proposed.
* A substantial contribution has been made to the training of manpower essential for quality monitoring in the water industry, as well as related health disciplines.

The objective of this project was to build on the above foundation in terms of additional support for the value of phages as water quality indicators, and to optimise technology for the application of phages as quality indicators in practice.

The merit of research along these lines was supported by:

* The Department of Water Affairs and Forestry has appointed a Task Force of experts to formulate guidelines for the quality of water intended for various purposes. This Task Force has included phages in guidelines for the quality of raw and treated drinking water supplies, as well as water used for recreational purposes. The Task Force identified further research on the application of phages as water quality indicators as priority.
* At international level interest in phage indicators is escalating. For instance, the International Organization for Standardization (ISO) is in the event of standardising techniques for world wide use of phages as indicators for a variety of purposes (ISO Committee Draft, 1991). The

Commission on Water Quality of the European Union is considering the inclusion of phages in quality specifications for drinking water and recreational waters. Growing world-wide interest in phage indicators has been outlined in more detail (IAWPRC Study Group on Health Related Water Microbiology, 1991).

TECHNICAL DETAILS OF PROJECT :

The agreement specified that the research would primarily be carried out in the laboratories of the Department of Medical Virology, University of Pretoria. This laboratory has the necessary facilities, expertise and materials for the study. The required infrastructure and arrangements for obtaining test samples from sites in and around Pretoria, as well as sites at Vereeniging, Bloemfontein, Pietermaritzburg, Cape Town and Windhoek, were in place.

The agreement furthermore specified that research would primarily focus on the following:

* Somatic coliphages, male-specific coliphages, and *B fragilis* HSP40 phages.
* Quantitative recovery of phages using adsorption-elution methods.
* Direct plaque assays on large volumes of water using single agar layers in large petri dishes.
* Qualitative presence-absence (P-A) tests on large volumes of water.
* Application of the new techniques in research on the incidence and behaviour of phages in selected water environments.
* Statistical evaluation of results was carried out according to procedures established with the Division on Biostatistics of the Medical Research Council.
* The study was carried out in close collaboration with international bodies such as ISO in order to ensure that technology and expertise are in line with the latest in the world.

RESEARCH OUTPUTS :

Details on some research outputs of the project have been recorded in the following publications, conference papers and reports:

Publications

Grabow W O K (1993a) Pathogenic organisms and indicators of faecal pollution. Proceedings: International Seminar on the Disinfection of Water and Wastewater in Developing Countries, Bello Horizonte, Brazil, 26-28 April. S471a Anais: Associacao Brasileira de Engenharia Sanitaria e Ambiental da Escola de Engenharia da UFMG, Bello Horizonte, Brasil. pp 17-31.

Grabow W O K (1993b) In: Specifications for enteric viruses and faecal coliforms in: South African Water Quality Guidelines, Volume 1 (Domestic Use) and Volume 2 (Recreational Use), Department of Water Affairs and Forestry, Pretoria.

Grabow W O K (1994a) Evaluation of the efficiency of "Stericlean". Hands-On (Newsletter of the Dental and Dental Technology Forum) 6/2, 7-9.

Grabow W O K (1994b) Health protection by disinfection of treated wastewater. In: Documentation Proceedings, International Conference on Integrated Wastewater Management - Collection, Treatment and Reuse, 203-214. European Water Pollution Control Association, Gesellschaft zur Förderung der Abwassertechnik, Hennef. 382 pp.

Grabow W O K, Wyn-Jones A P, Schildhauer C and Jofre J (1995a) Efficiency of the *Euroguard* domestic water treatment unit with regard to viruses, phages and bacteria. Water SA 21, 71-74.

Grabow W O K, Neubrech T E, Holtzhausen C S and Jofre J (1995b) *Bacteroides fragilis* and *Escherichia coli* bacteriophages: excretion by humans and animals. Water Science and Technology 31, 223-230.

Jagals P, Grabow W O K and De Villiers J C (1995) Evaluation of indicators for assessment of human and animal faecal pollution of surface run-off. Water Science and Technology 31, 235-241.

Grabow W O K (1995a) Progress in research on human viruses in water. In: Proceedings of the Fourth International Symposium on Contamination of the Environment by Viruses and Methods of Control held in Vienna on 2-4 September 1993, eds W Biffl and R Walter, University of Agriculture, Forestry and Renewable Resources, Vienna, Austria. Wiener Mitteilungen 128/2, 1-14.

Grabow W O K (1995b) Member of Working Group for ISO Standard: Water quality - Detection and enumeration of bacteriophages, Part 1: Enumeration of F-specific RNA bacteriophages. ISO 10705-1:1995. International Organization for Standardization, Geneva.

Grabow W O K (1995c) Member of Working Group for ISO Standard: Water quality - Detection and enumeration of bacteriophages, Part 2: Enumeration of somatic coliphages. ISO/CD 10705-2:1995. International Organization for Standardization, Geneva.

Grabow W O K (1995d) Member of Working Group for ISO Standard: Water quality - Detection and enumeration of bacteriophages, Part 3: Concentration of bacteriophages from water. International Organization for Standardization, Geneva.

Grabow W O K (1996a) Waterborne diseases: Update on water quality assessment and control. Water SA 22, 193-202.

Jagals P, Grabow W O K and Williams E (1996) The effects of supplied water quality on human health in an urban development with limited basic subsistence facilities. Water SA (in press).

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SUMMARY OF ACHIEVEMENTS

Methods for the quantitative enumeration of phages

Somatic coliphages

1. The double agar layer plaque technique specified in ISO (1995b) has been evaluated. This technique is intended for testing volumes of water up to about 10 ml. No evidence for improvement on the principles outlined in this technique has been found. There is no indication that a better host strain may be available. However, the project team believed that the ISO technique can be substantially simplified to render it more practical and user-friendly, and less expensive and labour intensive. These suggestions have been submitted to the ISO Working Group (WG) concerned. Some of the modifications have already been accepted, and others are under investigation. For instance, the proposal of accepting standard grade chemicals, which are much less expensive than analytical grade chemicals, provided they yield similar results, has now been accepted as general ISO policy. The proposal of the project to simplify the complicated, labour intensive and expensive procedure for the preparation of host inocula is being investigated by the ISO WG. The final report contains details on the technique recommended by the project team.

2. Possibilities for reducing the time required for the detection of phages have been investigated. Results revealed that using the above standard technique some plaques were visible after about 8 h incubation, but maximum plaques were visible only after some 20 h.

3. The value of 2,3,5-triphenyl-tetrazolium hydrochloride (tetrazolium) in plaque assays has been investigated. The results indicated that tetrazolium may enhance the early visibility of plaques. However, tetrazolium failed to reduce the time required for plaque assays to any meaningful extent.

4. Two rapid tests for the detection of phages described in the literature have been evaluated. Both techniques were rejected as impractical and failing to reduce the time required by the above plaque assay to meaningful extent. The results indicated that the development of meaningful rapid methods requires more intensive research, possibly using different approaches, which may be based on molecular techniques.

Male-specific coliphages

1. The double agar layer plaque assay specified in ISO (1995a) has been evaluated. This technique is intended for testing volumes of water up to about 10 ml. No evidence for improvement on the principles outlined in this technique has been found. In detailed studies on host strains the Havelaar strain specified in the ISO standard proved superior to the Cabelli strain. There is no indication that a better host strain may be available or readily developed. Unfortunately, however, the results and experience confirm that the time consuming, cumbersome and labour intensive procedure for the preparation of host cultures described in the ISO specification has to be followed closely. In the case of somatic coliphages, the ISO technique can be substantially simplified. The modified technique recommended is described in detail in the final report.

2. Research on rapid techniques and the use of tetrazolium yielded results similar to those for somatic coliphages.

3. Techniques for the confirmation of male-specific coliphages have been established. These are based on electron microscopy and RNase tests. Unfortunately the former requires sophisticated facilities and expertise, and the latter proved not easy to perform.

4. Typing of male-specific coliphages by gene probe hybridisation proved feasible. However, optimisation of technical details of the concept, assessment of the application of phage types for distinguishing between animal and faecal pollution, and application of the concept in practice, proved to require research beyond the objectives of this project.

***Bacteroides fragilis* HSP40 phages**

1. The double agar layer plaque assay described in the preceding WRC project has been evaluated. This technique is intended for testing volumes of water up to about 10 ml. Apart from minor modifications to ingredients of growth media, no evidence for improvement on the principles of the technique has been found. For instance, there is no indication of a host strain superior to HSP40 for the detection of phages excreted exclusively by humans.

2. As in the case of coliphages, tetrazolium proved to have certain benefits, and efforts to reduce the time required for the test were not successful.

Decontamination of water samples

Decontamination of water samples by means of chloroform treatment proved to slightly reduce counts of somatic coliphages and *B fragilis* HSP40 phages, and slightly increase counts of male-specific coliphages. Reasons for these observations, as well as alternative methods for decontamination such a membrane filtration, were not investigated in further detail because problems with contamination were successfully solved using aseptic working conditions and antibiotics for suppressing growth of contaminants.

Methods for the quantitative recovery of small numbers of phages from water

1. Amicon stirred cell units using membranes with molecular cut-off levels of 50 000 yielded an efficiency of recovery (EOR) in excess of 90% for all groups of phages in drinking water and environmental waters with limited pollution. The technique proved feasible for volumes of drinking water up to 1000 litres. Disadvantages included rapid clogging by turbid waters, and the cost of membranes.

2. The glass wool adsorption-elution procedure established in the laboratory has an EOR of 60-90 % for human viruses, but on average only about 1 % for a variety of phages tested. The results indicated that compared to human viruses, the adsorption of phages was much less efficient and so was the release of those phages that did adsorb. Efforts to improve the EOR by modifying the electrostatic charge on the glass wool using polyethylene-imine, and by increasing the pH of the elution buffer, made no meaningful difference.

3. The recovery of phages by adsorption-elution using conventional cellulose nitrate membrane filters with diameter 47 mm and pore size 0,45 μm as described by Sobsey, has been investigated in detail. Tests were carried out on 100 ml samples of a variety of environmental waters. Phage counts obtained were compared to those obtained by direct plaque assays. The latter yielded higher counts for waters which contained relatively high numbers of phages, but the membrane filtration procedure yielded positive results more often and higher counts for

waters which contained very low numbers of phages. This relatively inexpensive procedure would, therefore, seem to have merit for the recovery of phages from waters with low counts of phages such as drinking water.

Direct plaque assays on large volumes of water using large petri dishes

The merits of a single agar layer procedure using 140 mm diameter petri dishes described previously have been confirmed for groups of phages concerned. This procedure is intended for the detection of phages in 100 ml samples of water containing low numbers of phages such as drinking water supplies. Modification of the procedure by converting it into a double agar layer procedure substantially increased counts of phages obtained. This was confirmed for all three groups of phages concerned in comparative tests on a variety of water samples. The double agar layer procedure proved superior to the membrane filter adsorption-elution technique in terms of sensitivity and accuracy. Unfortunately, however, the technique is relatively expensive since the total cost of the ten disposable plastic petri dishes used amounts to some R 60. The recommended procedure for the new double agar layer plaque assay is described in detail in the final report.

Qualitative detection of phages in water using a presence-absence (P-A) procedure

These tests are based on adding nutrients and a culture of the appropriate host to a large volume of test water and overnight incubation. Phages present in the water, theoretically as few as a single viable particle, then infect the growing host and multiply. After overnight incubation these phages are present in large numbers which are readily detectable by conventional plaque assays. P-A procedures for all three groups of phages concerned were evaluated in tests on more than 50 samples of river water and more than 600 samples of treated drinking water. The results indicated that 500 ml was the optimum volume for samples of treated drinking water. Results for tests on 1000 ml did not differ significantly from those for 500 ml. The P-A tests proved more sensitive than direct plaque assays using large petri dishes or recovery of phages by membrane filter adsorption-elution. P-A tests are also relatively inexpensive and simple. However, since a single viable phage particle yields a positive result, the procedure is extremely sensitive to contamination, and the tests have to be conducted under absolutely aseptic conditions. P-A tests proved the method of choice for routine monitoring of the quality of treated drinking water supplies. The tests are suitable for monitoring the compliance of water supplies to a quality limit

based on the absence of phages from a particular volume of water. Details on numbers of phages present would require supplementary direct plaque assays. Recommended procedures for P-A tests are described in the final report.

Incidence of selected indicator phages in human and animal excreta

In an analysis of some 200 stool specimens from humans and a variety of domestic and wild animals, *B fragilis* HSP40 phages were detected only in 13 % of human stool specimens. Somatic and male-specific coliphages were excreted by considerably higher percentages of both humans and a variety of animals. These findings confirmed earlier observations that *B fragilis* HSP40 phages are excreted exclusively by humans and can be used to distinguish between faecal pollution of human and animal origin.

Incidence of phages, bacterial indicators and human viruses in waste and river water

Comparative analyses have been carried out on the following waters:

1. A total of 209 samples collected over a period of one year from a stream receiving diffuse effluents from an informal settlement in Mamelodi has been analysed. In the great majority of samples phages outnumbered human viruses. However, in small but meaningful number of samples human viruses were detected by conventional cell culture techniques in the absence of any of the groups of phages concerned. These results suggested that generally the absence of phages is a reliable indication of the absence of human viruses, but in exceptional cases phages may fail to reveal the presence of human viruses.

2. Basically similar results were obtained in studies on 150 samples of Klip River water and more than 50 samples of Vaal Dam sluice gate water. In this case, however, viruses were detected only in five samples which yielded negative results for *B fragilis* HSP40 phages in P-A tests on 500 ml volumes. Somatic and male-specific coliphages consistently outnumbered human viruses.

3. The above findings on natural water sources were confirmed in studies on waste water effluents from informal settlements at Botshabelo. The results highlighted the value of *B fragilis* HSP40 phages as indicators of human faecal pollution. The phages were detected only

in waste waters known to contain predominantly human wastes, and not in environmental waters known to contain predominantly animal wastes.

Incidence of phages, bacterial indicators and viruses in treated drinking water supplies

More than 600 samples of treated drinking water from various points in the Rand Water treatment system were analysed for human viruses, phages and bacterial indicators of faecal pollution, using the best available techniques. Human viruses were never detected, and phages rarely. The results showed that phages are valuable indicators of the absence of viruses, and of the efficiency of water treatment processes. The results indicated that P-A tests for somatic coliphages on 500 ml samples are practical, inexpensive and valuable components of systems for routine quality monitoring of treated drinking water supplies.

Survival of selected phages, bacterial indicators and viruses in water environments

The survival of phages, human viruses and bacterial indicators in various water environments has been compared. Samples of sea water, river water and buffer were seeded with human and animal stool specimens containing naturally occurring organisms. Reaction mixtures were seeded with laboratory cultures of polio viruses and selected phages which were not present in stool specimens. The survival of these organisms was determined under controlled laboratory conditions and in the open on roof top with or without exposure to sunlight. The results indicated that under most conditions resembling those in natural water environments at least some phages survived longer than laboratory strains of polio virus. Since polio virus is known as a relatively resistant member of enteric viruses, and phages have been shown to generally outnumber human viruses in most water environments, it would appear reasonable to conclude that the absence of phages is under most conditions a reliable indication of the absence of human viruses.

Survival of phages in water treatment processes

In routine analysis of water reclaimed from abattoir effluent by means of multiple-barrier system, viruses were detected only once in the absence of phages. These results indicated that enteric viruses are rarely detected in water treated by the processes concerned.

Practical aspects of phage indicators

Evidence has been presented that phages are detectable by relatively simple and inexpensive techniques. The following techniques which have been described and evaluated in detail for application in practice, proved reliable and sensitive:

* Double agar layer plaque assays using small petri dishes for samples up to 10 ml:
  Quantitative direct plaque assays for water which contains relatively high numbers of phages
* Double agar layer plaque assays using large petri dishes for samples up to 100 ml:
  Quantitative direct plaque assays for water which contains relatively small numbers of phages
* Presence-Absence (P-A) test for samples up to 500 ml or more:
  P-A test for water which contains very low numbers of phages

These techniques proved suitable for the quantitative enumeration of phages in waste water, river water, sea water and dam water, as well as the sensitive , routine qualitative monitoring of treated drinking water supplies.

International collaboration on phage technology and water quality assessment

Work on this project has been carried out in close collaboration with the International Organization for Standardization (ISO), and leaders in the field in a number of laboratories abroad. Techniques recommended are based on those standardised by ISO, with modifications for improvement according to results obtained in this study.

Recommended water quality guidelines

Data obtained in this study were used to formulate recommendations for drinking water (absence of phages from 500 ml samples) and environmental waters used for direct contact recreation (less than 10 phages per 10 ml). Recommended test methods for these guideline levels, application of the tests and the interpretation of results are discussed in the report.

Recommended procedures for routine water quality monitoring

Techniques recommended for the routine enumeration of all three groups of phages and monitoring of water supplies have been described in detail in the report.

Technology transfer and capacity building

Details on technology and expertise developed in this project have been forwarded to a number of laboratories for application in practice. These laboratories include Rand Water, Umgeni Water, University of the North West at Mmabathu, Johannesburg Municipal Laboratory and the Windhoek Municipal Laboratory. Results obtained in this study have been used by the Department of Water Affairs and Forestry in the formulation of water quality guidelines.

FUTURE RESEARCH NEEDS AND PRIORITIES

The project has revealed attractive possibilities for further development of technology and expertise to utilise phages as indicators in water qualitative assessment and monitoring. Challenges such as the following warrant further investigation:

* Development of practical techniques for the identification of serogroups of male-specific coliphages. Information presently available suggests that genetic hybridisation using appropriate gene probes may prove the most practical approach, but would require evaluation against neutralisation assays using specific antibodies.
* Determination of the specificity of serogroups of male-specific coliphages for excretion by humans and animals. This would include screening stools from humans and a variety of animals for the presence of the five serogroups of male-specific coliphages presently known, and possibly additional serogroups.
* Evaluation of the application in practice of serogroups of male-specific coliphages for distinction between faecal pollution of human and animal origin.
* Improvement of the sensitivity of techniques for the qualitative and quantitative detection of *B fragilis* HSP40 phages.
* Development of rapid techniques for the detection and enumeration of phages. Molecular techniques may offer feasible possibilities for this purpose.
* Development of practical techniques for the detection of specific members of the groups of somatic coliphages, male-specific coliphages and *B fragilis* HSP40 phages which may have indicator features superior to those of the groups of phages presently used.
* Research on shortcomings of phages and approaches to overcome these shortcomings. This would include an investigation of circumstances, such as outbreaks of viral diseases, which may result in situations where phages are outnumbered by human viruses.
* Improvement in practical details of phage technology including detection methods, host strains, the handling of host strains, and the characterisation of phage isolates.
* Correlation of the incidence of phages in water and the incidence of enteric infections in consumers of the water.

SUMMARY AND CONCLUSIONS

1. Practical techniques for the quantitative and qualitative detection of three groups of phages (somatic coliphages, male-specific coliphages and *B fragilis* HSP40 phages) have been optimised and evaluated for application in practice.

2. Practical procedures for routine water quality monitoring have been established and evaluated.

3. Evidence has been presented that phages generally outnumber human viruses in water environments, and that the absence of phages offers a valuable indication of the absence of human viruses as well as the efficiency of water treatment processes. However, since there is no direct correlation between numbers of any phages and the wide variety of human viruses that may be present in water, the absence of phages is no absolute guarantee of the absence of human viruses. Phages would, therefore, best be applied as an important component of an appropriately battery of tests for assessment of water quality.

4. Details have been obtained on the incidence and behaviour of phages in various water environments on which reliable quality guidelines can be based.

5. A contribution has been made to the establishment of technology and expertise which has valuable benefits for the water industry and related health disciplines. The technology and expertise has been transferred to other laboratories, and a contribution has been made to the training of manpower. An infrastructure of collaboration with laboratories locally and abroad has been established.

2. PROJECT RESEARCH

2.1. Introduction

Awareness of the value of phages as indicators of water quality is rapidly gaining ground (IAWPRC Study Group on Health Related Water Microbiology, 1991; Grabow, 1996; Müller, 1997). This is reflected by the formulation of standard phage detection techniques by the International Organization for Standardization (ISO, 1995, 1997a,b,c), and the inclusion of phages in water quality guidelines by bodies such as the World Health Organization (World Health Organization, 1993, 1996), American Public Health Association (Standard Methods, 1995), and the Department of Water Affairs and Forestry (Department of Water Affairs, 1993). The Commission of the European Communities is considering the replacement of specifications for human viruses by guidelines for phages (A H Havelaar, personal communication).

Since this is a new indicator concept, the development of detection techniques for various phages, and the application of the techniques in practice, are still to large extent in an infant stage. This is illustrated by substantial differences in techniques recommended by, for instance, ISO and the American Public Health Association. Many questions about the techniques and their application remain unanswered. This includes the preparation of host cultures, the composition of growth media and procedures for the detection of small numbers of phages in large volumes of water.

The objectives of this study include research on the optimisation of phage detection methods, assessment of the efficiency and reliability of these methods, and evaluation of the application of these methods in practice.

2.2. Methods for the Quantitative Enumeration of Phages

2.2.1. Somatic coliphages

2.2.1.1. Double agar layer plaque assay

A technique based on principles specified in ISO (1995) has been developed and optimised. Details of the technique are recorded in Appendix 1. The technique is less expensive, time consuming and labour intensive than the ISO technique. Although

the techniques have not been compared directly, there is no evidence of meaningful difference in sensitivity or accuracy. The ISO Working Group concerned is presently investigating the possibility of adapting the ISO technique accordingly. At this stage there is no indication of superior techniques or possibilities for improvement of the method. The developed and optimised technique differs from the ISO technique in terms of certain ingredients of growth media, including cations and sources of carbohydrates. Evaluation and optimisation of these differences would involve a substantial amount of work well beyond the scope of this project. Although there is no reason to believe that these variations may cause meaningful differences in results, detailed evaluation and optimisation is fully justified for the elimination of uncertainties, formulation of standardised techniques, simplification of techniques, and possibly also reducing the cost of analyses.

2.2.1.1.1. Time required for plaques to appear and effect of tetrazolium

Parallel plaque assays were carried out using plates with (300 μg/ml in top layer) and without 2,3,5-triphenyl- tetrazolium -hydrochloride (tetrazolium) in the top layer of double agar layer plaque assays as described in Standard Methods (1995). Comparative assays were carried out on dechlorinated tap water seeded with a laboratory culture of the somatic coliphage V1 (double-stranded DNA with short tail), and on samples of river water containing naturally occurring phages. Each assay consisted of three plates per dilution, and each test was repeated at least three times. Plates were incubated at 37°C and plaques were counted at time intervals with least possible exposure of plates to temperatures below 35°C. No effort was made to count plaques between 8 and 20 h because obtaining results in this period is impractical for a normal working day of 8 h.

The results of typical experiments in Table 2.1 indicate that:

a) Maximum plaque counts were obtained only after 20 h incubation, and these were more than twice as high as counts recorded after 8 h.
b) Counts tended to be higher on plates which contained tetrazolium. It would appear that the difference was primarily due to better visibility of plaques under certain circumstances. The reason for this phenomenon is not clear. In subsequent comparative tests on river water containing naturally occurring phages, differences between counts on plates with and without tetrazolium were not meaningful, and

plaques actually tended to be more clearly visible on plates without tetrazolium. The inclusion of tetrazolium in double agar layer plaque assays as previously suggested (Standard Methods, 1995) would, therefore not seem to be justified, particularly in view of the additional cost and labour. Similar results were recorded for phage V1 seeded into dechlorinated tap water and naturally occurring somatic phages in river water.

According to Hurst *et al* (1994) plaque formation by phages belonging to the families *Podoviridae* and *Siphoviridae* (both double-stranded DNA and long non-contractile tails) was inhibited by 300 μg/ml of tetrazolium in top layers. The initial findings fail to confirm these observations.

2.2.1.2. Rapid detection of somatic coliphages

Two techniques described for the rapid detection of phages (Armon and Kott, 1993; Ijzerman *et al*, 1994) were evaluated. Both proved complicated, labour intensive and expensive, while the extent to which optimum results were available sooner than conventional plaque assays was not meaningful. In addition, both are qualitative presence/absence (P-A) tests with no meaningful advantages over the P-A tests routinely used in our laboratory. The techniques were, therefore, not studied in further detail and rejected as impractical.

Table 2.1 Time required for detection of plaques and effect of tetrazolium on detection of plaques in double agar layer plaque assays for somatic coliphages

Test samples	Incubation time (h)	Average plaque count	
		Without tetrazolium	With tetrazolium
Suspension of V1 coliphage in PBS buffer	3,5	0	0
	4,0	0,3	2
	4,5	1	3
	5,5	2	5
	6,0	5	10
	6,5	6	11
	7,0	15	30
	7,5	22	49
	8,0	22	51
	20	73	119
	24	73	119
K19 Klip River water	3,5	0	0.3
	4,0	0	0,3
	4,5	0	0,3
	5,0	0,3	1
	5,5	0,3	1
	6,0	0,3	2
	6,5	1	3
	20	3	5
	24	3	5

2.2.2. Male-specific coliphages

The double agar layer plaque assay described in Appendix 1 is based on the standard method defined in ISO (1995). A detailed assessment of the ISO technique unfortunately confirmed that the cumbersome, time consuming and expensive procedure for the preparation of host inoculum cultures was essential. There is no indication of a feasible alternative for obtaining host cells at optimum levels of susceptibility to male-specific coliphages.

2.2.2.1. Selection of the host of choice for male-specific coliphages

Presently available results from research carried out world-wide indicate that the only two host strains worth considering are:

Havelaar host : *Salmonella typhimurium* strain WG49 phage type 3 Nal^r (F'lac: :Tn5) NCTC 12484

Cabelli host : *Escherichia coli* HS(pFamp)R

Further details on these hosts have been recorded elsewhere (ISO, 1995).

After optimisation of procedures for application of the Havelaar host, the Havelaar and Cabelli hosts have been compared in tests on naturally occurring male-specific phages in selected water environments.

In comparative tests on 34 water samples collected from the Klip River at sampling point K19 (Fig 1), the Havelaar host yielded higher average and median counts for male-specific coliphages than the Cabelli host (Table 2.2). The same trend was evident from similar tests on another 12 samples of K19 water, and 12 samples of Vaal Dam sluice gate (A18) water (Tables 2.3 and 2.4). The latter Table shows that the Havelaar host yielded positive results more often than the Cabelli host for water which contained low numbers of phages. At this stage there is no indication of host strains that may prove superior to the Havelaar host for the detection of male-specific coliphages.

