WATER RESEARCH COMMISSION

Research Project

RESEARCH ON BACTERIOPHAGES AS INDICATORS OF WATER QUALITY

1 January 1990 to 31 December 1992

F I N A L R E P O R T

W O K Grabow
C S Holtzhausen and J C de Villiers
assisted by other staff and post-graduate students

Department of Medical Virology, Faculty of Medicine
University of Pretoria
P O Box 2034, Pretoria 0001

321/1/93

ISBN 1 86845 023 6
WRC File : K5/321
WOKG:WR2/PH92.FR:93-08-02

ACKNOWLEDGEMENT

The research in this report emanated from a project funded by the Water Research Commission and entitled

RESEARCH ON BACTERIOPHAGES AS WATER QUALITY INDICATORS

The Steering Committee for this project consisted of the following persons:

Dr MJ Pieterse	Water Research Commission (Chairman)
Mr D Huyser	Water Research Commission (Secretary)
Prof WOK Grabow	University of Pretoria
Prof H Huismans	University of Pretoria
Mr GO Offringa	Water Research Commission
Dr R Kfir	Division of Water Technology, CSIR
Mrs CME de Wet	Rand Water
Mr FS Vivier	Department of National Health and Population Development

The financing of the project by the Water Research Commission and the contribution by the members of the Steering Committee are gratefully acknowledged (see further acknowledgements on p.5)

CONTENTS

ACKNOWLEDGEMENTS

Thanks are due to:

1. The Water Research Commission for financial support.

2. The University of Pretoria for facilities, staff and administrative services.

3. Prof J V van der Merwe, Dean of the Faculty of Medicine, for interest, support and encouragement.

4. Prof J Jofre, Department of Microbiology, University of Barcelona, for advice, assistance, participation in laboratory work, *Bacteroides fragilis* HSP40 host cultures, cultures of *B fragilis* phage strain B40-8, and for comments on parts of this report.

5. Dr A H Havelaar, Department of Microbiology, Rijksinstituut vorr Volksgezondheid en Milieuhygiene, Bilthoven, the Netherlands, for advice, a variety of hosts for coliphages, information on detection methods, and for comments on parts of this report.

6. Prof V J Cabelli, Department of Microbiology, University of Rhode Island, Kingston, Rhode Island, U S A, for a host for male-specific coliphages, and details on phage detection methods.

7. Staff and post-graduate students of our Department for interest, technical assistance and advice, notably Maureen B Taylor, Charl Swanevelder, Alette Pienaar, Andries Molefe and Belinda Brink.

EXECUTIVE SUMMARY

MOTIVATION

Water has a long history of involvement in the transmission of infectious diseases. Traditionally waterborne diseases are associated with contaminated or untreated supplies. However, evidence is accumulating that even drinking water supplies treated by methods generally accepted as sufficient may play a meaningful role in the transmission of certain infectious diseases. Viruses feature prominently among the pathogens involved, and the results disclose shortcomings in the reliability of conventional quality surveillance programmes. Viruses differ extensively from commonly used indicators of faecal pollution such as coliform bacteria, in terms of structure, composition, morphology, resistance, behaviour in water, and incidence in waste water. It is, therefore, not surprising that coliform and related faecal bacteria have certain shortcomings as indicators for the absence or survival of viruses.

Since the waterborne transmission of diseases continues, and in some situations even increases, attention is world-wide being given to the development of failsafe treatment processes, and the formulation of more reliable quality surveillance strategies. The challenges to accomplish these goals increase in complexity as world populations of humans and domestic animals increase, as the demand for potable water increases, and as the pollution of the limited water resources on earth increases. A special challenge is to meet the needs of developing countries and communities with limited financial and technical resources, which are most vulnerable to the transmission of disease by water.

Safety with regard to infectious diseases is the most important quality aspect of water supplies intended for human use for whatever purpose. Water quality surveillance programmes should, therefore, ideally include tests for human viruses. Unfortunately, however, these tests are still relatively expensive and require sophisticated laboratory facilities and technical know-how. In addition, the great majority of viruses of primary concern are not detectable by conventional virological laboratory techniques. Although intensive research is in progress world-wide to develop more practical, sensitive and reliable tests for human viruses, attention is also devoted to more reliable indicator systems.

In research on indicator systems more reliable for human viruses than faecal bacteria, the indicator features of bacteriophages (phages) are currently being investigated by many laboratories. Phages are viruses which infect bacteria. Their size, structure, morphology and composition closely resembles that of human viruses. The behaviour of phages in water and related environments, and their resistance to unfavourable conditions, treatment systems and disinfection processes do, therefore, more closely resemble those of human viruses than bacteria. An important advantage of phages is that many of them are detectable by relatively simple and inexpensive techniques which yield results in less than 24 hours. These techniques are within the capabilities of many laboratories. Growing international interest in the application of phages as

indicators is reflected by the International Organization for Standardization (ISO) which is in the event of formulating standardised techniques for the detection of certain phages, and the Commission of European Communities which has prepared a draft proposal for including phages in water quality guidelines.

OBJECTIVES

In view of observations and findings which suggest that phages may serve a valuable role as indicators of water quality, the main objective of this project was to investigate the possibility of using certain groups of phages as indicators of water quality, particularly with regard to human viruses. The following goals were set to obtain the required information:

1. **Development of techniques for the detection of phages.**
1.1. Establish laboratory facilities and recruit students and staff.
1.2. Obtain, establish and evaluate appropriate host cultures for detection of the following three groups of phages: somatic and male-specific coliphages, and *Bacteroides fragilis* phages.
1.3. Establish and optimise techniques for the detection of *B fragilis* phages, which are more complicated than those for commonly used coliphages (phages of *Escherichia coli*).
1.4. Develop and establish practical procedures for the routine detection of phages.
1.5. Assess techniques for the detection of small numbers of phages in large volumes of water.

2. **Investigate the incidence and behaviour of phages in water environments.**
2.1. Determine numbers of the three groups of phages in selected effluents and polluted waters.
2.2. Compare numbers of the three groups of phages in the above waters with those of bacterial indicators such as coliform bacteria and faecal streptococci, and in selected cases also with those of human viruses.

3. **Assess survival of phages in water treatment processes.**
3.1. Compare the survival of the three groups of phages with that of bacterial indicators, and in selected cases also with that of human viruses, in selected processes for the purification of waste water and treatment of drinking water.
3.2. Characterise selected phages by electron microscopy.

4. **Processing, evaluation and publication of results.**
4.1. Process results and evaluate pros and cons of the indicator value of phages.
4.2. Produce final report with recommendations.
4.3. Publish results.

ACCOMPLISHMENTS

1. **Development of techniques for the detection of phages.**

1.1. Laboratory facilities for research on phages have been established.

1.2. Staff and post-graduate students for research on phages have been recruited and trained.

1.3. Links of collaboration with international experts in the field have been established.

1.4. Participation in work on the formulation of phage techniques by the International Organization for Standardization (ISO) has been established.

1.5. A comprehensive literature review on phages and their potential value as indicators of water quality has been prepared.

1.6. Practical and reliable techniques for the detection of the three groups of phages concerned have been established.

1.7. Accuracy, reproducibility and reliability of phage detection techniques were confirmed by successful results obtained in an international calibration study conducted by Dr A H Havelaar, Rijksinstituut voor Volksgezondheid en Milieuhygiene, Bilthoven, The Netherlands.

1.8. An assessment of various techniques for the recovery of small numbers of phages from large volumes of water revealed that adsorption-elution techniques generally used for the recovery of human viruses from water had an unacceptably low efficiency of recovery for phages. These results imply that more detailed research on sensitive techniques for the recovery of phages is required.

1.9. Promising results were obtained with qualitative presence-absence tests for small numbers of phages in large volumes of water. The results suggest that this approach may profitably be applied for sensitive water quality assessment.

2. **Investigate the incidence and behaviour of phages in water environments.**

2.1. Evidence has been presented that *B fragilis* HSP40 phages were excreted only by a certain percentage of humans, and never by a variety of animals, including domestic and wild mammals and birds. Somatic coliphages were consistently excreted by all humans and animals, and male-specific coliphages by fluctuating percentages of humans and animals.

2.2. All three groups of phages were consistently detected in domestic waste water. *Bacteroides fragilis* HSP40 phages were present in relatively high numbers in hospital waste water (primarily of human origin), but were rarely if ever detected in abattoir waste water (primarily of animal origin). These results confirm findings that *B fragilis* HSP40 phages are excreted exclusively by humans.

2.3. The above results show that *B fragilis* HSP40 phages can be used to distinguish between faecal pollution of human and animal origin, which is most valuable for certain purposes of water quality assessment.

2.4. Studies on the incidence of the three groups of phages, faecal bacteria and human viruses in various water environments, including waste water, treated waste water, river water, polluted seawater and marine oysters and mussels, showed that there was no consistent or direct correlation in counts. Important, however, was that all three groups of phages consistently outnumbered human viruses. Results were used to calculate ratios in which the

groups of phages, bacterial indicators of faecal pollution and certain human viruses may occur in water environments.

3. **Assess survival of phages in water treatment processes.**

3.1. The survival of the three groups of phages, bacterial indicators and human viruses in various water treatment processes has been compared. The results indicate that the phages concerned are at least as resistant as bacterial indicators and human viruses.

3.2. Selected phage isolates have been investigated by electron microscopy. Valuable results have been obtain on the diversity of coliphages, and indications that *B fragilis* phages consist of a relatively homogenous morphological group, which has advantages for their application as indicators.

4. **Processing, evaluation and publication of results.**

4.1. Processing and evaluation of results supplied evidence in support of the indicator value of phages, at least in so far as that the absence of phages in all water environments tested is a reliable indication of the absence of those human viruses tested for in this study.

4.2. The project also supplied evidence that phages are detectable by relatively simple and inexpensive techniques which yield results within 24 hours. Techniques have been written up in cook-book style and supplied to some local laboratories together with appropriate host strains for evaluation and application.

4.3. Results obtained have been used as a basis for recommendations regarding the inclusion of phages in South African quality guidelines recommended for drinking water, recreational water, and shellfish meat intended for human consumption.

4.4. A final report with conclusions and recommendations has been prepared, results have been presented at conferences, and publications are in preparation.

CONCLUSIONS AND RECOMMENDATIONS

The main objectives of the project have been accomplished. Evidence has been presented that phages fulfil the fundamental requirements of indicators for faecal pathogens, notably human viruses. Indicator features of phages disclosed by this and other studies warrant further research regarding their application in practice for water quality assessment. One weakness revealed by the results is that male-specific coliphages and *B fragilis* phages tend to occur in many water environments in numbers too low for enumeration by direct plaque assays. This implies that future research should pay special attention to techniques for the detection of low numbers of phages in large volumes of water.

SUMMARY OF PHAGE INDICATOR FEATURES

Bacteriophages (phages) are viruses which infect bacteria. In terms of size, structure, morphology and composition they closely resemble human viruses. The behaviour of phages in water and related environments, and their resistance to unfavourable conditions, treatment systems and disinfection processes do, therefore, more closely resemble those of human viruses than bacterial indicators of faecal pollution.

Phages and human viruses cannot replicate themselves. They infect specific host cells and through their nucleic acid (RNA or DNA) they instruct these host cells to produce replicates of themselves. Phages and human viruses are host specific. In other words, different phages will only infect specific bacteria and different human viruses will only infect specific cells in the human body. This implies that phages and human viruses can to a large extent be identified by the host cells which they infect. The host specificity is determined by receptor sites (chemically different protein molecules) on the surface of host cells; various phages and human viruses can only adsorb to specific receptor sites. It is interesting, therefore, that phages could infect human cells if only these cells had phage receptor sites. Likewise, human viruses could theoretically infect bacteria if only they had the appropriate receptor sites and mechanisms for injecting their nucleic acid into the bacteria. However, there is some extent of diversity in host specificity, and certain phages can infect more than one species of bacteria, and certain viruses can infect humans, animals and even insects.

The host specificity implies that various phages and human viruses can be associated with environments in which their host cells occur. This implies, for instance, that phages which infect *Escherichia coli* can, like their hosts, be associated with faecal pollution.
As a result of their simple structure and rigid outer shell (capsid), many phages and certain human viruses tend to be more resistant to unfavourable conditions than bacteria generally used as indicators of faecal pollution, such as coliform bacteria. The most resistant human viruses are those commonly transmitted by water and food; these viruses are known as enteric viruses and the resistance is necessary for successful transfer by the faecal oral route. Human viruses which have other routes of transfer such as the human immunodeficiency, rabies, haemorrhagic fever, measles and influenza viruses, have low resistance to unfavourable environmental conditions.

Phages used as indicators of the sanitary quality of water and food have the following attractive indicator features:

1. Their behaviour in the environment resembles that of human enteric viruses.
2. Generally they are at least as resistant to unfavourable environmental conditions as human enteric viruses.
3. They generally outnumber human viruses in sewage and sewage-polluted environments, which implies that if phages are absent, human viruses are most likely also absent.
4. They are detectable by relatively simple and inexpensive methods which yield results in a short time, about 8 hours for many phages. The detection of human viruses is complex and expensive, and conventional cell culture methods may take more than a week.

5. They cannot infect humans and constitute no health risk.

In view of the above features the use of phages as models for human viruses, the application of phages for assessment of treatment efficiency, and the inclusion of phages in quality guidelines and specifications, are gaining ground world-wide.

Examples of phages used as indicators of sanitary quality

Coliphages
This term refers to a large group of phages which infect *Escherichia coli*. They can, therefore, to a reasonable extent be used as indicators of faecal pollution. However, there are limitations to this indicator value because some coliphages also infect other bacteria related to *E coli*, some of which can multiply and support the replication of coliphages in favourable environments other than the warm-blooded digestive tract. Counts of coliphages are, therefore, known to sometimes increase in environments such as wastewater and sand-filters where certain coliform and related bacteria thrive. Coliphages are detectable by very simple methods and results are available within about 8 hours. Generally *E coli* strain C is used as host. This particular strain is susceptible to an exceptionally wide range of coliphages because its natural defence mechanisms to phage infection have been impaired by genetic modification. The strain does, therefore, yield high counts of coliphages. Counts are generally lower than those of faecal coliforms by a factor of about 10.

Male-specific coliphages
The receptor sites for these coliphages are located on thread-like appendages known as sex fimbriae produced by *E coli* and related bacteria which carry a particular genetic element known as the *E coli* fertility plasmid (F Factor) which plays a role in the transfer of nucleic acid (genetic material) among *E coli* bacteria. Important in terms of indicator features is that sex fimbriae are only produced at temperatures above about 32°C, which implies that male-specific coliphages infect their hosts and multiply in the gastro-intestinal tract of humans and warm-blooded animals (37°C), while chances for replica-tion in natural water and related environments are negligible. All of this implies that male-specific coliphages are highly specific indicators for sewage pollution. Another important indicator feature of male-specific coliphages is that many of them, such as the phages designated MS, are almost identical to human enteric viruses like polio, coxsackie and hepatitis A in terms of structure, composition and morphology. Male-specific coliphages are, therefore, ideal models or indicators for human enteric viruses. Although male-specific coliphages generally also outnumber human viruses in water environments, their numbers are usually lower than those of somatic coliphages by the order of 10 to 100. Male-specific coliphages are not as easily detectable as somatic coliphages. The main challenge is to obtain host cultures with high densities of sex fimbriae, which are produced only for a short period at the right temperature during the logarithmic growth phase of cultures of the host bacteria.

Bacteroides fragilis HSP40 phages

Bacteroides fragilis is a normal inhabitant of the gastro-intestinal tract of humans and warm-blooded animals. It is a strict anaerobe, which means that, for all practical purposes, it cannot multiply or support the replication of phages in water or related environments. This implies that *B fragilis* phages are specific indicators of faecal pollution. Phages which infect *B fragilis strain* HSP40 are highly specific for human excreta, which has the important advantage that they can be used to distinguish between faecal pollution of human and animal origin (Tartera and Jofre, 1987). Numbers of *B fragilis* HSP40 phages in domestic wastewater are generally lower than those of somatic coliphages by a factor of 10 to 100. The detection of *B fragilis* HSP40 phages is relatively complicated because maintenance of the host culture, cultivation under strict anaerobic conditions and control of contamination require relatively advanced facilities and know-how. Results of plaque assays are usually available only after 48 hours. A disadvantage of the relatively low numbers of the phages is that meaningful assessment in many water environments requires the inclusion of recovery or enrichment procedures.

PRESENTATION OF RESULTS AT SCIENTIFIC MEETINGS

HOLTZHAUSEN CS, JOFRE J and GRABOW WOK (1991).
The incidence of *Bacteroides fragilis* and *Escherichia coli* bacteriophages in human and animal faeces.
Poster: Annual Congress of the Federation of South African Societies of Pathology, Warmbaths Overvaal, 1-3 July.

HOLTZHAUSEN CS, JOFRE J and GRABOW WOK (1992).
***Bacteroides fragilis* and *Escherichia coli* bacteriophages: excretion by humans and animals.**
Paper: Seventh Biennial Congress, South African Society for Microbiology, University of the Orange Free State, Bloemfontein, 30 March to 1 April.

POTGIETER N, HOLTZHAUSEN CS, GRUNDLINGH A and GRABOW WOK (1990).
Evaluation of two methods for the recovery of viruses from water.
Poster: Faculty Day, Faculty of Medicine, University of Pretoria, 1 Aug.
Abstract: **South African Medical Journal 79**, 338 (1991).

GLOSSARY

Bacteriophage (Phage)
Virus which infects bacteria. Infection consists of: Phage adsorbs to specific receptor site on bacterium (host); phage injects its nucleic acid (RNA or DNA) into the bacterial host; the phage nucleic acid (genome) is a genetic message which instructs the host to produce large numbers of new phages similar to the one which caused the infection; typically (but not always) the new phages are released by rupture (lysis) of the host cell; the host is destroyed in the process. A replication cycle of 20-30 min is typical of coliphages under optimal conditions of nutrients and temperature; at suboptimal conditions the time needed for one cycle may be significant longer: other bacteriophages may also have longer "burst-times". Since different species and strains of bacteria have different receptor sites, members of different bacterial species or strains can be infected only by certain phages; this determines the host-specificity of phages, and implies that various bacterial host strains can be used to detect specific groups of phages.

***Bacteroides fragilis* phages**
Phages which infect *Bacteroides fragilis* bacteria. In this report the term refers most of the time to phages which infect *B fragilis* strain HSP40. In this report the term refers to, unless otherwise specified, phages which infect *B fragilis* strain HSP40. These phages have so far only been found in the stool of humans and no animals, which implies that they may be used to distinguish between faecal pollution of human and animal origin.

Coliphages
Phages which typically infects *Escherichia coli* and certain closely related species of bacteria.

Fertility
Refers to the state of bacteria (typically coliforms) in which they carry the so-called fertility plasmid. This plasmid codes for the production of pili, which are tube-like structures capable of attaching to other related bacteria. The pili have the function to bring about contacts between F^+ and F^- cells, and is not involved in the transfer of genetic material. The production of pili takes place only at elevated temperatures (about 30-45°C). An important feature of pili is that they carry phage receptor sites which occur nowhere else on the surface of the bacteria; this implies that certain phages can only infect bacteria with pili; these phages are known as male-specific phages.

F-specific phages
Male-specific phages.

Hfr phages
Male-specific phages.

Host
Bacterial species or strain which is susceptible to a particular phage; each phage has particular host bacteria; selected host bacteria can, therefore, be used to detect specific groups of phages. Host specificity is determined by the phage receptor sites which occur on the surface of the host.

High frequency (Hfr)
The F-factor in Hfr-strains is integrated into the bacterial chromosome and consequently certain genetic markers (close to the site of integration) are trensferred with higher frequency than when the F-factor is a plasmid. Hfr strains do not produce more pili and are equally sensitive to F-specific phages as cells carrying the F-plasmid. However, the F-factor is a more stable trait of Hfr strains, hence they are more reliable host strains to work with.

Male-specific (MS) phages
Phages which specifically adsorb to receptor sites on pili. Since pili are produced only at elevated temperatures (above 30°C), these phages can only infect and replicate at elevated temperatures and not at temperatures below 30°C as they normally prevail in the environment. Male-specific coliphages consist of two typical groups, one of which comprises small icosahedral single stranded RNA phages which in structure and composition closely resemble human enteroviruses (receptor sites on sides of pili), and the other group which comprises long filamentous single-stranded DNA phages (receptor sites on tips of pili).

Phage
Short for "bacteriophage".

Pili
Tube-like structures (fimbriae) on the surface of bacteria. Fertility plasmids (factors, genetic elements) code for the production of pili as part of a gene transfer process resembling a sexual process in higher organisms. Pili carry phage receptor sites which occur nowhere else on the surface of bacteria. This implies that certain phages can only infect bacteria in the state where they carry pili.

Plasmid
Short piece of nucleic acid (DNA) which can code for certain functions; eg the F (fertility) plasmid (factor) codes for the production of pili which facilitate conjugation and render the bacterium susceptible to male-specific phages.

Plaque
Clear area in a lawn of bacteria in a layer of sloppy agar caused by infection and lysis of the bacteria by a phage. When a phage is added to sloppy agar in the presence of an appropriate number of host bacteria, and the sloppy agar is poured into a petri dish to solidify, plaques will develop during incubation as a result of infection of a host bacterium by the phage and release of the phages, each of which will again infect a neighbouring host bacterium; this process will continue for as long as the bacteria multiply because only metabolically active bacteria can replicate phages; plaques are generally visible within about eight hours; due to various factors, including termination in the growth of host bacteria, the size of the circular plaques does not increase any further after about 20h. Different phages produce plaques which differ

in size and appearance as a result of differences such as the numbers of phages released during each replication cycle and the time of each replication cycle.

Plaque assay
Method for enumerating phages by the number of plaques which they produce. Typically an appropriate volume of a sample (eg 1ml) which contains phages is mixed with an appropriate number of host bacteria in molten sloppy agar and poured onto a layer of bottom agar in a petri dish; during incubation (37°C for coliphages) plaques can typically be counted from 8h onwards. Each viable phage in the test sample will cause infection which results in a plaque; therefore, the number of plaques is typically a direct indication of the number of viable phages in the test sample.

Plaque forming units (PFU)
The number of phages which can cause plaques; typically this refers to the number of viable (infectious) phages; generally one phage will cause one plaque; therefore, generally speaking one PFU is the equivalent of one viable phage. There are exceptions, however, where more than one viable phage may be required to form one plaque. It may be possible that one plaque represents more than one viable phage because they were physically aggregated or adsorbed to a particle.

Receptor site
Chemically distinct molecular structures which occurs on the surface of bacteria. Different species and strains of bacteria carry different receptor sites which are chemically distinguishable. Each phage can recognise and adsorb only to certain receptor sites; receptor sites do, therefore, determine the host specificity of phages.

ABBREVIATIONS

BE	=	beef extract
CFU	=	colony forming units
F	=	fertility, as it refers to coliform bacteria
HAV	=	hepatitis A virus
MEM	=	Eagle's minimal essential medium
nmwl	=	nominal molecular weight limit
PBS	=	phosphate-buffered saline
PFU	=	plaque forming units
PEI	=	polyethylenimine

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

GENERAL INTRODUCTION AND OBJECTIVES

A wide variety of pathogenic micro-organisms may be transmitted by water and food contained by human excreta. Diseases caused by these pathogens have far-reaching public health and sosio-economic implications, particularly in developing countries. The pathogens concerned represent various groups of infectious agents including viruses, bacteria, protozoa, intestinal parasites and fungi.

Viruses are particularly important in this regard mainly because they are excreted in large numbers by infected persons, their infectious dose is small, they remain infectious for relatively long periods in the environment, and they are resistant to treatment and disinfection processes.

Assessment of the virological safety of water and food is difficult because tests for viruses are complex, expensive, time consuming, labour intensive, require special laboratory facilities and highly trained staff, and many of the viruses are not detectable by convential virus tests. For this reason indicators are generally used for assessment of the safety of water and food. Coliform bacteria and faecal streptococci are among the most commonly used indicators. These bacteria are excreted in large numbers in the faeces of man and animals. Although some of them may be able to replicate in water environments, their presence in water or food suggests faecal pollution, while their absence indicates the absence of faecal pollution or the inactivation of faecal pathogens. These indicator organisms have the advantage of being detectable by simple and inexpensive tests which yield results in a relatively short time. However, these bacterial indicators have shortcomings, particularly with regard to viruses. These shortcomings are mainly related to the higher resistance to unfavourable conditions of at least some viruses, the lack of correlation between numbers of the indicators of viruses, and the ability of some indicators to multiply in environments other than the gastrointestinal tract.

In order to overcome the shortcomings of commonly used indicator bacteria, additional indicators such as bacteriophages are often used. Bacteriophages are viruses which infect bacteria. In terms of composition, structure and size they resemble human viruses. The fundamentally most important difference between bacteriophages (phages) and human viruses is that they use different hosts. Phages have the important advantage of being detectable by relatively simple, rapid and inexpensive methods. They are, therefore, often used as models or indicators for research on viruses, and available evidence suggests that phages may serve a valuable purpose as indicators for the virological safety of water and food.

Many aspects of the validity and reliability of phage indicators, and their application for this purpose in practice, remain to be elucidated. Thus far phages which infect *Escherichia coli* (coliphages) have most commonly been used as indicators of sanitary quality because the host primarily multiplies, and replicates these phages, in the gastrointestinal tract of warm-blooded animals. Coliphages are excreted in faeces. Somatic coliphages which infect *E coli* bacteria are most commonly used because they represent a large group and are easily detectable. However, some somatic coliphages

may also be replicated by coliform bacteria in certain water and food environments, which implies that their presence does not necessarily indicate faecal pollution or the presence of enteric viruses. Members of the group of male-specific coliphages, on the other hand, only infect *E coli* and related bacteria which have sex fimbriae. These fimbriae, on which the receptor sites for the male-specific phages occur, are produced only above 30°C. This implies that male-specific coliphages are much more specific for faecally polluted environments than somatic coliphages.

According to recent findings phages which infect a specific strain of *Bacteroides fragilis*, designated strain HSP40, may have particularly attractive features as indicators for human enteric viruses. The phages which infect these organisms would appear to be excreted only by humans and no other animals, probably because the host is highly specific for humans. Since the organism is a strict anaerobe and requires some growth factors that will very seldom be present in the environment, it will not multiply or replicate in any environment other than the gastrointestinal tract. In addition, the phages would appear to be exceptionally resistant to unfavourable conditions. These features imply that *B fragilis* HSP40 phages are highly specific for human faecal pollution and may be most valuable indicators for human enteric viruses.

The primary objective of this study was to assess the value of *B fragilis* HSP40 phages as indicators for human enteric viruses in water and food. Accomplishment of this goal required the following tasks:

1. Develop practical and reliable techniques for the detection of *B fragilis* HSP40 phages, somatic and male-specific coliphages.
2. Select practical and reliable methods for the recovery of small numbers of phages from large volumes of water.
3. Assess the specificity of *B fragilis* HSP40 phages by comparing their insidence as well as that of coliphages in stool samples from humans and a variety of animals.
4. Compare the insidence and survival of *B fragilis* HSP40 phages in various water environments and in shellfish meat with that of coliphages, bacterial indicators and enteric viruses.
5. Assess the indicator value of *B fragilis* HSP40 phages on the basis of results obtained.

This study was intended to make a contribution to the selection of practical and reliable indicator systems for assessment of the virological safety of water and food. The information may make a useful contribution to the formulation of quality guidelines, procedures for routine quality assessment of water and food, and to strategies aimed at the control of diseases transmitted by water and food.

CHAPTER 2

LITERATURE REVIEW

2.1 GENERAL BACKGROUND

Bacteriophages (phages) are viruses which infect bacteria. They were discovered independently by Frederick W Twort in England in 1915 and by Felix d'Herelle at the Pasteur Institute in Paris in 1917 (Pelczar *et al*, 1988). Bacteriophages were the last of the three major classes of viruses to be discovered during World War I. The other two classes were the plant viruses and animal viruses. It was then hoped that their ability to kill bacteria could be used for the prevention and treatment of bacterial disease, but this did not prove successful due to the rapid selection of resistant bacteria (Goyal, 1987).

Since the bacterial hosts of phages are easily cultivated under controlled conditions, demanding relatively little in terms of time, labor, and space compared with the maintenance of plant and animal hosts, bacteriophages have received considerable attention in viral research (Pelczar *et al*, 1988). It would be difficult to say who first considered using phages to study the basic properties of living organisms. However, studies of the basic genetic properties of phages led to the development of an entirely new science - that of molecular biology - which is allowing unprecedented advancements in all the biological and medical sciences. In addition, the way all viruses reproduce was first indicated by work with phages (Ackermann, 1969).

2.2 STRUCTURE AND MORPHOLOGY OF BACTERIOPHAGES

They basically consist of a nucleic acid molecule (genome) surrounded by a protein coat (capsid). The capsid is made up of morphological subunits called capsomeres. The capsomeres consist of a number of protein subunits or molecules called protomers. Some phages also contain lipid and additional structures such as tails and spikes.

Bacteriophages may be grouped into the following six morphological types (Fig 2-1) (Pelczar *et al*, 1988):

A This most complex type has a hexagonal head, a rigid tail with a contractile sheath, and tail fibers.
B Similar to A, this type has a hexagonal head. However, it lacks a contractile sheath, its tail is flexible, and it may or may not have tail fibers.
C This type is characterized by a hexagonal head and a tail shorter than the head. The tail has no contractile sheath and may or may not have tail fibers.
D This type has a head made up of large capsomeres, but has no tail.
E This type has a head made up of small capsomeres, but has no tail.
F This type is filamentous.
G These phages have a lipid envelope and are pleomorphic.

The tail may be contractile or noncontractile (flexible or not). If contractile the tail may be stretched or contracted (Goyal, 1987). The length and width of the head may

either be the same or the length may be greater than the width; however, short fat heads are not seen. The least common form is a filament in which the protein molecules form a tubular structure in which the nucleic acid is embedded. Phages are known that contain double-stranded DNA (most common), single-stranded DNA, single-stranded RNA, and double-stranded RNA (least common) (Freifelder, 1987).

2.3 REPLICATION OF PHAGES

By itself, a phage can persist, but it can neither grow nor replicate except within a bacterial cell. Phages possess genes which code for a variety of proteins. However, all known phages use the ribosomes, protein-synthesising factors, amino acids, and energy-generating systems of the host-cell to replicate, and hence a phage can grow only in a metabolising bacterium. Some phage species have fewer than 10 genes and use almost all of the cellular functions, whereas others have 30-100 genes and are less dependent on the host. A few of the large phage particles have so many of their own genes that, for certain functions such as DNA replication, they need no host genes (Freifelder, 1987).

Virulent bacteriophages usually kill the cells they infect. Temperate bacteriophages, however, have more subtle effects although also often able to kill cells (Brock and Madigan, 1988).

The basic sequence of events during phage replication is similar for most phages. All phages infect bacteria by attaching to specific receptor sites on the cell wall, surface layers, flagellae or fimbriae, and by injecting their nucleic acid through a puncture hole into the host. In contrast to viruses which infect animal cells, the capsid remains outside. Variations occur with respect to the number of phage proteins made and the degree of utilising host functions (Pelczar *et al*, 1988).

Virulent or lytic phages differ from temperate phages as follows:
After infection the viral nucleic acid of lytic phages immediately starts to code for the production of new viruses. The viral nucleic acid takes control of the cell metabolism and 'directs' the bacterium to synthesise new viral nucleic acid and other materials required for the production of complete viral particles. The newly formed virus particles are released by a sudden rupture of the cell wall (lysis) and are free to infect other susceptible bacteria (Fig 2-2) (Pelczar *et al*, 1988).

Virulent bacteriophages have large burst sizes and relatively short infectious cycles, and rapidly infect and destroy a given bacterial population (Davis *et al*, 1990). In contrast to lysogenic phages, lytic phages produce clear plaques because all host bacteria in the plaque are destroyed by lysis.

After infection the nucleic acid of temperate (lysogenic) phages does not immediately code for the production of new phages but becomes integrated into the genome of the host cell. As the bacterium reproduces, the phage genome remains integrated and replicates together with the host genome. Only upon induction by various agents and conditions such as ultraviolet light, will the genome start to code for the production of new phages. Generally these phages are released at low frequency with limited impact on host populations. In the case of some temperate phages, the phage

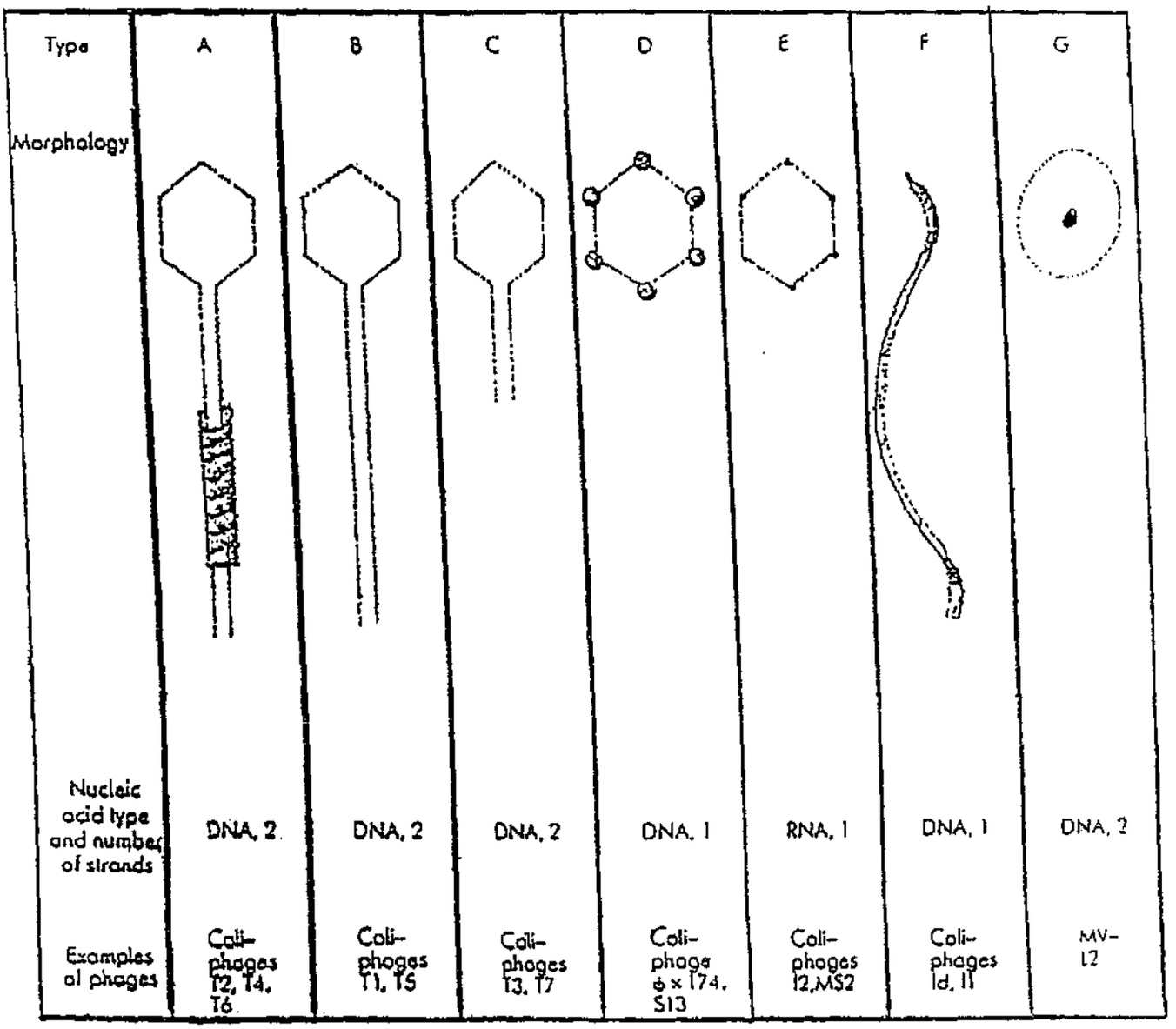


FIGURE 2-1 Schematic representation of morphological types of bacteriophages (Bradley, 1967)