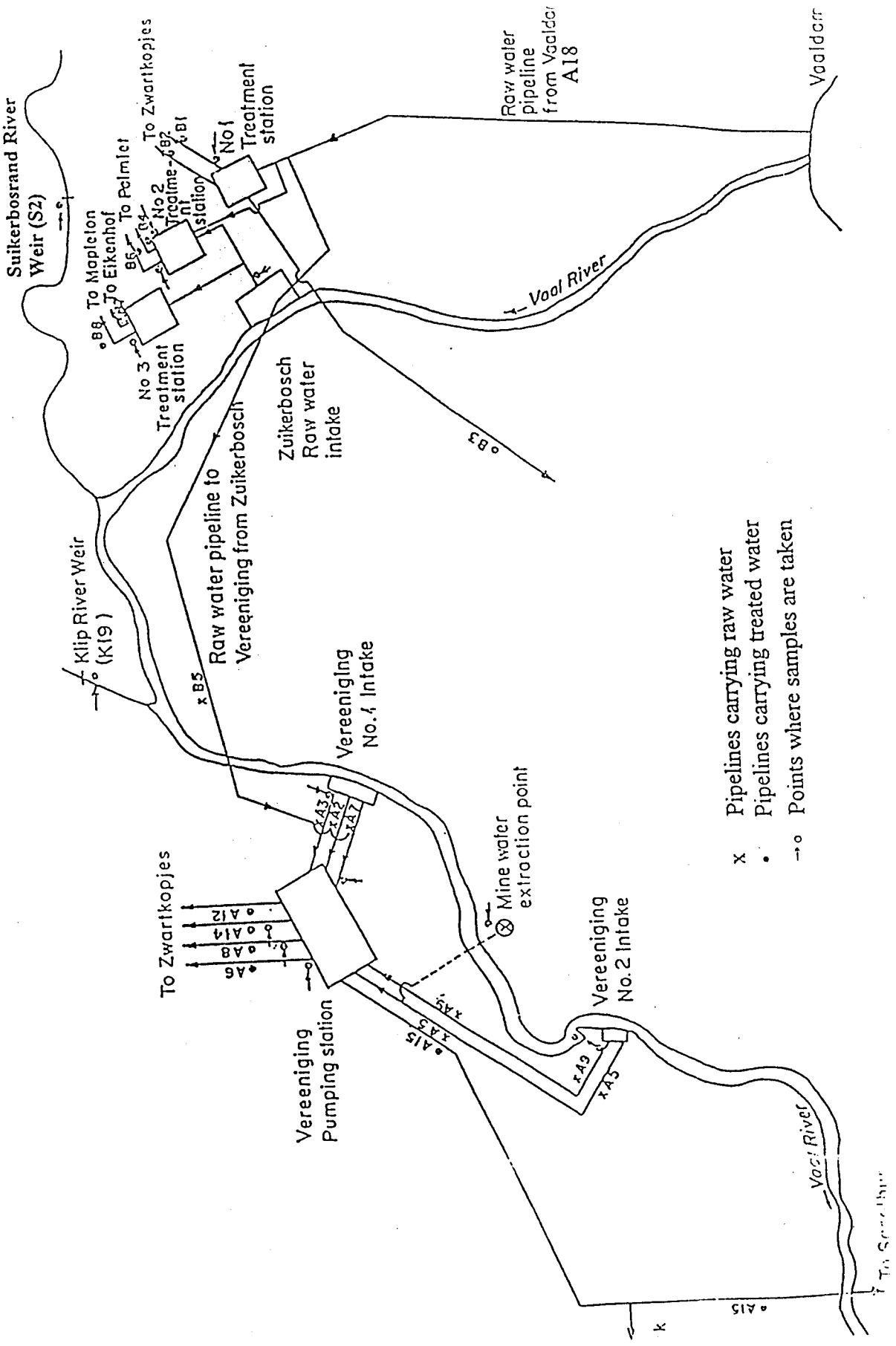

Figure 1. Map showing the location of sampling point K19 on the Klip River which feeds the Vaal River

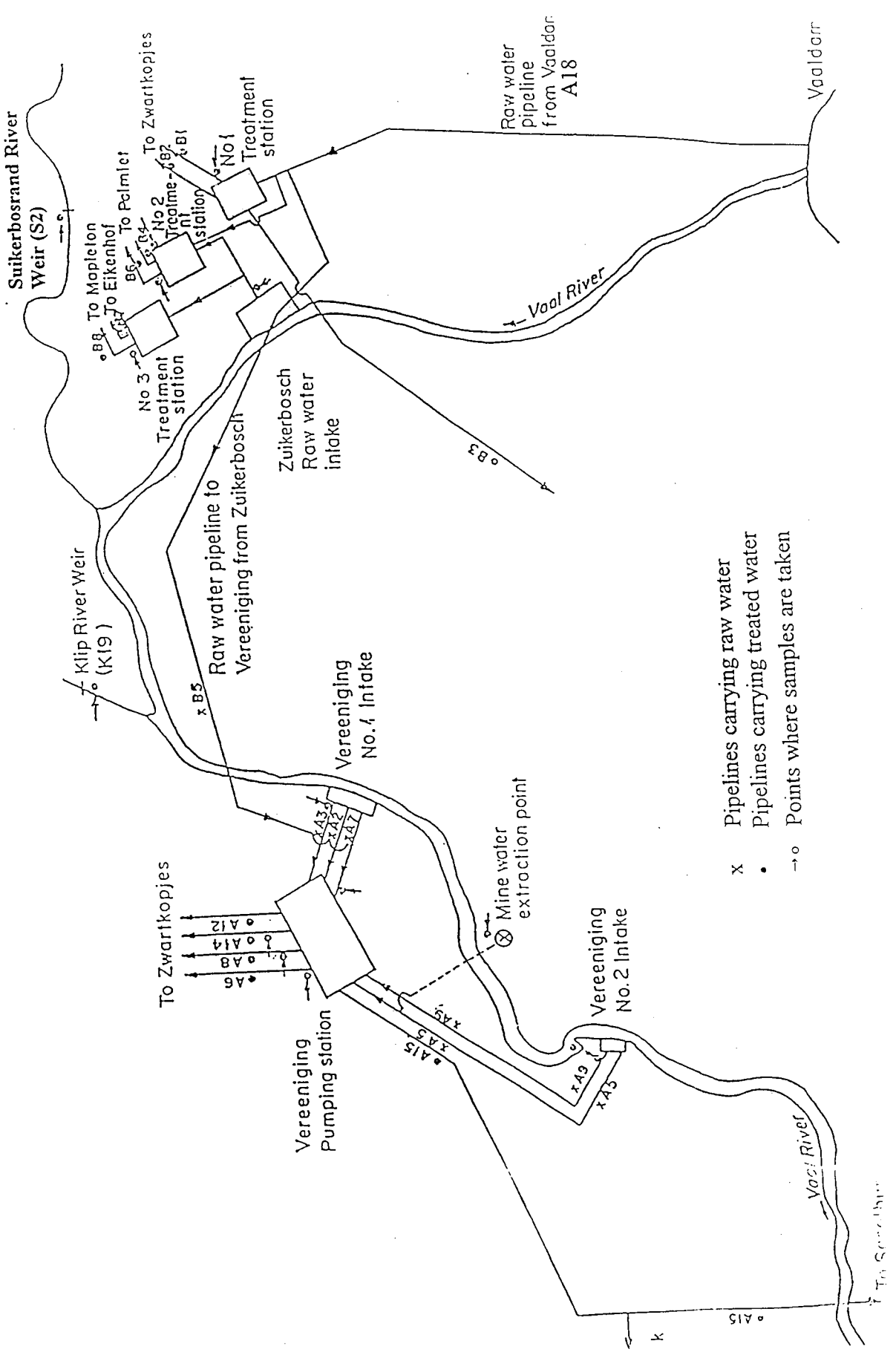

Figure 1. Map showing the location of sampling point K19 on the Klip River which feeds the Vaal River

Table 2.2 Comparison of the Cabelli and Havelaar hosts for the enumeration of male-specific coliphages in Klip River water at sampling point K19 using the direct double agar layer plaque assay

	Plaque forming units per 1 ml	
	Cabelli host	Havelaar host
No of tests	34	34
Range	0-24	0-28
Average	3,76	5,02
Median	1,0	2,0

2.2.2.2. Confirmation of male-specific coliphages

2.2.2.2.1. Electron microscopy

The distinctive morphology of male-specific coliphages was clearly visible by electron microscopic procedures routinely used in the laboratory.

2.2.2.2.2. RNase test

Test procedures based on the selective inactivation of male-specific coliphages by the RNase enzyme have been optimised and successfully established. The test has been used to characterise phages isolated on the Havelaar and Cabelli hosts. Phages from 116 plaques obtained in four tests on K19 river water using the Havelaar host, and the same number of plaques obtained in tests on the same water samples using the Cabelli host, were purified and cultivated for characterisation. Plaques were picked at random from both hosts. All plaques on the Havelaar host were faint plaques typical of male-specific coliphages. Many plaques on the Cabelli host were clear plaques typical of somatic phages. In total 93% of phages on the Havelaar host were RNase sensitive, ie RNA phages. The percentage of RNase sensitive plaques on the Cabelli host was much lower. These findings show that many plaques on the Cabelli host are not male-specific RNA phages, which includes the clear plaques. All RNase sensitive phages isolated from the Cabelli host, produced plaques on the Havelaar host. However, some RNase sensitive phages isolated from the Havelaar host failed to produce plaques on the Cabelli host, which suggests that the Cabelli host may not be susceptible to all male-specific RNA coliphages. RNase sensitive phages were confirmed as male-specific RNA (F-RNA) phages by electron microscopy. Exact

details of results are not presented because the results of RNase tests tended to prove difficult to interpret and were not particularly reliable. The findings were not investigated in further detail because the results left no doubt that the Havelaar host was more specific for F-RNA phages than the Cabelli host. These observations, together with results in Tables 2.2 to 2.4 according to which the Havelaar host yields higher plaque counts than the Cabelli host for phages in a variety of water environments, strongly suggest that the Havelaar host is superior to the Cabelli host for general purposes of the detection of F-RNA phages in water environments.

2.2.2.3. Typing of male-specific coliphages by gene probe hybridisation

Prof Sobsey kindly supplied materials and details on techniques for the typing serogroups of male-specific RNA coliphages by means of hybridisation using digoxigenin labelled oligonucleotide probes as described by Hsu *et al* (1995). Indications are that this procedure is more practical for the confirmation of male-specific RNA coliphages than conventionally used RNase inactivation, electron microscopy and serotyping. This procedure is expected to prove of major benefit in assessment of the value of male-specific RNA coliphages for water quality assessment. A particularly important advantage of the gene probe hybridisation procedure is that it offers a practical method for typing the five serotypes of male-specific RNA coliphages. Indications are that certain serogroups of these phages are specific for faecal pollution of human origin and others for faecal pollution of animal origin (Hsu *et al*, 1995). This implies that typing of male-specific coliphages may prove a useful tool for distinguishing between faecal pollution of human and animal origin. Details on serogroups and probes are summarised in the diagram below. The oligoprobes (base length 25-30) are end-labelled with digoxigenin for colorimetric immuno-enzymatic detection. Optimisation of techniques for the typing of the serogroups of male-specific coliphages requires inputs beyond the scope of this project.

Diagram: 2.1 Details on serogroups of male-specific (F-RNA) (F^+ RNA) coliphages and gene probes for their typing (Hsu *et al*, 1995)

Serogroup I	Serogroup II	Serogroup III	Serogroup IV
Phage MS2	Phage GA	Phage Qß	Phages FI & SP
Animals	Humans	Humans	Animals
Probe 1	Probe II	Probe III	Probe IV
Probe A		Probe B	

2.2.3. *Bacteroides fragilis* HSP40 phages

The double agar layer plaque assay described previously (Grabow *et al*, 1993, 1995) has been re-evaluated, modified and optimised . The latest version is described in Appendix 1. The P-A test procedure for the phages is also described in this Appendix, and is based on the same principles as those described for somatic and male-specific coliphages. Although it has been confirmed that these phages are highly specific for human faecal pollution, data obtained in this study on their incidence in various water environments show that their numbers tend to be rather low, in many cases lower than those of human viruses. This implies that although they specifically indicate faecal pollution of human origin, their absence from water environments does not necessarily imply the absence of human viruses, at least in terms of technology for the detection of these phages presently available. According to Prof J Jofre (personal communication) there is reason to believe that numbers of *B fragilis* HSP40 in sewage and polluted water environments are considerably higher than indicated by presently available techniques. There is, therefore, a need to upgrade techniques for the detection of *B fragilis* HSP40 phages because they serve a most valuable role in water quality assessment. One disadvantage of presently available techniques is that they are time consuming, cumbersome, labour intensive and relatively expensive, primarily because strict anaerobic incubation is required. Prof Jofre and his team are presently working on the possibility of cloning the gene that codes for the receptor site of *B fragilis* HSP40 phages into an aerobic bacterium such as *E coli* which may support the replication of the phages and can be used as host in convenient assays similar to those for coliphages.

2.2.4. Decontamination of water samples for phage testing

Chloroform decontamination was investigated in three comparative tests on hospital waste water. These samples were used to investigate the effect of chloroform on naturally occurring phages in waste water samples. Homogenised samples were divided into two, one was exposed to chloroform according to common procedure, and phage titrations were carried out on both. On average counts of somatic coliphages and *B fragilis* HSP-40 phages tended to be higher in chloroform treated samples, and counts of male-specific coliphages higher in untreated samples. Higher counts were probably due to the elimination of contaminant bacteria which overgrow the host organisms, which may apply in particular to the *B fragilis* phages. Reasons for the lower counts of male-specific coliphages in chloroform-treated samples were not clarified.

No further time was spent on questions regarding the decontamination of samples because problems with contamination were solved by rigorous application of aseptic laboratory procedures and antibiotic treatment. However, the observations that chloroform treatment may increase phage counts, at least under some circumstances, warrants further investigation. Decontamination of samples by membrane filtration would also seem to warrant consideration (Jofre, personal communication).

2.3. Methods for the Recovery of Small Numbers of Phages

2.3.1. Ultrafiltration

The efficiency of ultrafiltration for the recovery of phages has been evaluated. Tests were carried out on samples of phosphate buffered saline (PBS) and dechlorinated tap water seeded with known numbers of type-specific representatives of somatic and male-specific coliphages, and *B fragilis* HSP-40 phages. Tests were carried out on 200 ml, 300 ml and 1000 ml seeded test samples. Titres of phages in the seeded test samples ranged from 1×10^3 to 5×10^4 per ml. An Amicon stirred cell unit with 47 mm diameter membranes of 50 000 molecular cut-off level, was used. Test samples were filtered to a final volume of 20-35 ml. This concentrate was thoroughly stirred to suspend all phages which may have adsorbed to the membrane, and then transferred

to a measuring cylinder. Another 5 ml of PBS was added to the filter chamber and thoroughly stirred to remove remaining phages. This was then added to the concentrate, and the volume of the concentrate made up with PBS to a fixed volume for titration of the phages. In tests on seven 1000 ml samples seeded with MS2 male-specific coliphages, the average EOR was 94% (range 61-124%). Similar results were obtained for smaller volumes of test samples. Comparable results were obtained for the other two phages.

The results show that ultrafiltration can be used for the highly efficient recovery of phages from volumes of water of up to 1000 ml and possibly even more. The most important disadvantage of the method is that meaningful application is limited to water with low turbidity because the membranes clog readily and the presence of concentrated organic material in the final test sample may interfere with plaque assays. Contamination was a typical problem of ultrafiltration concentrates of river water. The technique proved well suited for drinking water. However, the procedure is relatively expensive. The membranes cost about R 500 a piece and are not reusable.

2.3.2. Glass wool adsorption-elution

The efficiency of recovery (EOR) of glass wool adsorption-elution for human enteric viruses is in the order of 60-95% according to reports from various laboratories (Grabow, 1996). The efficiency of this procedure for the recovery of phages has, therefore, been investigated. The MS2 male-specific coliphage was used as model in these experiments. In one series of experiments five 5-litre samples of dechlorinated tap water were seeded with MS2 phage. The range of phage counts in the water samples was 5,0 x 10^4 to 1,2 x 10^6 per ml. The preparation of filters containing 10 g of glass wool, the filtration, and the recovery by means of pH 9,5 beef extract-glycine buffer, were carried out as described for human viruses. The average EOR was 1,1% (range 0,3-1,8%). Titration of phages in the filtrates indicated that on average 72% (range 64-83%) of phages failed to adsorb, which implies that on average only 28% of phages adsorbed. These results suggest that adsorption of phages to the glass wool was poor, and that the release of adsorbed phages was also inefficient. Inactivation of phages by the pH 9,5 elution buffer was not tested but

appears unlikely in view of details on the resistance of human viruses to this buffer.

In an attempt to increase the efficiency of adsorption of phages, the glass wool was treated in packed columns with polyethylene-imine (PEI), according to procedures described for human viruses. PEI is used to change the electrostatic charge on conventional glass wool from negative to positive for recovery of negatively charged human viruses at neutral pH levels. Basically PEI was passed through a column to completely saturate the glass wool, the column stopcock was then closed with sufficient PEI left to cover the glass wool, and left like this for 24 h on the laboratory bench. The PEI was then drained and the column rinsed with 100 ml of distilled water. Tests were carried out using five 5-litre samples of dechlorinated tap water seeded with MS2 phage to counts of 5,0 x 10^4 to 4,6 x 10^5 per ml. Phages were eluted with the conventional pH 9,5 buffer. In this case the average percentage of phages in the filtrate was only 9% (range 0-21%), suggesting that on average 91% of phages had adsorbed. However, the average EOR was still only 6,4% (range 5,2-7,1%). These results show that PEI increases the adsorption of MS2 phage to the glass wool, but that the phages are not readily released by the elution buffer and procedure used. The eventual EOR is not much higher than without PEI treatment.

In an attempt to increase the release of phages from PEI-treated glass wool columns, a number of experiments were carried out in which the elution process was modified by increasing the volume of elution buffer, changing the pH of the elution buffer to pH 10,0 or 9,0, and increasing or decreasing the flow rate of the elution buffer. None of these modifications made any significant difference to the EOR. Since other possibilities for the detection of small numbers of phages in large volumes of water are available, further attempts to upgrade the recovery of phages by glass wool adsorption-elution were abandoned.

The results are interesting in terms of the following: Since the recovery process depends on the adsorption of viruses and phages to a particular surface, and subsequent release from that surface, the difference in EOR indicates differences in adsorption and elution properties between phages and human viruses. Since

adsorption to surfaces plays an important role in the behaviour and survival of human viruses in the environment, these differences in adsorption and elution properties may imply differences in the behaviour of viruses and phages in the environment, which would have implications for the indicator value of phages.

2.3.3. Membrane filter adsorption-elution

The method described by Sobsey *et al* (1990) has been evaluated. The method is based on adsorption-elution using conventional membrane filtration (Gelman GN-6 Metricel; 47 mm; 0,45 μm) and elution with 3% beef extract. Any volume of water that will pass through a membrane (100 ml or more) can be filtered and phages can afterwards be recovered by appropriate procedure using 5 ml beef extract.

In comparative tests on 22 samples of Klip River water at sampling point K19, direct double agar layer plaque assays using small dishes yielded higher average and median counts of somatic and male-specific coliphages than similar plaque assays on membrane filter concentrates of 100 ml samples. In the case of direct plaque assays the count per 100 ml was calculated from counts obtained in tests on 1 ml samples (Table 2.3 and 2.4). In similar comparative tests on 12 samples of Vaal Dam A18 water which contained considerably lower numbers of phages, the membrane filter concentrates yielded positive results more often for male-specific coliphages, and average and median counts were higher (Table 2.5). However, counts of somatic coliphages in Vaal Dam A18 water were higher by direct plaque assays (Table 2.6).

The higher counts obtained by direct plaque assays compared with plaque assays on 100 ml filter concentrates of K19 water, may be due to failure of the filters to recover all phages, which is reasonable to expect. However, the higher counts obtained by direct plaque assays may also at least to some extent be due to error introduced by multiplication of counts for 1 ml test samples by 100 to calculate counts per 100 ml for comparison to results obtained by membrane filter tests. Despite potential shortcomings in tests on water containing numbers of phages large enough for direct titration, the value of membrane filter recovery was clearly illustrated in test on A18 water which contained low numbers of male-specific coliphages (Table 2.5). These

findings indicate that the membrane filter method has merit for the detection of small numbers of phages in large volumes of water. Counts of somatic coliphages in A18 water were probably high enough for direct plaque assays (Table 2.6) eliminating the benefits of membrane filter recovery. This possibility is supported by results of tests on alternative methods for the detection of low numbers of phages described in the next section.

2.4. Direct Plaque Assay for Small Numbers of Phages in Water

Direct plaque assays on 100 ml samples of water using single agar layers in 10 large petri dishes according to the principles described earlier (Grabow *et al*, 1993) have been re-evaluated and optimised.

In comparative tests on naturally occurring phages in waters from various environments phages were enumerated by conventional double agar layer plaque assays using small petri dishes (90 mm diameter) in threefold on 1 ml samples (SP-DL), and single agar layer assays on 100 ml samples using 10 large petri dishes (140 mm diameter) (LP-SL). Counts per 100 ml for assays using small petri dishes were calculated by multiplying average counts for 1 ml by 100. Results show that LP-SL yielded higher counts and positive results more often than SP-DL for waters which contained very low numbers of somatic coliphages but not for waters which contained relatively high counts of phages (Tables 2.6 to 2.9).

NOTE:

SP-DL = Small Plates with Double agar Layer
LP-SL = Large Plates with Single agar Layer
LP-DL = Large Plates with Double agar Layer

Comparative tests similar to those recorded in Tables 2.6 to 2.9 were carried out in tests on dechlorinated tap water seeded with somatic coliphage V1, male-specific coliphage MS2 and *B fragilis* phage B40-8. Results were similar to those recorded for naturally occurring phages in Tables 2.6 to 2.9. Reasons for the lower numbers of plaques on the large petri dishes remain to be explained, but may be due to less

ideal host infection and replication conditions on the large petri dishes. The difference may also at least in part be due to error introduced by multiplying counts for 1 ml by 100 to obtain a count per 100 ml for SP-DL. However, the results still imply that the procedure offers a useful means for the direct quantitative enumeration of very low numbers of all three groups of phages in 100 ml volumes of water. The volume of water to be analysed can obviously be increased by increasing the number of plates. The procedure would remain relatively simple. In the case of tests for *B fragilis* HSP-40 phages in large volumes of water, this approach may even be superior to P-A tests, taking into account the shortcomings of P-A tests for these phages outlined in the next section.

Unfortunately cost is a factor in using large petri dishes for direct plaque assays on large volumes of water. The cost of a single disposable large petri dish (140 mm) is about R 5,00, which implies that the total cost for a test using 10 plates amounts to R 50,00 for petri dishes alone. The cost of small petri dishes (90 mm) is about R 0,50, which implies that the cost of petri dishes for a three-fold test using three dilutions amounts to R 4,50. Cost would, therefore, be another reason for using large plates only for the quantitative enumeration of very small numbers of phages, as may be expected in drinking water supplies and environmental waters with limited pollution. The cost of using large petri dishes could, of course, be reduced by using reusable glass petri dishes.

Reasons for the differences in counts obtained by small and large plates were investigated, and attempts were made to increase the sensitivity and accuracy of counts obtained by large plates. One difference between plaque assays using large and small petri dishes is the double agar layer used with the small plates. The possibility of the double layer playing a role in plaque formation was investigated by comparing SP-DL, LP-SL and large plates with double agar layers (LP-DL). Comparative tests on various waters showed that LP-DL yielded higher counts and positive results more often than LP-SL (Tables 2.7 to 2.11). The LP-DL approach has, therefore, been optimised and investigated in further detail. A recommended procedure for LP-DL is described in Appendix 1. Although LP-DL yielded higher counts than LP-SL,

counts for waters which contained relatively high numbers of phages were still lower than those obtained by SP-DL. The agar medium used for the bottom layer in LP-DL plates represents a minor factor in the cost of the assays, which implies that the difference in the cost of LP-DL and LP-SL is minimal. The additional labour required for preparing the large plates with bottom agar is likewise minimal.

Reasons for higher counts obtained by SP-DL than LP-DL are not clear and were not investigated in further detail. Theoretically both may be expected to detect all plaque forming units because the only difference is the diameter of the plates. The most likely reason for the differences would, therefore, appear to be associated with error introduced by the calculation of counts per 100 ml for SP-DL test carried out on 1 ml test samples.

Whatever the reason for differences in counts per 100 ml obtained by SP-DL and LP-DL, the results clearly illustrate the value of LP-DL for the direct enumeration of very low numbers of phages in water. In order to determine levels of phage numbers best suited for SP-DL and LP-DL, comparative plaque assays were carried out on dilutions of Klip River K19 water. The results in Tables 2.12 and 2.13 indicate a cut-off level at counts of about 1000 plaques per 100 ml, with SP-DL yielding higher counts above this level and LP-DL below this level.