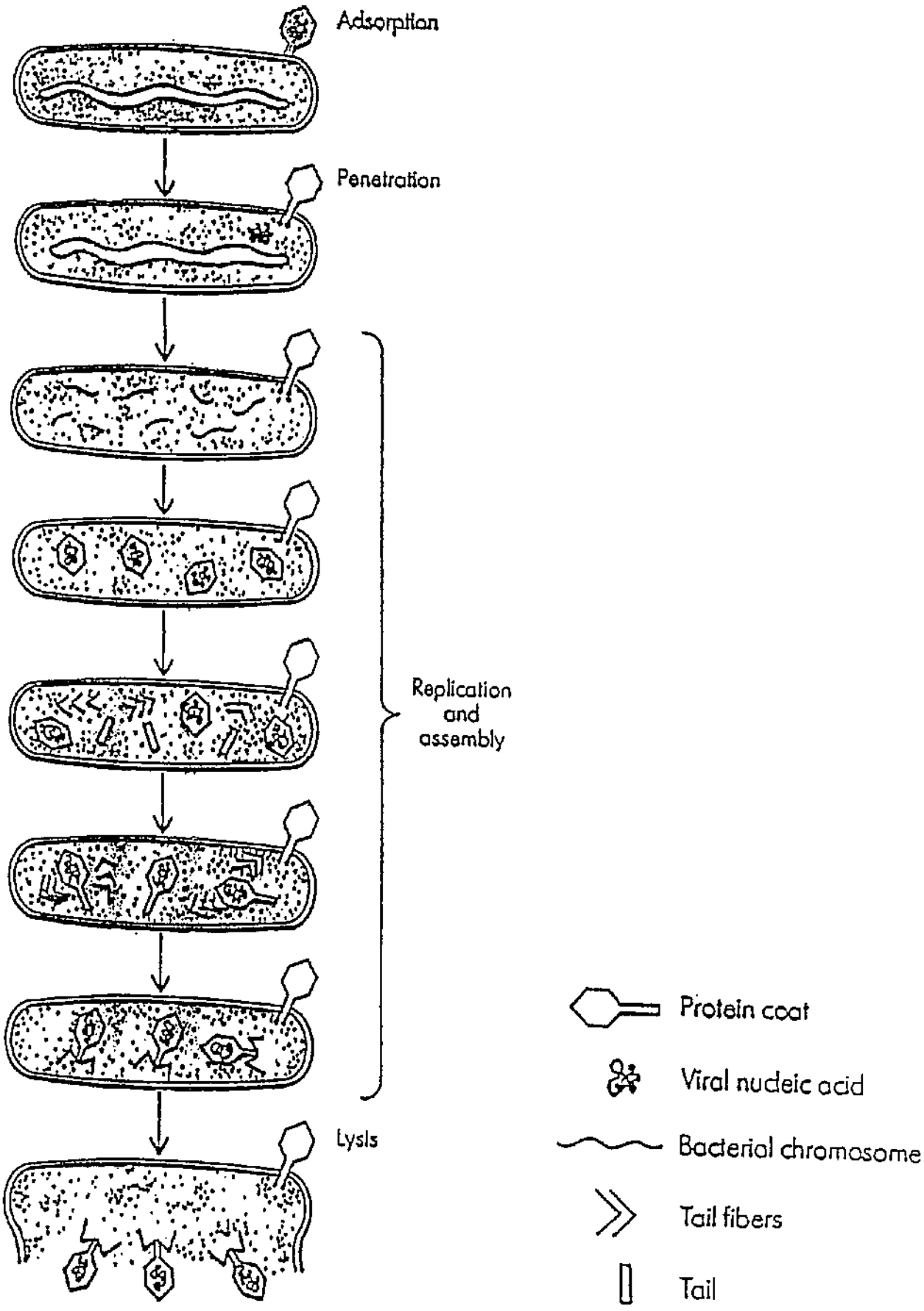


FIGURE 2-2 Replication of lytic phages (Pelczar *et al*, 1988).

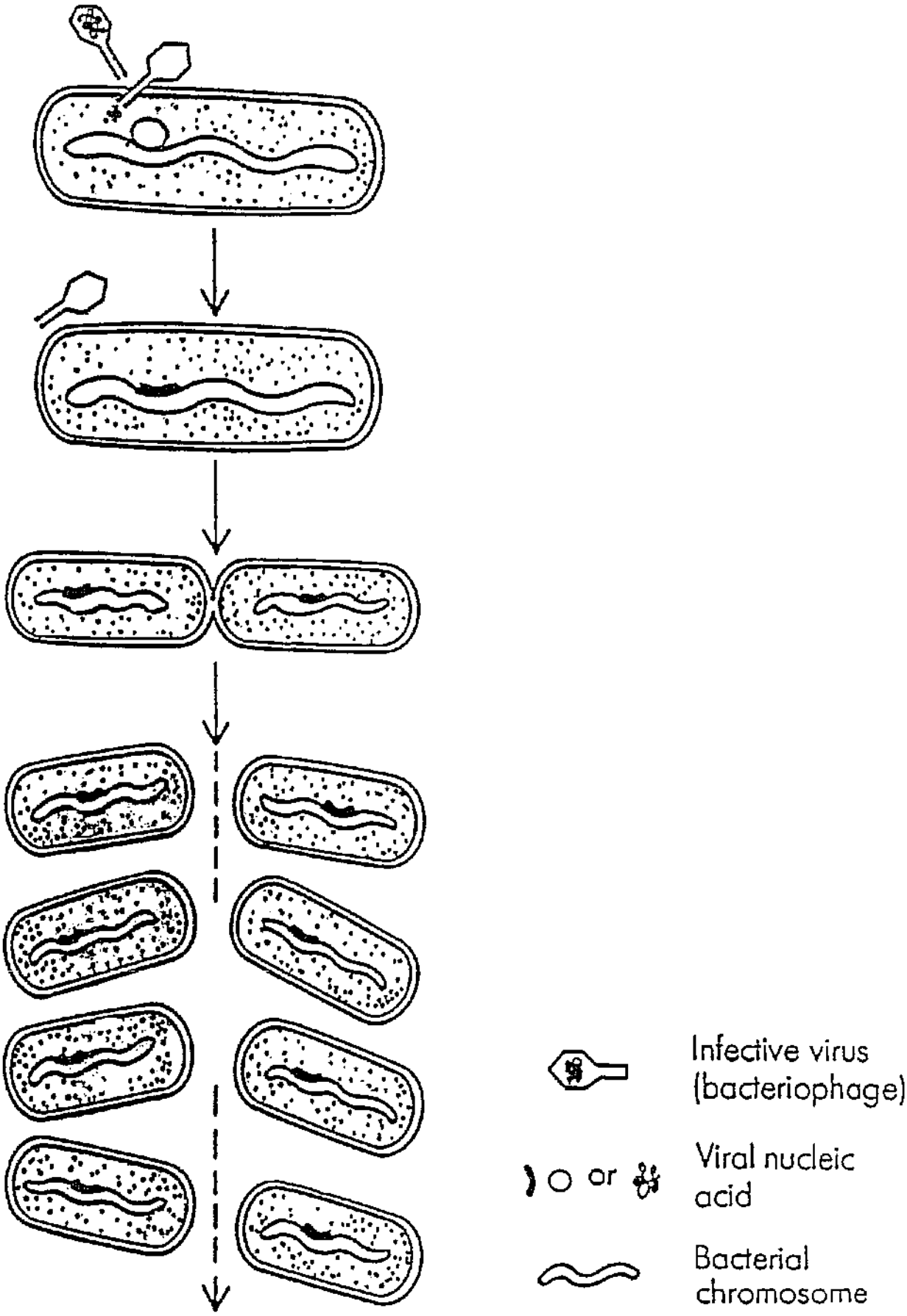


FIGURE 2-3 **Replication of lysogenic phages** (Pelczar *et al*, 1988).

genome is not integrated into the host genome, but remains free in the cytoplasm as an independently replicating plasmid (episome) which at low frequency codes for the production and release of new phages. In contrast to lytic phages, lysogenic phages produce faint or mottled plaques because many host bacteria in the plaque area remain viable (Hayes, 1968).

2.4 BACTERIOPHAGE CLASSIFICATION

2.4.1 Bacteriophage Taxonomy

Adams (1953) was the first phage taxonomist of note. He considered all the available criteria for classification and concluded that a Linnaean system, although desirable, was premature. He also warned against grouping phages together with other viruses because doing so would imply a common origin.

Bacteriophages are associated with almost all bacterial genera, including the cyanobacteria, archaebacteria and mycoplasma (Francki *et al*). A phage may readily be grouped by the use of a few gross characteristics (Abeles *et al*, 1984):
(a) host range,
(b) morphology of virions,
(c) nucleic acid of virions,
(d) strategies of infection and morphogenesis,
(e) phylogeny,
(f) serology,
(g) sensitivity to physical and chemical agents,
(h) host- and environment-dependent properties.

Six major families, each divided into several subgroups, are currently recognized. They are schematically shown in Fig 2-4.

Goyal *et al* (1987) classified bacteriophages into three groups according to their mode of interaction with the surface structure of the host bacteria, that is:
(a) those that infect by recognising the appendages of the host bacteria such as pili and flagella (appendage phages),
(b) those recognising the outer layer such as the polysaccharide capsule (capsule phages),
(c) those recognising the cell wall (somatic phages).

2.4.2 Groups of phages of interest

2.4.2.1 Somatic coliphages

This term somatic coliphages is used to describe the phages that infect F- *Escherichia coli* host strains, which are infected by phages adsorbing to receptors situated on the cell-wall (somatic receptors). Phage V_1, which is used in this study as control for somatic coliphages, is a typical isolate from a sewage-polluted river (Grabow, 1986).

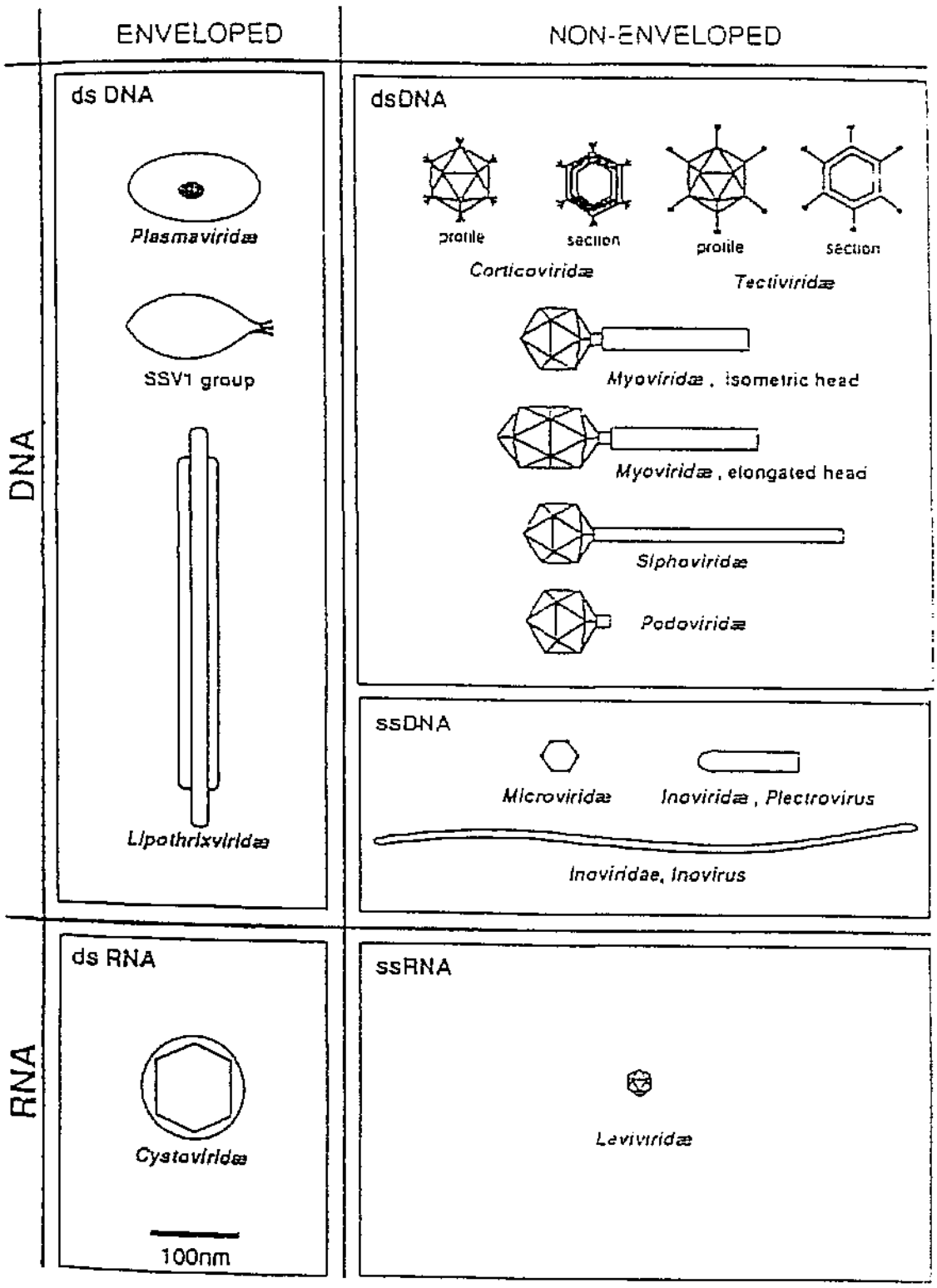


FIGURE 2-4 Major families of bacteriophages.

2.4.2.2 Male-specific coliphages

Male-specific coliphages are single-stranded RNA phages which represent the simplest phages and so constitute a convenient model system for observing biological events such as viral adsorption and penetration, replication and translation of the viral genome, assembly, and viral release. The F-specific bacteriophages specifically adsorb to F- or sex-pili coded by the classical F-plasmid of *E coli* K-12 and related plasmids of the IncF-incompatibility group (Havelaar and Pot-Hogeboom, 1988). They are classified into four major groups according to their serological properties. All RNA phages isolated in America in the early 1960s belong to group 1, whereas those isolated in Japan, belong to groups 2, 3 and 4. The F-specific RNA phage MS-2 (ATCC 15597-B1) (Havelaar and Hogeboom, 1984), used in this study is a member of group 1 (Valegard *et al*, 1990).

The group of F-specific RNA phages (QB, f2 and MS-2 are the best known members of this group) have particularly attractive features as indicators of human enteric viruses because their physical structure closely resembles that of enteroviruses and also because model experiments have shown them to belong to the more resistant types of virus. These phages consist of a simple cubic capsid of 24-27nm diameter, single-stranded RNA as their nucleic acid and they will only infect cells bearing the F or sex pili on which the receptor sites for these phages occur (Havelaar *et al*, 1984).

Phage MS-2 is a typical representative of small-plaquing (Parker, 1981) spherical single-stranded RNA phages (Strauss and Sinsheimer, 1963). In terms of morphology, structure and composition phage MS-2 closely resembles human picornaviruses (Grabow, 1984). MS-2 belongs to the morphological group E (Bradley, 1967; Ackermann, 1978) that is also known for its resistance to various chemicals (Kruse, 1968; Shah and McCamish, 1972; Bitton, 1980; Snead *et al*, 1980); heat treatment (Burge *et al*, 1981); sunlight (Kapuscinski and Mitchell, 1983) and ultraviolet-radiation (Harm, 1980; Havelaar *et al*, 1990).

The entire genome of MS-2 bacteriophage has been sequenced. It is a positive-sense single-stranded RNA molecule of 3 569 nucleotides (Bulmer, 1989). The icosahedral protein shell with triangulation number T = 3 is composed of 180 copies of the coat protein (relative molecular mass M_r 13 700); the virion also contains one copy of a maturation protein (the A-protein; M_r 44 000) (Atkins *et al*, 1979; Beremand and Blumenthal, 1979; Fiers, 1979). The coat protein of these bacteriophages serves to protect the viral RNA and acts as a translational repressor of the phage-encoded replicase gene (Sugiyama and Nakada, 1970).

The host-range of pilus-specific bacteriophages is not necessarily restricted to one or a few closely related species. Production of pili is usually encoded on the F (fertility) plasmid and the host-range of pilus-dependent phages depends primarily on the successful transfer and expression of the plasmid. Since F encoded pili are only synthesised at temperatures above 30°C, F-specific coliphages are not likely to replicate in environments other than the gastro-intestinal tract of warm-blooded animals. The classical F-plasmid of *E coli* K-12 has successfully been transferred to *Salmonella typhimurium, Shigella* and *Proteus*, causing the recipient cells to become sensitive to the F-specific phage MS-2 (Birge, 1981).

2.4.2.3 *Bacteroides fragilis* phages

Bacteriophages infecting the main genera of anaerobic bacteria that are found in the human gastrointestinal tract have been reported, including phages which infect Bacteroides, mainly *Bacteroides fragilis* (Nacescu *et al*, 1972; Keller and Traub, 1974; Booth *et al*, 1979; Kai *et al*, 1985; Jofre *et al*, 1987; Tartera and Jofre, 1987; Tartera *et al*, 1989). The genus *Bacteroides* is found in the human gastrointestinal tract in large numbers (more than 10^9-10^{10} CFU/g faeces) in comparison to coliform bacteria which range from 10^6-10^8 CFU/g (Salyers, 1984). *Bacteroides* bacteria have limited value as indicators because they are relatively sensitive to unfavourable environmental conditions. However, the phages of at least certain strain of *Bacteroides* bacteria would seem to be resistant and may, therefore, prove valuable indicators (Tartera and Jofre, 1987; Tartera *et al*, 1988; Jofre *et al*, 1986). Even when using *B fragilis* indicator strains with a relatively broad phage susceptibility, the percentage of excretors among the human population is low; around 5% according to Kai *et al* (1985) and around 10% according to Tartera and Jofre (1987). Tartera and Jofre (1987) found that phages which infect a certain strain of *Bacteroides fragilis* designated HSP40 were not detected in the faeces of warm blooded animals such as cows, pigs, rabbits, mice, poultry or quail, but only in human faeces. In this study phage B40-8 was used as type-specific control for phages infecting *B fragilis* HSP40 (Tartera and Jofre, 1987). *Bacteroides* spp are strict anaerobes and are a major component of human faeces (Geldreich, 1978; Salyers, 1984). Tartera *et al* (1989) studied the ability of bacteriophage B40-8 to replicate at either 22 or 30°C in freshwater and sediment samples in the presence of host bacteria. No increase in phage number was observed under either aerobic or anaerobic conditions in any kind of environmental samples. Only in the presence of necessary nutrients for the host bacteria and under anaerobic conditions were the bacteriophages able to multiply. This implies that phages of *Bacteroides fragilis* HSP40 can be use to distinguish between human and animal faecal pollution.

2.5 PHAGE ECOLOGY

2.5.1 Introduction

Because phages have the potential to influence the numbers of bacteria in a given environment, and because of their potential use as indicators of human enteric viruses, their interactions with their host bacteria are of interest to microbial ecologists (Goyal *et al*, 1987).

Like natural freshwater environments, marine environments also contain astonishingly large numbers of phages which infect organisms which form part of the natural flora of these environments. For instance, by using special detection techniques, Berg *et al* (1989) have found that natural freshwater environments may contain total numbers of the order of $2,5 \times 10^8$ phages per ml, and Suttle *et al* (1990) have detected phage counts of the order of 10^8 to 10^9 per ml in seawater. These phages are highly specific for natural inhabitants of water environments and will not infect organisms of human or animal origin.

2.5.2 Phages of human and animal faecal origin in water environments

2.5.2.1 Replication

Studies on the effect of temperature on bacteriophage adsorption to host bacteria and on bacteriophage replication have yielded interesting and potentially important results. Anderson (1957) found that high titers of phage against *Salmonella typhi* were produced when the phage and bacteria were incubated at 38,5°C. No phages were produced when the cultures were incubated at room temperature, 18-20°C. Seeley and Primrose (1980) reported that bacteriophages could be classified by the effect of temperature on their efficiency of plating. These authors found three types of coliphages:

(a) low-temperature (LT) phages with optimum plating temperatures at or below 30°C,
(b) mid-temperature (MT) phages in the range of 15-42 °C,
(c) high-temperature (HT) phages in the range of 25-42°C or 30-45°C.

2.5.2.2 Survival

Several factors have the potential to influence the numbers and behaviour of bacteriophages in water. These include the densities of both host bacteria and their phages; the association of phages and bacteria with solids; the presence of organic matter, especially organic matter that influences the metabolic activity of the host bacteria; ultraviolet and visible light; temperature; pH; the concentration and type of ions, and the metabolic activities of micro-organisms other than the host bacteria. The effects of several of these variables on phage survival have been studied under laboratory conditions. Less information is available on their survival under natural conditions (Goyal *et al*, 1987).

Yates *et al* (1985) found that viral survival in ground water did not correlate with the pH, nitrate concentration, turbidity, or hardness of ground water samples obtained from several sources in the United States. These authors found that the temperature of incubation was the only variable that correlated with the survival of phage MS-2.

Niemi (1976) studied the survival of phage T7 in different types of water, including river, lake and ground water, and concluded that the temperature and type of water influence phage survival.

Scheuerman *et al* (1987) reported that the inactivation rate of phage MS-2 was similar to that of poliovirus and was lower than the rates for indicator bacteria in survival chambers placed in a cypress strand under natural conditions. They also found higher levels of coliphages in waters of a cypress strand than in the underlying sediment.

Because some freshwater environments contain highly coloured water with relatively high concentrations of humic and fulvic acids, it may be expected that these compounds would influence the attachment of bacteriophages to sediments and other solids and possibly also to their host bacteria. Humic acids have been shown to influence enterovirus attachment to microporous filters (Sobsey and Hickey, 1985). Fulvic acid was found to complex with viruses and reduce their ability to adsorb to soils (Bixby and O'Brien, 1979).

The presence of organic matter may also influence phage numbers by influencing the metabolic rate of the host bacterium. Anderson (1957) found that the titre of phage produced by *Salmonella typhi* growing in sewage was similar to titres produced by its growth in laboratory media, namely 10^9-10^{10} plaque forming units (PFU)/ml.

The presence of certain metallic ions, particularly calcium and magnesium ions, has been found to influence phage adsorption under laboratory conditions (Havelaar and Hogeboom, 1983).

Studies on the distribution of phages and its relationship to the pH of the water have not been reported (Goyal *et al*, 1987). Phages are only affected by either high or low pH, therefore, the pH of water would probably not affect the phages.

The numbers of bacteriophages and their host bacteria are likely to be important factors in the ecology of phages in fresh water environments. At low levels of host bacteria, the probability of a phage encountering a susceptible host is low and productive infection may not occur. Bacteria in fresh water environments may be under more nutrient-limiting conditions and may be concentrated at the surface of solids rather than in the overlaying water (Goyal *et al*, 1987). It has been shown that only when the count of host bacteria exceeds 10^4/ml, the phages will replicate successfully (Goyal *et al*, 1987).

According to Goyal (1987) the following factors may influence the survival of coliphages in marine environments:
(a) the ionic environment,
(b) temperature,
(c) hydrostatic pressure,
(d) anaerobiosis,
(e) bacterial hosts.

2.5.2.3 Distribution

2.5.2.3.1 Freshwater

In most of the studies on the distribution of phages in freshwater environments strains of *E coli* have been used as host bacteria. All available data indicate that coliphages survive better in freshwaters than coliform bacteria. As shown in Table 2-1, coliphages have been isolated from lakes, rivers, and other types of fresh water environments in several parts of the world (Goyal *et al*, 1987).

Enteric bacteria other than *E coli* and indigenous coliform bacteria may also be present in freshwater environments. Therefore, it can be expected that phages active against these bacteria would also be recovered from these waters. Although limited information is available, phages which infect *Klebsiella pneumoniae* and *Salmonella* spp have been recovered from fresh water. They have been found in numbers comparable to those of phages which infect *E coli* (Table 2-2) (Goyal *et al*, 1987).

Samples of water and sediments taken in a park with low levels of faecal pollution from wildlife, and where no foci of human faecal pollution are known, were negative for phages of *B fragils* HSP40. Forty-six percent of the samples from the Llobregat

River, which receives mainly domestic sewage, contained *Bacteroides* phages. The levels of *Bacteroides* phages in these waters ranged from 0 to 43 with a mean value of 6,7 per 100ml of sample (Tartera *et al*, 1989).

2.5.2.3.2 Seawater

Wastewater discharges to the sea may introduce phages to marine enviroments which indicate pollution of human and/or animal pollution. This is illustrated by many studies in which somatic and male-specific coliphages, as well as other phages such as those which infect *Bacteroides fragilis* HSP40, have been detected in seawater and shellfish meat (Tartera and Jofre, 1987; Grabow *et al*, 1989; IAWPRC 1991; Kfir *et al*, 1991).

2.5.2.3.3 Shellfish

Of all seafoods, bivalve molluscs such as oysters and mussels, are most frequently associated with infections in human consumers because they feed by filtering large volumes of water, up to 20 litres per hour. As part of this feeding process, micro-organisms are accumulated together with food particles faster than they are inactivated or excreted. Bivalves can concentrate micro-organisms in their gastro-intestinal tract to more than six times the level in the surrounding water (Brown, 1987). Oysters, mussels and clams constitute a higher risk of infection than gastropods, because they are eaten raw or lightly cooked.

Elimination of viruses from contaminated shellfish is reported to be less efficient than that of bacterial indicators of faecal pollution, particularly if the initial contamination level is low and if the contaminating viruses originate from natural sewage. Consequently, low levels of viruses may be retained in shellfish meat for relatively long periods of time and bacteriological tests do not satisfactorily reflect the virus content of shellfish (Goyal *et al*, 1979; Johnson, 1979; Brodisch *et al*, 1986; Grabow *et al*, 1989; Idema *et al*, 1991).

Vaughn and Metcalf (1975) were the first to demonstrate that the retention of coliphage T4 by oysters suspended in clean estuarine waters was similar to that of an enterovirus, Coxsackie B3.

Mesquita (1988) demonstrated that in mussels, naturally occurring F-specific bacteriophages and somatic coliphages are eliminated at a significantly lower rate than *E coli* with virtually no phage reduction within 72 hours but a 1-3 $\log_{10}$-unit reduction of *E coli*, faecal streptococci and spores of clostridia.

From these few laboratory experiments it appears that bacteriophages may be good indicators to monitor virus removal in depuration tanks and other systems for shellfish depuration.

TABLE 2-1 Levels of *Escherichia coli* phages in freshwater environments (Goyal *et al*, 1987)

SOURCE OF WATER	HOST	PHAGE/LITRE	REFERENCE
Polishing pond, Florida	*E coli* B	$1,9 \times 10^4$	(1)
Lake Alice, Florida	*E coli* B	$1,1 \times 10^3$	(1)
Lake Alice, Florida	*E coli* C	$1,5 \times 10^2$	(2)
Lake Alice, Florida	*E coli* B	$1,5 \times 10^1$	(2)
River Avon, England	*E coli* W 3110	$6,5 \times 10^5$	(3)
River water, England	*E coli* HfrH	$1,8 \times 10^4$	(4)
River water, England	*E coli* W 3110	$3,5 \times 10^3$	(5)
Lake Alice, Florida	*E coli* C	$1,3 \times 10^4$	(6)
Apies River, South Africa	*E coli* K12 Hfr	$1,7 \times 10^5$	(7)
Vaal River, South Africa	*E coli* K12 Hfr	$4,0 \times 10^3$	(7)
Hudson River, New York	*E coli*	$5,0 \times 10^1$	(8)
Potamic River, Washington, DC	*E coli* C	$1,0 \times 10^4$	(9)
Danube River, Czechoslovakia	*E coli* B-39	$2,0 \times 10^4$	(10)
Cypress strand, Florida	*E coli* C-3000	$2,4 \times 10^5$	(11)

(1) Bitton *et al* (1981); (2) Kennedy *et al* (1985); (3) Logan *et al* (1980); (4) Primrose and Day (1977); (5) Seeley et al (1979); (6) Shields and Farrah (1986); (7) Grabow *et al* (1984); (8) Hilton and Stotsky (1973); (9) Kenard and Valentine (1974); (10) Simkova and Servenka (1981); (11)Scheuerman *et al* (1987)

TABLE 2-2 Phages of enteric bacteria other than *Escherichia coli* in freshwater environments (Goyal *et al*, 1987)

SOURCE OF WATER	HOST BACTERIA	PHAGE/LITRE	REFERENCE
River Avon, England	*K pneumoniae* 889	$1,0 \times 10^6$	(1)
River Avon, England	*S typhimurium* G46	$8,5 \times 10^3$	(1)
River water, England	*S typhimurium* G46	$8,2 \times 10^2$	(2)
River water, England	*K pneumoniae*	$1,2 \times 10^3$	(2)
River water, England	*K pneumoniae* 889	$1,1 \times 10^3$	(3)

(1) Logan *et al* (1980); (2) Primrose and Day (1977); (3) Seeley *et al* (1979)

2.5.2.3.4 Sewage

Domestic sewage harbours a wide range of phage strains, the detection of which is carried out by using a variety of host bacteria. According to Dhillon and Dhillon (1974) and Bell (1976), wild-type *E coli* strains are poor hosts for enumerating coliphages naturally occurring in sewage. This is in accordance with the results of Hilton and Stotzky (1973) who could only detect phages infectious to a wild-type *E coli* strain after enrichment of relatively large volumes of polluted river water. Wild *E coli* strains normally have a complete O-antigen that masks the majority of phage receptors situated in the R-core of the lipopolysaccharide. Rough or semi-rough laboratory strains are therefore much more productive hosts (Havelaar and Hogeboom, 1983). Those used most extensively for analysis of sewage are *E coli* B (Kott, 1966; Dhillon *et al*, 1970; Bell, 1976; Ayres, 1977; Gerba *et al*, 1978; Ignazitto *et al*, 1980; Nupen *et al*, 1980); *E coli* C (Kenard and Valentine, 1974; Dhillon *et al*, 1976; Wentsel *et al*, 1982) or derivatives of *E coli* K-12 like W3110 (Primrose *et al*, 1982). In addition to these so-called female host strains, male derivatives of K-12 are also used (Dhillon *et al*, 1970; Kott *et al*, 1978; Glass and O'Brien, 1980; Primrose *et al*, 1982). With the use of the specially constructed host strain WG49, Havelaar *et al* (1984) were able to demonstrate the consistent presence of FRNA phages in sewage from domestic, hospitals and poultry processing plants. Plaque counts usually varied between 10^2 and 10^4 PFU/ml, while those of somatic coliphages with host strain *E coli* CN (Havelaar and Hogeboom, 1983) were on the average two to four times higher.

Counts of coliphages in raw domestic wastewater are of the order of 10^5-10^7 PFU/L (Bell, 1976; Ignazzitto *et al*, 1980; Havelaar and Hogeboom, 1984; Havelaar *et al*, 1984). However, it seems that these levels represent only a relatively small portion of the actual number of phages in sewage. Using electron microscopy (EM), Ewert and Paynter (1980) demonstrated that plaque assays using *E coli* as host detected only 3,8% of the EM count. The total EM count in sewage varied between $1,6 \times 10^8$ and $1,2 \times 10^{10}$ phage particles/L.

2.5.2.3.4.1 Somatic coliphages in sewage

Tartera *et al* (1989) detected somatic coliphages (host *E coli* C600) in numbers that ranged from $1,4x10^4$ to $2,4x10^7$ per 100ml and an average of $1,2x10^6$ and $6,1x10^6$ per 100ml in samples collected from domestic raw sewage and a mixture of domestic and industrial sewage respectively. Somatic coliphages were detected in 55% of water and in 89% of sediment samples collected from a natural park, although mean values were low: 3,4 per 100ml and 7,2 per 100g respectively. Somatic coliphages were found in 73% of the river water samples tested, with an average of $9,06x10^2$ per 100ml. The samples obtained from slaughterhouses contained somatic coliphages in numbers that ranged from $2,4x10^7$ to $2,4x10^9$ per 100ml and an average of $5,3x10^8$.

2.5.2.3.4.2 Male-specific coliphages in sewage

Male-specific coliphages have been detected in a wide variety of wastewaters and polluted water environments (Grabow *et al*, 1980; IAWPR, 1991; Kfir *et al*, 1991).

Male-specific coliphages have been found to constitute the predominant phage species in sewage from domestic drainage (Dhillon *et al*, 1970; Furuse *et al*, 1973; Dhillon and Dhillon, 1974; Furuse *et al*, 1978). Havelaar *et al* (1984) found male-specific phages always present in domestic sewage, usually in numbers above 10^3PFU/ml.

Some studies have focused on F-specific RNA phages in sewage because these phages have been recommended as indicators of faecal pollution (Havelaar *et al*, 1984). This suggestion is based on the knowledge that the F pili are not produced below 30°C, thus preventing these phages from multiplying in environmental waters. Methods have been developed to assay specifically for these phages (Havelaar *et al*, 1984). RNA phage levels in raw sewage vary between 10^4 and 10^7PFU/L, using F^+ *Escherischia coli* or *Salmonella typhimurium* strains (Dhillon *et al*, 1970; Havelaar and Hogeboom, 1984).

Male-specific coliphages are not consistently present at high levels in human faecal wastes, but they are consistently and abundantly present in sewage and sewage-polluted waters (Havelaar *et al*, 1990; Debartolomeis and Cabelli, 1991). Male-specific phage counts in domestic sewage and other wastewaters cannot be explained solely by direct input from faecal sources (Havelaar *et al*, 1990). These phages should be considered as indicators of sewage pollution rather than faecal pollution. An unresolved question is by what mechanism these phages multiply in the sewage environment (Havelaar *et al*, 1990). Laboratory studies indicate that host-cells will be sensitive to infection only when cultured at temperatures of 30°C or above and that organisms replicating in the sewage environment cannot act as natural hosts. Possibly, *E coli* strains originating directly from faeces fulfil this role (Havelaar and Pot-Hogeboom, 1988).

2.5.2.3.4.3 *Bacteroides fragilis* bacteriophages in sewage

Bacteroides bacteria are obligatory anaerobes and sporeless, therefore their die-off is rapid under oxygen exposure (Tartera and Jofre, 1987) and as such, unsuitable as an indicator. However, their bacteriophages are of promising potential as indicators with much higher resistance, similar or higher to coliphages and human viruses (Tartera *et al*, 1988; Tartera and Jofre, 1989; Jofre *et al*, 1989).

Tartera *et al* (1989) detected *B fragilis* phages in all of the raw sewage samples they tested. Numbers of phages infecting *B fragilis* ranged from 23 phages to $4,6 \times 10^4$ phages per 100ml, and averages of $5,3 \times 10^3$ and $1,3 \times 10^3$ were observed in samples collected from raw domestic sewage and samples collected from a mixture of domestic and industrial wastewater. In wastewater from two slaughterhouses only 2 of 20 samples were positive for *Bacteroides* phages after enrichment of 100ml of wastewater. These bacteriophages could be either of animal origin or due to low input of human contamination. However, the numbers

were very small in comparison with the values in domestic sewage. In contrast, numbers of coliphages were nearly two logarithms higher than the numbers found in domestic sewage (Tartera *et al*, 1989).

2.6 PHAGES AS INDICATORS

2.6.1 Phages as indicators of human enteric viruses

There are a large number of potentially pathogenic micro-organisms in raw sewage and treatment process effluents (Funderburg and Sorber, 1985). Water-borne diseases can result from the discharge of pathogenic organisms from sewage into water (Feachem *et al*, 1981). Pathogens of concern in wastewater can be devided into two broad categories: bacteria and viruses. More than 100 human virus types have been reported to be associated with wastewater discharges (Grabow, 1968). Their pathological effects differ widely. Clinical levels may be evident as mild, cold-like symptoms or as the severe manifestations evoked by infectious hepatitis or paralytic poliomyelitis (Safferman and Morris, 1976). The most prevalent and important sewage-related waterborne diseases in developed countries are acute gastroenteritis and infectious hepatitis (Cabelli, 1983; Cabelli, 1989). Given the health risk that viruses represent, it is essential to develop more information on the nature and extent of viral contamination in the environment. Ideally, isolation of the viruses themselves is the most appropriate means of virus detection. However, viral isolation of wastewater has the following disadvantages (Kott *et al*, 1974):

(a) much time may elapse before one strain of virus is accepted as the virus of water pollution,
(b) isolation techniques are costly, delicate and time consuming,
(c) facilities of a central laboratory are required,
(d) working with primary tissue culture is expensive,
(e) days elapse between inoculation and obtaining results.

Consequently, it is desirable to establish reliable indicators for determining the presence of viruses.

The evaluation of the virological quality of water heavily depends upon the use of indicator organisms, because currently available virological techniques are impractical and have too many shortcomings (Grabow *et al*, 1984).

The basic requirements of indicators are (Grabow, 1986):

(a) they should be present in water environments whenever enteric viruses are present,
(b) they should be present in the same or higher numbers than viruses,
(c) they should be at least as resistant as viruses to water purification and disinfection processes as enteric viruses,
(d) they should be specific for faecal or sewage pollution,
(e) they should preferably be non-pathogenic and detectable by simple, rapid and inexpensive methods.

Guelin (1948) was the first to propose the use of bacteriophages as indicators of enteric bacteria and viruses. His results suggested that the number of coliphages in fresh and marine waters were related to the number of coliforms.