Table 2.3 Counts of somatic coliphages in Klip River K19 water and Vaal Dam sluice gate A18 water obtained by plaque assays directly on water samples and on 100 ml membrane filter concentrates

	Plaque forming units per 100 ml			
	Klip River K19		Vaal Dam A18	
	Direct	Concentrate	Direct	Concentrate
Test series	95-07-10 to 96-07-22		96-01-08 to 96-08-19	
No of tests	22	22	19	19
No positive	22	20	14	7
Range	200-6030	0-2170	0-700	0-210
Average	2182	598	107	15
Median	1180	150	30	0
Standard deviation	2104	710	180	48

Direct plaque assays : Count per 100 ml calculated from results of tests on 1 ml samples
Membrane filters : Plaque assays on phages recovered from 100 ml samples

Table 2.4 Counts of male-specific coliphages in Klip River K19 water obtained by plaque assays directly on water samples and on 100 ml membrane filter concentrates

	Plaque forming units per 100 ml			
	Cabelli host		Havelaar host	
	Direct	Concentrate	Direct	Concentrate
Test series 1:	95-07-10 to 96-04-01			
No of tests	16	16	16	16
No positive	13	14	16	16
Range	0-2400	0-1500	60-2800	45-500
Average	371	255	553	234
Median	80	70	250	150
Standard deviation	696	429	804	187
Test series 2:			95-07-10 to 96-07-22	
No of tests	-	-	27	27
No positive	-	-	27	27
Range	-	-	60-4800	45-700
Average	-	-	761	262
Median	-	-	300	200
Standard deviation	-	-	1068	205

Direct plaque assays : Count per 100 ml calculated from results of tests on 1 ml samples
Membrane filters : Plaque assays on phages recovered from 100 ml samples
- = Not done

Table 2.5 Counts of male-specific coliphages in Vaal Dam sluice gate (A18) water determined by plaque assays directly on water samples and on 100 ml membrane filter concentrates

	Plaque forming units per 100 ml			
	Cabelli host		Havelaar host	
	Direct	Concentrate	Direct	Concentrate
Test series 1:	96-01-08 to 96-04-01			
No of tests	12	12	12	12
No positive	0	3	1	4
Range	0	0-25	0-60	0-45
Average	0	3	5	8
Median	0	0	0	0
Standard deviation	0	8	17	15
Test series 2:			96-01-08 to 96-08-19	
No of tests	-	-	24	24
No positive	-	-	1	7
Range	-	-	0-60	0-45
Average	-	-	3	7
Median	-	-	0	0
Standard deviation			12	14

Direct plaque assays : Count per 100 ml calculated from results of tests on 1 ml samples
Membrane filters : Plaque assays on phages recovered from 100 ml samples
- = Not done

Table 2.6 Counts of somatic coliphages in Klip River K19 water and Vaal Dam sluice gate A18 water obtained by using double agar layer small plates, membrane filter concentrates and single agar layer large plates

	Plaque forming units per 100 ml		
	Small plates	Concentrates	Large plates
Klip River K19	96-01-08 to 96-07-08		
No of tests	15	15	15
No positive	15	13	15
Range	200-5900	0-1835	50-3760
Average	2030	416	1117
Median	12	100	600
Standard deviation	2048	646	1209
Vaal Dam A18	96-01-08 to 96-07-22		
No of tests	16	16	16
No positive	11	5	13
Range	0-700	0-210	0-300
Average	125	16	63
Median	47	0	47
Standard deviation	192	52	80

Small plates : 90 mm diameter disposable plastic petri dishes:
Count per 100 ml calculated
Large plates : 140 mm diameter disposable plastic petri dishes : 100 ml tested

Table 2.7 Counts of somatic coliphages in Klip River K19 water obtained by plaque assays using double agar layer small plates, and single or double agar layer large plates

	Plaque forming units per 100 ml		
	Small plates	Large plates	
	Double layer	Single layer	Double layer
Test series 1	95-10-09 to 95-12-04		
No of tests	7	7	-
No positive	7	7	-
Range	200-1700	100-500	-
Average	1100	271	-
Median	1300	300	-
Standard deviation	597	180	
Test series 2	95-12-11 to 96-05-20		
No of tests	21	21	21
No positive	21	21	21
Range	80-4700	18-1700	50-1900
Average	1256	565	651
Median	900	400	500
Standard deviation	1099	461	508
Test series 3	96-06-10 to 96-10-14		
No of tests	10	-	10
No positive	10	-	10
Range	467-5900	-	740-3760
Average	3582	-	1970
Median	4450	-	1890
Standard deviation	2013	-	1000

Small plates : 90 mm diameter disposable plastic petri dishes
Count per 100 ml calculated

Large plates : 140 mm diameter disposable plastic petri dishes : 100 ml tested - =Not done

Table 2.8 Counts of somatic coliphages in Vaal Dam sluice gate A18 water obtained by plaque assays using double agar layer small plates, and single or double agar layer large plates

	Plaque forming units per 100 ml		
	Small plates	Large plates	
	Double layer	Single layer	Double layer
Test series 1	95-10-09 to 95-12-04		
No of tests	7	7	-
No positive	2	7	-
Range	0-60	3-13	-
Average	17	7	-
Median	0	6	-
Standard deviation	29	4	-
Test series 2	95-12-11 to 96-05-20		
No of tests	21	21	21
No positive	15	11	15
Range	0-700	0-300	0-400
Average	143	51	61
Median	70	10	13
Standard deviation	202	90	108
Test series 3	96-06-10 to 96-08-07		
No of tests	8	-	8
No positive	6	-	8
Range	0-170	-	8-93
Average	49	-	47
Median	33	-	47
Standard deviation	55	-	25

Small plates : 90 mm diameter disposable plastic petri dishes
Count per 100 ml calculated

Large plates : 140 mm diameter disposable plastic petri dishes : 100 ml tested

- = Not done

Table 2.9 Counts of somatic coliphages in Apies River water obtained by using double agar layer small plates, and single or double agar layer large plates

	Plaque forming units per 100 ml		
	Small plates	Large plates	
	Double layer	Single layer	Double layer
No of tests	10	10	10
No positive	10	10	10
Range	1000-1600	80-1000	200-400
Average	1250	228	260
Median	1150	105	200
Standard deviation	207	276	84

Small plates : 90 mm diameter disposable plastic petri dishes:
Count per 100 ml calculated
Large plates : 140 mm diameter disposable plastic petri dishes : 100 ml tested

Table 2.10 Counts of somatic coliphages in treated waste water obtained by plaque assays using double agar layer small plates and double agar layer large plates

	Plaque forming units per 100 ml	
	Small plates	Large plates
Secondary treated effluent	96-07-09 to 96-08-27	
No of tests	5	5
No positive	5	5
Range	200-3830	532-2600
Average	1460	1510
Median	870	1506
Standard deviation	1467	932
Chlorinated sand filter effluent	96-07-09 to 96-08-27	
No of tests	5	5
No positive	5	5
Range	533-3000	312-2510
Average	1167	1299
Median	667	755
Standard deviation	1051	1113

Small plates : 90 mm diameter disposable plastic petri dishes:
Count per 100 ml calculated
Large plates : 140 mm diameter disposable plastic petri dishes : 100 ml tested

Samples collected at the Daspoort sewage treatment works in Pretoria

Table 2.11 Counts of somatic coliphages in diffuse effluents from an informal settlement obtained by plaque assays using double agar layer small plates and double agar layer large plates

	Plaque forming units per 100 ml	
	Small plates	Large plates
Secondary treated effluent	96-07-15 to 96-08-07	
No of tests	4	4
No positive	4	4
Range	1100-4400	222-3000
Average	2200	1022
Median	1650	432
Standard deviation	1500	1327
Chlorinated sand filter effluent	96-07-15 to 96-08-07	
No of tests	5	5
No positive	3	5
Range	0-60	15-87
Average	20	44
Median	10	39
Standard deviation	25	26

Small plates : 90 mm diameter disposable plastic petri dishes:
Count per 100 ml calculated
Large plates : 140 mm diameter disposable plastic petri dishes : 100 ml tested

Samples collected at two sites from diffuse effluents from an informal settlement in Atteridgeville

Table 2.12 Counts of somatic coliphages in dilutions of Klip River K19 water obtained by plaque assays using double agar layer small plates, and double agar layer large plates

Date	Plaque forming units per 100 ml	
	Small plates	Large plates
96-08-19		
200:0	2 000	1 740
180:20	1 800	1 680
160:40	1 800	1 670
140:60	1 900	1 637
120:80	1 400	1 265
100:100	1 000	1 087
80:120	900	864
60:140	533	715
40:160	560	557
20:180	300	456
96-08-26		
200:0	467	740
180:20	400	697
160:40	100	778
140:60	200	613
120:80	300	596
100:100	167	510
80:120	167	466
60:140	100	352
40:160	100	202
20:180	67	147

Small plates : 90 mm diameter disposable plastic petri dishes:
Count per 100 ml calculated
Large plates : 140 mm diameter disposable plastic petri dishes : 100 ml tested
Dilutions of Klip River K19 water in sterile phosphate buffered saline (PBS): 200:0 = undiluted K19 water

Table 2.13 Counts of somatic coliphages in dilutions of Klip River K19 water obtained by plaque assays using double agar layer small plates, and double agar layer large plates

Date	Plaque forming units per 100 ml	
	Small plates	Large plates
96-09-16		
200:0	4 700	2 149
180:20	4 100	1 834
160:40	4 200	1 783
140:60	4 100	1 673
120:80	3 400	1 446
100:100	2 800	1 237
80:120	2 300	975
60:140	1 400	954
40:160	1 400	712
20:180	500	400
96-10-14		
200:0	800	940
180:20	567	925
160:40	567	934
140:60	367	766
120:80	500	612
100:100	267	540
80:120	200	436
60:140	233	372
40:160	133	190
20:180	100	128

Small plates : 90 mm diameter disposable plastic petri dishes:
Count per 100 ml calculated

Large plates : 140 mm diameter disposable plastic petri dishes : 100 ml tested

Dilutions of Klip River K19 water in sterile phosphate buffered saline (PBS) : 200:0 = undiluted K19 water

2.5. Qualitative Detection of Small Numbers of Phages

2.5.1. Qualitative presence/absence (P-A) tests

P-A test procedures for somatic and male-specific coliphages, and *Bacteroides fragilis* phages, have been standardised for routine application in water quality monitoring. Details on the recommended test procedures are recorded in Appendix 1. An evaluation of various procedures indicated that the optimum volume of water to be tested in the quality assessment of drinking water supplies was 500 ml. Results of tests on 1000 ml samples did not differ significantly from those on 500 ml samples. Testing of larger volumes is possible, but becomes impractical and expensive, and the need for that level of sensitivity in routine monitoring of the quality of drinking water supplies seems unlikely.

The standardised P-A test procedures for all three groups of phages were applied in tests on more than 50 samples of river water (mainly Klip River at site K19), and more than 600 samples of treated drinking water, most of which supplied by Rand Water. In a substantial number of tests positive results were obtained by P-A tests but not by conventional direct plaque assays (SP-DL). These results confirm that P-A tests were much more sensitive than direct plaque assays for all three groups of phages. Details of results are presented in 2.7.

The findings confirm that P-A tests offer a simple and inexpensive means for the qualitative detection and screening of small numbers of phages in large volumes of water. Since many water quality guidelines and specifications are based on the presence, and not quantitative numbers, of indicators or pathogens in a specified volume of test water, these P-A tests for phages fulfil a useful role in water quality assessment.

The team experienced that these highly sensitive P-A tests have to be applied with caution, particularly in routine quality monitoring of treated drinking water supplies. Positive results tend to be inter-preted as an indication of unacceptable quality. This may, however, not be the case because the health risks reflected by the presence of

phages at that level have not yet been established. In most situations these tests should be applied as a highly sensitive screening component of a battery of tests including additional tests for appropriately selected indicators and pathogens. The test procedure serves a most valuable purpose as highly sensitive monitoring procedure for the efficiency of drinking water treatment processes. The tests are, of course, useful tools for various research purposes.

In view of their basic sensitivity the P-A tests are obviously vulnerable to contamination. The tests do, therefore, have to be carried out under carefully controlled laboratory conditions, and samples have to be collected aseptically, because contamination with a single viable phage particle will lead to a positive result.

Some questions about the reliability of P-A tests for *B fragilis* phages remain to be clarified. Reason is that results of tests in which known numbers of phages were seeded into samples of water, indicate that P-A tests failed to detect all viable phages. Theoretically this may be due to problems with the inoculation of viable host bacteria into the test suspension (Jofre, personal communication). With the facilities presently available in our laboratory, anaerobic host cultures are inoculated into test water samples which contain oxygen. This exposure to oxygen probably inactivates a large number of the host bacteria. This implies that many phages in the test sample may adsorb to inactive host bacteria or receptor material of lysed host bacteria. These phages would fail to replicate. The test water becomes anaerobic only during anaerobic incubation after inoculation of the host culture. During these fully anaerobic conditions host bacteria which have survived the oxygen shock become metabolically active and phages which have adsorbed to these bacteria are replicated. The problem may be solved by prior removal of oxygen from the test sample of water and inoculation of the host culture under anaerobic conditions. This could possibly be carried out in an anaerobic inoculation cabinet which is expensive and presently not available to us. Alternative solutions should be investigated.

Theoretically it is possible to obtain quantitative estimates from P-A tests on large volume dilution series of test samples. However, this has not been investigated

because the procedure is cumbersome, labour intensive, expensive and subject to the statistical shortcomings of all MPN assays. In addition, more practical alternatives for the quantitative enumeration of small numbers of phages in large volumes of water are available.

2.6. Incidence of Indicator Phages in Human and Animal Wastes

Details on studies along these lines have been published (Grabow *et al*, 1995) (Appendix 2). The results indicate that *B fragilis* HSP40 phages are highly specific for humans and excreted by about 14% of individuals. Somatic and male-specific coliphages were excreted by considerably higher percentages of both humans and a variety of animals. Since that time another 100 human stool specimens have been analysed. *Bacteroides fragilis* HSP40 phages were detected in 13% of these specimens by P-A tests on suspensions containing 1,0 g wet mass stool. In no case were phages detected by direct plaque assays which indicates that the phages were present in low numbers in all positive specimens, at least by the plaque assays used.

2.7. Phages, Bacteria and Viruses in Waste and Environmental Waters

The results of a study on waste water from an informal settlement have been published (Grabow *et al*, 1996) (Appendix 3). In an Analysis of 209 samples collected over a period of one year, human enteric viruses were recovered from a number samples which yielded negative results in conventional tests for somatic and male-specific coliphages, *B fragilis* HSP40 phages, and even faecal bacteria such as faecal coliforms and enterococci. The results reveal shortcomings in the indicator value of these commonly used indicators when using conventional methods.

Some 150 samples of water from the Klip River collected at weekly intervals at sampling point K19, and more than 50 samples of Vaal Dam sluice gate water collected at sampling point A18, have been analysed for phages, bacterial indicators of faecal pollution and human viruses. Typical results are presented in Appendix 4. The consistent presence of somatic and F-RNA coliphages confirms faecal and sewage pollution. The detection of *B fragilis* HSP40 phages in some of the samples confirms faecal pollution specifically of human origin. However, these phages were rarely detected and only by presence-absence tests on 500 ml samples. This suggests the phages were present in low numbers or the detection methods have shortcomings.

The isolation of viruses from five samples which yielded negative results for *B fragilis* HSP40 phages in presence-absence tests, implies that these phages are not reliable indicators for the presence of viruses in water environments. Cytopathogenic viruses were never detected in the absence of somatic coliphages, F-RNA coliphages, faecal coliforms or enterococci. However, viruses were occasionally isolated from water in which *B fragilis* HSP40 phages were not even detected by P-A tests on 500 ml samples. Although counts of all phages and bacterial indicators were considerable lower in Vaal Dam sluice gate (A18) water than in Klip River (K19) water, evidence of faecal pollution was consistently present. Appendix 3 also shows a graphical presentation of somatic coliphage counts recorded by the team's and Rand Water's laboratories. Despite all efforts to standardise laboratory procedures as far as possible, variation in results is evident. This variation may at least in part be due to inherent variation in technology for enumerating micro-organisms, the fact that the samples analysed were not identical even though they were collected from the same water at the same site and time, and the longer transit period for samples analysed in our laboratory.

Details on phages in diffuse effluents from the Botshabelo township have been published (Jagals *et al*, 1995). Among other things, this study confirms the value of *B fragilis* HSP40 phages as indicators of human faecal pollution.

2.8. Phages, Bacteria and Viruses in Drinking Water Supplies

More than 600 samples of treated drinking water from various points in the Rand Water treatment system were analysed for human viruses, phages and bacterial indicators of faecal pollution. Samples were collected each week over a period of three years. Tests included recovery of viruses by glass wool adsorption-elution, P-A tests on 500 ml samples for somatic coliphages, male-specific coliphages and *B fragilis* HSP40 phages, and conventional membrane filter tests for coliform bacteria. Details of the study have been reported (Grabow, 1997). In summary, human viruses were never detected in the treated drinking water, and phages rarely if ever. This implies that P-A test on 500 ml samples are feasible and a valuable component of indicator systems for routine monitoring of the quality of treated drinking water

supplies. These phage results indicate the absence of human viruses as well as efficient treatment of the water.

2.9. Survival of Phages, Bacteria and Viruses in Water Environments

2.9.1. Laboratory experiments on the survival of phages and other indicators in sea water

Experiments were carried out on sea water seeded with human stool and marine guano to investigate the survival of naturally occurring organisms under controlled laboratory conditions. Seeded suspensions were continuously stirred by magnetic stirrer on the laboratory bench, at temperatures of 22-28°C, and pH levels of 7,4-8,0. A laboratory strain of poliovirus was seeded into the suspensions. Laboratory experiments were carried out on one litre of sea water samples each seeded with 1,0 g of fresh human stool or marine bird droppings. The results of typical experiments are presented graphically in Appendix 5. In summary, these results indicate that somatic coliphages survived longest, and were still detectable after 22 days. Enterococci in human stool seemed to survive longer than faecal coliforms, and in guano it was the other way round. Survival of male-specific coliphages was relatively short. All organisms except male-specific coliphages survived for longer than poliovirus. Neither the human stool nor the guano contained *B fragilis* HSP40 phages, and the human stool contained no somatic or male-specific coliphages. Differences in numbers of organisms in the suspensions may partly explain differences in survival times.

2.9.2. Survival of phages and indicators in river water exposed to sunlight

Experiments have been carried out using water from the Apies River. Seeded water was stirred in shallow glass bowls on roof-top. Controls were run under identical conditions except that the beakers were protected from sunlight. Results of a typical experiment in Appendix 5 show that exposure to sunlight has a major effect on the survival of all organisms tested. The tests on survival in river water were supplemented by similar tests using PBS buffer and sea water. the results indicate a general trend for phages to survive at least as long as human viruses under conditions resembling those in natural water environments.

2.9.3. Survival of phages in water treatment processes

The survival of phages, bacterial indicators of faecal pollution and human/animal viruses in a multiple barrier system for the reclamation of water from abattoir effluent has been investigated. No *B fragilis* HSP40 phages were detected in the abattoir effluent which confirms the specificity of the phages for human excreta. Enteric viruses were isolated from occasional samples which yielded negative results for somatic coliphages even in P-A tests on 500 ml samples.

2.10. Indicator Value of Phages in Terms of Numbers and Survival

Data obtained in this study show that:

* Under most circumstances the phages covered by this study outnumbered at least those human viruses which are detectable by conventional techniques.
* The phages concerned tended to survive at least as long as human viruses in a variety of natural water environments and laboratory conditions resembling natural environments.
* The phages were at least as resistant as some enteric viruses to water treatment and disinfection processes.

These features meet fundamental requirements of indicators for routine monitoring of water quality and assessment of the efficiency of treatment processes (Grabow, 1996). However, human viruses were occasionally detected in the absence of at least some of the phages concerned. This is not altogether surprising since viruses may be excreted by infected individuals in numbers of 10^{10} or more per gram of faeces. In situations such as disease outbreaks or peaks of seasonal incidence viruses may outnumber many phages just like they may outnumber commonly used bacterial indicators of faecal pollution. These results and considerations underline the value and importance of using phages as an important component of appropriately selected combinations of indicators for water quality monitoring.

2.11. Practical Aspects of Phage Indicators

Evidence has been presented that:

* Phages are detectable by relatively simple and inexpensive techniques.
* Practical techniques have been established for the sensitive and accurate qualitative

and quantitative detection of phages in a water containing phages in numbers ranging from high to very low.

* Phage tests do not require sophisticated and costly laboratory facilities or equipment, and can be applied by most water quality laboratories.
* Results of many phage tests are available within 24 h.
* Phages are not pathogenic and constitute no health hazard to laboratory workers.

These features meet fundamental requirements of indicators for water quality assessment (Grabow, 1996).

2.12. International Collaboration

An infrastructure for collaboration with international experts and research bodies has been established including the International Organization for Standardization, Universities of Barcelona and North Carolina, and the Netherlands National Institute for Environmental Hygiene (Grabow, 1996).

2.13. Recommended Water Quality Guidelines

Data on the following features of phages indicators obtained in this study have been used to formulate recommendations for water quality guidelines:

* Relative incidence of the phages concerned, human viruses and commonly used bacterial indicators of faecal pollution, in water environments.
* Relative resistance of phages to water treatment and disinfection processes.
* Feasibility of phage detection methods. This includes cost, time, labour, expertise and facilities required for carrying out the tests.

The following quality guidelines are based on the above considerations:

Drinking water : Absence of any phages from 500 ml samples.

Test method : Presence-Absence test on 500 ml samples.
Positive results to be followed up by quantitative tests on at least 100 ml samples using double agar layer plaque assays on large petri dishes (LP-DL), or conventional plaque assays (SP-DL) on membrane filter concentrates of 500 ml samples.

Environmental waters (ie rivers, dams, sea water) intended for direct contact recreation (ie swimming) : Less than 10/10 ml of any phages.

Test method : Direct quantitative plaque assay on 10 ml samples using conventional small plates with double agar layer (SP-DL).

Interpretation:

* The recommended guidelines are no absolute guarantee for the absence of pathogens such as human viruses and protozoan parasites.
* In view of potential shortcomings under certain conditions, the phage tests should be used as one component of a combination of tests. The combination of tests for indicators and pathogens should be appropriately selected for the purpose concerned.
* The recommended guidelines are not based on data directly related to health risks. The guide-lines do, therefore, not give any direct indication of risks of infection by any pathogens.
* Data recorded in this study could also be used to formulate guidelines for treated waste water to be discharged into environmental water sources such as rivers, dams and the sea.
* Techniques defined in this study could be used to distinguish between faecal pollution of human and animal origin. Under circumstances this would cast valuable light on risks of infection.

2.14. Recommended Procedure for Routine Water Quality Monitoring

Techniques recommended for general and routine use in water quality monitoring and research are described in Appendix 1. These techniques are based on technology development and research carried out in this project. The techniques are representative of the best technology internationally recommended at the time of printing.

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Appendix 1

Methods for the detection of phages

Plaque Assay for Somatic Coliphages
using
Small Petri Dishes with Double Agar Layer (SP-DL)

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Principle

1. Conventional plaque assay for somatic coliphages in small volumes of water (generally 1,0 ml) using small petri dishes (90 mm diam) based on principles originally formulated by Adams.
2. *Escherichia coli* strain C is exceptionally susceptible to a wide range of somatic coliphages largely because it has an impaired nucleic acid restriction enzyme.
3. The media described here differ somewhat from those specified in ISO/DIS 10705-2:1997. There is no evidence that one may be superior to the other. In terms of basic theoretical considerations there is no reason to expect meaningful differences in results.

Test procedure

1. Steam the required number of test tubes with top agar to liquefy agar and adjust to 48°C in a heating block.
2. Add 0,5 ml of the host culture to the top agar.
3. Add 1,0 ml of the test sample, or an appropriate dilution of the test sample, to the top agar in each test tube.
4. Mix gently and pour the top agar mixture with minimum delay onto the bottom agar layer in a 90 mm phage agar plate.
5. Repeat the above in tenfold to obtain counts per 10 ml. If tenfold dilutions are required, three plates should preferably be used for each dilution to obtain meaningful results.
6. Incubate inverted plates overnight at 35-37°C and count plaques of somatic coliphages.

Materials

Growth medium (Nutrient broth)
Prepare ordinary nutrient broth (Difco or equivalent) according to the manufacturer's instructions, heat to dissolve, dispense in convenient containers, ie, 100 ml quantities in 200 ml medical flats, autoclave, store at about 4°C for not longer than 30 days.

Phage bottom agar

Bacto agar	14,0 g
Tryptone	13,0 g
NaCl	8,0 g
Na_2CO_3 solution	5,0 ml
Glucose	1,5 g
Dist water	1000 ml

Heat to dissolve agar, and autoclave.
Pour about 20 ml in 90 mm diameter petri dishes.
Store at 4±2 °C: maximum 10 days.

Phage top agar

Bacto agar	8,0 g
Tryptone	10,0 g
NaCl	8,0 g
Glucose	3,0 g
Na_2CO_3 solution	5,0 ml
$MgCl_2$ solution	1,0 ml
Dist water	1000 ml

Autoclave and cool to 55-60 °C.
Add 6,0 ml $CaCl_2$ solution.
Add nalidixic acid solution if considered necessary (1,0 ml/100 ml).
Distribute 2,5 ml aliquots into test tubes with caps.
Store at 4±2 °C: maximum 30 days.

Host culture

Escherichia coli strain C (ATCC 13706)	=	WG4
Nalidixic acid resistant mutant of WG4	=	WG5

Preparation of host culture

Inoculate typical colonies from a stock agar plate, or a loopful of growth from a stock agar slant, into 50 ml of prewarmed growth medium and incubate for 3±1 h at 36±2 °C with gentle shaking. Use immediately or take the inoculum culture from the incubator and quickly cool to 5-10 °C, preferably by placing onto melting ice. Use this inoculum culture the same working day. The inoculum culture should ideally have a count of 1-10 x 10^8 per ml.

Test sample

Water (eg drinking water, wastewater, river water, seawater) or liquid suspension (eg suspension of shellfish meat). Make tenfold dilution in peptone saline solution as necessary.

Nalidixic acid solution

Dissolve 0,5 g of nalidixic acid in 4 ml of 1 M NaOH. Add 16 ml of sterile water, mix well. Decontaminate by membrane filtration, eg syringe filter, 0,22 μm membrane.
Store at 4±2 °C : maximum 4 weeks.

$MgCl_2$ solution

Prepare 4 M stock solution by dissolving 820 g of $MgCl_2.6H_2O$ crystals in 1000 ml of water; sterilise by autoclaving; store at room temp in the dark.

Na_2CO_3 solution

Dissolve 150 g of Na_2CO_3 in 1000 ml of water by gentle heating. Store at 4±2 °C : maximum 6 months.

$CaCl_2$ solution

Prepare 1 M stock solution by dissolving 147 g of $CaCl_2.2H_2O$ in 1000 ml water by gentle heating. Decontaminate by membrane filtration, eg syringe filter, 0,22 μm membrane.
Store at 4±2 °C : maximum 6 months.

Peptone saline solution

Dissolve 1,0 g peptone and 8,5 g sodium chloride in 950 ml water by boiling. Adjust pH to 7,0 ± 0,1 using 1 M NaOH or HCl. Make up to 1000 ml with water, and dispense in convenient volumes.

Autoclave. Store at 4±2 °C : maximum 6 months.

Notes

1. Tests to be carried out according to basic principles outlined in:

1.1. Grabow W O K, Holtzhausen C S and de Villiers C J (1993) Research on Bacteriophages as Indicators of Water Quality. WRC Report No 321/1/93. Water Research Commission, Pretoria. 147 pp.

1.2. ISO 10705-1:1995. Water Quality - Detection and Enumeration of Bacteriophages. Part 1: Enumeration of F-specific RNA bacteriophages. International Organization for Standardization, Geneva. 15 pp.

1.3. ISO/DIS 10705-2:1997. Water Quality - Detection and Enumeration of Bacteriophages. Part 2: Enumeration of somatic coliphages. International Organization for Standardization, Geneva. 17 pp.

2. In the case of heavily contaminated test samples (eg waste water), interfering microbial growth may be suppressed by the addition of 1,0 ml of the nalidixic acid solution to 100 ml of molten phage top agar.

   Final concentration of nalidixic acid in phage top agar = 250 μg/ml.

   The resistant mutant WG5 must be used as host in these assays.

   Nalidixic acid is generally not necessary for testing treated drinking water.

3. A heating block should be used for tubes with top agar instead of a water bath if possible in order to avoid contamination by phages in water bath water.

4. Always use distilled water for the preparation of media and reagents.

5. Autoclaving = 121°C/15 min.

Commercial media and reagents

The following or equivalent are recommended:

Difco: Bacto Agar, Nutrient Broth, Tryptone, Peptone

Sigma: Glucose, nalidixic acid, NaCl, $CaCl_2.2H_2O$, $MgCl_2.6H_2O$

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Plaque Assay for F-RNA (male-specific) Coliphages
using
Small Petri Dishes with Double Agar Layer (SP-DL)

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Principle

1. Conventional plaque assay for F-RNA coliphages in small volumes of water (generally 1,0 ml) using small petri dishes (90 mm diam).
2. *Salmonella typhimurium* strain WG49 (Havelaar) has been selected in detailed evaluations by laboratories world-wide as the host of choice.
3. F-RNA coliphages share a number of fundamentally important features with enteric viruses and meet important requirements of indicators for water quality assessment and routine monitoring.

Test procedure

1. Steam the required number of test tubes with top agar (ssTYGA) to liquefy agar and adjust to 48°C in a heating block.
2. Add 1,0 ml of the test sample, or an appropriate dilution of the test sample, to the top agar in each test tube.
3. Add 1,0 ml of the host culture to the top agar and mix gently.
4. Pour the top agar mixture with minimum delay onto the bottom agar layer (TYGA) in a 90 mm phage agar plate.
5. Repeat the above in tenfold to obtain counts per 10 ml. If tenfold dilutions are required, three plates should preferably be used for each dilution to obtain meaningful results. Use peptone-saline solution for dilutions.
6. Incubate inverted plates overnight (18±2 h) at 35-37°C and count plaques of F-RNA coliphages (faint lysogenic plaques).