The reasons for proposing coliphages as indicators of enteric viruses have been detailed as follows by Kott (1981), namely:

(a) coliphages occur in abundance in wastewater and polluted water,
(b) the populations of coliphages exceed those of enteric viruses,
(c) coliphages are incapable of reproduction outside of the host organism,
(d) coliphages can be isolated and enumerated or quantitated by simple methods,
(e) the time interval between sampling and final result is shorter than that for enteric viruses,
(f) many coliphages are more resistant to inactivation by adverse environments and disinfection than enteroviruses.

Laboratory studies with individual coliphages have demonstrated that many of these phages survive longer in natural waters and are more resistant to common disinfectants such as chlorine than are many enteroviruses (Kott *et al*, 1974; Simkova and Cervenka, 1981; Grabow *et al*, 1983; Stetler, 1984; Yates *et al* 1985; Grabow, 1986).

However, Vaughn and Metcalf (1975) and Borrego *et al* (1990) suggested that the use of coliphages as indicators was not appropriate because of their poor correlation with the presence of human enteric viruses in water and because of the possible multiplication of the coliphages in natural waters in certain seasons. Observations regarding poor correlations with human enteric viruses may, however, largely be due to technical difficulties in the enumeration of viruses.

Enteric viruses have been detected in the absence of coliphages (Montgomery, 1982; Deetz *et al*, 1984) and Seeley and Primrose (1980) detected autochthonous coliphages in unpolluted waters.

Yates *et al* (1985) stated that studies with individual phages do not prove the usefulness of the coliphage indicator concept because many coliphages are inactivated more readily than enteroviruses under the same conditions. In addition, one cannot depend on the same phage being consistently present in the environment.

Primrose *et al* (1982) proposed male-specific phages and Havelaar *et al* (1986) proposed RNA bacteriophages belonging to the E morphological group as models of enteric viruses. Certain findings, however, question the absolute indicator value of male-specific coliphages. For instance, Morinigo *et al* (1992) detected enteric viruses in certain water environments but no male-specific coliphages.

An innovative and new concept for bacteriophage indicators was introduced by workers at the Univeristy of Barcelona (Tartera and Jofre, 1987). The exciting features of their findings are basically that phages which infect a particular strain of *Bacteroides fragilis*, which they have designated strain HSP40, would appear to be highly specific for human stool and survive in the environment for at least as long as human viruses.

These bacteriophages were detected in 10% of faecal samples from human origin; they were also detected in sewage and contaminated natural waters but never in

non-faecally contaminated samples. Moreover, *B fragilis*, a predominant intestinal bacterium, is a strict anaerobe, and thus phage multiplication in the aquatic environment is unlikely (Tartera *et al*, 1989).

Bosch *et al* (1989) found that the *B fragilis* bacteriophage B40-8 was more resistant to inactivation by chlorine than poliovirus type 1, simian rotavirus SA11, coliphage f2, *Escherichia coli* and *Streptococcus faecalis*. They suggested the use of bacteriophages infecting *B fragilis* to monitor the efficiency of disinfection processes for viruses in the environment.

2.6.2 Phages as indicators of faecal pollution

Several authors have proposed the use of coliphages as faecal indicators because of their consistent presence in the faeces of man and other warm-blooded animals, sewage and sewage-polluted waters (Vaughn and Metcalf, 1975; Dhillon *et al*, 1976; Kott, 1984; Borrego *et al*, 1987; Goyal, 1987).

Lower animals, such as cows and pigs, generally contain higher numbers of coliphages than humans (Dhillon *et al*, 1976; Osawa *et al*, 1981; Furuse *et al*, 1983). The microflora of the gut, diet, and physiological state of the animal could potentially affect the numbers of coliphages in faeces. Osawa *et al* (1981) found a higher percentage of stools from zoo animals to be positive for phages than from domestic farm animals. The absence of phages in stools and/or their occurence at varying numbers may only be a reflection of the limitations of the specificity of the bacterial host and not necessarily the absence of phages *per se* in each individual (Goyal *et al*, 1987).

Furuse et al (1983) found qualitative and quantitative differences between phages isolated from faecal samples from healthy individuals and from patients. Lower phage titres of patients treated with several antibiotics could be due to changes in the microbial flora of their intestinal tract. Leukaemic patients nursed in an open ward and who had received no antibiotic treatment showed higher phage titres in their faeces than those in an protected environmental ward, thus suggesting a correlation between bacterial and phage titres in faecal samples.

The most commonly used indicators are total and faecal coliforms (Kenard and Valentine, 1974; APHA, 1989). However, several authors have pointed out that these indicators have shortcomings for indicating the incidence and behaviour of enteric viruses and other enteric pathogens in water, and in addition, their survival capability in aquatic environments is low (Marzouk *et al*, 1980; Borrego *et al*, 1983; Goyal, 1983; Geldenhuys and Pretorius, 1989). Bacteriophages were proposed as potential model viruses in water quality studies. Most attention has been directed to organisms capable of infecting commonly used *Escherichia coli* host strains by primary adsorption to the cell wall (somatic coliphages). However, these are a heterogeneous group of organisms, which may originate from faecal and non-faecal sources (Seeley and Primrose, 1980) and may be inactivated by widely different rates in water treatment processes (Gerba, 1987). Male-specific bacteriophages which enter the host cell via primary adsorption to F- or sex-pili are a more homogeneous group of relatively resistant organisms, which makes them

an attractive model virus (Havelaar *et al*, 1990). Tartera and Jofre (1987) have proposed the *Bacteroides fragilis*-specific bacteriophages as viral indicators. Phages which infect *Bacteroides fragilis* HSP40 have the same origin as human viruses and they will not replicate significantly in the environment (Tartera *et al*, 1989).

2.7 RECOVERY METHODS

In many water environments phages are present in numbers too low for detection by direct plaque assays. Low numbers of phages are generally detected by either or both of the following two procedures:

(a) Quantitative method in which viruses are recovered from large volumes of water by means of various techniques followed by plaque assays to detect and enumerate the phages.

(b) Qualitative method in which hosts and nutrients are added to large volumes of water which are then incubated to allow replication of phages which may be present. The presence of phages is then detected by plaque assays or qualitative spot tests.

Methods for the recovery of phages should ideally be:

(a) rapid,
(b) inexpensive,
(c) simple,
(d) capable of yielding high recoveries of a wide range of bacteriophages,
(e) adaptable for use with large volumes of different types of water, for example, freshwater, estuarine water, seawater and wastewater.

The best possible recovery or enrichment methods are necessary for purposes such as the determination of:

(a) the effect of environmental factors, such as temperature, pH, and organic compounds on phage distribution,
(b) seasonal and spatial distribution and survival of phages in the environment,
(c) the adequacy of water treatment processes by using phages as indicators,
(d) the extent of faecal pollution in natural water sources.

2.7.1 Quantitative methods based on adsorption-elution

2.7.1.1 Filter adsorption-elution

A commonly employed recovery procedure is called viradel (virus adsorption elution) (Goyal and Gerba, 1983), which consists of virus adsorption to filters with their subsequent elution in a small volume of buffer. The viradel procedure is considered to be among the best methods for detecting small numbers of viruses in large volumes of water (Sobsey and Jones, 1979; Wallis *et al*, 1979; Goyal and Gerba, 1982).

A factor that might affect the efficiency of phage adsorption to filters is the amount of organic material in the water sample under investigation. It seems likely that not

only viruses but that all other proteinaceous materials in the water will adsorb to the filter medium (Logan *et al*, 1980). Logan *et al* (1980) also described a 'volume effect' on phage adsorption to filters, whereby an increase in the sample volume led to a progressive decrease in the amount of phages adsorbing to the filter.

The presence of di- and trivalent cations has been found to promote virus adsorption by membrane filters (Valentine and Allison, 1959; Wallis and Melnick, 1967; Rao and Labzoffsky, 1969; Wallis *et al*, 1972; Metcalf *et al*, 1974; Fields and Metcalf, 1975; Wallis *et al*, 1979; Farrah *et al*, 1981). The mechanism of cation enhancement of viral adsorption to membrane filters has been examined in several studies. It has been suggested that multivalent cations promote adsorption of viruses to membrane filters by altering the charge on the filter (Metcalf *et al*, 1974; Kessick and Wagner, 1978; Wallis *et al*, 1979), by altering the thickness of the layer of charges surrounding the surface of the filter (Valentine and Allison, 1959), or by forming salt bridges between the viruses and the filters (Mix, 1974; Kessick and Wagner, 1978). Farrah (1981) found that magnesiumsulphate promotes hydrophobic interactions between MS-2 and membrane filters. This is consistent with previously published results with other viruses (Rao and Labzoffsky, 1969; Wallis *et al*, 1972; Fields and Metcalf, 1975).

Two general types of filters have been used:

2.7.1.1.1 Negatively charged filters

Electronegative filters have net negative charges at pH values between 4 and 6. Optimal adsorption of viruses to electronegative filters is obtained by lowering the pH to 3,5 and by adding aluminium chloride (0,001M) prior to filtration (Borrego *et al*, 1991). This conditioned water is then passed through virus-adsorbent filters. Under these conditions the viruses acquire a net positive charge, enabling them to adsorb to the negatively charged membrane filters (Gerba *et al*, 1978). Adsorbed viruses are subsequently eluted by using high pH buffer, generally more than pH 9,5 (Gerba *et al*, 1978; Shields and Farrah, 1986). An alkaline pH results in reversal of charge on the phage particle so that it is rapidly eluted from the filter. Although these negatively charged filters yield relatively high efficiences of recovery with human and animal viruses, they have been shown to be unsuitable for bacteriophage recovery, primarily because phages are not as stable as enteroviruses at the low pH values required for adsorption of viruses to these filters (Seeley and Primrose, 1979; Sabatino and Maier, 1980; Primrose *et al*, 1981; Seeley and Primrose, 1982; Shields *et al*, 1986). Also, the use of high pH buffers as eluents may result in additional inactivation (Primrose *et al*, 1981).

2.7.1.1.2 Positively charged filters

Electropositive filters have net positive charges or slight negative charges at pH values between 4 and 6 (Sobsey and Jones, 1979; Sobsey and Glass, 1980). Investigators have recently explored the use of electropositive membrane filters, which allow for the adsorption of viruses over a broader pH range without the addition of salts and pretreatment of the water (Sobsey and Jones, 1979; Sobsey and Glass, 1980; Chang *et al*, 1981; Seeley and Primrose, 1982; Singh and

Gerba, 1983). Several positively-charged filters of varying composition are now commercially available (Shields *et al*, 1986). Such filters have been shown to be excellent for the adsorption of many types of viruses and phages from water at near-neutral pH levels (Logan *et al*, 1980; Sobsey and Glass, 1980; Sobsey *et al*, 1980; Garelick and Scutt, 1981). Efficient elution from these positively charged filters has been accomplished using proteinaceous solutions, such as beef extract (Landry et al, 1978; Sobsey and Jones, 1979; Goyal *et al*, 1980; Sobsey and Glass, 1980; Hejkal *et al*, 1982; Seeley and Primrose, 1982).

Primrose and Day (1977) used hydroxylapatite (calcium phosphate) for phage recovery from 2L volumes of distilled water, and were able to recover 33-90% of selected phages.

Seeley and Primrose (1979) observed that methods used for recovery of human viruses from drinking water were not suitable for the recovery of bacteriophages from natural waters because the adsorption conditions in these methods are optimized for animal viruses, not phages. Additionally, elution conditions at high pH resulted in phage inactivation. These authors, therefore, devised a procedure for phage recovery which consisted of pretreatment of water by passage through a sand and an anion-exchange resin; adsorption of phages to fiberglass and cellulose nitrate filters by the addition of 5×10^{-4}M magnesium chloride and adjustment of pH to 3,8; elution of adsorbed phages with 3% beef extract solution at pH 8,5; and further concentration of the resulting eluate by ultrafiltration. Using this procedure, they were able to process 4L of water and end up with 5ml at recoveries of 18-80%. Although this method is attractive, it has the disadvantage of being complicated, costly, and time consuming.

Logan *et al* (1980) evaluated positively charged Zeta Plus 60-S filters for recovery of bacteriophages from natural water. They found that a variety of phages adsorbed efficiently to these filters at water pH levels below neutrality, but that adsorption was reduced above pH 7. Based on these data, they devised a system that consisted of:

(a) prefiltration of water through a filter of 5um porosity,
(b) adjustment of water to pH 5,5-6,0,
(c) adsorption of phages on to Zeta Plus 60-S filters,
(d) elution of bound phages in a small volume of arginine/1% beef extract solution (pH 9,0),
(e) secondary concentration of the resulting eluate by ultrafiltration.

Using this procedure, phages in 65L of river water were concentrated to 35ml with recoveries in the range of 50-60%. Later, Logan *et al* (1981) scaled up this procedure to process volumes of up to 500L of water.

Goyal *et al* (1980) used positively charged Zeta Plus filters for the recovery of coliphages MS-2, ϕX-174, T2 and T4 from 17L volumes of tap water, sewage and lake water. Using this method, they were able to recover coliphages from 17L volumes of tap water with recoveries ranging between 34 and 100%. Recoveries from raw and secondarily treated sewage were approximately 55%.

Singh and Gerba (1983) evaluated the fine and coarse grades of filter aid for coliphage recovery. They used AP-20 prefilters as support for overlaying the slurry

of filter aid in 47-mm-diameter polypropylene filter housings. The recovery from 10-20L samples of tap water was 11-70% for T2 and 43-70% for MS-2.

Rose *et al* (1984) studied the comparative efficiency of fibre glass and Virosorb 1-MDS for the recovery of coliphages from primary wastewater effluents, and obtained a higher recovery of phages with fibre glass filters (17% versus 14% on Virosorb). However, these results were reversed in tests on secondary effluents (22% of recovery from fibre glass versus 61% from Virosorb).

Nupen and Bateman (1985) reported a mean recovery of phages using 1-MDS cartridges of 31%, with range between 18 and 42%. Havelaar (1986) obtained mean recoveries between 31 and 76% using Virosorb 1-MDS filters.

Shields and Farrah (1986) described a procedure in which phages were adsorbed to positively charged Virosorb 1-MDS filters followed by their elution with a solution of 10% beef extract, pH 9. This eluate was further concentrated by the addition of two volumes of saturated ammonium sulfate to beef extract eluate. This resulted in the formation of inorganic floc that was recovered by centrifugation and resuspension in a small volume of distilled water. Using this procedure, 2-3L of lake water and sewage effluent was concentrated to 3,5ml with an average phage recovery of more than 70%.

Goyal *et al* (1987) stated that the following factors should be kept in mind in order to optimise the adsorption-elution technique:

(a) prefiltration of a sample may be necessary if it contains a large amount of suspended matter which may clog the virus-adsorbing filter, thus decreasing its efficiency. It should be realised, however, that most viruses have affinity with solids and may thus be removed by prefiltration of suspended matter; in such cases, both prefilter and the virus-adsorbing filter should be eluted,
(b) the type of adsorbent filter used,
(c) effect of pH and ionic conditions on adsorption,
(d) flow rate,
(e) choice of eluent and pH,
(f) reconcentration procedure.

Electropositive filters may easily be blocked by colloidal matter in the sample, which limits the processing of large volumes of water (Borrego *et al*, 1991). Borrego *et al* (1991) developed a new method for virus elution, consisting of the slow passage of the eluent through the filter, thus increasing the contact time between eluent and virus adsorbed on the filters. The use of this technique yielded a recovery of 71,2% compared with 46,7% recovery of phages obtained by the standard elution procedure. High percentages (over 83%) of phage adsorption were obtained with nine different filters from 1L aliquots of the samples, except for Virosorb 1-MDS filters which adsorbed only between 1,6 and 32% of phages. Phage recovery by using the method of slow elution depended on the filter type, with recovery ranging between 1,6% for Virosorb 1-MDS filters treated with polyethelenimine to 103,2% for diatomaceous earth filters treated with 0,1% Nalco (Borrego *et al*, 1991).
Electropositive filters have some disadvantages. Asbestos-containing and diatomaceous earth filters have slow flow rates, which prohibit the analysis of large volumes of water, whereas charge-modified, resin-containing filters are expensive

relative to other filter types (Preston *et al*, 1988). Dissolved organic compounds significantly affect virus adsorption to electropositive filters (Sobsey and Glass, 1984).

Secondary concentration

When large volumes of water are filtered in adsorption-elution procedures, and relatively large volumes (about 100ml or more) of eluent are used to recover the viruses/phages, a second step of concentration is generally applied to recover the viruses/phages into a smaller volume of final concentrate. Reconcentration procedures used for virus recovery include adsorption to and subsequent elution from a smaller membrane filter (Farrah and Bitton, 1979; Shields *et al*, 1985), hydroextraction (Farrah *et al*, 1977), ultrafiltration (Selna and Miele, 1977), and inorganic (Wallis *et al*, 1972; Farrah *et al*, 1976) or organic (Katzenelson *et al*, 1976) flocculation.

Katzenelson *et al* (1976) developed an organic reconcentration procedure in which a buffer containing beef extract is used to elute viruses/phages. After elution the pH of the buffer is reduced to 3,5 which triggers flocculation of the beef extract. Viruses in the eluate adsorb to the flocs and are recovered by centrifugation. The pellet is then resuspended in neutral pH buffer. Poor efficiency of this method for phages has been reported by several researchers (Hurst *et al*, 1984; Payment *et al*, 1984; Payment and Trudel, 1987). Poor recovery has been reported for reconcentration with organic flocculation due to the fact that bacteriophages are more sensitive to adverse environmental factors than enteric viruses (Katzenelson *et al*, 1976; Dahling and Wright, 1986; Jothikumar *et al*, 1990). Shields and Farrah (1986) developed a reconcentration step for bacteriophages in protein solutions which does not involve drastic changes in pH. They compared the ammonium sulfate flocculation procedure with the organic flocculation procedure for the recovery of bacteriophages MS-2, ϕX174 and T3. They found that optimum precipitation occured when beef extract was mixed with saturated ammonium sulfate. The overall mean recovery of bacteriophages with the ammonium sulfate flocculation was 85%, compared with 12% for the organic flocculation method.

2.7.1.2 Glass powder adsorption-elution

Adsorption to and elution from negatively or positively charged glass powder was shown to be effective for the recovery of enteroviruses (Sarrette *et al*, 1977; Schwartzbrod and Lucena, 1978; Bosch *et al*, 1986) rotaviruses (Bosch *et al*, 1988) and HAV (Bosch *et al*, 1991; Gajardo *et al*, 1991) from large volumes of water. A disadvantage of negatively charged glass powder is that the pH of the sample has to be lowered to facilitate the adsorption of viruses (Gajardo *et al*, 1991). The efficiency of negatively or positively charged glass powder for the recovery of phages has not yet been investigated.