When high bacterial background flora may interfere with growth of the host and replication of phages, the addition of nalidixic acid is recommended to suppress contaminant growth.
Add 0,2 ml of stock nalidixic acid solution to 50 ml top agar (ssTYGA): final conc 100μg/ml.
Nalidixic acid is heat resistant and can be added to the top agar prior to autoclaving.

Confirmatory test

When there is reason to suspect that F-RNA phages may constitute less than 90 % of plaques obtained by the above procedure, or absolute identity is essential, the identity of F-RNA phages should be confirmed as follows:
Prepare plates in parallel to those described above but add 5,0 ml RNase solution to 50 ml top agar just after the addition of the calcium-glucose solution, to obtain a final RNase concentration of 100 μg/ml. In some cases it may be necessary to increase the RNase concentration to 400 μg/ml. F-RNA phages may also be confirmed by electron microscopy, and serological and molecular techniques.

Materials

Host for F-RNA coliphages:
Salmonella typhimurium WG49 (Havelaar):
Salmonella typhimurium strain WG49 phage type 3 Nal^r (F'lac: :Tn5)
Reference: NCTC 12484

Inoculum culture of WG49 for F-RNA phages

1. **Working culture**

Thaw one vial of stock culture and streak for single colonies on selective lactose-containing agar medium such as McConkey.
Incubate at 37±1 °C for 18±2 h.
Pick 3-5 typical lactose-positive colonies and add to 50 ml TYGB in a conical flask (about 300 ml).
Incubate at 37±1 °C for 5±1 h while shaking at 100±10/min.
Add 10 ml of glycerol (870 g/l) and mix well.
Distribute 1,2 ml aliquots into plastic vials (Eppendorf).
Store at -70±10 °C: maximum 2 years.

2. **Inoculum culture**

Add 0,5 ml of working culture thawed at room temp to 50 ml of TYGB in a conical flask (about 300 ml) or nephelometric conical flask with side arm for optical density reading.
Incubate at 37±1 °C while shaking at 100±10/min.
Measure turbidity every 30 min using a spectrophotometer.
When a turbidity equivalent to approximately 10^8 colony forming units (cfu) per ml has been reached*, cool culture quickly on melting ice and use within 2 h.

* The required density of host cells is generally reached at an optical density (OD) reading of 0,8-1,0 after 2-4 h incubation. However, this is variable and a growth curve has to be prepared for each batch of working cultures using procedures described in detail (ISO, 1995).

Tryptone - Yeast extract - Glucose - Broth (TYGB)

1. **Basal medium**

Trypticase peptone (BBL)	10,0 g
Yeast extract (Difco)	1,0 g
NaCl (Sigma)	8,0 g
Dist water	1000 ml

Dissolve with gentle heating if necessary.
Adjust pH to be 7,2±0,1 at 25 °C after autoclaving.
Distribute 200 ml volumes into bottles and autoclave (121±1 °C for 15 min).
Store at 4±2 °C in dark: maximum 6 months.

2. **Complete medium**

Basal medium	200 ml
Calcium-glucose solution	2,0 ml

Add and mix aseptically
Store at 4±2 °C in dark: maximum 6 months.

Tryptone - Yeast extract - Glucose - Agar (TYGA)
Prepare basal medium as for TYGB but add 15 g of agar (depending on the gel strength of the agar used the concentration may have to be adjusted to 12-20 g).
After autoclaving cool to 50-55 °C, add prewarmed calcium-glucose solution as for TYGB, mix gently, and pour 20 ml quantities into 90 mm diameter petri dishes.
Store plates at 4±2 °C: maximum 6 months.

Top agar (ssTYGA)
Prepare basal medium as for TYGA but use 8 g of agar instead of 15 g (depending on the gel strength of the agar used, the concentration may have to be adjusted in the range 6-10 g).
Autoclave and cool to 55-60 °C.
Add calcium-glucose solution: 0,5 ml/50 ml.
Distribute 2,5 ml aliquots into test tubes with caps.
Store tubes at 4±2 °C: maximum 6 months.

Calcium-glucose solution

$CaCl_2 \cdot 2H_2O$ (Sigma)	3,0 g
Glucose (Sigma)	10,0 g
Dist water	100 ml

Dissolve with gentle heating if necessary
Decontaminate by filtration: 0,22 μm membrane
Store at 4 °C: maximum 6 months

Nalidixic acid stock solution

Nalidixic acid (Sigma)	2500 mg
NaOH (1M)	2,0 ml
Dist water	98,0 ml

Dissolve nalidixic acid in NaOH
Add water
Decontaminate by filtration: 0,22 μm membrane
Store at 4 °C: maximum 2 weeks

Peptone saline solution
Dissolve 1,0 g peptone and 8,5 g sodium chloride in 950 ml water by boiling. Adjust pH to 7,0 ± 0,1 using 1 M NaOH or HCl. Make up to 1000 ml with water, and dispense in convenient volumes.
Autoclave.
Store at 4±2 °C : Maximum 6 months.

RNase solution

RNase	100 mg
Dist water	100 ml

Dissolve RNase by heating for not more than 10 min at 100 °C.
Distribute 0,5 ml aliquots into plastic vials (Eppendorf).
Store at -2 °C: maximum 12 months.
Recommended RNase: RNase A from bovine pancreas (Boehringer Mannheim).

Notes

1. Tests to be carried out according to basic principles outlined in:

1.1. Grabow W O K, Holtzhausen C S and de Villiers C J (1993) Research on Bacteriophages as Indicators of Water Quality. WRC Report No 321/1/93. Water Research Commission, Pretoria. 147 pp.

1.2. ISO 10705-1:1995. Water Quality - Detection and Enumeration of Bacteriophages. Part 1: Enumeration of F-specific RNA bacteriophages. International Organization for Standardization, Geneva. 15 pp.

3. Water baths are a common source of phage contamination unless carefully controlled. A heating block is, therefore, recommended for tubes with top agar in order to avoid risk of contamination.
4. Always use distilled water for the preparation of media and reagents.
5. Autoclaving = 121°C/15 min.
6. The names of commercial media and reagents are indicated merely as a guide. A variety of alternative commercial equivalents may yield similar and acceptable results. Analytical grade products are required only when proven to yield results superior to those obtained by means of standard grade reagents.

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Plaque Assay for *Bacteroides fragilis* HSP40 Phages
using
Small Petri Dishes with Double Agar Layer (SP-DL)

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Principle

1. Conventional plaque assay for *B fragilis* HSP-40 phages in small volumes of water (generally 1,0 ml) using small petri dishes (90 mm diam) based on principles originally formulated by Adams.
2. As far as is known, these phages are excreted only by about 14 % of humans and not by any animals. The phages can, therefore, be used to distinguish between human and animal faecal pollution.
3. *Bacteroides fragilis* is a strictly anaerobic bacterium, which requires incubation under absolutely anaerobic conditions. Implications are that these plaque assays are more complicated, labour intensive and expensive in terms of facilities and materials than corresponding tests for phages of aerobic bacteria such as *Escherichia coli*. Also, production of visible plaques is slower, and generally requires 2 days of incubation instead of 1 day as with coliphages.

Test procedure

1. Steam the required number of test tubes with top agar to liquefy agar and adjust to 48 °C in a heating block.
2. Add 1,0 ml of the host culture to the top agar in each tube.
3. Add 1,0 ml of the test sample, or an appropriate dilution of the test sample, to the top agar in each tube.
4. Pour the top agar mixture with minimum delay onto the bottom agar layer in a 90 mm phage agar plate.
5. Repeat the above in tenfold to obtain counts per 10 ml. If tenfold dilutions are required, three plates should preferably be used for each dilution to obtain meaningful results.
6. Incubate inverted plates anaerobically at 35-37°C for 2 days and count plaques.

Materials

Growth medium (BPRM)

Tryptone	(Difco)	10,0 g
Peptone	(Difco)	10,0 g
Yeast extract	(Difco)	2,0 g
NaCl	(Sigma)	5,0 g
$CaCl_2 \cdot 2H_2O$ (0,5% in H_2O)	(Merck)	10,0 ml
$MgSO_4 \cdot 7H_2O$ (1,2% in H_2O)	(Merck)	10,0 ml
L-Cysteine monohydrochloride	(Sigma)	0,5 g
Dist water		955 ml

Dissolve ingredients and autoclave (121°C for 15 min).
Keep autoclaved medium at 60-80°C for further processing.
Add the following:

Glucose (1M in distilled H_2O)	(Sigma)	10,0 ml
Haemin (0,1% in 0,02% NaOH)	(Sigma)	10,0 ml
Na_2CO_3 (1M)		25,0 ml

Adjust pH to 7,0 using concentrated HCl, and add:

Kanamycin sulphate	(Sigma)	final concentration	100 μg/ml
Vancomycin	(Sigma)	final concentration	7,5 μg/ml

Bottom agar
Prepare like growth medium but add 17,0 g of Bacto agar (Difco) and autoclave basic ingredients.
Cool to about 60-80 °C, and add glucose, haemin, Na_2CO_3 and antibiotics as for BPRM.
Pour 20 ml volumes into 90mm diameter petri dishes.
Store plates at 4±2 °C: Maximum 10 days.

Top agar
Same as bottom agar, except for 7,0 g instead of 17,0 g agar (depending on the gel strength of the agar used, the concentration of the agar may have to be adjusted to obtain a suitable top layer).
Distribute 2,5 ml aliquots into test tubes with caps.
Store at 4±2 °C: Maximum 10 days.

Host culture
Bacteroides fragilis strain HSP40.
Reference: ATCC 51477.
Streak reference ATCC or stock culture on bottom agar for single colonies.
Incubate anaerobically at 35-37 °C for 2 days.
Stock cultures:
Inoculate 50 ml of BPRM medium with 3 typical colonies.
Incubate anaerobically at 35-37 °C for 2 days.
Add 10 ml of glycerol (870 g/l) and mix well.
Distribute to 1,5 or 2,0 ml plastic vials (Eppendorf).
Fill vials to top and seal tightly to promote anaerobic conditions.
Store at -70±10 °C: maximum 2 years.
Regularly check the host for the following properties:

Pale colonies, pleomorphic or uniform Gram-negative rods with irregular staining.
Susceptible to type specific phage B40-8.
Growth stricly anaerobic.

Working culture:
Thaw one vial of stock culture and streak for single colonies on bottom agar.
Incubate anaerobically at 37±1 °C for 2 days.
Store at 4±2 °C: maximum 7 days.
Inoculum culture:
Pick 3 typical colonies of *B fragilis* HSP40 from working culture plate and inoculate required volume of BPRM broth, ie 50 ml.
Incubate anaerobically at 37±1 °C for 2 days.

Notes

1. Tests to be carried out according to basic principles outlined in:
1.1. Grabow W O K, Holtzhausen C S and de Villiers C J (1993) Research on Bacteriophages as Indicators of Water Quality. WRC Report No 321/1/93. Water Research Commission, Pretoria. 147 pp.
1.2. Tartera C, Araujo R, Michel T and Jofre J (1992) Culture and decontamination methods affecting enumeration of phages infecting *Bacteroides fragilis* in sewage. Appl Environ Microbiol 58, 2670-2673.
2. Success of the method heavily depends upon the extent to which strict anaerobic conditions are maintained during incubation, as well as the extent to which exposure of host cultures, growth media and test samples to oxygen is restricted. Application of the commercial BBL Gaspak Anaerobic System is recommended. However, similar or superior results may be obtained by alternative systems. Ideally, the plaque assays should be carried out in anaerobic inoculation cabinets, and oxygen should be removed from test samples.
3. A heating block should be used for tubes with top agar instead of a waterbath if possible in order to avoid contamination by phages in waterbath water.
4. Always use distilled water for the preparation of media and reagents.
5. Autoclaving = 121°C/15 min.
6. Names of commercial media and reagents are mentioned merely as a guide. Alternative products may yield similar results. Analytical grade reagents are required only when proven to yield results superior to those obtained by standard grade reagents.

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Direct Plaque Assay for Somatic Coliphages in 100 ml Water Samples

using

Large Petri Dishes with Double Agar Layer (LP-DL)

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Principle

1. Intended for the enumeration of small numbers of somatic coliphages in 100 ml water samples.
2. Intended for water with low numbers of phages such as treated drinking water or environmental water with limited pollution.
3. Based on the addition of cations, host nutrients, nalidixic acid (if necessary), and host inoculum to 100 ml of water, mixing and pouring in equal volumes into 140 mm petri dishes with bottom agar, and counting of plaques after overnight incubation.
4. The procedure could be adapted to accommodate other volumes of water, but tends to become impractical for volumes of more than 100 ml in terms of labour and cost. There would, however, not seem to be meaningful need for direct plaque assays for somatic coliphages in volumes of water in access of 100 ml.

Test procedure

1. Steam a bottle of PAC to liquefy agar and adjust temperature to 48 °C (ie water bath).
2. Pour 100 ml of water to be tested into a convenient sterile container, eg 200 ml screw cap medical flat.
3. Add 1,0 ml Ca solution.
4. If interference by microbial growth is expected, add 1,0 ml of NA solution.
5. Adjust temperature of water sample to 35-37 °C (ie water bath or incubator).
6. Add 5,0 ml of host culture to water sample, using WG4 in the absence of NA, and WG5 in the presence of NA.
7. Keep inoculated water sample for about 3 min at 35-37 °C.
8. Add inoculated water sample gently to the bottle of PAC; mix gently to avoid the formation of bubbles by turning bottle end over end once.
9. Pour mixture swiftly but gently in equal volumes into ten 140 mm diameter plastic petri dishes with bottom layer and let solidify with lids partly open.
10. Incubate inverted plates overnight at 35-37 °C and count plaques.

Materials

PAC (Phage Agar Concentrate)

Meat extract powder (Lab Lemco, Oxoid)	14,0 g
Yeast Extract Powder (Oxoid)	4,0 g
NaCl	4,0 g
Peptone (Difco)	12,0 g
Na_2CO_3	1,0 g
$MgCl_2.6H_2O$	1,0 g
Agar (Bacto)	14,0 g
Dist water	1000 ml

Heat to dissolve agar.
Adjust pH to 7,2.
Dispense 100 ml quantities into suitable bottles, eg, 250 ml screw cap medical flats.
Autoclave.
Store at 4±2 °C: maximum 4 months.

Bottom agar

Agar (Bacto, Difco)	14,0 g
Tryptone (Bacto, Difco)	13,0 g
NaCl	8,0 g
Glucose	1,5 g
Na_2CO_3	1,0 g
Dist water	1000 ml

Autoclave.
Cool to 45-50 °C and pour 30 ml into each of 140 mm diameter petri dishes. Allow to solidify.
Store at 4±2 °C: maximum 2 months.

Ca solution

$CaCl_2.2H_2O$	13,0 g
Dist water	100 ml

Autoclave.
Store at 4 °C or room temperature: maximum 4 months.

NA solution

Nalidixic acid (Sigma)	0,7 g
Sterile dist water	20 ml

If the nalidixic acid does not dissolve directly in water, it can first be dissolved in 5 ml 1M NaOH and then added to 15 ml water.
Decontaminate by filtration using a 0,22 μm pore size membrane.
Store at 4±2 °C: maximum 2 weeks.

Host culture

Escherichia coli strain C (ATCC 13706), nalidixic acid sensitive = WG4
Nalidixic acid resistant mutant of WG4 = WG5
Inoculum:
Inoculate colony from stock agar plate or slant into 50 ml nutrient broth (Difco).
Incubate at 35-37 °C with shaking for 4-5 h to obtain cultures in the logarithmic growth phase.

Notes

1. Tests to be carried out according to basic principles outlined in:

1.1. Grabow W O K, Holtzhausen C S and de Villiers C J (1993) Research on Bacteriophages as Indicators of Water Quality. WRC Report No 321/1/93. Water Research Commission, Pretoria. 147 pp.

1.2. ISO 10705-1:1995. Water Quality - Detection and Enumeration of Bacteriophages. Part 1: Enumeration of F-specific RNA bacteriophages. International Organization for Standardization, Geneva. 15 pp.

1.3. ISO/DIS 10705-2:1997. Water Quality - Detection and Enumeration of Bacteriophages. Part 2: Enumeration of somatic coliphages. International Organization for Standardization, Geneva. 17 pp.

2. This double agar layer plaque assay using 140 mm petri dishes (LP-DL), tends to yield slightly lower counts than conventional double agar layer plaque assays using 90 mm petri dishes (SP-DL) in assays on waters which contain relatively high numbers of somatic coliphages. Therefore, LP-DL is recommended only for assays on waters which contain numbers of phages below the reliable detection limit of SP-DL. This implies that LP-DL would primarily be used for treated drinking water and environmental waters with low levels of sewage pollution. SP-DL would primarily be used for waste water and environmental waters with relatively heavy faecal pollution.

3. Commercial products other than those mentioned may yield similar results. Analytical grade reagents are recommended only if evidence is available that they yield results superior to those obtained by using standard grade reagents from reliable manufacturers.

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Somatic Coliphages, F-RNA Coliphages and *Bacteroides fragilis* HSP40 Phages

Detection by

Qualitative Presence-Absence Tests on 500 ml Samples of Water

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Principle

These qualitative tests are intended for the detection of low numbers of phages in 500 ml samples of water. This would include routine monitoring of treated drinking water. The procedure is based on:

1. Neutralise chlorine residuals in 500 ml samples of test water.
2. Add nutrients for cultivation of the appropriate hosts to the water samples.
3. Add cations to promote adsorption of phages to host bacteria.
4. Add antibiotics to select for bacterial host concerned.
5. Add inoculum of host bacteria in broth culture.
6. Incubate overnight at 35-37°C.
7. Test for presence of phages by conventional plaque assay or qualitative spot test.

Test procedures

The following procedures are for testing 500 ml samples. Theoretically any volume of water can be tested by corresponding adjustment of nutrients and inocula.

Chlorine residuals in test samples of treated drinking water may be neutralised by means of sodium thiosulphate. The addition of 1 ml of a 1 % solution of thiosulphate in water, to 500 ml samples of conventional treated drinking water supplies, is generally sufficient. However, this should be confirmed by testing samples for chlorine residuals.

1. Somatic coliphages and F-RNA coliphages

1.1. Weigh the following nutrients into an appropriate container, ie 500-1000 ml glass flask:

Trypticase peptone (BBL)	6,0 g
Yeast extract (Difco)	0,5 g
NaCl	4,0 g

1.2. Add test water 500 ml

1.3. Add Calcium-glucose solution 1,0 ml

1.4 Add nalidixic acid stock solution:

Somatic coliphages	(Final concentration 250 μg/ml)	5,0 ml
F-RNA coliphages	(Final concentration 100 μg/ml)	2,0 ml

1.5. Add inoculum culture of host 3,0 ml

1.6. Incubate overnight (20±2 h) at 35-37°C

1.7. Test for presence of somatic or F-RNA coliphages using plaque assay (tenfold dilutions: 0 to at least -8) or spot test.

2. *Bacteroides fragilis* HSP40 phages

Add the following to a suitable container, ie 500 ml glass bottle with screw cap:

Tryptone	(Difco)	5,0 g
Peptone	(Difco)	5,0 g
Yeast extract	(Difco)	1,0 g
NaCl	(Sigma)	2,5 g
$CaCl_2 \cdot 2H_2O$ (0,5% in H_2O)	(Merck)	5,0 ml
$MgSO_4 \cdot 7H_2O$ (1,2% in H_2O)	(Merck)	5,0 ml
L-Cysteine monohydrochloride	(Sigma)	0,3 g
Glucose (1M in distilled H_2O)	(Sigma)	5,0 ml
Haemin (0,1% in 0,02% NaOH)	(Sigma)	5,0 ml
Na_2CO_3 (1M)		13,0 ml
Test water sample		400 ml

Mix thoroughly and adjust pH to 7,0 using concentrated HCl.

Add the following:

Kanamycin sulphate	(Sigma)	final concentration	100 µg/ml
Vancomycin	(Sigma)	final concentration	7,5 µg/ml
Inoculum culture of host			60 ml

Mix thoroughly, fill bottle to top with test water.

Put parafilm over mouth of bottle and screw cap on tightly to promote anaerobic condition.

Incubate overnight (20±2 h) at 35-37°C.

Test for presence of *B fragilis* HSP40 phages using plaque assays on 0 to at least -8 tenfold dilutions.

Materials

Host cultures:

Somatic coliphages:

Escherichia coli strain C (WG4, Havelaar): Nalidixic acid sensitive.

Reference: ATCC 13706.

Escherichia coli WG5 (Havelaar): Nalidixic acid resistant mutant of *E coli* strain C

F-RNA coliphages:

Salmonella typhimurium WG49 (Havelaar):

Salmonella typhimurium strain WG49 phage type 3 Nal^r (F'lac: :Tn5)

Reference: NCTC 12484

Inoculum cultures of WG4 and WG5 for somatic coliphages

Inoculate 3-5 typical colonies from a stock agar plate, or a loopful of growth from a stock agar slant, into nutrient broth (Difco) or TYGB and incubate at 35-37 °C for 4-5 h with shaking.

Inoculum culture of WG49 for F-RNA phages

1. **Working culture**

   Thaw one vial of stock culture and streak for single colonies on selective lactose-containing agar medium such as McConkey.
   Incubate at 37±1 °C for 18±2 h.
   Pick 3-5 typical lactose-positive colonies and add to 50 ml TYGB in a conical flask (about 300 ml).
   Incubate at 37±1 °C for 5±1 h while shaking at 100±10/min.
   Add 10 ml of glycerol (870 g/l) and mix well.
   Distribute 1,2 ml aliquots into plastic vials (Eppendorf).
   Store at -70±10 °C: maximum 2 years.

2. **Inoculum culture**

   Add 0,5 ml of working culture thawed at room temp to 50 ml of TYGB in a conical flask (about 300 ml) or nephelometric conical flask with side arm for optical density reading.
   Incubate at 37±1 °C while shaking at 100±10/min.
   Measure turbidity every 30 min using a spectrophotometer.
   When a turbidity equivalent to approximately 10^8 colony forming units (cfu) per ml has been reached*, cool culture quickly on melting ice and use within 2 h.

   * The required density of host cells is generally reached at an optical density (OD) reading of 0,8-1,0 after 2-4 h incubation. However, this is variable and a growth curve has to be prepared for each batch of working cultures using procedures described in detail (ISO, 1995).

Inoculum culture of *Bacteroides fragilis* HSP40 host

Host culture

Bacteroides fragilis strain HSP40.
Reference: ATCC 51477.
Streak reference ATCC or stock culture on bottom agar for single colonies.
Incubate anaerobically at 35-37 °C for 2 days.

Stock cultures:

Inoculate 50 ml of BPRM medium with 3 typical colonies.
Incubate anaerobically at 35-37 °C for 2 days.
Add 10 ml of glycerol (870 g/l) and mix well.
Distribute to 1,5 or 2,0 ml plastic vials (Eppendorf).
Fill vials to top and seal tightly to promote anaerobic conditions.
Store at -70±10 °C: maximum 2 years.
Regularly check the host for the following properties:

Pale colonies, pleomorphic or uniform Gram-negative rods with irregular staining.
Susceptible to type specific phage B40-8.
Growth strictly anaerobic.

Working culture:

Thaw one vial of stock culture and streak for single colonies on bottom agar.
Incubate anaerobically at 37±1 °C for 2 days.
Store at 4±2 °C: maximum 7 days.

Inoculum culture:

Pick 3 typical colonies of *B fragilis* HSP40 from working culture plate and inoculate required volume of BPRM broth, ie 100 ml.
Incubate anaerobically at 37±1 °C for 2 days.

Calcium-glucose solution

$CaCl_2 \cdot 2H_2O$	(Sigma)	3,0 g
Glucose	(Sigma)	10,0 g
Dist water		100 ml

Dissolve with gentle heating if necessary
Decontaminate by filtration: 0,22 μm membrane
Store at 4 °C: maximum 6 months

Nalidixic acid stock solution

Nalidixic acid	(Sigma)	2500 mg
NaOH (1M)		2,0 ml
Dist water		98,0 ml

Dissolve nalidixic acid in NaOH
Add water
Decontaminate by filtration: 0,22 μm membrane
Store at 4 °C: maximum 2 weeks

Tryptone - Yeast extract - Glucose - Broth (TYGB)

1. **Basal medium**

Trypticase peptone	(BBL)	10,0 g
Yeast extract	(Difco)	1,0 g
NaCl	(Sigma)	8,0 g
Dist water		1000 ml

Dissolve with gentle heating if necessary.
Adjust pH to be 7,2±0,1 at 25 °C after autoclaving.
Distribute 200 ml volumes into bottles and autoclave (121±1 °C for 15 min).
Store at 4±2 °C in dark: maximum 6 months.

2. **Complete medium**

Basal medium	200 ml
Calcium-glucose solution	2,0 ml

Add and mix aseptically
Store at 4±2 °C in dark: maximum 6 months.

Growth medium (BPRM)

Tryptone	(Difco)	10,0 g
Peptone	(Difco)	10,0 g
Yeast extract	(Difco)	2,0 g
NaCl	(Sigma)	5,0 g
$CaCl_2 \cdot 2H_2O$ (0,5% in H_2O)	(Merck)	10,0 ml
$MgSO_4 \cdot 7H_2O$ (1,2% in H_2O)	(Merck)	10,0 ml
L-Cysteine monohydrochloride	(Sigma)	0,5 g
Dist water		955 ml

Dissolve ingredients and autoclave (121°C for 15 min).
Keep autoclaved medium at 60-80°C for further processing.
Add the following:

Glucose (1M in distilled H_2O)	(Sigma)	10,0 ml
Haemin (0,1% in 0,02% NaOH)	(Sigma)	10,0 ml
Na_2CO_3 (1M)		25,0 ml

Adjust pH to 7,0 using concentrated HCl, and add:

Kanamycin sulphate	(Sigma)	final concentration	100 μg/ml
Vancomycin	(Sigma)	final concentration	7,5 μg/ml

Notes

1. Tests to be carried out according to basic principles outlined in:

1.1. Grabow W O K, Holtzhausen C S and de Villiers C J (1993) Research on Bacteriophages as Indicators of Water Quality. WRC Report No 321/1/93. Water Research Commission, Pretoria. 147 pp.

1.2. ISO (1995) ISO 10705-1:1995, Water quality - Detection and enumeration of bacteriophages - Part 1: Enumeration of F-specific RNA bacteriophages. International Organization for Standardization, Geneva. 11 pp.

1.3. ISO (1997) ISO/DIS 10705-2, Water quality - Detection and enumeration of bacteriophages. Part 2: Enumeration of somatic coliphages. International Organization for Standardization, Geneva. 17 pp.

1.4. Tartera C, Araujo R, Michel T and Jofre J (1992) Culture and decontamination methods affecting enumeration of phages infecting *Bacteroides fragilis* in sewage. Appl Environ Microbiol 58, 2670-2673.

2. The P/A test is extremely sensitive to contamination. A single phage contaminant will yield a false positive result. Therefore, the utmost care must be taken to avoid contamination. Water baths should be used with special caution because these are often sources of phage contamination.

3. Commercial names of media and chemicals are mentioned merely as guide. Any alternative media or chemicals which yield equivalent results are acceptable. Analytical grade reagents are required only when standard grade reagents have been proved inferior.