2.7.1.2.1 Negatively charged glass powder

Vilagines *et al* (1978) reported 30% recovery of enteroviruses from river water using glass powder. They used glass powder to adsorb enteroviruses suspended in water at pH 3,5 and eluted at pH 11,5. These adsorption-elution conditions are quite unsuitable for many phage types and the method would be suitable for use only with particularly stable phages (Seeley and Primrose, 1982).

Negatively charged glass powder yielded recoveries of HAV from 20L samples at efficiencies of 100% for tap water, 80% for freshwater, 75% for seawater and 61% for sewage (Bosch *et al*, 1991).

2.7.1.2.2 Positively charged glass powder

Recently it has been shown that the charge of glass powder can be changed to positive by treatment with polyethylenimine (PEI) (Gajardo *et al*, 1991). Positively charged glass powder yielded recoveries of HAV from 20L samples at effiencies of 100% for tap water, 94% for seawater and 61% for freshwater and sewage (Bosch *et al*, 1991).

2.7.2 Enrichment

With this qualitative method, phages are isolated from large volumes by adding growth media and cultures of the host bacterium to the test sample. After incubation for 24h these cultures are then tested for the presence of phages by conventional methods.

Guelin (1952) was the first to propose enrichment methods for the detection of low numbers of phages. Kott (1966) described a most probable number (MPN) method for detecting phages that used enrichment of serial dilutions of a water sample to detect and estimate phages. This method made it possible to detect numbers as low as two PFU per 100ml of sample.

Hilton and Stotsky (1973) were unable to isolate coliphages from river water by concentrating the sample sixfold by ultracentrifugation or by direct plating. However, by using an enrichment procedure they were able to detect phages in that sample.

In most cases, phages obtained in enrichment cultures are detected by subsequent plaque assays. However, the phages produced by enrichment can also be examined by electron microscopy (Ackermann and Nguyen, 1983) or by spot tests (Kott, 1966).

The enrichment technique is useful for the rapid detection of low numbers of a specific phage in a water sample.

A disadvantage of this method is that only the phage for a given bacterial host can be isolated (Seeley and Primrose, 1982; Goyal *et al*, 1987). Where a number of

different phages for a particular host are present in the inoculum the most vigorous will come to predominance. If rare or less productive phages are being sought their presence may well be masked by overgrowth. A furher disadvantage of the enricment technique is that large quantities of media are required.

2.7.3 Ultrafiltration

Ultrafiltration involves the selective removal of small molecules from a water sample, including the water molecules themselves, and leaving behind large particles such as viruses, because they are too large to penetrate the membrane pores (Strohmaier, 1967; Sweet *et al*, 1974). This technique uses filters that may be configured either as hallow fibers through which the water sample is passed, or sheet filters against which the water is kept in motion by means of a recirculating pump or stirring apparatus (Berman *et al*, 1980; Logan *et al*, 1980; Jansons and Bucens, 1986). Membranes of 10 000 nominal molecular weight limit (nmwl) are generally used for the recovery of viruses and phages (Divizia *et al*, 1989). Tangential flow retention is a separation technique whereby flow is directed across the membrane surface in a sweeping motion. This sweeping action helps keep material retained by the membrane from creating a layer on the filter surface, a condition known as concentration polarisation. Tangential flow filtration is used to recover material retained by the membrane (retentate) or to recover material passing through the membrane (filtrate). Particles smaller than the pore size or nominal molecular weight cutoff are able to pass through the membrane and may be separated from higher molecular weight or larger particles. Particles larger than the pore size or molecular cutoff are 'rejected' by the membrane and are recovered (Divizia *et al*, 1989). Tangential flow and hollow fibre ultrafiltration systems have a large filtration surface area, the purpose of which is to alleviate clogging, which is a serious problem with flat sheet membranes and extensively restricts the volume of water that can be filtered. The membranes may be pretreated with beef extract solutions before they are used on a water sample to suppress virus adsorption and to facilitate the eventual recovery of the viruses/phages from the membrane (Berman *et al*, 1980).

High efficiencies of recovery have been reported for ultrafiltration. Nupen *et al* (1980) found recovery efficiencies of 94% for the recovery of a wide variety of viruses and phages from tap water or equivalent water using sheet membranes. The average recovery efficiency of male-specific coliphages from 1L samples of activated sludge effluent was 69%, and from 10L samples of the effluent after primary clarification in a wastewater reclamation plant 72%, after secondary clarification 83%, and after sand filtration 91%. Polio 1, 2 and 3 viruses, reovirus, a rotavirus and seven different morphological types of coliphages were recovered from seeded 100L samples of distilled, tap or reclaimed water at an average efficiency of 94%. Divizia *et al* (1989) evaluated the recovery by ultrafiltration using a polysulphonate flat membrane of 10 000nmwl of poliovirus and hepatitis A (HAV) in 1L samples of dechlorinated tap water. The recovery of HAV under standard conditions i.e 12psi inflow, retentate : filtrate ratio 6 : 7, was 100%. This was reduced to 50% when the inflow pressure was increased to 26psi. Poliovirus recovery was 15% under standard conditions. When the membranes were pretreated with beef extract at neutral pH, the efficiency of poliovirus

recovery was 100%. When the membranes were pretreated with other buffers such as tap water; 0,1% glycine; 1% glycine; beef extract pH 9,5, the recovery was less efficient.

Ultrafiltration (molecular filtration) has a particular use for the recovery of viruses in a water sample (Berg *et al*, 1971; Wallis *et al*, 1972; Sobsey *et al*, 1973; Farrah *et al*, 1978; Sobsey and Jones, 1979). It is not dependant on virus adsorption to a matrix and consequently minimises virus loss resulting from competition with other organic matter for adsorption sites (Berman *et al*, 1980). Some recovery procedures also require pH changes and polycation additions to obtain more efficient virus adsorption. Recovery by molecular filtration, however, is not based on the net charge of the virus particle, thus eliminating the need for acidifying or adding polycation salts (Berman *et al*, 1980; Nupen *et al*, 1980). In addition, ultrafiltration is relatively simple, standard equipment is available, no chemicals have to be added to water samples, no secondary concentration step is required, and sterilisation of equipment is simple and reliable (Nupen *et al*, 1980). However, disadvantages include high cost of capital equipment and clogging of membranes which limits the volume of water that can be processed.

2.8 CONCLUSIONS

This literature review shows that assessment of the microbiological safety of water and food heavily depends on the use of indicators. However, commonly used indicators such as coliform bacteria have shortcomings, particularly with regard to human viruses. Indications are that bacteriophages, which closely resemble human viruses in terms of size, composition and morphology, may have valuable indicator features. Phages which infect *Bacteroides fragilis* HSP40 seem to have the additional advantage of distinguishing between faecal pollution of human and animal origin. However, techniques for the recovery and detection of low numbers of phages need to be improved, and much more information on the reliability of phages as indicators of viruses is required.

The objectives of this study are, therefore, to improve methods for the recovery and detection of phages, and to assess in more detail the reliability of phages as indicators of human viruses in selected environments. In view of features such as their specificity for human excreta, special attention will be given to phages which infect *Bacteroides fragilis* HSP40.

CHAPTER 3

PHAGE DETECTION METHODS

3.1 INTRODUCTION

Guelin (1948), Coetzee (1962; 1966) and Kott (1966) were the first to suggest the assessment of sewage pollution of water environments by analysis for bacteriophages. Since then, many authors have developed phage detection methods using a variety of host strains, culture media etc. (IAWPRC, 1991).

Bacteriophages are easily isolated and cultivated in logarithmic phase cultures of bacteria in broth or on agar plates. In liquid cultures, lysis of the bacteria may cause a cloudy culture to become clear, whereas in agar-plate cultures, clear zones, or plaques, become visible to the unaided eye (Pelczar *et al*, 1988).

The principle requirement for isolation and cultivation of phages is that optimal conditions for growth of the host organisms are provided. The best and most usual source of phages is sewage. Phages which infect intestinal bacteria such as coliforms and *Bacteroides fragilis*, can also be isolated from faeces, although the population of phages is highly variable in faeces. For example, coliphages or other bacteria found in the intestinal tract can best be isolated from sewage or manure. This is done by centrifugation or filtration of the source material and addition of chloroform to kill the bacterial cells. A small amount of this preparation is generally mixed with the host organism in a sloppy top agar layer which is spread on an agar medium. Growth of phage is indicated by the appearance of plaques in the otherwise opaque growth of the host bacterium (Pelczar *et al*, 1988).

The most important factor in defining a phage-assay method is the choice of the host strain (IAWPRC, 1991). In my study the host bacterium *Escherichia coli* C (ATCC 13706)(WG4) and its nalidixic acid resistant mutant WG5 were used for the enumeration of somatic phages; *Salmonella typhimurium* WG49 phagetype 3 Nal^r(F'lac::Tn5) (Havelaar and Hogeboom, 1984) and *Escherichia coli* HS(pFamp)R (Debartolomeis and Cabelli, 1991) for the enumeration of male-specific phages; and *Bacteroides fragilis* strain HSP40 (Tartera and Jofre, 1987) for the enumeration of phages of anaerobic bacteria.

E coli C (ATCC 13706)(WG4) (Havelaar and Hogeboom, 1983) detects a variety of somatic coliphages. The bacterium is lactose positive, sensitive to nalidixic acid and is an effective host strain for many bacteriophages. This can partly be explained by the absence of a DNA-restriction system in this bacterium (does not destroy foreign DNA), and partly by the broad range of receptor sites available (Havelaar and Hogeboom, 1983).

Escherichia coli C (WG5) (Havelaar and Hogeboom, 1984) detects somatic coliphages, is lactose-positive and resistant to nalidixic acid (330*u*g/ml).

Escherichia coli HS (pFamp)R, as modified in the laboratory of Debartolomeis and Cabelli (1991), satisfies most of the requirements for the enumeration of male-specific bacteriophages in environmental waters. In one study more than 95% of the phages from sewage samples which plaqued on it were male-specific, and it had a significantly higher plaquing efficiency than *E coli* K-12 Hfr for the male-specific phages when stock suspensions or sewage effluents were examined. If an *E coli* K-12 Hfr strain (Kott *et al*, 1974) is used for the examination of faecally polluted water, the resulting plaque count will reflect both the number of somatic phages (i.e those finding a receptor on the cell wall such as the T-phages) and the number of F-specific phages (Havelaar and Hogeboom, 1984). The host strain *E coli* HS(pFamp)R is resistant to streptomycin and ampicillin which can be included in the plaquing medium to prevent overgrowth by bacteria present in the sample. Resistance to ampicillin is encoded by the F plasmid, and inclusion of ampicillin (0,015g/L) and streptomycin (0,015g/L) in stock cultures selects against any varients which may have lost the plasmid. Finally, the host strain is easily grown and is not derived from a pathogen (Debartolomeis and Cabelli, 1991).

Salmonella typhimurium Nal^r (F'lac::Tn5)(WG49) is sensitive to male-specific coliphages (DNA and RNA phages), as well as somatic *Salmonella* phages. It is lactose-positive and resistant to nalidixic acid (100*u*g/ml) and kanamycin sulphate (20*u*g/ml). This strain was constructed by the introduction of F'42 lac::Tn5 plasmid into *Salmonella typhimurium* WG45 strain (Cornax *et al*, 1991). This *Salmonella* host carries *E coli* fimbriae, which implies that it is susceptible to male-specific coliphages but not to most somatic coliphages (Havelaar and Hogeboom, 1984). Since numbers of somatic *Salmonella* phages are generally low in the environment, this *Salmonella* strain is ideally suited for the relatively selective detection of male-specific coliphages (IAWPRC, 1991).

Bacteroides species constitute the most frequent and dominant anaerobic bacterial species in the intestinal tract of humans and warm-blooded animals (Buchanan *et al*, 1974). These organisms may also be found in smaller numbers in the female genital tract but not commonly in the mouth or upper respiratory tract (Balows *et al*, 1991). Kai *et al* (1979) were the first to succeed in isolating phages for *Bacteroides* species. Booth *et al* (1979) isolated a number of *Bacteroides* phages from sewage. In a series of parallel tests of twelve *Bacteroides* strains (fresh human isolates and collection strains) *B fragilis* HSP40 was found to give consistently higher counts for sewage samples than other strains (Tartera and Jofre, 1987) and *Bacteroides fragilis* HSP40, therefore, has been used in this study to examine the ecology of *Bacteroides* phages.

Assays of phages by plaque counts were conducted by the double-agar-layer method (Adams, 1959). The principle of this technique involves mixing of a sample of water or shellfish meat suspension (concentrated or diluted) with a small volume of a molten semi-solid top agar nutrient medium, addition of a culture of the host-strain, and plating on a solid nutrient agar medium. The relationship between plaques formed in such assays and the phage number used for inoculation has been reported to be linear (Luria, 1953).

The aim of this study was to describe methods used in this study for the isolation, cultivation and preparation of phages for a variety of phage-host systems.

3.2 MATERIALS AND METHODS

3.2.1 Double-agar-layer plaque assay for somatic coliphages (According to Grabow *et al*, 1984)

Growth medium (Nutrient broth)

Nutrient broth (dehydrated)	(Difco)	8,0g

The nutrient broth was dissolved in 1 000ml distilled water and sterilised in the autoclave for 15min at 121°C.

Phage bottom agar

Bacto agar	(Difco)	11,0g
Tryptone	(Difco)	13,0g
NaCl	(Holpro)	8,0g
Glucose	(Sigma)	1,5g
Distilled H_2O		1 000ml

The ingredients were dissolved in distilled water, autoclaved for 15min at 121°C and 20ml volumes were poured into 90mm diameter Petri dishes.
When interference by growth of bacteria in the test sample was expected, the nalidixic acid-resistant strain *E coli* WG5 and plates containing 330*u*g/ml nalidixic acid in the bottom agar were used (Havelaar and Hogeboom, 1983).
The phage bottom agar plates were stored at 4°C for not more than 10 days.

Phage top agar

Same as phage bottom agar, except for 7,0g instead of 11,0g Bacto agar per 1 000ml.
When interference by growth of bacteria in the test sample was expected, top agar containing 330*u*g/ml nalidixic acid was used.
The phage top agar was dispensed in 2,5ml volumes into sterile tubes and stored at 4°C for not more than 30 days.

Host culture

Escherichia coli strain C WG4(ATCC 13706) or its nalidixic acid resistant mutant WG5 were used. These hosts were kindly supplied by Dr A H Havelaar.
A lyophilised culture of the host bacterium was rehydrated in nutrient broth, and incubated for 24h at 37°C without shaking.
A plate of MacConkey agar was inoculated from this overnight culture in such a way that single colonies were obtained.
The plate was incubated for 24h at 37°C.
A lactose-positive colony was selected and subcultured on nutrient agar slopes for 24h at 37°C.
The agar slopes were stored at 4°C and subcultured every 2-4 weeks.
Sensitivity to phage V_1 was checked regularly.

Preparation of inocula for plaque assays

During the late afternoon of the day before the assays were to be carried out, an inoculum of the stock culture was transferred to tubes with 10ml quantities of nutrient broth.
The cultures were incubated overnight at 37°C without shaking before mixing with test samples and top agar.

3.2.2 Double-agar-layer plaque assay for male-specific coliphages

3.2.2.1 *E coli* HS(pFamp)R
(According to Debartolomeis and Cabelli, 1991).

Growth medium (Tryptone broth)

Tryptone	(Difco)	10,0g
Dextrose	(PAL)	1,0g
NaCl	(Holpro)	5,0g
Distilled H_2O		1 000ml

Tryptone, dextrose and NaCl were dissolved in distilled water, and sterilised in the autoclave for 15min at 121°C.

Antibiotic solution

Ampicillin	(Sigma)	150,0mg
Streptomycin	(Sigma)	150,0mg
Distilled H_2O		100ml

The solution was decontaminated by filtration through an 0,22*u*m membrane filter and kept at 4°C for not longer than 1 week.

Phage bottom agar

Tryptone	(Difco)	10,0g
Dextrose	(Sigma)	1,0g
NaCl	(Holpro)	5,0g
Bacto agar	(Difco)	12,0g
Distilled H_2O		1 000ml

The ingredients were dissolved in distilled water, and autoclaved for 15min at 121°C. The temperature was lowered to 50°C, 20ml antibiotic solution was added and 20ml volumes were poured into 90mm diameter Petri dishes.
The plates were stored at 4°C for not more than 10 days.

Phage top agar
Same as phage bottom agar, except for 7,0g instead of 12,0g Bacto agar per 1 000ml.
Volumes of 2,5ml were dispensed into sterile tubes.
The phage top agar was stored at 4°C for not more than 30 days.

3.2.2.2 *Salmonella typhimurium* WG49
(According to ISO, 1991).

Calcium-glucose solution

$CaCl_2.2H_2O$	(Holpro)	3,0 g
Glucose	(Sigma)	10,0 g
Distilled H_2O		100 ml

The ingredients were dissolved in distilled water while heating gently, cooled to room temperature and decontaminated by filtration through an 0,22*u*m membrane filter.
The medium was stored in the dark at 4°C for not longer than 6 months.

Nalidixic acid solution

Nalidixic acid	(Sigma)	250 mg
NaOH-solution (1 mol/L)	(Holpro)	2,0 ml
Distilled H_2O		8,0 ml

The nalidixic acid was dissolved in the NaOH-solution, distilled water added and mixed well.
The solution was decontaminated by filtration through an 0,22*um* membrane filter and stored at -20°C for not longer than 1 month.

Growth medium (TYGB)

Trypticase peptone	(Difco)	10,0 g
Yeast extract	(Difco)	1,0 g
NaCl	(Holpro)	8,0 g
Distilled H_2O		1 000 ml

The ingredients were dissolved in distilled water, and the pH adjusted to 7,2 at 25°C.
The medium was autoclaved at 121°C for 15min and the pH checked to be 7,2 after autoclaving.
Calcium-glucose solution was added aseptically and the medium was mixed well.

Phage bottom agar (TYGA)

Trypticase peptone	(Difco)	10,0 g
Yeast extract	(Difco)	1,0 g
NaCl	(Holpro)	8,0 g
Bacto agar	(Difco)	14,0 g
Distilled H_2O		1 000 ml

The ingredients were dissolved in distilled water, and the pH was adjusted to 7,2 at 25°C.
The medium was autoclaved for 15min at 121°C and the pH checked to be 7,2 after autoclaving.
When interference by microbial growth was expected, 4ml nalidixic acid solution was added to obtain a final concentration of 100*um*/ml.
Calcium-glucose solution was aseptically added, the medium was mixed well and 20ml volumes were poured into 90mm diameter Petri dishes.
The plates were stored in the dark at 4°C for not longer than 1 month.