4. The addition of nalidixic acid is required only when the bacterial flora of the water sample may interfere with replication of the host inoculum, multiplication of phages, or subsequent detection of phages.

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Appendix 2

Phages excreted by humans and animals

Pergamon

Wat. Sci. Tech. Vol. 31, No. 5-6, pp. 223–230, 1995.

0273-1223/95 $9·50 + 0·00

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BACTEROIDES FRAGILIS AND *ESCHERICHIA COLI* BACTERIOPHAGES: EXCRETION BY HUMANS AND ANIMALS

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ABSTRACT

The faecal excretion of somatic and male-specific coliphages, and phages of *Bacteroides fragilis* strain HSP40, by humans and a variety of animals has been investigated as part of research on indicator features of phages. Ninety human stool specimens were obtained from black and white male and female individuals varying in age from 6 months to 85 years. Sixty-five faecal samples from domestic animals including cattle, sheep, pigs, horses, dogs, cats, geese and rabbits, were obtained from various agricultural and domestic sources. The National Zoological Gardens in Pretoria kindly supplied 38 stool specimens from higher primates (gorilla, orang-outang and chimpanzee). Thirty-seven stool specimens from chacma baboons and vervet monkeys were obtained from the Zoological Gardens and our animal research centre. Five specimens of seabird droppings were obtained from the west coast of South Africa. The qualitative presence of phages was determined by an enrichment procedure followed by a plaque spot test. Double agar layer plaque assays were used to titrate phages. *Bacteroides fragilis* phages were detected in 13% of human stool samples, but not in any animal faeces. Somatic coliphages were detected in 54% of human, 56% of domestic animal, 57% of monkey and baboon, 53% of higher primate, and 60% of seabird specimens. Male-specific coliphages were detected in 26% of human, 90% of domestic animal, 76% of monkey and baboon, 63% of higher primate, and 20% of seagull faecal samples. Titres of phages in selected samples varied from undetectable by direct plaque assay to 4.5×10^6 somatic and 3.2×10^4 male-specific coliphages per gram of seabird droppings. Faecally polluted environments may, therefore, contain substantial numbers of somatic and male-specific coliphages of human and animal origin. The results confirm earlier observations that *B fragilis* phages can be used to distinguish between faecal pollution of human and animal origin.

KEYWORDS

Bacteriophages, *Bacteroides fragilis*, *Escherichia coli*, humans, animals, indicators

INTRODUCTION

Bacteriophages (phages) are viruses which infect bacteria. These bacterial viruses share properties with human viruses in terms of structure, composition, size and morphology. In view of this resemblance some phages have attractive features for application as indicators or models of human viruses (IAWPRC Study Group on Health Related Water Microbiology, 1991; Grabow *et al*, 1993). An important indicator feature of phages is that many of them are detectable by simple, rapid and inexpensive techniques. In addition, phages constitute no human health risk. Since phages can, like human viruses, only multiply in specific metabolically active host cells, the incidence in the environment of phages which infect bacteria such as *Escherichia coli* (coliphages), is as specific for faecal pollution as the host cells. A group of coliphages known as somatic coliphages is, therefore, widely being used as indicator of water quality and the potential presence of human viruses. These phages occur in sewage-polluted environments in numbers which usually exceed those of cytopathogenic human enteric viruses by several orders of magnitude. In addition, coliphages tend to be at least as resistant to unfavourable environmental conditions as human viruses.

One indicator shortcoming of somatic coliphages is that at least some of them can also infect bacteria related to *E coli*. Some of these hosts are metabolically active in certain water environments to the extent that they can multiply and support the replication of somatic coliphages. This implies that the presence and numbers of somatic coliphages in water environments are neither qualitatively nor quantitatively related to faecal pollution. Another group of coliphages known as male-specific or F-specific RNA coliphages can only infect hosts with sex fimbriae (pili) coded for by the fertility (F) genetic factor. These fimbriae, which carry the receptor sites for male-specific phages, are produced only at temperatures above about 30°C. Since sewage or waters in the open environment rarely reach this temperature, possibilities for male-specific coliphages to multiply in the environment are negligible which implies that these phages are specific indicators of faecal pollution. Another important indicator feature of male-specific coliphages, such as the type-specific phage MS2, is that their size and structure very closely resemble that of typical enteroviruses like poliovirus, and they even have a single-stranded RNA genome like enteroviruses. In view of these features male-specific coliphages are rapidly gaining ground as water quality indicators, and the International Organization for Standardization has formulated a standard procedure for their detection and enumeration (ISO, 1993).

Phages which infect *Bacteroides fragilis* bacteria also have attractive indicator features. These bacteria are strictly anaerobic and only multiply in the gastrointestinal tract of warm-blooded animals (where they outnumber *E coli* by 100-fold or more), which implies that the phages are specific for faecal pollution. In an elaborate search for the best possible host strain for these phages, Tartera and Jofre (1987) discovered that one particular strain designated *B fragilis* HSP40, only detected phages in 10% of 40 human stool specimens, and not in faecal specimens from 40 cows, 50 pigs, 21 rabbits, 28 mice, 20 hens or 10 quail. This suggested that *B fragilis* HSP40 phages were highly specific indicators of human faecal pollution, which, among other things, implied that the phages could be used to distinguish between faecal pollution of human and animal origin.

The ability to distinguish between faecal pollution of human and animal origin has valuable benefits for water quality assessment and control (Jagals *et al*, 1995). The objective of this study was, therefore, to confirm the specificity of *B fragilis* HSP40 by testing excreta from humans and animals in another part of the world, and screening

animals not previously tested, including higher primates closely related to humans. The stool specimens were also screened for somatic coliphages, and male-specific coliphages using two host strains, in order to compare their incidence to that of *B fragilis* HSP40 phages, to study their incidence in excreta in a part of the world not previously investigated, and to investigate their incidence in the excreta of animals not previously studied. This information is essential for assessing the value of all these phages as indicators of faecal pollution.

MATERIALS AND METHODS

Stool specimens

Human stool specimens were obtained at our two teaching hospitals and a private pathology firm, all in Pretoria, from black and white male and female individuals. Stool specimens from domestic animals (cattle, sheep, pigs, horses, dogs, cats, geese and rabbits) were obtained from various farms, private homes and the National Zoological Gardens in Pretoria. Stool specimens from chacma baboons (*Papio ursinus*) and vervet monkeys (*Cercopithecus aethiops*) were collected at our animal research centre and the National Zoological Gardens. Stool specimens from higher primates (gorilla, orang-outang and chimpanzee) were kindly supplied by the National Zoological Gardens. Specimens of seabird droppings were collected from mariculture shellfish rafts on the West coast of South Africa, and were predominantly from cape cormorant (*Phalacrocorax capensis*), southern blackbacked gull (*Larus dominicanus*), Hartlaub's gull (*Larus hartlaubii*) and related species of seabirds. Human stool specimens were from different individuals. Specimens from animals were also from different individuals, except for the higher primates of which only limited numbers were available. In the case of these animals, specimens were collected from the same individual not more than once a week, and half the batch was collected 10 months after the other, in order to cover natural changes in intestinal flora. Apart from the seabird droppings, all human and animal stool specimens were collected in and near Pretoria. Fresh specimens of faeces and droppings were collected in sterile MacCartney bottles. Specimens were tested within 5 h of collection or frozen at -20°C until examination.

Bacterial host strains

Somatic coliphages:
Escherichia coli strain C (ATCC 13706) (WG5)
kindly donated by AH Havelaar (Grabow *et al*, 1993)
Male-specific coliphages:
1. *Salmonella typhimurium* strain 3 Nal^r (Flac::Tn5) (WG49) (NCTC 12484)
   kindly donated by AH Havelaar (ISO, 1993)
2. *Escherichia coli* strain HS(pFamp)R
   kindly donated by VJ Cabelli (Debartolomeis and Cabelli, 1991)

***Bacteroides fragilis* HSP40 phages:**
Bacteroides fragilis strain HSP40
from our own culture collection (Tartera and Jofre, 1987)

Enrichment procedure

Stool specimens (1 g wet mass) were incubated in 22 ml volumes of growth medium inoculated with 3 ml quantities of host cultures in the exponential growth phase. Separate enrichments were carried out for each host, using the growth medium specified for the host (Debartolomeis and Cabelli, 1991; Grabow *et al*, 1993; ISO, 1993; Tartera and Jofre, 1987). Enrichment cultures were incubated at 37°C for 24 h, *B fragilis*

anaerobically and the rest aerobically. The qualitative presence of phages in the enrichment cultures was then tested by means of a spot test.

Spot test

Enrichment cultures (1 ml) were decontaminated by adding 0.4 ml chloroform, brief vortexing, leaving on the bench for 10 min, and centrifugation at 7000 rpm for 5 min. Plates for spot tests for each host were prepared as for double agar layer plaque assays except that no test suspension was added to the top layer. Plates were left on the bench with lids removed for about 60 min to allow excess surface moisture of the seeded overlay to dry. A sterile pasteur pipette was then used to spot one drop from each decontaminated enrichment culture onto the top agar of a plate seeded with the corresponding host culture. Not more than three evenly distributed spots were placed on each plate, and allowed to dry. Plates were then incubated at 37°C for 24 h, *B fragilis* anaerobically and the rest aerobically. The presence of phages in the spot was visible from lysis of the host bacteria which caused clearly visible plaques, generally confluent. Phages present in positive enrichment cultures were further investigated by plaque assays. Individual plaques were picked for purification and propagation. Phages which yielded plaques on one or more of the two hosts for male-specific coliphages, were tested for ability to produce plaques at both 25°C and 37°C. Phages which produced plaques at 25°C were not considered male-specific coliphages (Debartolomeis and Cabelli, 1991) and discarded.

Double agar layer plaque assays

These assays were basically carried out in triplicate on tenfold physiological saline dilutions of test samples as described by Grabow and Coubrough (1986) using logarithmic phase cultures of hosts. Growth and plating media used for each host were as previously described: *E coli* C (Grabow and Coubrough, 1986), *S typhimurium* WG49 (ISO, 1993), *E coli* HS(pFamp)R (Debartolomeis and Cabelli, 1991), *B fragilis* HSP40 (Tartera and Jofre, 1987).

RESULTS

The 90 human stool specimens were from 62 white and 28 black individuals, 48 males and 42 females, varying in age from 6 months to 85 years. Of these stool donors 48 suffered from gastroenteritis, diarrhoea, dysentery or related intestinal infections, while 42 were not known to have any intestinal disorders. The results summarised in Table 1 did not show any meaningful relationship between the incidence of phages and the variables of ethnic group, age, sex or intestinal illness.

The results summarised in Table 1 show that the incidence of somatic coliphages in human stools (54%) was similar to that of higher primates (53%) and primates (57%), but lower than that of domestic animals (70%) and higher than that of birds (48%). The incidence of male-specific coliphages as determined by means of the host strain *E coli* HS(pFamp)R, was lower in human stool specimens (26%) than in those of higher primates (63%), primates (76%), domestic animals (60%) and birds (36%). The incidence of phages in different animals varied. For instance, somatic coliphages were present in 100% of specimens from pigs and cats, but only in 38% of gorilla and rabbit specimens. The incidence of male-specific coliphages displayed similar variation.

An outstanding feature of the results is that *B fragilis* HSP40 phages were detected only in 13% of human stool specimens and not in excreta of any animals or birds.

Table 1. Qualitative detection of somatic coliphages, male-specific coliphages and *Bacteroides fragilis* phages in stool specimens of humans and animals

Specimens	n	Percent positive faecal specimens		
		Somatic	Male-specific	*B fragilis*
Human	90	54	26	13
Higher primates				
Gorilla	16	38	63	0
Chimpanzee	15	40	47	0
Orang-outang	12	92	83	0
Total	43	53	63	0
Primates				
Chacma baboon	18	61	67	0
Vervet monkey	19	53	84	0
Total	37	57	76	0
Domestic animals				
Pig	8	100	50	0
Sheep	10	40	60	0
Horse	7	86	29	0
Cattle	9	78	78	0
Rabbit	8	38	63	0
Dog	4	75	75	0
Cat	4	100	75	0
Total	50	70	60	0
Birds				
Goose	20	45	40	0
Seabird	5	60	20	0
Total	25	48	36	0

The *E coli* host strain HS(pFamp)R was used for the detection of male-specific phages.

Results of comparative tests summarised in Table 2 show that the *E coli* host strain HS(pFamp)R yielded positive results for male-specific coliphages in 62 (57%) and *S typhimurium* WG49 in 31 (29%) of 108 randomly selected human and animal faecal specimens tested. The *E coli* host yielded positive results for 39 specimens for which the *S typhimurium* host yielded negative results, and the *S typhimurium* host yielded positive results for 8 specimens for which the *E coli* host yielded negative results.

Table 2. Male-specific coliphages detected in human and animal faeces using host strains *Escherichia coli* HS(pFamp)R and *Salmonella typhimurium* WG49

Host	Percentage of specimens positive for male-specific coliphages				
	Human n = 2	Higher Primates n = 16	Primates n = 24	Domestic Animals n = 46	Birds n = 20
E coli	50	56	75	57	40
S typhimurium	50	19	58	20	20

Primates = chacma baboons and vervet monkeys; Birds = geese

Titres (plaques per gram wet mass) of phages recorded in typical faecal specimens were:

Somatic coliphages:	Human	= 290 000; 4 600; 4 300; 140
	Orang-outang	= 110 000; 180 000
	Seabird	= 4 500 000
Male-specific coliphages:	Gorilla	= 56
	Chimpanzee	= No plaques detected by direct titration
	Orang-outang	= 130; 46
	Chacma baboon	= 360
	Vervet monkey	= 36; 53
	Seabird	= 32 000

DISCUSSION

This study represents one of the most extensive investigations which has to date been carried out on the incidence of somatic coliphages, male-specific coliphages and *B fragilis* HSP40 phages in human and animal excreta. The incidence of these phages in the excreta of humans and animals in this part of the world, and in the excreta of some of the animals, notably primates (chacma baboon and vervet monkey), higher primates (gorilla, chimpanzee and orang-outang) and seabirds, has not previously been investigated. Although the number of samples tested is not sufficient for statistically significant conclusions on the excretion of each of the groups of phages by humans and individual species of animals, the results do give a valuable indication of tendencies of phage excretion, which casts valuable light on the value of these phages as indicators of faecal pollution.

An important finding is the confirmation of earlier observations (Tartera and Jofre, 1987) that *B fragilis* HSP40 phages are indeed highly specific for human faeces, and that these phages were not even detectable in the excreta of closely related higher primates (Table 1). The incidence of *B fragilis* HSP40 phages in 13% of stool specimens of 90

individuals in and near Pretoria, is close to the 10% reported by Tartera and Jofre (1987) for specimens collected in Barcelona. The marginal difference may partly be due to a more sensitive enrichment procedure used in the present study.

Reasons for the human specificity of *B fragilis* HSP40 phages remain to be elucidated. One possibility may be that the host or hosts of these phages are highly specific for the human intestinal tract. Specificity of certain species or strains of enteric bacteria for either humans or animals is known (Jagals *et al*, 1995). Research on differences between *B fragilis* strain HSP40 and other members of the *Bacteroides fragilis* species which occur in large numbers in the intestinal tract of both humans and animals, may cast light on this phenomenon.

Results on the incidence of phages in stool specimens from man and animals obtained in various studies are difficult to compare due to many variables such as differences in phage host strains and phage detection methods used, statistical fluctuations due to small numbers of samples tested, different populations studied, and differences in diet of study subjects. However, some tendencies would seem apparent. For instance, the incidence of somatic coliphages in the stool of domestic animals (Table 1) which ranged from 38% to 100%, is similar to that reported by Dhillon *et al* (1976), Osawa *et al* (1981) and Havelaar *et al* (1986). The incidence of somatic coliphages in the faeces of primates and birds would seem to fall into the same order (Table 1). The 54% incidence of somatic coliphages in human stool samples (Table 1) is similar to results of Dhillon *et al* (1976) and Havelaar *et al* (1986), but higher than the 24% of Osawa *et al* (1981), and the 1,6% to 14% of Furuse *et al* (1983).

The incidence of up to 75% of male-specific coliphages in domestic animal stool samples (Table 1) is considerably higher than the 0% and 0-33% reported by Dhillon *et al* (1976) and Osawa *et al* (1981), respectively, but would appear to be in agreement with the findings of Havelaar *et al* (1986). The incidence of the phages in primates and birds would seem to be similar to that in domestic animals (Table 1). The 26% incidence of male-specific coliphages in human stool samples is considerably higher than the 0% of Dhillon *et al* (1976) and the low incidence reported by Havelaar *et al* (1986), but similar to the 0-33% of Osawa et al (1981). The sensitive enrichment procedure used in this study for the detection of phages may at least in part account for some of these differences. The absence of a relation between the incidence of both somatic and male-specific coliphages and age, state of health, sex or ethnic groups with differences in diet and lifestyle, is interesting because Furuse *et al* (1983) detected coliphages in 1.6% of stools from healthy individuals and in 14% of those of hospitalised patients.

Reasons for the more frequent detection of male-specific coliphages by means of the *E coli* host HS(pFamp)R than by *S typhimurium* WG49 have not been investigated. The percentage of false positive isolations may be higher with the former host. This difference should be investigated because it has implications for the standardisation of detection methods, and because in our hands the *E coli* host proved more user-friendly (unpublished observations).

The variation in incidence and counts of phages in excreta of humans and animals may be related to the natural fluctuation in the bacterial flora of humans and animals. As the colonisation of a particular bacterial host in the gastrointestinal tract reaches a maximum, the production of its phages reaches a maximum, and as the host disappears, the phages disappear. The high counts of phages in some specimens, notably those of seabirds, would seem to be in agreement with counts of 10^6 to 10^8 per gram of faeces

reported by Dhillon *et al* (1976) and Havelaar *et al* (1986). This and earlier studies do, therefore, show that both somatic and male-specific coliphages are excreted in substantial numbers by many humans and animals, and that *B fragilis* HSP40 phages are excreted only by humans, which adds to evidence supporting their use as indicators.

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Appendix 3

Phages in waste water from an informal settlement

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VIRUSES IN WASTE WATER FROM AN INFORMAL SETTLEMENT

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SUMMARY

Viruses are a major cause of waterborne diseases. Details on viruses in waste water are essential for management strategies aimed at the protection of water sources and the control of waterborne diseases. Virological screening of waste water is also carried out for monitoring enteric viruses circulating in communities, and assessment of public health campaigns such as poliomyelitis vaccination. In this study information has been obtained on viruses in diffuse effluents from a typical low socio-economic informal settlement with restricted sanitation and water supply. Sewage from an adjacent community with a sewerage system has likewise been analysed. The study area was selected as representative of communities which tend to be exceptionally vulnerable to enteric infectious diseases, and waste water from the settlements may be expected to contain high levels of waterborne pathogens. A total of 209 samples collected over a period of 9 months in 1994 were analysed for cytopathogenic enteric viruses and related indicators. Conventional cell culture propagation using three cell types yielded 486 isolates of viruses which consisted of 263 coxsackie B viruses (54%), 109 polioviruses (22%), 101 untyped enteroviruses (21%), 9 adenoviruses (2%), 2 echoviruses (<1%), and 2 reoviruses (<1%). All six types of coxsackieviruses were isolated, but 48% were type B2. All three types of polioviruses were isolated, all of them vaccine strains. Counts of faecal indicators and the incidence of viruses tended to increase after rainfall, which indicates that stormwater run-off was heavily polluted. Even though viruses were not enumerated, the isolation of viruses from 72 % of stream samples and 96% of sewage samples, suggests that the numbers of viruses in the waters were exceptionally high. No viruses were detected in the stream upstream of the settlement. Viruses outnumbered faecal bacteria and phages commonly used as indicators in a substantial number of stream water samples. This confirms the exceptional high incidence of viruses in the waters concerned as well as shortcomings of faecal bacteria and phages commonly used as indicators of water quality. The high incidence of all three vaccine strains of polioviruses in the absence of wild-type strains, confirms the success of poliomyelitis vaccination in the communities concerned. The results show that diffuse effluents from the settlement heavily pollute the stream with viruses and other faecal organisms. This has major implications for risks of infection constituted by the stream. The findings underline the need for efficient sanitary services in communities of this kind in order to protect water sources and to control waterborne diseases.

INTRODUCTION

Viruses are excreted in large numbers by infected individuals and may remain infectious for days, weeks or months in water environments (Grabow, 1996). In addition, the minimum infectious dose of viruses may be as low as a single particle. Viruses are, therefore, a common cause of waterborne disease. Details on the incidence and behaviour of viruses in water environments are limited because their enumeration requires advanced technology, and commonly used faecal bacteria have shortcomings as indicators for viruses. Details on viruses in waste water are essential for health risk assessment and management strategies aimed at the protection of water sources and the control of waterborne diseases. Virological screening of wastewater is also used for other purposes such as the investigation of enteric viruses circulating in communities, and monitoring of public health campaigns such as poliomyelitis vaccination (Tambini *et al*, 1993; Van der Avoort *et al*, 1995). This study focuses on viruses in diffuse effluents from a typical low socio-economic informal settlement with restricted sanitation and water supply. These communities are exceptionally vulnerable to enteric infections (Von Schirnding *et al*, 1993), and waste water effluents are likely to contain high levels of faecal organisms.

MATERIALS AND METHODS

A total of 209 samples were collected over a period of 9 months in 1994 and analysed for enteric viruses and related indicators. These samples were collected at regular intervals from a stream which runs through the settlement and a sewerage pipeline which serves an adjacent community. The stream was sampled about 3 km upstream of the settlement, right in the settlement and about 1 km downstream of the settlement. Cytopathogenic viruses were isolated from unconcentrated samples by conventional cell culture propagation using the BGM monkey kidney and PLC/PRF/5 human liver cell lines, and primary vervet kidney cells (Grabow and Taylor, 1993). Faecal coliform bacteria, enterococci, somatic and male-specific coliphages, and phages infecting *Bacteroides fragilis* HSP40 were enumerated by conventional methods (Grabow, 1996). Viruses were typed by cytopathogenic effect (CPE) in cell cultures and stained cover slips, inoculation of newborn mice, neutralisation tests using the Lim Benyesh-Melnick antiserum pools for enteroviruses, and molecular techniques using routine procedures (Grabow *et al*, 1992). The National Institute for Virology, Johannesburg, kindly rendered assistance in the typing of certain isolates, notably the confirmation of vaccine strains of polioviruses. Adenovirus typing was limited to distinction between enteric and non-enteric types using the commercial adenoclone-type 40/41 immunoassay (Cambridge Biotech, Worcester, MA).

RESULTS

No viruses were detected in samples of water from the stream upstream of the settlement. In these samples counts of faecal coliforms were in the range of 100 to 2800 per 100 ml. Somatic and male-specific coliphages were detected only on rare occasions, and counts were in the range of 0 to 200 per 100 ml. A total of 486 viruses were isolated from 72% of 142 samples of stream water collected in and below the settlement, and from 96% of 53 sewage samples. The isolates consisted of 263 coxsackie B viruses (54%), 109 polioviruses (22%), 9 adenoviruses (2%), 2 echoviruses (0,3%) and 2 reoviruses (0,4%). The remaining isolates consisted of 42 viruses only typed as enteroviruses (9%), and 59 only typed as enteroviruses other than coxsackie- or polioviruses (12%). All six types of coxsackie B viruses were isolated, but 48% were coxsackie B2. All three types of polioviruses were isolated. All the poliovirus isolates were vaccine strains. All 9 adenovirus isolates were non-enteric types. Details on viruses isolated from the stream and sewage samples are jointly summarised in Table 1, because the relative proportions of various viruses isolated from the stream and sewage did not differ significantly. Table 2 compares the percentages of viruses isolated on each of the three cell culture types, which gives an indication of the efficiency of the cells for the isolation of viruses from environmental waters. Many of the viruses only yielded a CPE after two or three blind passages.

Maximum levels of faecal coliforms, enterococci, somatic coliphages and male-specific (F-RNA) coliphages are recorded in Table 3. These results show that maximum levels of enterococci and coliphages in the stream were close to those in sewage. Counts of indicator organisms and the incidence of viruses tended to increase after rainfall, which shows that stormwater run-off contained heavy loads of faecal excreta. The following numbers of samples had counts of faecal bacteria or phages lower than those of viruses: faecal coliforms (2 samples), enterococci (14 samples), somatic coliphages (4 samples), male-specific coliphages (25 samples) and *B fragilis* HSP40 phages (many samples). The latter number of samples is not clearly defined because low counts of phages could at times have been due to malfunctioning of the phage test. Counts of faecal bacteria and phages were lower in the stream at the sampling site 1 km downstream of the settlement. In this area the stream slowly passed through some reed-beds and stagnant patches of water which clearly reduced counts of the organisms. The percentage of samples from which viruses were isolated was, however, the same (72%) for samples collected from the stream inside and downstream of the settlement, suggesting no significant reduction in numbers of viruses.

Table 1. Viruses isolated from 142 samples of polluted stream water and 53 samples of sewage

Virus	Isolates on each cell culture			Total
	PLC	BGM	VK	
Coxsackie B1	9	9	1	19
Coxsackie B2	47	43	37	127
Coxsackie B3	16	10	14	40
Coxsackie B4	16	26	18	60
Coxsackie B5	2	0	1	3
Coxsackie B6	3	5	6	14
Total coxsackie B	93	93	77	263
Polio Sabin 1	39	26	15	80
Polio Sabin 2	6	10	2	18
Polio Sabin 3	6	2	3	11
Total polio	51	38	20	109
Echo 7	0	0	1	1
Echo 27	0	0	1	1
Entero non-cox/polio	22	24	13	59
Entero untyped	10	17	15	42
Adeno	9	0	0	9
Reo	0	0	2	2
Total isolates	185	172	129	486

Samples collected from stream inside settlement and about 1 km downstream.

Entero non-cox/polio = enteroviruses other than coxsackie or polio.

Entero untyped = enteroviruses not typable by available neutralising typing antisera.

Relative composition of viruses isolated from the stream polluted by diffuse effluents from the informal settlement did not differ significantly from that of sewage of an adjacent community.

Table 2. Comparison of three cell culture types for the isolation of cytopathogenic viruses from a polluted stream and sewage in developing communities

Virus	Percentage isolates on each cell culture			Percentage of total
	PLC	BGM	VK	
Coxsackie B1	47	47	6	4
Coxsackie B2	37	34	29	26
Coxsackie B3	40	25	35	9
Coxsackie B4	27	43	30	13
Coxsackie B5	67	0	33	1
Coxsackie B6	21	36	43	3
Total coxsackie B	35	35	30	54
Polio Sabin 1	49	33	18	17
Polio Sabin 2	33	56	11	1
Polio Sabin 3	55	18	27	2
Total polio	41	38	21	22
Echo 7	0	0	100	0,3
Echo 27	0	0	100	0,3
Entero non-cox/polio	37	41	22	12
Entero untyped	24	40	36	9
Adeno	100	0	0	2
Reo	0	0	100	0,4
Total isolates	38	35	27	100

Total number of isolates = 486.

Percentages calculated from numbers of isolates presented in Table 1.

PLC = PLC/PRF/5 human liver cell line.

BGM = Buffalo Green Monkey kidney cell line.

VK = Primary vervet kidney cells.

Table 3. Maximum levels of faecal bacteria and coliphages in the stream and sewage

Indicator	Count/ml	
	Stream	Sewage
Faecal coliforms	733 000	30 000 000
Enterococci	33 300	62 000
Somatic coliphages	20 000	22 500
F-RNA coliphages	1016	1200

DISCUSSION

This study represents the most comprehensive survey of viruses in water recorded to date in South Africa, and probably the most detailed investigation of viruses in diffuse effluents from an informal settlement anywhere in the world. Although viruses have not been enumerated, the high percentage of samples from which viruses were isolated without the application of recovery techniques suggests that the incidence of viruses in the stream downstream of the settlement as well as the sewage were exceptionally high compared with data on the general incidence of viruses in sewage and waste water, according to which numbers of cytopathogenic viruses are generally about 200 to 11 000 per litre, but may exceed 100 000 per litre (WHO Scientific Group, 1979; Dahling *et al*, 1989). The viruses isolated in this study are limited to viruses which cause a cytopathogenic effect in cell cultures. These viruses probably only represent the tip of the iceberg of the total number of viruses in the waters because the great majority of viruses concerned are not detectable by cell culture propagation (Bern and Glass, 1994; Grabow, 1996).

The finding that viruses outnumbered faecal coliforms, enterococci and all three phages in a substantial number of river water samples, confirms the exceptionally high numbers of viruses, as well as shortcomings of commonly used indicators for indicating the presence of viruses. The high proportion of coxsackie B viruses (54% of all isolates, Table 2) is in agreement with results reported for sewage in other parts of the world (Dahling *et al*, 1989). The finding that the three vaccine strains of polioviruses represented 22% of all viruses isolated (Table 2), in the absence of wild type strains, confirms the success of poliomyelitis vaccination campaigns in the communities concerned and indicates that the risk of poliomyelitis in the communities is low.

A total of 101 isolates (Table 1) have only been typed as far as enteroviruses, or enteroviruses other than coxsackie or polio. These isolates probably belong to the wide range of 33 different serological types of echoviruses. This has been confirmed by typing two of the isolates in this group, which turned out to be echovirus types 7 and 27. Typing of these viruses is extremely labour intensive and expensive, and contributes little to the objectives of this particular study. The nine isolates of adenoviruses (Table 1) were likewise not typed any further than confirming that they were not enteric adenoviruses, ie types 40 or 41. These adenoviruses do, therefore, belong to any of the remaining 44 serological types.

The PLC/PRF/5 cell line proved most useful for the isolation of viruses from environmental waters because 38% of viruses were isolated on these cells, compared with 35% on BGM cells and 27% on primary vervet kidney (VK) cells (Table 2). PLC/PRF/5 cells were particularly susceptible to polioviruses, and all 9 adenoviruses were isolated on these cells. The susceptibility of PLC/PRF/5 cells to adenoviruses would seem to be in agreement with their ability to support the replication of enteric adenoviruses (Grabow *et al*, 1992). However, primary vervet kidney cells would seem to be more susceptible to echoviruses and reoviruses than the other two cell types. In combination the data on viruses isolated (Table 2) suggest that ideally all three cell types should be applied in parallel for the isolation of viruses from environmental waters. In a previous comprehensive evaluation of a wide variety of cell culture systems, the three cell culture types used in this study have been selected as the most sensitive for the isolation of viruses from environmental waters (Potgieter and Grabow, 1995). The low number of reoviruses isolated in this study would appear surprising because relatively higher numbers of reoviruses were recorded in studies on other waters (Grabow and Nupen, 1981; Dahling *et al*, 1989). This may imply variation in the incidence of reoviruses because the cell cultures and conditions of viral propagation used in this study are ideally suited for the isolation of reoviruses.

This study shows that diffuse effluents from the settlement heavily polluted the stream with viruses and other faecal organisms. At peak levels of pollution, particularly when stormwater run-off from the settlement entered the stream, counts of enterococci and phages in the stream were close to those in raw sewage (Table 3). These observations have major implications for the downstream utilisation of the water in the stream, because the quality by far exceeded levels considered acceptable for the utilisation of water for domestic purposes, recreation and irrigation. Since the study site was selected as typical of similar situations elsewhere, the results underline the importance of appropriate sanitary services for informal settlements.

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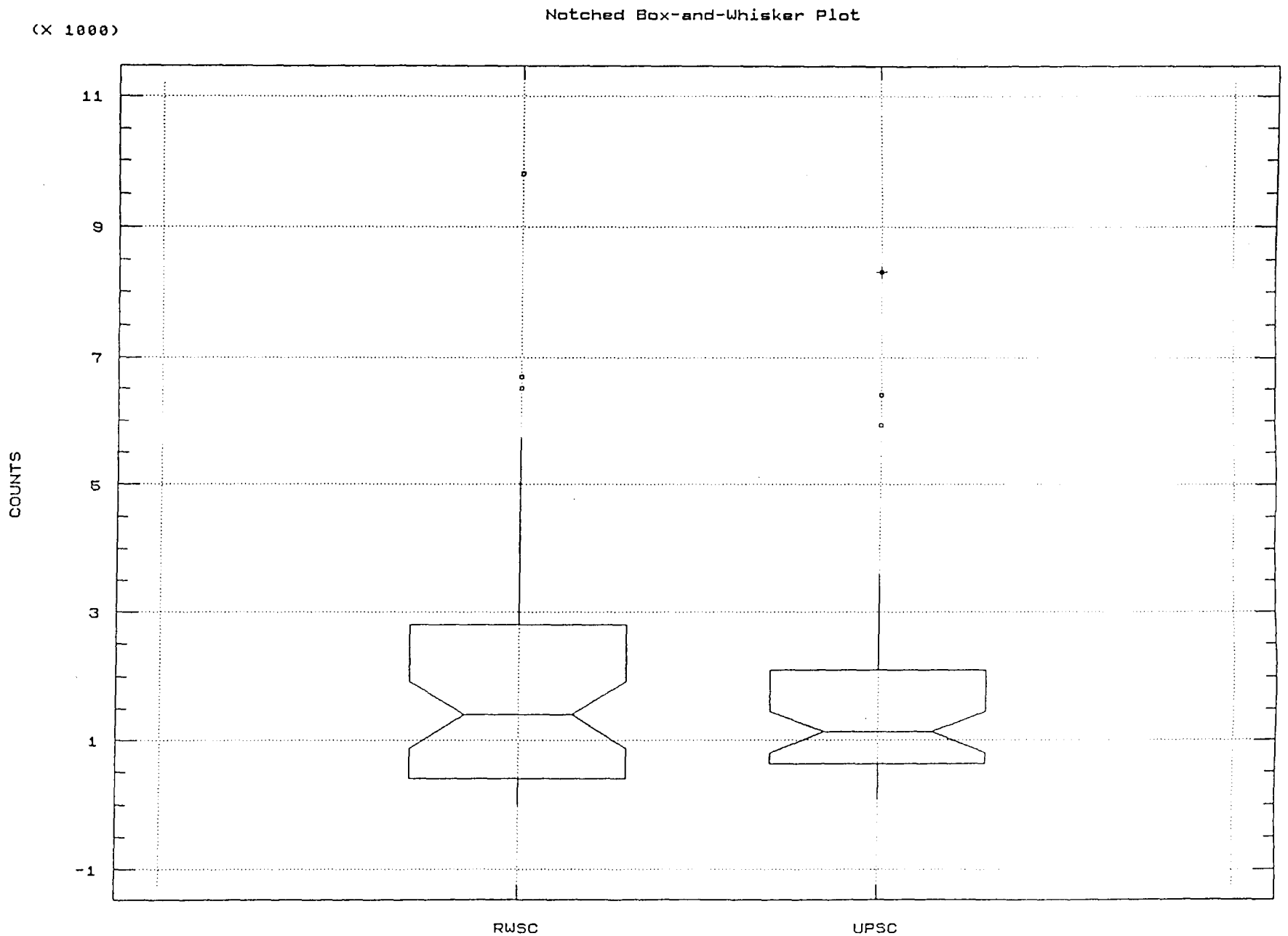
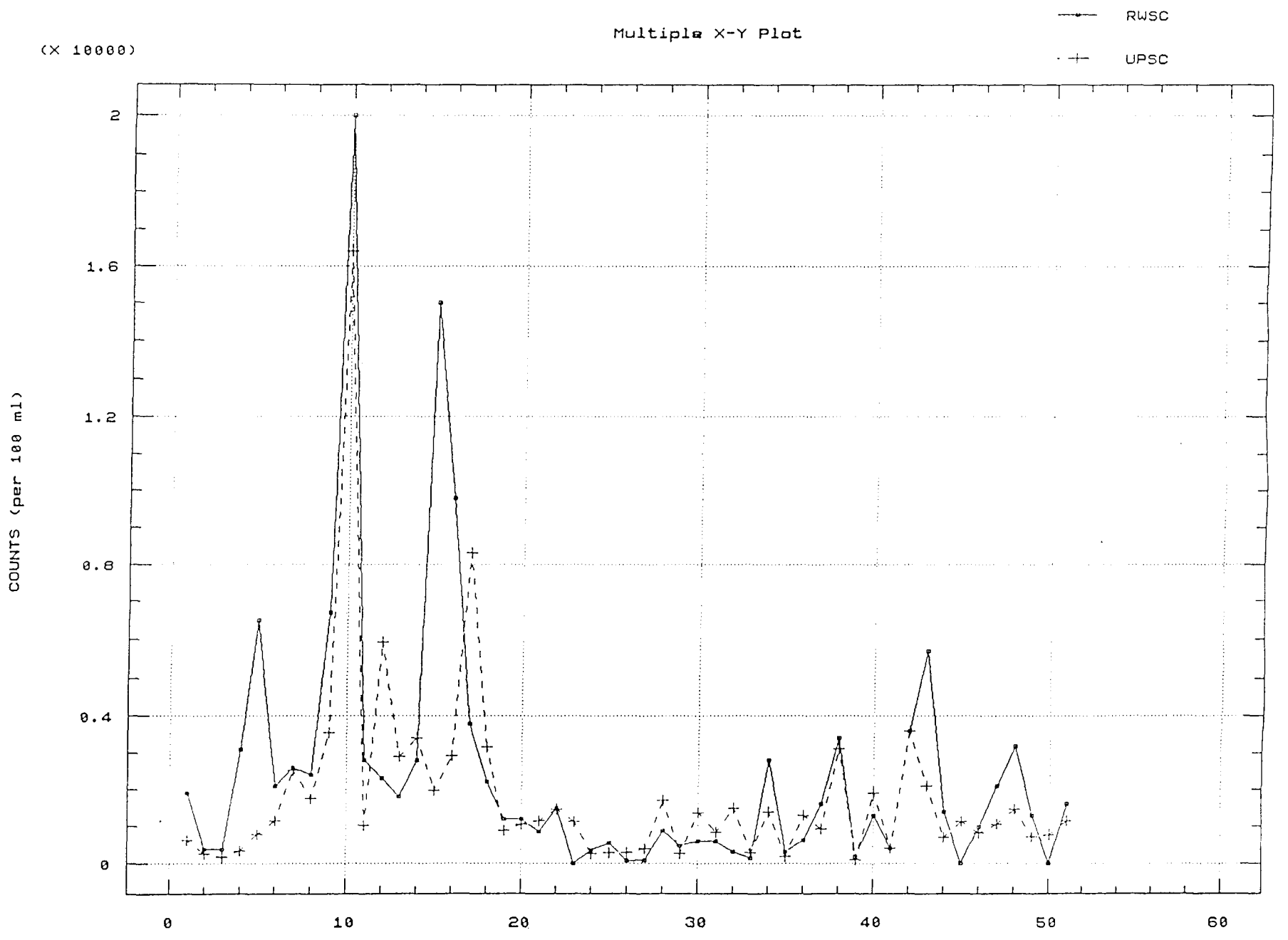


Counts of somatic coliphages (SC) recorded by Rand Water (RW) and the University of Pretoria (UP) laboratories for 51 samples of Klip River (K19) water collected from April 1995 to March 1996



Counts of somatic coliphages (SC) recorded by Rand Water (RW) and the University of Pretoria (UP) laboratories for 51 samples of Klip River (K19) water collected from April 1995 to March 1996

Appendix 4

Phages in Klip River and Vaal Dam water

Table 1. Klip River (K19) water:
Human viruses and related indicators

Date	Count per 100 ml											
	Faecal coliforms		Enterococci	Somatic coliphages			MS phages		*Bact* phages		Viruses	
	RW	UP	UP	RW		UP	UP		UP		UP	
95-04-03	19 000	1000	350	1900	-	633	-	100	A	-		0
95-04-10	6500	463	230	380	-	267	-	67	A	-		0
95-04-18	2700	210	70	380	-	167	-	100	A	-		0
95-04-24	8100	657	120	3100	-	333	-	167	A	-		0
95-05-02	6000	203	110	6500	-	800	-	233	A	-		Adeno
95-05-08	42 000	353	180	2100	-	1160	-	630	A	-		0
95-05-15	7700	1600	133	2600	-	2530	P	0	A	-		0
95-05-22	1500	403	226	2400	-	1760	-	230	A	-		0
95-05-29	4200	576	180	6700	-	3560	-	200	A	-		0
95-06-05	8600	1560	300	20 000	-	16 400	-	5130	P	-		0

- = not done or results not available
Counts = average of tests in threefold
Presence/Absence tests on 500 ml samples: P = Present, A = Absent
? = test in progress

continued

Date	Count per 100 ml											
	Faecal coliforms		Enterococci	Somatic coliphages			MS phages		*Bact* phages		Viruses	
	RW	UP	UP	RW		UP	UP		UP		UP	
95-06-12	6000	1030	136	2800	-	1030	-	260	A	-	0	
95-06-19	7400	560	50	2300	-	5930	-	960	A	-	0	
95-06-26	3000	446	203	1800	-	2900	-	930	A	-	0	
95-07-03	27 000	2730	166	2800	-	3400	-	2100	A	-	0	
95-07-10	2500	400	103	15 000	-	2960	-	960	A	-	0	
95-07-17	4600	600	203	9800	-	2930	-	2360	A	-	0	
95-07-24	2000	1300	166	3800	-	8300	-	1760	A	-	0	
95-07-31	750	450	150	2200	-	3160	-	1030	A	-	0	
95-08-07	1200	380	156	1200	-	900	-	260	A	-	0	
95-08-14	680	446	170	1200	-	1060	-	600	A	-	0	
95-08-21	4700	2760	636	870	-	1160	-	430	A	-	0	
95-08-28	6100	1200	63	1500	-	1460	-	230	P	-	0	
95-09-04	4900	1130	57	-	-	1130	-	30	A	-	0	
95-09-11	5200	1116	400	370	-	260	-	30	A	-	0	
95-09-18	3600	1830	256	550	-	300	-	60	A	-	0	

continued

Date	Count per 100 ml													
	Faecal coliforms		Enterococci	Somatic coliphages			MS phages		*Bact* phages		Viruses			
	RW	UP	UP	RW		UP	UP		UP		UP			
95-09-26	1000	646	120	80	-	300	-	33	A	-	0			
95-10-02	8300	2500	246	110	-	400	-	33	A	-	0			
95-10-09	3000	1433	427	900	-	1700	-	100	A	-	Reo			
95-10-16	3800	21 600	353	480	-	260	-	100	A	-	0			
95-10-23	3000	1866	1110	600	-	1360	-	430	A	-	0			
95-10-30	190	3660	512	600	-	850	-	67	A	-	0			
95-11-06	2500	1123	330	310	-	1500	-	530	A	-	0			
95-11-13	3200	3500	406	150	-	300	P	0	A	-	0			
95-11-20	7400	3130	1560	2800	-	1400	-	60	A	-	0			
95-11-27	5000	3000	670	320	-	200	-	30	P	-	0			
95-12-04	1300	7630	610	630	-	1300	-	60	A	-	0			
95-12-11	6100	2870	970	1600	-	930	-	100	A	-	0			
95-12-18	13 000	1070	720	3400	-	3130	-	100	A	-	Reo			

continued

Date	Count per 100 ml													
	Faecal coliforms		Streptococci	Somatic coliphages			MS phages		*Bact* phages		Viruses			
	RW	UP	UP	RW		UP	UP		UP		UP			
96-01-02	820	270	120	180	-	100	-	60	A	-	0			
96-01-08	7200	-	-	1300	-	1900	-	160	A	-	0			
96-01-15	2100	-	-	400	-	400	P	0	A	-	0			
96-01-22	7000	-	-	3600	-	3600	-	60	A	-	0			
96-01-29	7900	-	-	5700	-	2100	P	0	A	-	0			
96-02-05	1600	-	-	1400	-	700	P	0	A	-	0			
96-02-12	-	-	-	-	-	1130	P	0	P	0	Reo			
96-02-19	4000	-	-	980	-	830	-	60	A	-	Reo			
96-02-26	66	-	-	2100	-	1060	P	0	A	-	0			
96-03-04	4800	-	-	3200	-	1460	-	300	P	0	0			
96-03-11	8400	-	-	1300	-	730	-	60	A	-	0			
96-03-18	26 000	-	-	-	-	760	-	160	A	-	Reo			
96-03-25	6800	-	-	1600	-	1160	-	100	A	-	0			
96-04-01	24 000	-	-	6300	-	2630	-	300	A	-	Reo			
96-04-09	4100	-	-	2600	-	760	-	100	A	-	0			

continued

Date	Count per 100 ml								
	Faecal coliforms		Streptococci	Somatic coliphages		MS phages	*Bact* phages		Viruses
	RW	UP	UP	RW	UP	UP	UP		UP
96-04-15	44 000	-	-	1700	- 700	- 200	A	-	0
96-04-22	9000	-	-	5300	- 2860	- 830	A	-	Reo
96-04-29	8700	-	-	1300	- 1230	- 260	A	-	Reo
96-05-06	4100	-	-	580	- 1560	- 330	A	-	Reo
96-05-13	33 000	-	-	1470	- 930	- 260	A	-	0
96-05-20	10 000	-	-	4200	- 4700	- 1800	A	-	Reo
96-05-27	5800	-	-	7000	- 1530	- 1330	A	-	Reo
96-06-03	1300	-	-	2200	- 2500	- 200	A	-	0
96-06-10	12 000	-	-	1620	- 5260	- 1330	A	-	0
96-06-18	3900	-	-	5300	- 5900	- 830	A	-	Adeno
96-06-24	4200	-	-	1900	- 4060	- 1160	A	-	Reo
96-07-01	4700	-	-	3300	- 5530	- 4800	A	-	Reo
96-07-08	2200	-	-	3100	- 5530	- 800	A	-	0
96-07-15	2900	-	-	1800	- 4300	P 0	A	-	Reo
96-07-22	3600	-	-	3000	- 6030	- 1070	A	-	Reo

continued

Date	Count per 100 ml								
	Faecal coliforms		Streptococci	Somatic coliphages		MS phages	*Bact* phages		Viruses
	RW	UP	UP	RW	UP	UP	UP		UP
96-07-29	940	-	-	4600	- 13 400	- 11 800	A	-	Reo
96-08-05	790	-	-	1700	- 7270	- 2100	A	-	Reo
96-08-12	1500	-	-	1000	- 2300	- 900	A	-	0
96-08-19	1400	-	-	2200	- 2000	- 470	A	-	0
96-08-26	660	-	-	100	- 470	- 170	A	-	0
96-09-02	1500	-	-	1750	- 2070	- 630	A	-	0
96-09-09	1400	-	-	580	- 700	- 500	A	-	0
96-09-16	1500	-	-	3100	- 3600	- 330	A	-	0
96-09-23	3600	-	-	3600	- 2400	- 1400	A	-	0
96-09-30	920	-	-	870	- 700	- 130	A	-	0
96-10-07	6400	-	-	3200	- 1300	- 100	A	-	0
96-10-14	3200	-	-	610	- 1600	- 900	A	-	0
96-10-21	600	-	-	2500	- 1300	- 400	A	-	?
96-10-28	130	-	-	1300	- 1300	- 600	A	-	0
96-11-04	6200	-	-	1000	- 1100	- 500	A	-	0

continued

Date	Count per 100 ml							
	Faecal coliforms		Streptococci	Somatic coliphages		MS phages	*Bact* phages	Viruses
	RW	UP	UP	RW	UP	UP	UP	UP
96-11-11	2500	-	-	840	- 1600	- 1200	A -	0
96-11-18	4200	-	-	2400	- 1300	- 800	A -	?
96-11-25	4600	-		1800	- 3500	- 700	A -	?
96-12-02	2500	-	-	770	- 800	- 100	A -	?
96-12-09	7800	-	-	2700	- 1800	- 1100	A -	?
96-12-17	2200	-	-	2690	- 1200	- 400	A -	?
97-01-06	-	-	-	-	- 500	- 100	A -	?

Table 2. **Vaal Dam sluice gate (A18) water: Human viruses and related indicators**

Date	Count per 100 ml									
	Faecal coliforms		Enterococci	Somatic coliphages			MS phages		*Bact* phages	Viruses
	RW	UP	UP	RW	UP		UP		UP	UP
95-07-31	-	51	84	-	P	0	P	0	A -	0
95-08-07	-	9	30	-	P	0	P	0	A -	0
95-08-14	-	33	70	-	P	0	P	0	A -	0
95-08-21	-	32	70	-	P	0	P	0	A -	0
95-08-28	-	35	72	-	P	0	P	0	A -	0
95-09-04	-	62	85	-	P	0	P	0	A -	0
95-09-11	-	12	34	-	P	0	P	0	A -	0
95-09-18	-	43	129	-	P	0	P	0	A -	0
95-09-26	-	49	82	-	P	0	P	0	A -	0
95-10-02	-	40	75	-	P	0	P	0	A -	0
95-10-09	-	55	59	-	P	0	P	0	A -	0
95-10-16	-	95	140	-	P	0	P	0	A -	0
95-10-26	-	153	178	-	P	0	P	0	A -	0

- = not done or results not available; counts = average of tests in threefold; *Bact* = *Bacteroides fragilis* HSP40 phages;
Presence/Absence tests on 500 ml samples: P = Present, A = Absent, ? = test in progress

continued

Date	Count per 100 ml											
	Faecal coliforms		Enterococci	Somatic coliphages			MS phages		*Bact* phages		Viruses	
	RW	UP	UP	RW	UP		UP		UP		UP	
95-10-30	-	166	165	-	P	0	P	0	A	-		0
95-11-06	-	53	105	-	P	0	P	0	A	-		0
95-11-13	-	193	122	-	P	0	P	0	A	-		0
95-11-20	-	130	110	-	P	0	P	0	A	-		0
95-11-27	-	108	85	-	-	60	P	0	A	-		0
95-12-04	-	56	40	-	P	0	P	0	A	-		0
95-12-11	-	64	49	-	P	0	P	0	A	-		0
95-12-18	-	147	92	-	-	33	P	0	A	-		0
96-01-02	-	43	30	-	-	640	P	0	A	-		0
96-01-08	-	-	-	-	-	360	P	0	A	-		0
96-01-15	-	-	-	-	-	60	P	0	A	-		0
96-01-22	-	-	-	-	-	30	P	0	A	-		0
96-01-29	-	-	-	-	P	0	P	0	A	-		0

continued

Date	Count per 100 ml											
	Faecal coliforms		Enterococci	Somatic coliphages			MS phages		*Bact* phages		Viruses	
	RW	UP	UP	RW	UP		UP		UP		UP	
1996-02-05	-	-	-	-	P	0	P	0	A	-		0
1996-02-12	-	-	-	-	-	100	P	0	A	-		0
1996-02-19	-	-	-	-	-	730	P	0	A	-		0
1996-02-26	95	-	-	310	-	360	P	0	A	-		0
1996-03-04	610	-	-	-	-	130	P	0	A	-		0
1996-03-11	10	-	-	30	-	130	P	0	A	-		0
1996-03-18	18	-	-	0	P	0	P	0	A	-		0
1996-03-25	190	-	-	30	P	0	P	0	A	-		0
1996-04-01	30	-	-	20	P	0	P	0	A	-		0
1996-04-09	180	-	-	0	P	0	P	0	A	-		0
1996-04-15	57	-	-	60	P	0	P	0	A	-		0
1996-04-22	73	-	-	0	P	0	P	0	A	-		0

continued

Date	Count per 100 ml											
	Faecal coliforms		Enterococci	Somatic coliphages			MS phages		*Bact* phages		Viruses	
	RW	UP	UP	RW	UP		UP		UP		UP	
1996-04-29	130	-	-	60	-	100	P	0	A	-		0
1996-05-06	61	-	-	0	-	100	P	0	A	-		0
1996-05-13	0	-	-	0	P	0	P	0	A	-		0
1996-05-20	0	-	-	2500	-	30	P	0	A	-		Ent
1996-05-27	130	-	-	20	-	60	P	0	A	-		0
1996-06-03	62	-	-	10	-	100	P	0	A	-		0
1996-06-10	52	-	-	20	-	30	P	0	A	-		0
1996-06-18	76	-	-	0	P	0	P	0	A	-		0
1996-06-24	51	-	-	510	-	30	P	0	A	-		0
1996-07-01	28	-	-	10	P	0	P	0	A	-		0
1996-07-08	110	-	-	10	-	170	P	0	A	-		0
1996-07-15	61	-	-	10	-	30	P	0	A	-		0

continued

Date	Count per 100 ml											
	Faecal coliforms		Enterococci	Somatic coliphages			MS phages		*Bact* phages		Viruses	
	RW	UP	UP	RW	UP		UP		UP		UP	
1996-07-22	-	-	-	-	-	30	P	0	A	-		0
1996-07-29	24	-	-	10	-	70	P	0	A	-		0
1996-08-05	30	-	-	0	-	60	P	0	A	-		0
1996-08-12	35	-	-	0	P	0	P	0	A	-		0
1996-08-19	22	-	-	0	-	100	P	0	A	-		0
1996-08-26	1	-	-	0	P	0	P	0	A	-		0
1996-09-02	20	-	-	0	-	130	P	0	A	-		0
1996-09-09	32	-	-	10	P	0	P	0	A	-		0
1996-09-16	120	-	-	0	P	0	P	0	A	-		0
1996-09-23	48	-	-	0	P	0	P	0	A	-		0

continued

Date	Count per 100 ml													
	Faecal coliforms		Enterococci	Somatic coliphages			MS phages			*Bact* phages		Viruses		
	RW	UP	UP	RW	UP		UP			UP		UP		
1996-09-30	14	-	-	0	P	0	P	0		A	-	0		
1996-10-07	32	-	-	0	P	0	P	0		A	-	0		
1996-10-14	72	-	-	30	-	100	P	0		A	-	0		
1996-10-21	22	-	-	10	P	0	P	0		A	-	?		
1996-10-28	1500	-	-	30	P	0	P	0		A	-	0		
1996-11-04	67	-	-	-	-	-	-	-		-	-	-		
1996-11-11	92	-	-	0	P	0	P	0		A	-	0		
1996-11-18	26	-	-	20	P	0	P	0		A	-	0		
1996-11-25	-	-	-	-	P	0	P	0		A	-	?		
1996-12-02	19	-	-	0	P	0	P	0		A	-	0		
1996-12-09	110	-	-	10	P	0	P	0		A	-	?		
1996-12-17	150	-	-	0	P	0	P	0		A	-	?		