Phage top agar

Same as phage bottom agar, except for 7,0g instead of 14,0g Bacto agar per 1 000ml, and 2,5ml volumes were dispensed into sterile tubes.
The phage top agar was stored in the dark at 4°C for not more than 30 days.

Host cultures

(a) ***Escherichia coli*** **HS (pFamp)R**

This host was kindly supplied by Prof V J Cabelli.
A lyophilised host culture was rehydrated in tryptone broth, and incubated for 24h at 37°C. A loopful of this overnight culture was streaked onto a MacConkey agar plate for single colonies. The plate was incubated for 24h at 37°C. A lactose-negative colony was selected, subcultured onto a tryptone agar slope with ampicillin (1,5 mg/ml) and streptomycin (1,5 mg/ml) and incubated for 24h at 37°C.

The cultures were stored at -4°C and subcultured every 2-4 weeks. Sensitivity for phage MS-2 was checked regularly.

Preparation of inocula for plaque assays
Six hours before the assays were to be carried out, 0,3ml of the antibiotic solution was aseptically added to tubes containing 10ml of tryptone broth.
An inoculum of the stock culture was then transferred to the tubes.
The cultures were incubated at 37°C for 6h without shaking.

(b) *Salmonella typhimurim* Nal^r (F'lac :: Tn5) (WG49)
(According to ISO, 1991)
This host was kindly supplied by Dr A H Havelaar.
A lyophilised host culture was rehydrated in 5ml of TYGB using a Pasteur pipette.
The suspension was transferred to 50ml TYGB in a 300ml Erlenmeyer flask.
The culture was incubated for 18h at 37°C while shaking at 100min^{-1}.
A loopful of this overnight culture was streaked onto a MacConkey agar plate for single colonies. The plates were incubated for 24h at 37°C.
A lactose-positive colony was selected and inoculated into 50ml of TYGB in a 300ml Erlenmeyer flask.
The Erlenmeyer flask was incubated for 7h at 37°C while shaking at 100min^{-1}.
After addition of 10ml of glycerol and thorough mixing 1,2ml aliquots were dispensed into cryo tubes which were then stored at -70°C.

Quality control of host strain WG49 and calibration of turbidity measurements
One vial of host culture was taken from the freezer and thawed at room temperature.
TYGB (50ml) was added to a nephelometric Erlenmeyer-flask (Figure 3-1) and rewarmed to 35-37°C.
After the spectrophotometer reading was adjusted to 0 on the filled side-arm, 0,5ml of host culture was inoculated.
The host culture was incubated at 37°C while shaking at 150min^{-1} up to 3h.
Turbidity was measured every 30min and a 1ml sample withdrawn for viable counts, assuring that the flask was taken from the incubator as short as possible.
Samples were diluted to 10^{-6} and 0,1 volumes of the 10^{-4}, 10^{-5} and 10^{-6} dilutions were spread on TYGA plates, and incubated at 37°C for 18h.
At t=0h and t=3h, two plates of MacConkey agar were inoculated with the same dilutions and incubated similarly.
The total number of colonies on each plate containing between 30 and 300 colonies was counted and the number of CFU/ml calculated.
The number of lactose-positive and lactose-negative colonies was also counted.
At times t=0 and t=3h, 0,1ml of the 10^2 dilution was spread on a plate of MacConkey agar. One disc with nalidixic acid (Nal)(30*u*g) and one disc with kanamycin (Km)(1 000*u*g) were placed on the plates and incubated for 18h at 37°C.
The zones of inhibition around the antibiotic discs were measured.
The host-strain culture was accepted if the following criteria were met:
Plate count on TYGA at 0 h: 1 - 3 x 10^7 CFU/ml
Plate count on TYGA at 3 h: 7 - 40 x 10^7 CFU/ml
Lactose-negative colonies (plasmid segregation) < 5 %
Inhibition zone around Nal-disc : absent
Km-disc : < 15mm diameter
The host-strain culture was checked for sensitivity to F-specific RNA bacteriophages

regularly.

Preparation of inocula for plaque assays
One vial of host culture was taken from the freezer and thawed at room temperature.
TYGB medium (50ml) was added to a nephelometric Erlenmeyer-flask, warmed to 35-37°C, and the spectrophotometric reading adjusted to 0 using the filled side-arm for reading.
A 0,5ml inoculum of the thawed host culture was then added to the TYGB medium.

This host culture was incubated at 37°C while shaking at 150 min^{-1} up to 3h.
Turbidity was measured every 30min.
At a turbidity corresponding to a cell-density of approximately 10^8 CFU/ml, the culture was taken from the incubator and quickly cooled on melting ice.
The culture was used within 2h.

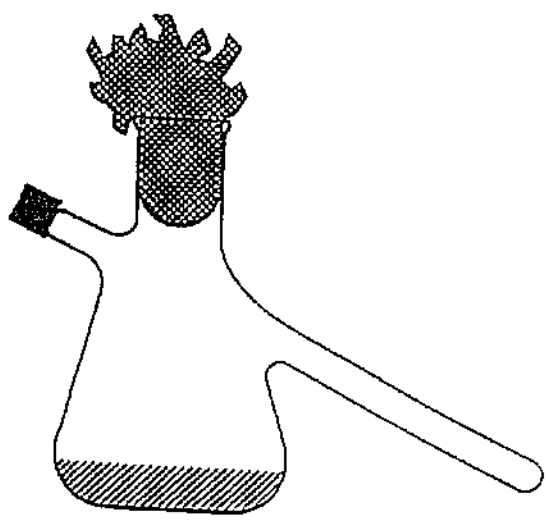


FIGURE 3-1 Nephelometric Erlenmeyer flask for the culturing of the host (ISO, 1991)

3.2.3 Double-Agar-Layer Plaque Assay for phages of *Bacteroides fragilis* HSP40

Growth medium (MBB)

Tryptone	(Difco)	10,0 g
Beef extract	(Difco)	10,0 g
Yeast extract	(Difco)	2,0 g
NaCl	(Holpro)	5,0 g
$CaCl_2.2H_2O$ (0,5% in H_2O)	(Merck)	10,0 ml
$MgSO_4.7H_2O$ (1,2% in H_2O)	(Merck)	10,0 ml
Cysteine HCl.$9H_2O$	(Sigma)	0,5 g
Distilled H_2O		1 000 ml

The ingredients were dissolved in distilled water and autoclaved for 15min at 121°C.
The medium was kept at 60-80°C for further processing.
The following were added to the medium:

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

The pH was adjusted to 7,0 with concentrated HCl.
The following were then added to the medium:

Kanamycin sulphate	(Sigma)	100 *u*g/ml
Vancomycin	(Sigma)	7,5 *u*g/ml

Phage bottom agar

Bacto agar	(Difco)	12,0 g
Tryptone	(Difco)	10,0 g
Yeast extract	(Difco)	2,0 g
NaCl	(Holpro)	5,0 g
$CaCl_2$ (0,5% in H_2O)	(Merck)	10,0 ml
$MgSO_4.7H_2O$ (1,2% in H_2O)	(Merck)	10,0 ml
Cysteine HCl.$9H_2O$	(Sigma)	0,5 g
Distilled H_2O		1 000 ml

The ingredients were dissolved in distilled water, and autoclaved for 15min at 121°C.
The medium was kept at 60-80°C for further processing.
The following were added:

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

The pH was adjusted to 7,0 using concentrated HCl.
The following were then added:

Kanamycin sulphate	(Sigma)	100 *u*g/ml
Vancomycin	(Sigma)	7,5 *u*g/ml

20ml volumes were poured into 90mm diameter Petri dishes.
Plates were stored at 4°C for not more than 4 days.

Phage top agar

Same as phage bottom agar, except for 7,0g instead of 12,0g Bacto agar per 1000ml, and 2,5ml volumes were dispensed into sterile tubes.
The phage top agar was stored at 4°C and used within the following 24h.

Host culture
Cultures of *Bacteroides fragilis* HSP40 were kindly supplied by Prof J Jofre, University of Barcelona (Spain).
Agar plates containing kanamycin and vancomycin were inoculated from these cultures in such a way that single colonies were obtained.
The plates were incubated for 48h under anaerobic conditions at 37°C.
a Gram-negative colony (pale, pleomorphic or uniform gram-negative rods with irregular staining), was inoculated into growth medium and incubated anaerobically for 48h at 37°C.
Isolated colonies suitable for single colony subcultures were available after 48h of incubation.
Agar to agar subcultures of the host bacterium were made by transferring heavy inocula with a sterile needle into agar slopes containing kanamycin sulphate (100*u*g/ml) and vancomycin (7,5*u*g/ml).
The agar slopes were stored at 4°C in anaerobic jars and subcultured every 2 weeks.
Sensitivity for phage B40-8 was checked regularly.

3.2.4 Plaque assay for phages
(Adams, 1959)
Tubes with phage top agar were steamed to liquify agar and adjusted to 48°C in a thermostatic waterbath.
A volume of 1,0ml of the test sample, or of an appropriate dilution of the test sample, was added to the top agar in a test tube, followed by the addition of 0,3ml of an appropriate logarithmic growth phase broth culture of the host.
The test sample or appropriate dilutions of phage suspension were added to the series of tubes of molten top agar first as the phages are likely to be less heat-sensitive than the host.
The above was repeated in threefold to obtain phage counts per 3ml.
Exposure of inoculated tubes to 48°C was as short as possible in order to limit inactivation of phages or hosts.
The contents of the tubes were gently mixed, avoiding bubble formation.
The top agar mixture was poured onto the bottom agar layer in a phage agar plate, distributed evenly and allowed to solidify on a perfectly horizontal, cool surface.
Inverted plates were incubated overnight at 37°C under aerobic conditions for male-specific coliphages and somatic coliphages, and under anaerobic conditions for *Bacteroides fragilis* phages.
The plaques were counted with a counting apparatus with indirect, oblique light, selecting plates with 30-100 plaques.
The results were expressed as the number of plaque-forming units per volume (PFU/ml).

3.2.5 Preparation of phage stock
Two methods were used to prepare stock suspensions of phages - the plate method and the broth method.

Plate method
A dilution of the specific phage that produced semi-confluent lysis was inoculated with the appropriate bacterial host into tubes containing phage top agar and poured onto phage bottom agar plates.
Following 18h of incubation, 2,5ml of broth medium was dispensed onto each plate.

The broth with top agar was collected by scraping from the bottom agar using a glass rod.
This suspension was homogenised by vigorous shaking and then centrifuged at 7 000rpm for 10min.
Most bacteria were deposited by the centrifugation, leaving the phages in the supernatant.
Chloroform (10% vol/vol) was added to the supernatant and the stock solutions of phages were stored at 4°C.

Broth method
Phages were added to the host culture near the end of the log phase to ensure that nearly every cell is infected simultaniously.
The suspensions were centrifuged at 7 000rpm for 10min after lysis had occured (visible as a marked drop in turbidity).
Chloroform treatment was applied after centrifugation as with the plate method.

3.2.6 Effect of naladixic acid on phage assays
Two strains of *E coli* C, namely WG4 and the naladixic acid-resistant mutant WG5, were evaluated to determine the effect of the addition of antibiotics on the number of plaques formed.

3.2.7 Effect of inoculum size on plaque assays
To evaluate the effect of the inoculum size of the host bacterium on plaque assays, 0,3ml, 0,5ml and 1,0ml quantities of host bacterium were added to dilutions of the stock cultures of the phages. The aim was to obtain a layer of bacterial cells which could multiply and produce a confluent lawn and not separate colonies.

3.3 RESULTS

3.3.1 Plaque assays
Plaques were successfully obtained with all the host bacteria used. Figure 3-2 shows plaques obtained with the bacterial host *Bacteroides fragilis* HSP40 and the phage B40-8.

3.3.2 Comparison of broth and plate methods for the preparation of phage stock
The plate method usually gave higher titres of phages (Table 3-1).

3.3.3 Effect of nalidixic acid on plaque assays
In comparitive tests done on 88 sewage samples using the nalidixic acid-sensitive (WG4) and -resistant (WG5) strains of *E coli* C, the addition of nalidixic acid markedly reduced bacterial background growth of contaminant bacteria without any effect on the number of plaques formed (See Chapter 6). Figure 3-3(a) shows plaque assays without the addition of antibiotics and by using the bacterial host WG4. Figure 3-3(b) shows plaque assays with the addition of 330 *u*g/ml nalidixic acid and by using the bacterial host WG5.

3.3.4 Effect of inoculum size on plaque assays

The different quantities of host bacterium yielded no differences in the number of plaques formed. The volume of inoculum that gave the best lawn was 0,3ml for the *E coli* C and *B fragilis* host preparations. If more of the host bacteria were added, the hosts tended to fail to multiply because of a lack of nutrients. In the case of *S typhimurium* WG49 1ml of host culture preparation yielded the best results, while single colonies were obtained when smaller quantities of host bacteria were used.

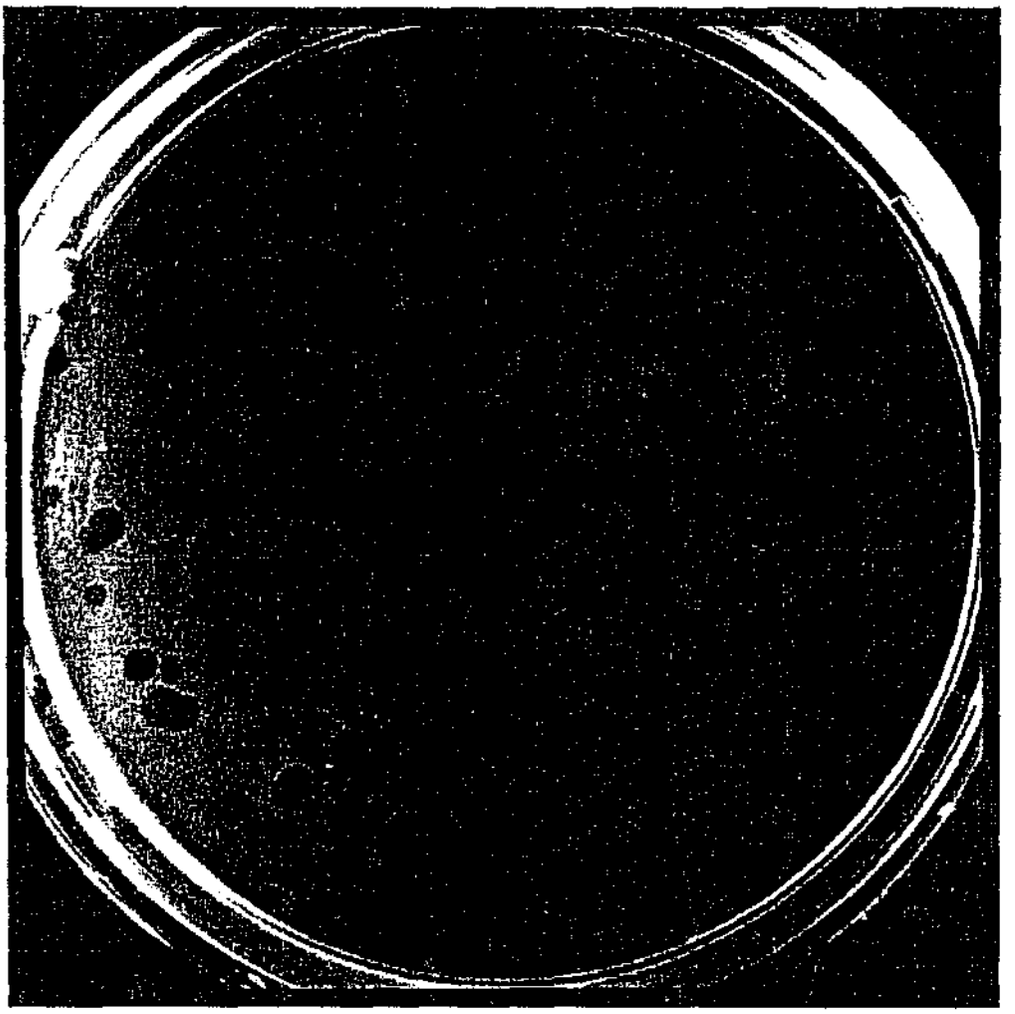
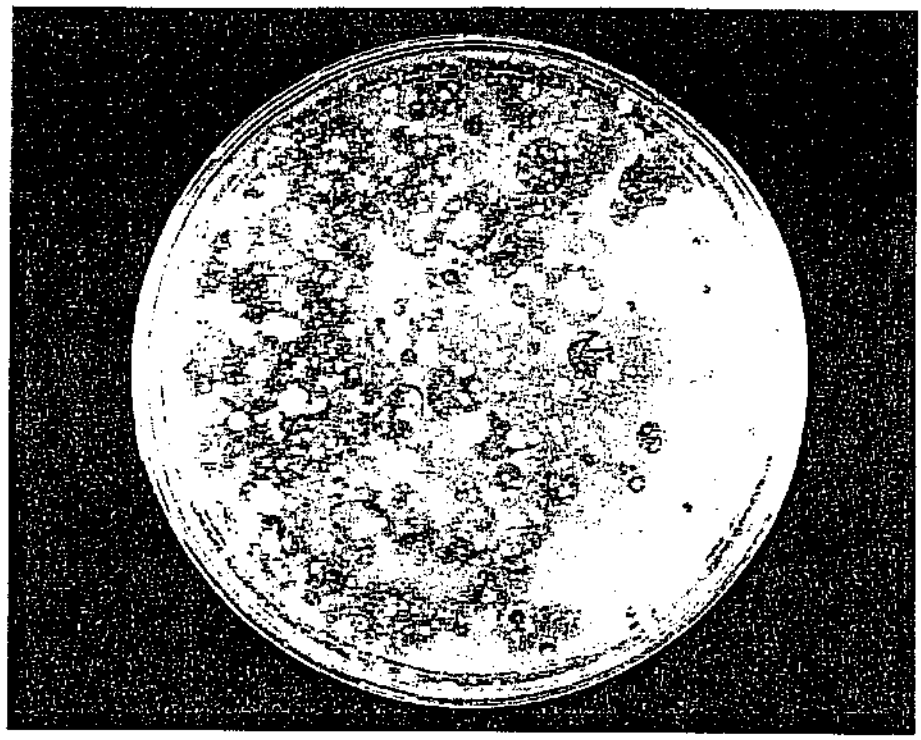


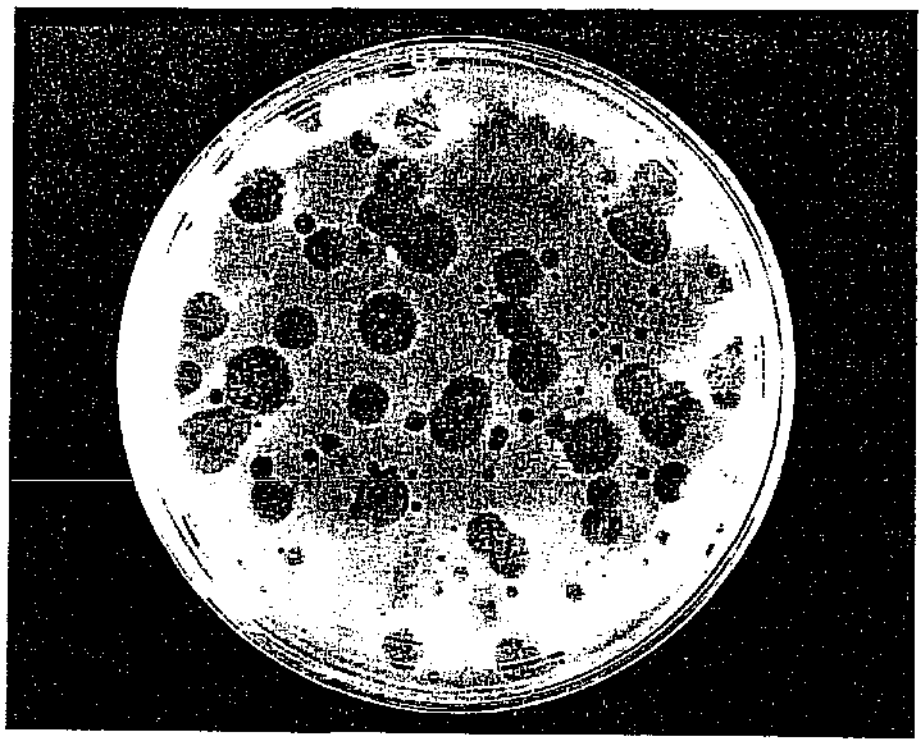
FIGURE 3-2 Plaques obtained with the bacterial host *B fragilis* HSP40. Plaques (clear zones) are formed when bacterial growth in a Petri dish is lysed by bacteriophages.