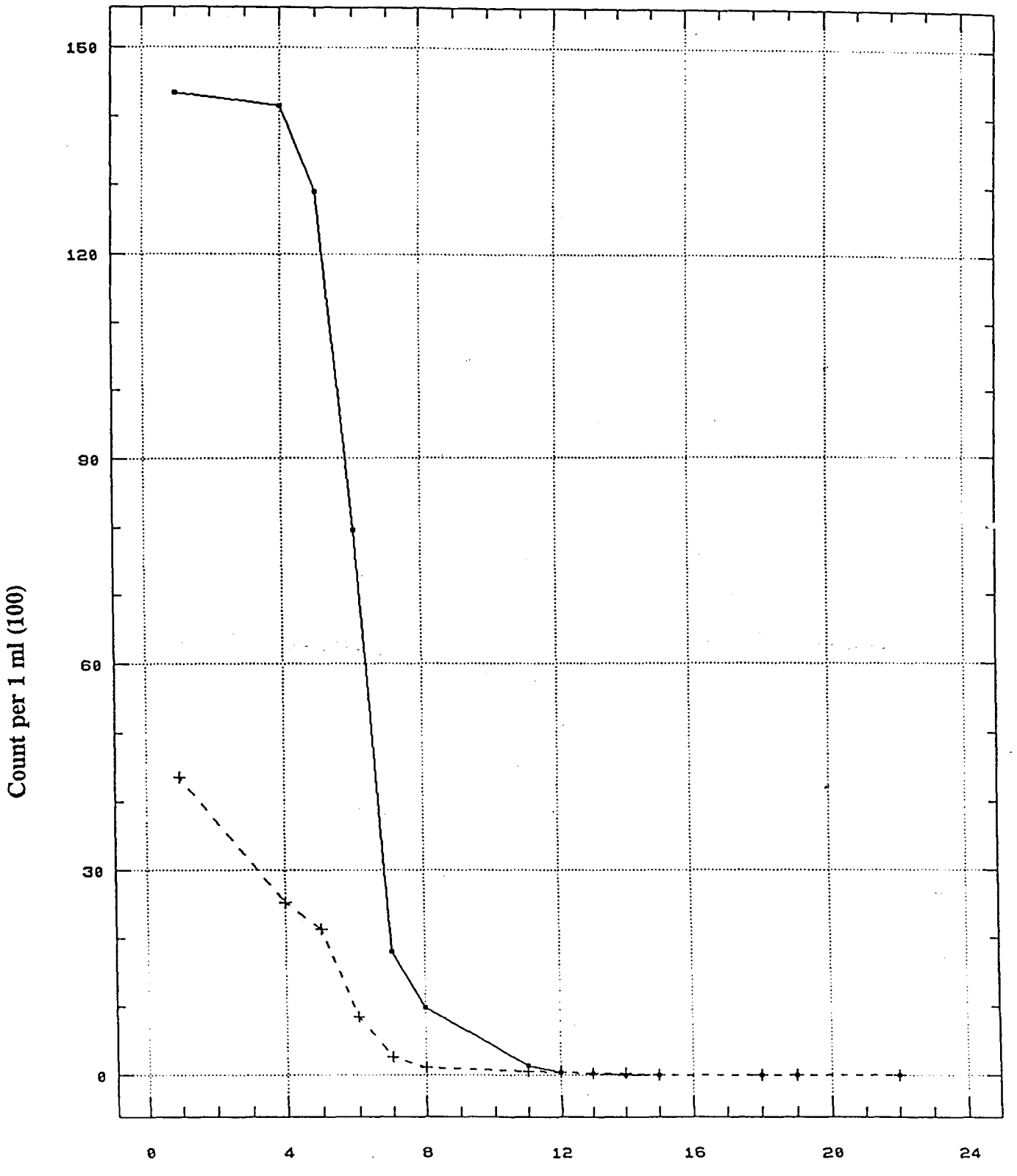
continued

Date	Count per 100 ml							
	Faecal coliforms		Enterococci	Somatic coliphages		MS phages	*Bact* phages	Viruses
	RW	UP	UP	RW	UP	UP	UP	UP
1997-01-06	-	-	-	0	P 0	P 0	A -	?

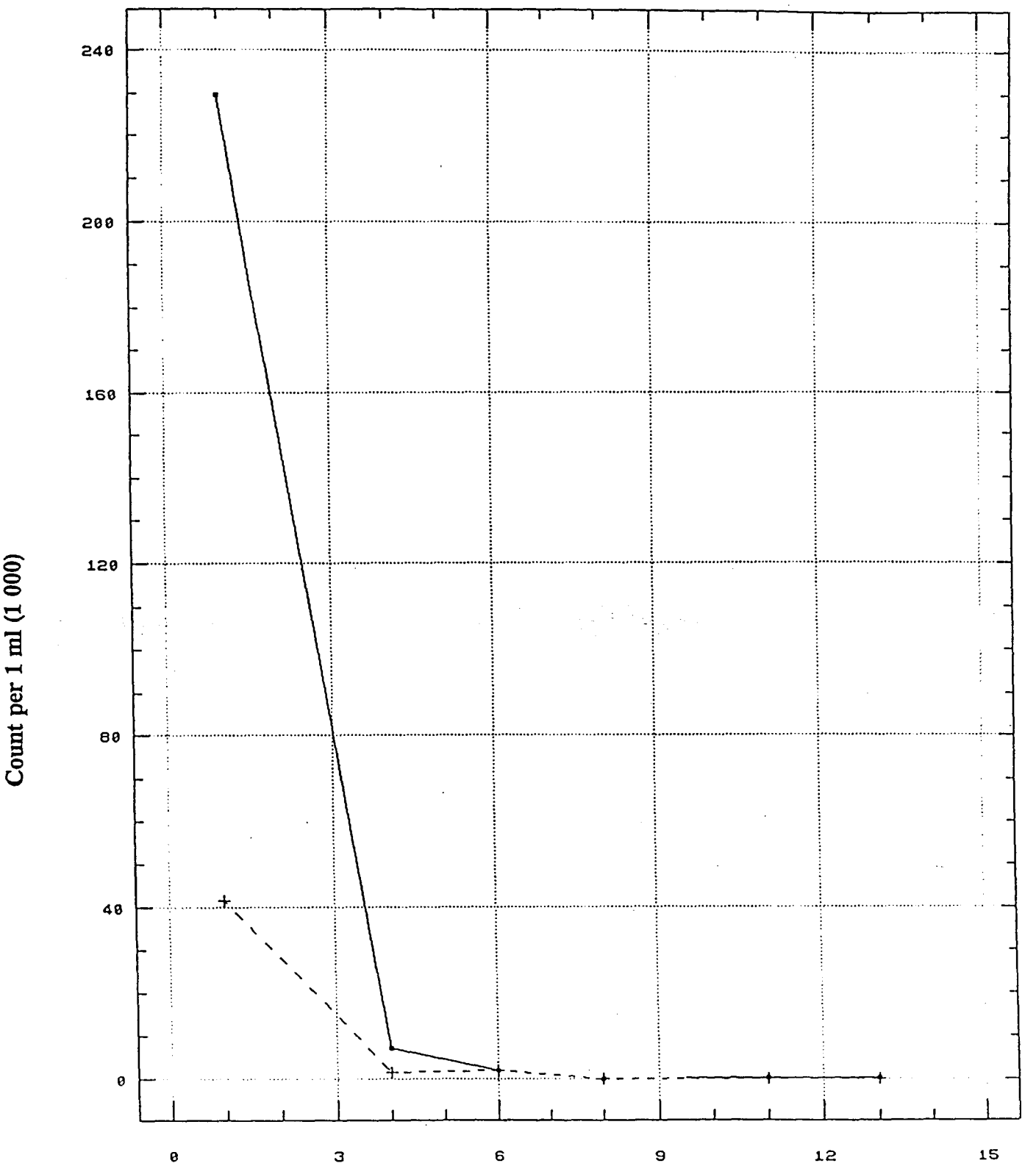
C:\wpd\vaal-18.97-01-24.

Appendix 5

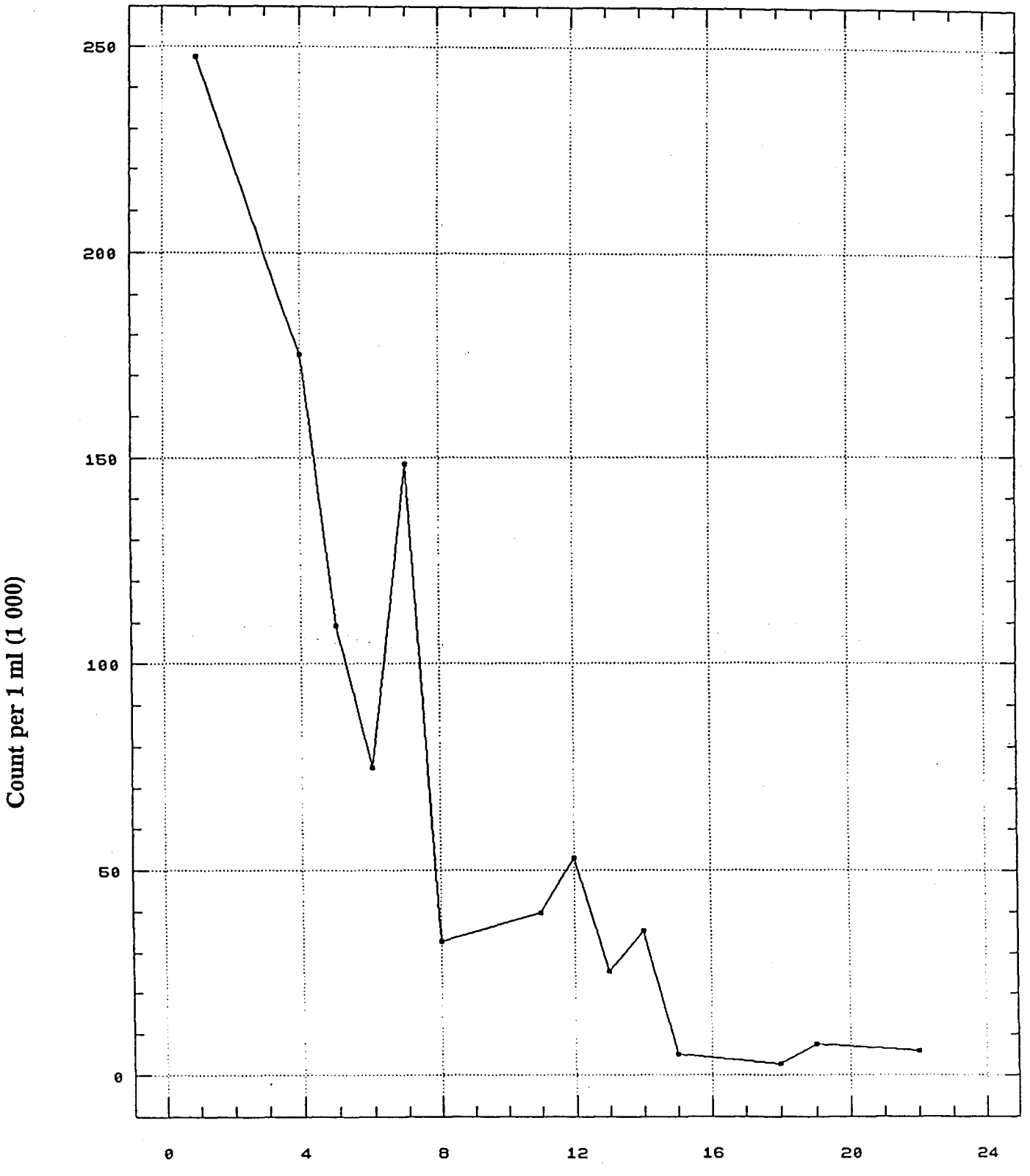
Survival of phages, viruses and bacteria in water



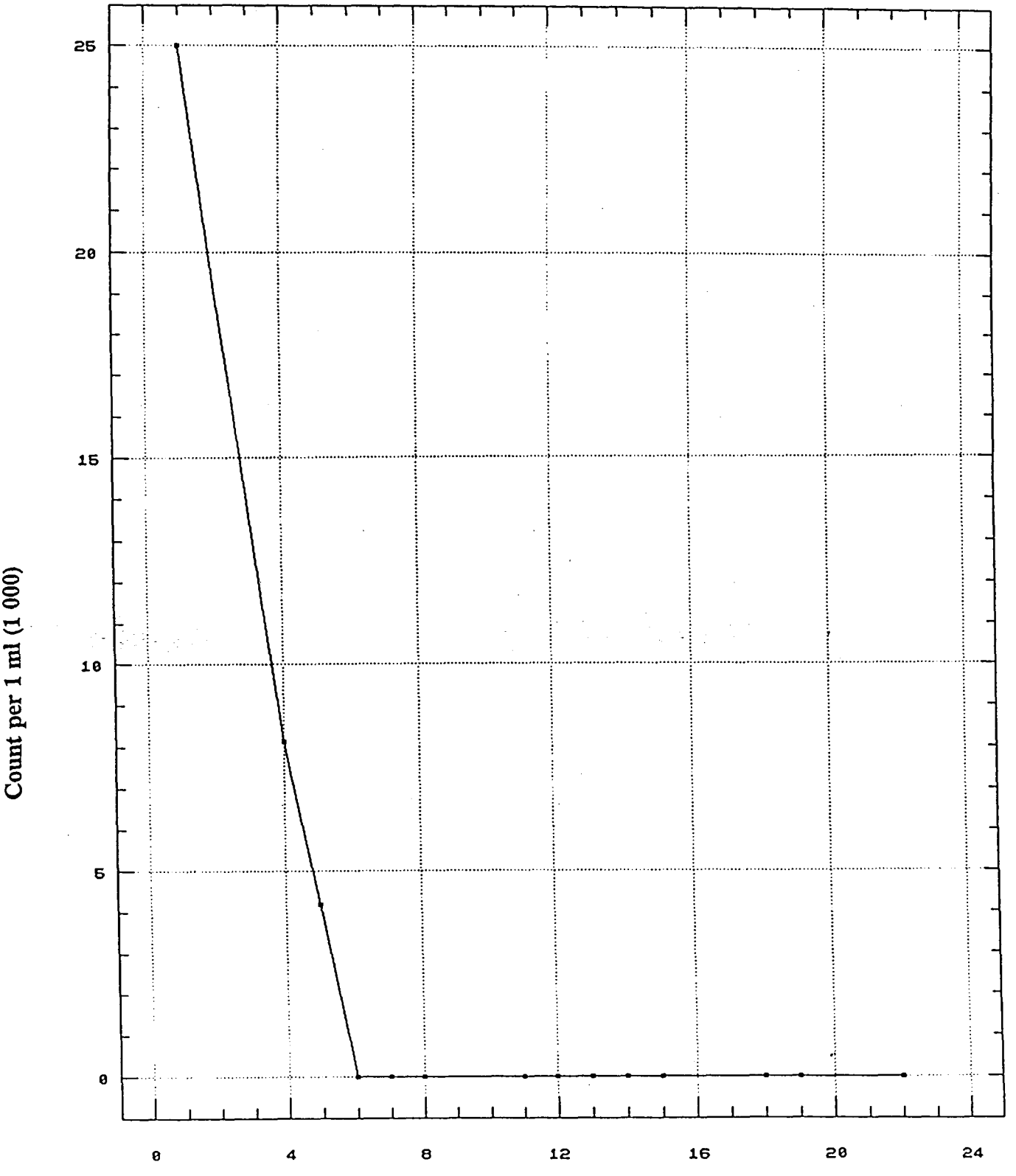
Survival on the laboratory bench in sea water seeded with bird droppings and human stool: Enterococci



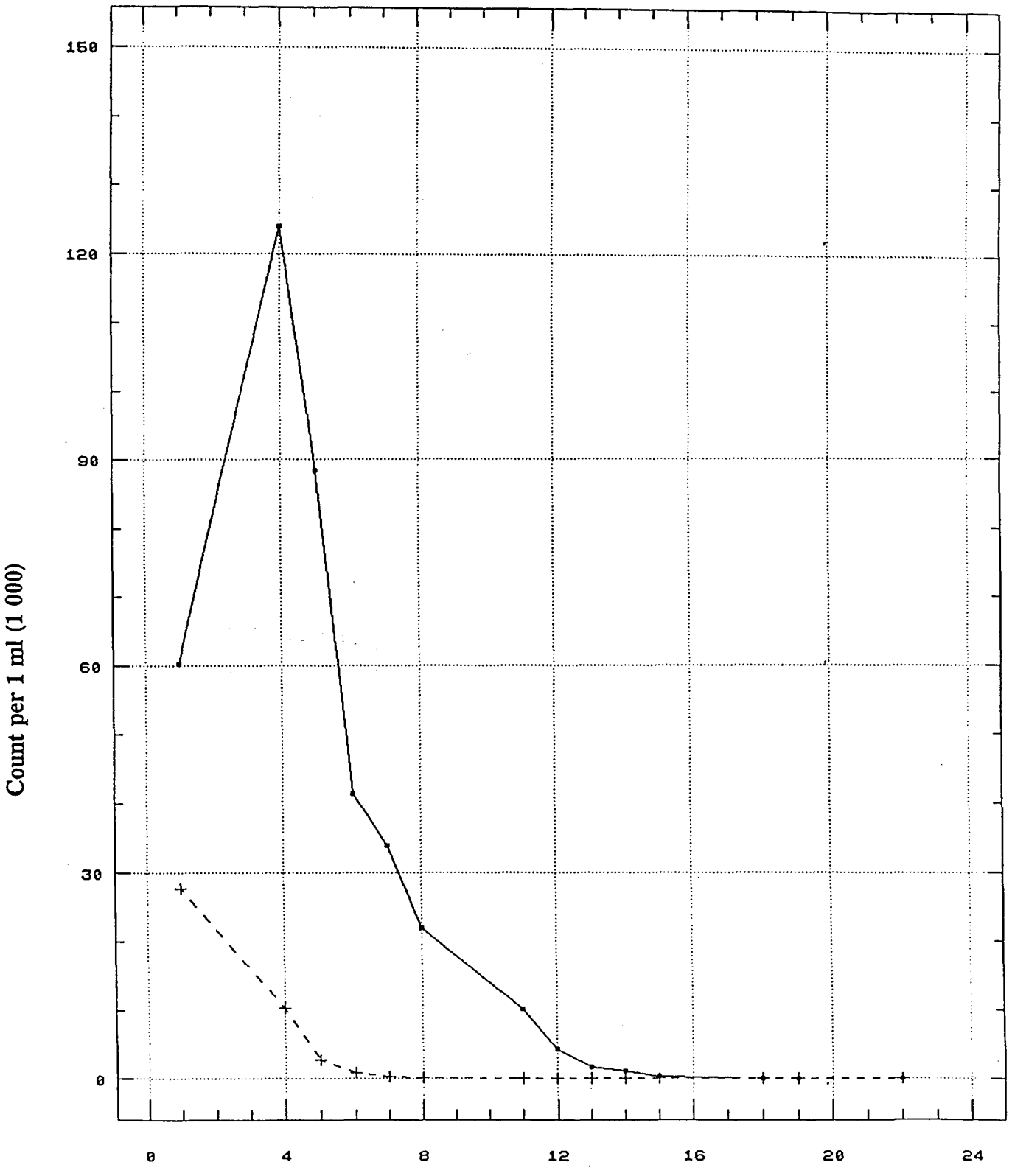
Survival on the laboratory bench in sea water seeded with bird droppings and human stool: Seeded poliovirus



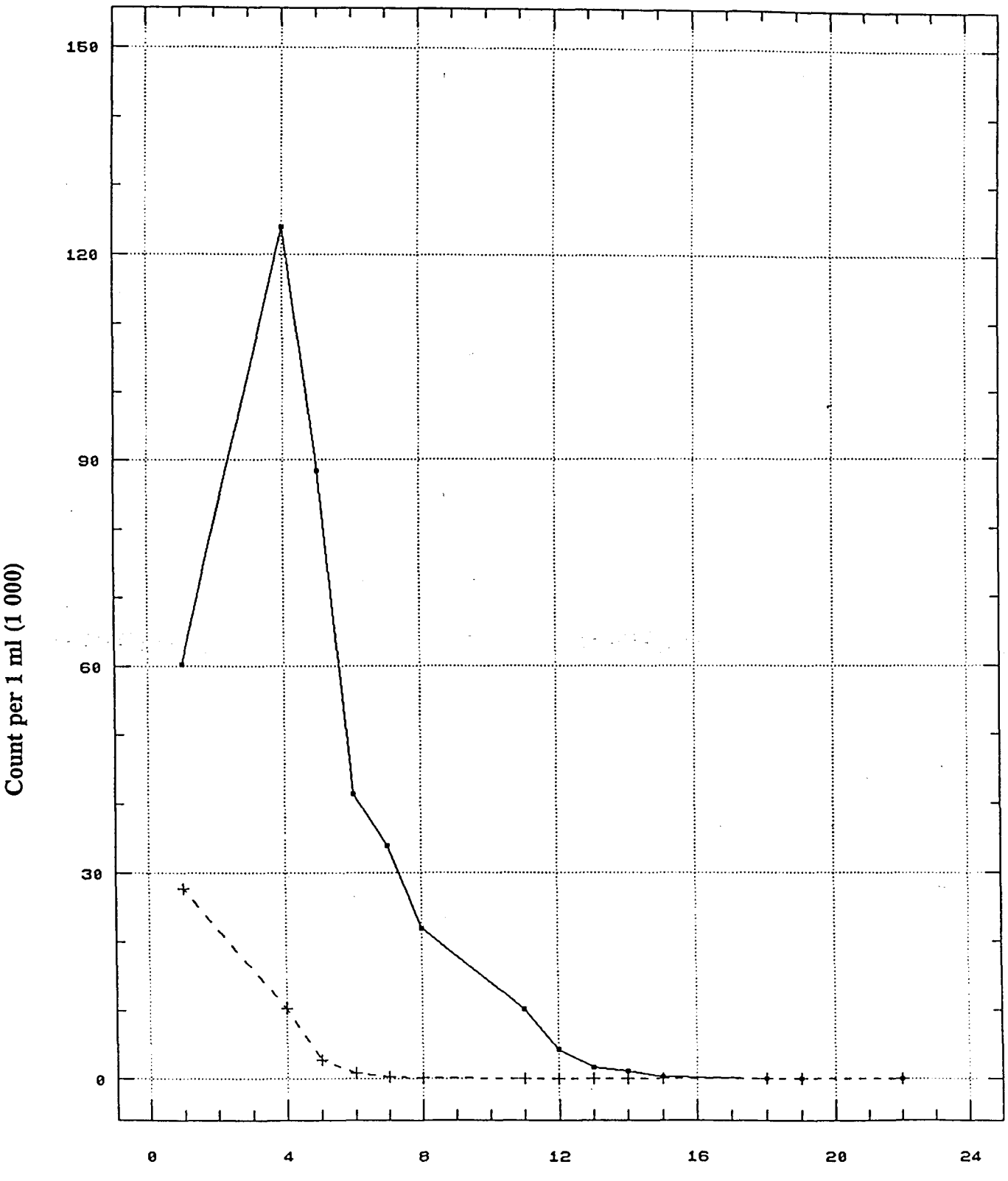
Survival on the laboratory bench in sea water seeded with bird droppings and human stool: Somatic coliphages



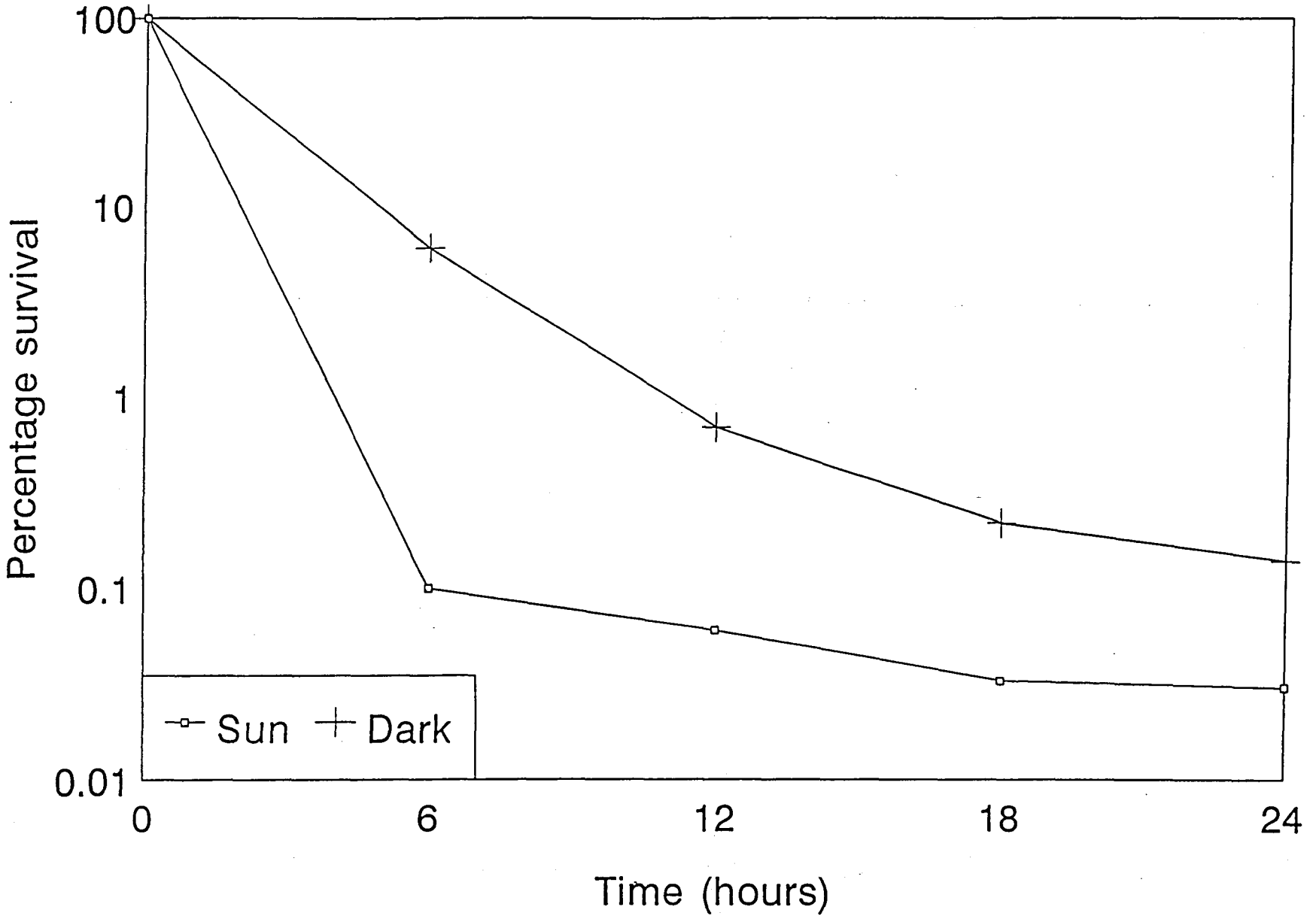
Survival on the laboratory bench in sea water seeded with bird droppings and human stool: Male-specific coliphages



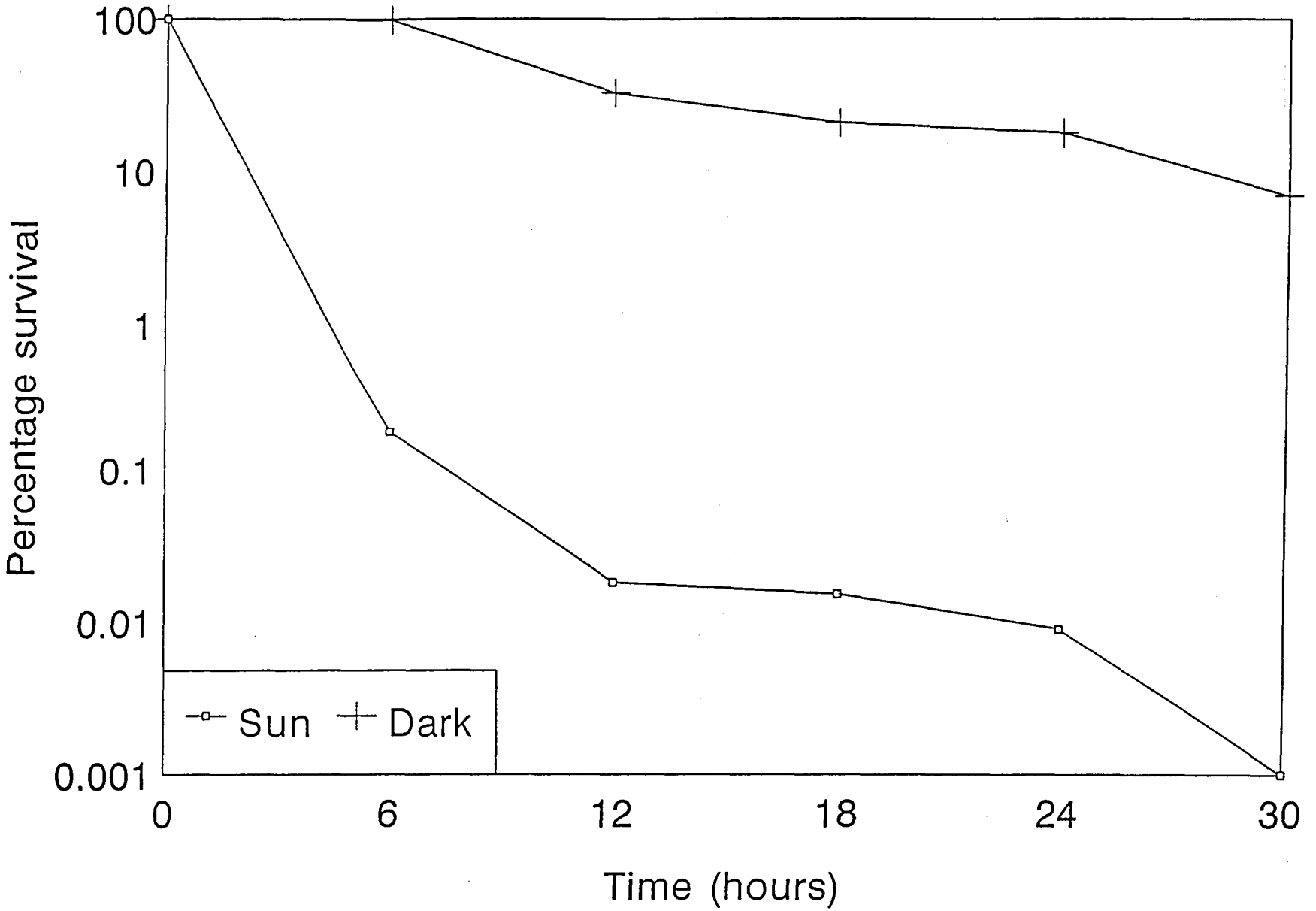
Survival on the laboratory bench in sea water seeded with bird droppings and human stool: Faecal coliforms



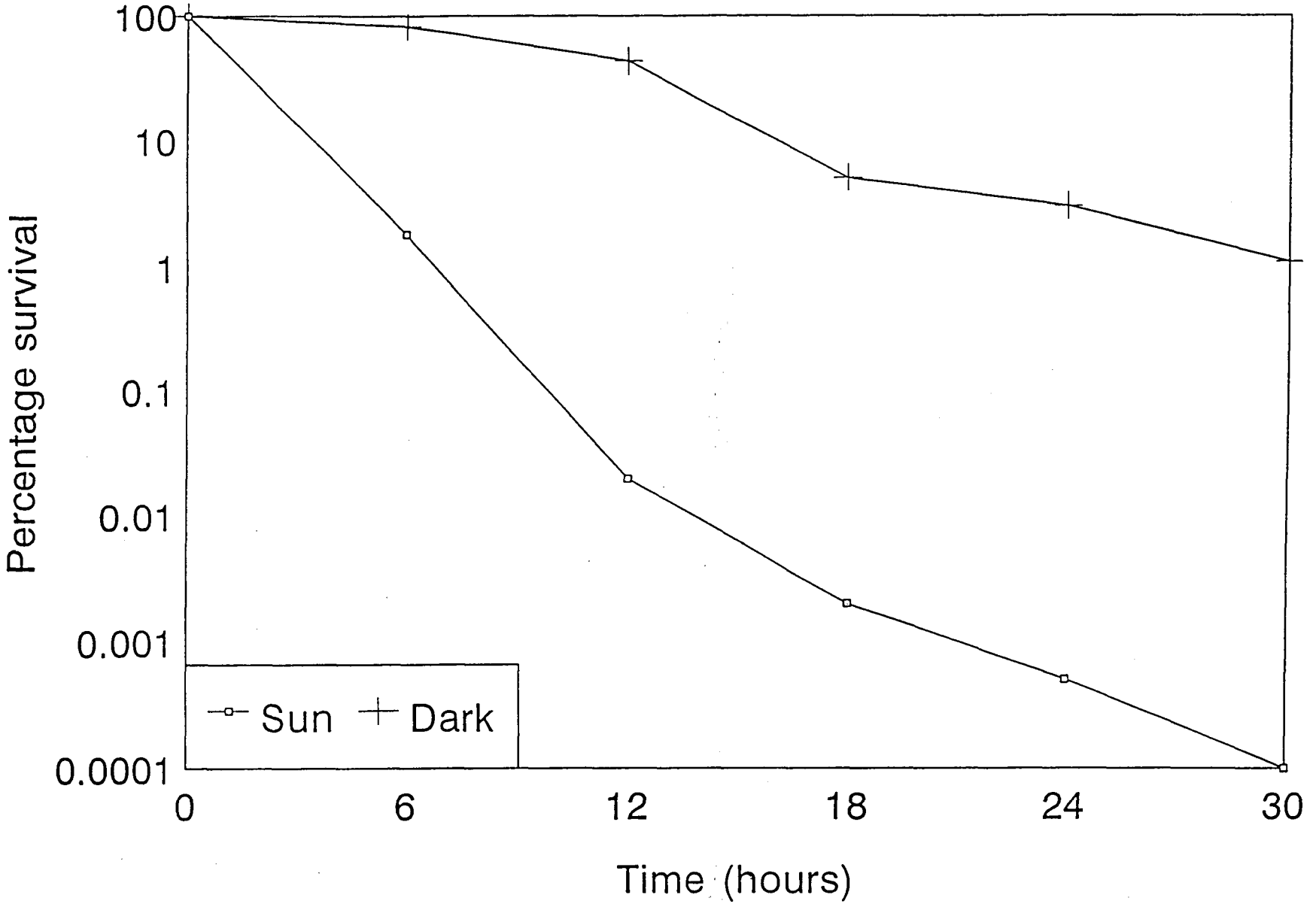
Survival on the laboratory bench in sea water seeded with bird droppings and human stool: Faecal coliforms



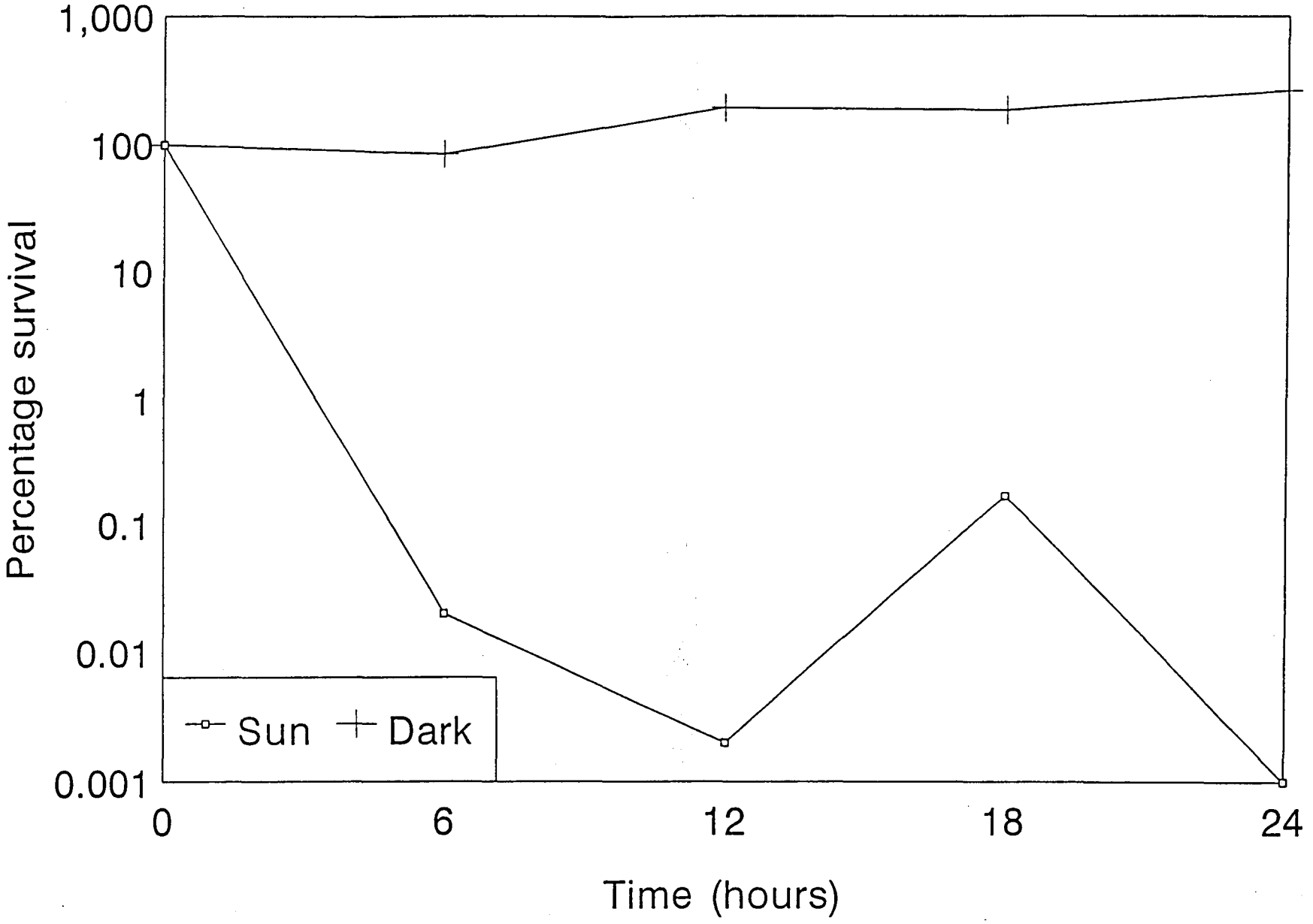
Survival in Seeded buffer in the presence and absence of sunlight:
Poliovirus type 1



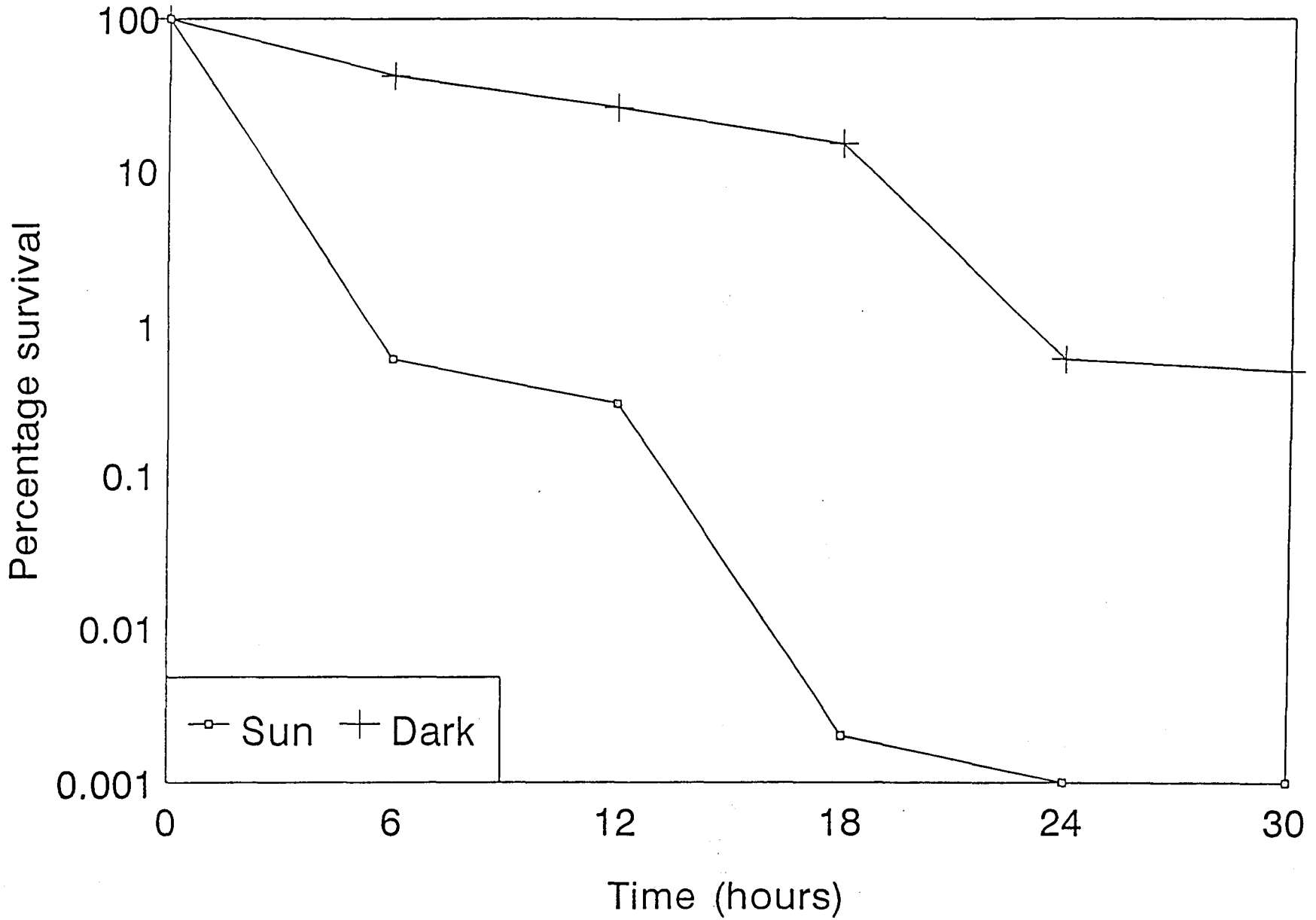
Survival in Seeded buffer in the presence and absence of sunlight:
Somatic coliphage V1



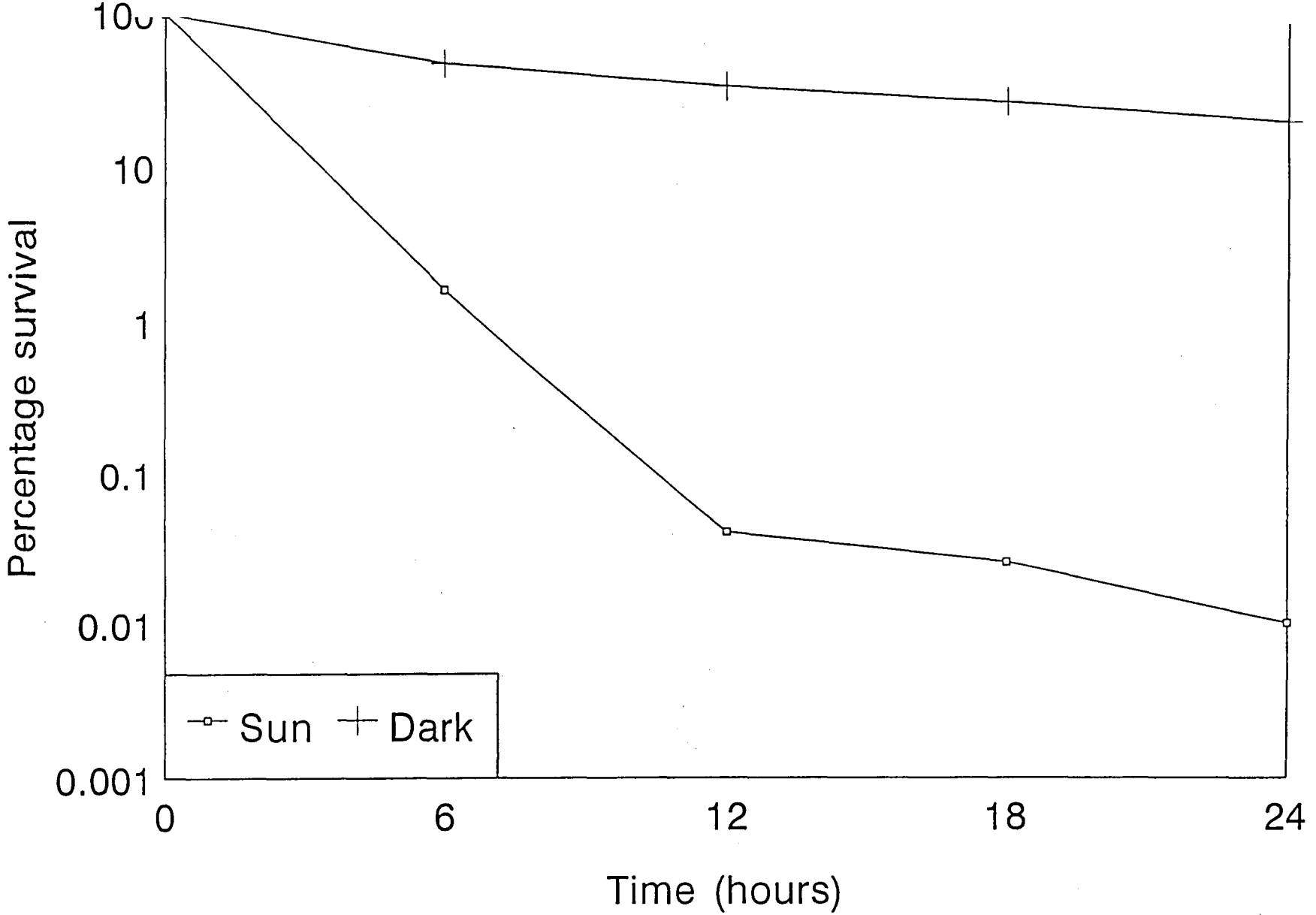
Survival in Seeded buffer in the presence and absence of sunlight:
Male-specific coliphage MS2



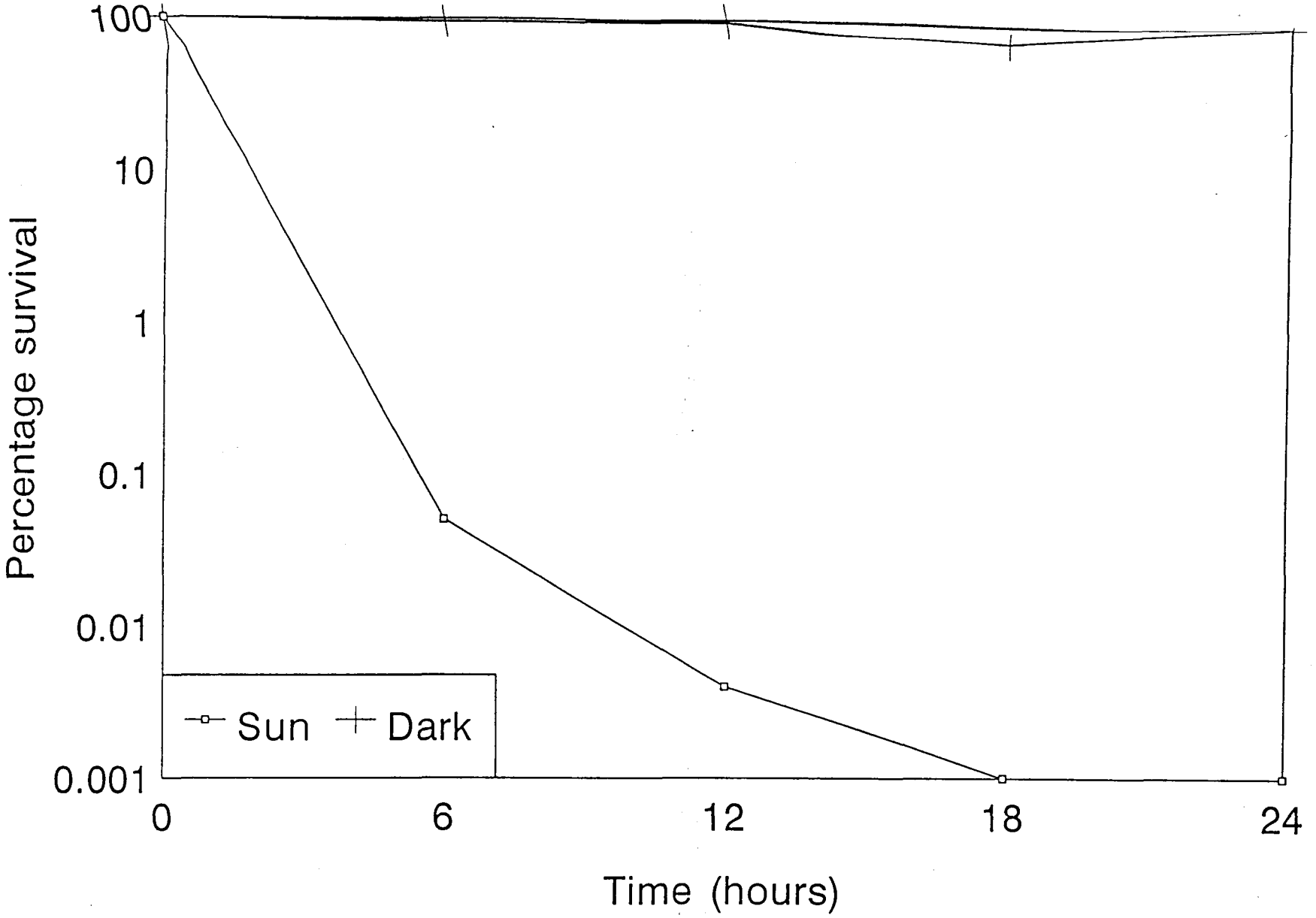
Survival in Seeded buffer in the presence and absence of sunlight:
Enterococcus faecalis



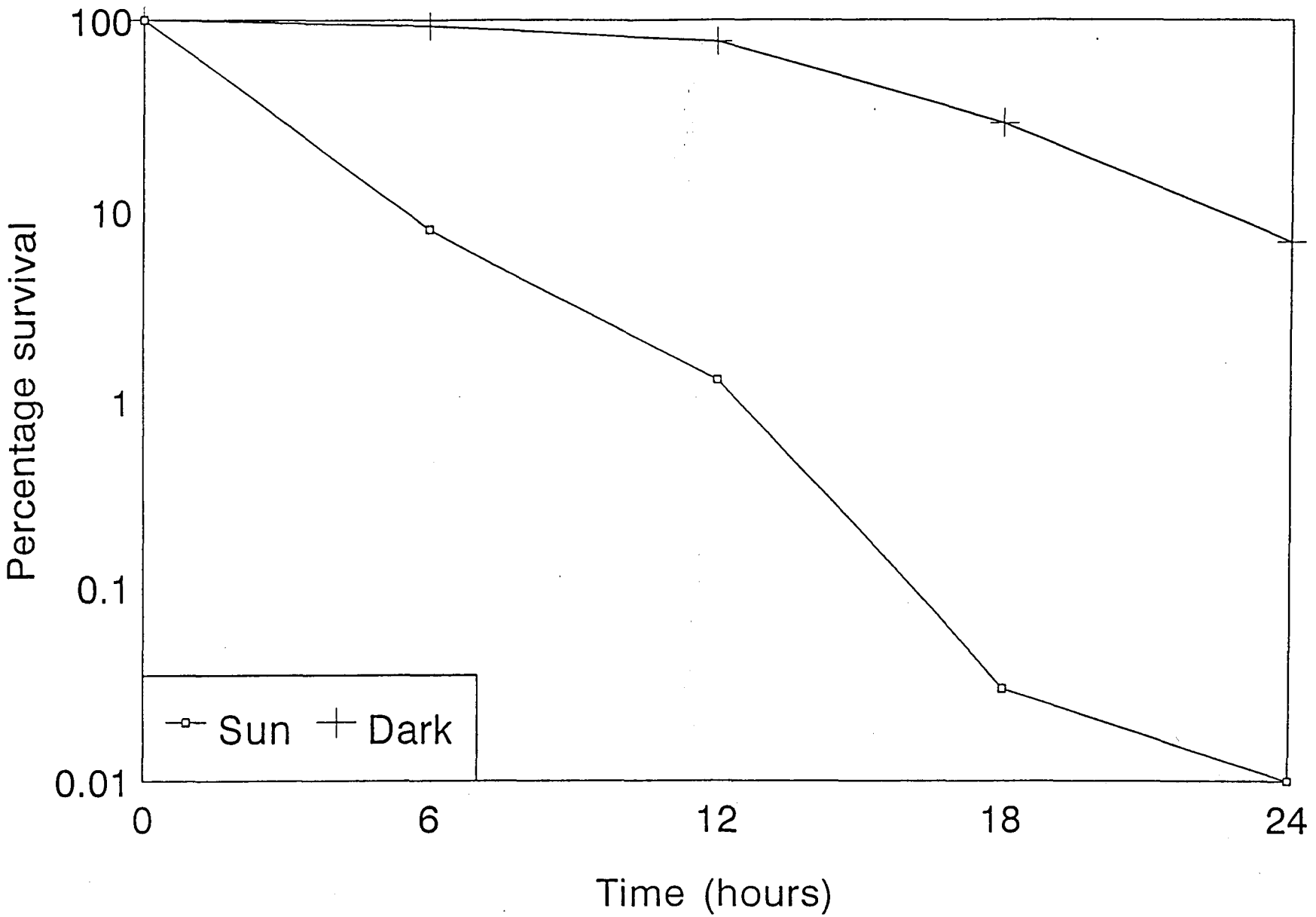
Survival in seeded Apies River water in the presence and absence of sunlight:
Enterococcus faecalis



Survival in seeded Apies River water in the presence and absence of sunlight: Poliovirus type 1



Survival in seeded Apies River water in the presence and absence of sunlight: Somatic coliphage V1



Survival in seeded Apies River water in the presence and absence of sunlight: Male-specific coliphage MS2