TABLE 3-1 Average counts of phages obtained with the broth and the plate methods.

Method	Phage (titre/ml)		
	V_1 (n = 5)	MS-2 (n = 4)	B40-8 (n = 3)
Broth	$4,6 \times 10^{7}$	$8,0 \times 10^{5}$	$6,9 \times 10^{8}$
Plate	$3,8 \times 10^{12}$	$3,1 \times 10^{12}$	$4,7 \times 10^{10}$



(a) without nalidixic acid



(b) with nalidixic acid

FIGURE 3-3 Effect of nalidixic acid on bacterial background:
(a) without nalidixic acid
(b) with nalidixic acid

3.4 DISCUSSION

In studies covered by this chapter methods have been established and optimised for the detection of somatic coliphages, male-specific coliphages and the bacteriophages of *Bacteroides fragilis*. This work included procedures for the handling, storage and preservation of internationally recognised and applied host strains for detection of the phages, as well as procedures for the cultivation, typing, storage and titration of type-specific phages for each of the three groups of viruses. The procedures which have been established are in agreement with internationally accepted methodology.

The observation that plate cultures yielded higher phage titres than broth cultures has valuable implications for the preparation of high titre phage stocks. The finding that the nalidixic acid-resistant mutant (WG5) of the *E coli* C (WG4) host strain yields counts of phages in the presence of nalidixic acid comparable to those of WG4 in the absence of nalidixic acid, implies that WG5 can be used conveniently and accurately for the detection of somatic coliphages in waters which contain high numbers of bacteria which may interfere with the plaque detection of phages.

Objectives accomplished in this Chapter imply that the materials and methods are available for research on the phages concerned.

CHAPTER 4

RECOVERY OF PHAGES

4.1 INTRODUCTION

The bacteriophages selected for research in this study generally occur in sewage in numbers high enough for enumeration by direct plaque assays. However, in environments such as purified sewage, river water, shellfish meat and drinking water, numbers of the phages are too low for detection by direct plaque assays (Sorber *et al*, 1971; Divizia *et al*, 1989; Payment *et al*, 1989; Borrego *et al*, 1991). In order to use the phages as indicators of sewage pollution or the presence of human viruses it is, therefore, essential to have reliable and accurate methods for the detection of small numbers of phages in large volumes of water (Hill *et al*, 1971; Goyal and Gerba, 1982). The detection of small numbers of phages in large volumes of water or food such as shellfish meat may be accomplished by the following two approaches:

(a) The quantitative recovery of phages from large volumes followed by plaque assay detection, which yields a quantitative assessment of numbers of phages.

(b) The qualitative enrichment of phages in large volumes by the addition of appropriate hosts and nutrients, incubation and eventual detection of phages by plaque assays, which yields a qualitative presence/absence result for the volume tested.

Available data on the wide variety of recovery techniques described in the literature as reviewed in Chapter 2, show that the most successful approaches are based on ultrafiltration and adsorption-elution principles. Research on the selection and optimisation of appropriate recovery techniques in this study is, therefore, restricted to these two approaches.

4.2 MATERIALS AND METHODS

4.2.1 Indicator bacteria and phages

The host bacteria *E coli* C(ATCC 13706), *E coli* HS(pFamp)R and *B fragilis* HSP40 were used for the enumeration of the phages V_1, MS-2 and B40-8, respectively. Stocks of all bacteriophages were prepared by lysis of liquid culture. Cultures were clarified by centrifugation at 7 000rpm for 10min and the chloroform-treated supernatants stored at 4°C.

4.2.2 Phage assay procedure

Phages were assayed by the double-agar-layer method described in Chapter 3.

4.2.3 Ultrafiltration recovery

10 and 20L volumes of treated drinking water were dechlorinated with 10% sodium thiosulphate and seeded with 1ml of stock phages of known titre. The molecular filtration unit (Pellicon cassette TFF system, Millipore corp., Bedford, Mass.) was

equipped with a PTGC (polyethersulfone), pH range 2-14, membrane which had a total surface area of 12,5m^2 and a nominal molecular weight limit of 10 000. The membrane retained components with a minimum size of 0,005 to 0,02*um*. The membrane was stored at 4^{0}C in 0,1% of formaldehyde. Before each run the membrane was washed with distilled water to remove any trace of formaldehyde. The water to be processed was pumped through the inlet port. Retained material flowed tangentially across the membrane, out of the filter holder through the retentate outlet port, and returned to the original sample flask. As the water passed across the membrane surface, a certain fraction of the volume passed through the two filtrate outlet ports for separate collection. Figure 4-1 illustrates the tangential flowpath of the Pellicon cassette unit. For the best separation performance, a balance of pump rate and pressure was required. The filtrate and retentate volumes were recorded, and a portion of each was assayed. Sterilisation was achieved by recirculating a 0,1% solution of NaOCl for 10min. This was followed by a sterile water wash which was continued until water leaving all ports was free of chlorine by the Palin test using DPD no4 tablets. The results were expressed in terms of the percentage phage recovery from initial suspension to final retentate.

4.2.4 Positively-charged cartridge filter recovery

Zetapor positively charged microporous cartridge filters with a pore size of 0,45*um* were used (CUNO, Inc., Meriden, U.S.A). Test samples were dechlorinated by adding 2,5ml of a 10% sodium thiosulphate solution to 10L of tap water (APHA, 1989). The pH was adjusted by slow addition of 1N HCl to 5,5 with constant stirring. Samples of 20L of treated drinking water were seeded with 1ml of stock phages of known titre. Thorough mixing of the phages was accomplished with a portable Gallenkamp magnetic stirrer and pH levels were monitored using a pH meter. After mixing, a 5ml aliquot was removed to determine the input number of phages. The samples were then pumped through the cartridges. Elution from the cartridge was accomplished by recirculation of 500ml of eluent for 5min, using a highspeed peristaltic pump. The pH of the eluate was immediately adjusted to pH 7,0 by slow addition of 1N HCl with constant stirring. After measuring the total volume, the eluate was assayed for phages. The results were expressed in terms of the percentage phage recovery from initial suspension to final eluate.

4.2.5 Glass powder recovery

The charge of 100g of glass powder was modified by polyethylenimine (PEI) (Sigma) treatment (Preston *et al*, 1988). 100g of glass powder was mixed with 200ml of 2% PEI solution in distilled water. The mixture was shaken for 5h, then the glass powder was allowed to settle and the supernatant was decanted. The glass powder was air dried overnight at room temperature and then used for recovery of phages. The glass powder apparatus described by Schwartzbrod and Lucena (1978) was used in some experiments. In the other experiments a small modified version (Grabow, unpublished) based on the same principle was used. Chlorine in the water samples was neutralised by adding 2,5ml of a 10% sodium thiosulphate solution to 10L of water. The samples were seeded with phages V_1, MS-2 and B40-8. Aliquots of 5ml were removed to determine the input number of bacteriophages. The water was passed under negative pressure through the glass powder in the apparatus.

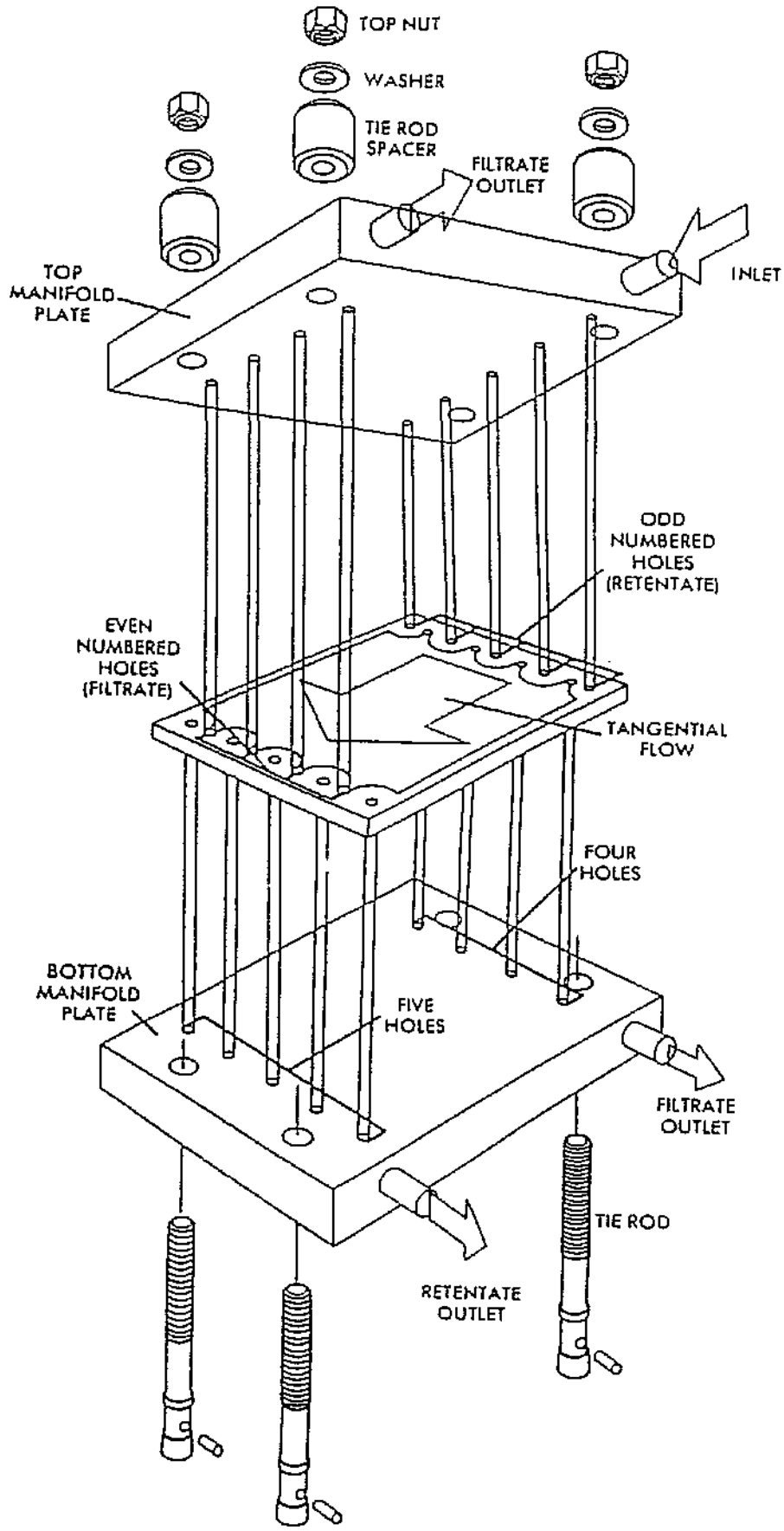


FIGURE 4-1 The tangential flowpath of the Pellicon cassette unit.

Phages were eluted with 100ml of buffer. The buffers used were 0,05M glycine (pH 11,5), 0,05M glycine-3% beef extract (pH 9,5) and 1M NaCl-3% beef extract (pH 9). The eluant was aspirated by negative pressure and vigorously bubbled through the glass powder for 1min. Neutralised eluates were assayed for phages. The results were expressed in terms of the percentage bacteriophage recovery from initial suspension to final eluate.

4.2.6 Enrichment

Nutrients for individual hosts in concentrations as described in Chapter 3 were added to 100ml of test water. Log-phase cultures (3ml) of the appropriate host bacteria were added to these suspensions and incubated overnight at 37°C. After incubation the cultures were tested for the presence of phages by plaque assays or spot tests as described in Chapters 3 and 5, respectively. Results were expressed as positive or negative.

4.3 RESULTS

Results for the recovery of phages with the Pellicon cassette system are shown in Tables 4-1 and 4-2. When 20 litres were processed, the recovery of phage V_1 averaged 30% (n=7) with a range of 11 to 57%; phage MS-2 averaged 19% (n=7) with a range of 0,01 to 72%; and phage B40-8 averaged 34% (n=7) with a range of 1 to 74%. When 10 litres were processed, the recovery of phage V_1 averaged 40% (n=5) with a range of 4 to 73%; phage MS-2 averaged 58% (n=6) with a range of 30 to 93%; and phage B40-8 averaged 28% (n=3) with a range of 22 to 39%. The substantial difference in the efficiency of recovery of MS-2 phage from 10L and 20L samples cannot be explained.

Results for the recovery of phages with the positively-charged cartridge filter are shown in Table 4-3. The recovery of phage V_1 averaged 18% (n=8) with a range of 0 to 48%; phage MS-2 averaged 17% (n=8) with a range of 0 to 48%; and phage B40-8 averaged 31% (n=7) with a range of 0 to 83%.

Results for the recovery of phages V_1 and B40-8 with PEI-modified glass powder using 1M NaCl-3% beef extract buffer (pH 9,0) are shown in Table 4-4. The adsorption of phage V_1 averaged 97% with a range of 94 to 98%. The recovery of phage V_1 averaged 20% (n=6) with a range of 0 to 38%. The adsorption of phage B40-8 averaged 98% with a range of 93 to 100%. The recovery of phage B40-8 averaged 18% (n=6) with a range of 2 to 38%.

Results for the recovery of phages V_1 and B40-8 with PEI-modified glass powder using 0,05M glycine buffer (pH 11,5) are shown in Table 4-5. The adsorption of phage V_1 averaged 95% with a range of 86 to 99%. The recovery of phage V_1 averaged 2% (n=4) with a range of 0,02 to 6%. The adsorption of phage B40-8 averaged 79% with a range of 71 to 84%. The recovery of phage B40-8 averaged 0,5% (n=3) with a range of 0 to 1%.

Results for the recovery of phages V_1 and B40-8 with PEI-modified glass powder using 0,05M glycine-3% BE buffer (pH 9,5) are shown in Table 4-6. The adsorption of

phage V_1 averaged 97% with a range of 93 to 100%. The recovery of phage V_1 averaged 6% (n=9) with a range of 1 to 17%. The adsorption of phage B40-8 averaged 97% with a range of 92 to 100%. The recovery of phage B40-8 averaged 6% (n=5) with a range of 2 to 14%.

4.4 DISCUSSION

On average Pellicon ultrafiltration had a higher efficiency of recovery for the three phages tested (35%) than the positively charged cartridge filters (22%) or positively charged glass powder (9%). However, the ultrafiltration system is extremely expensive, and in addition the membrane needs to be replaced at very high cost after every six to eight experiments. It should also be kept in mind that all tests have been carried out on seeded tap water with a turbidity of less than 0,5NTU. In tests on water with higher turbidity, or tests on larger volumes, clogging may have substantial adverse effects on the efficiency of recovery and life-span of the ultrafilter membrane.

Adsorption of phages with PEI-treated glass powder was more than 95%, except for one set of experiments done with phage B40-8 (79%). However, problems were encountered with the elution of the phages and poor efficiencies of recovery were obtained. Elution with 1M NaCl-3% beef extract buffer (pH 9,0) yielded a substantially higher recovery of the phages than did elution with 0,05M glycine-3% beef extract buffer (pH 9,5) or 0,05M glycine buffer (pH 11,5). These findings are in agreement with those of Goyal *et al* (1980) who found that either 3% beef extract (pH 9,0) or 0,5M NaCl containing 4% beef extract (pH 9,0) to be the best eluant for recovery of phages MS-2, ϕX174, T2 and T4. The use of high-pH eluents (pH 10,0 to 11,5) may inactivate phages (Primrose *et al*, 1981). However, phage B40-8 is not affected by exposure to pH 10,5 (Jofre, personal communication). The low efficiency of recovery for phage B40-8 may be due to a joint effect of PEI and high pH.

The results of this evaluation of methods for the recovery of phages from seeded tap water indicate that positively charged cartridge filters have the most attractive features in terms of overall consideration of efficiency of recovery, cost and ease of operation. If the release of phages from positively charged glass powder could be improved, this very simple and inexpensive method would be the best of them all.

The efficiency of recovery of *B fragilis* phages and male-specific coliphages by the three methods investigated here has not previously been described.

The flow rate (tempo) of the Pellicon ultrafiltration system very heavily depended upon the condition of the membrane, primarily in terms of the state of clogging. As the membrane was reused, the flow rate decreased rapidly. When the flow rate decreased to virually zero, the experiment was terminated. In some experiments additional time was allowed to assess the feasibility of prolonged filtration. These are the reasons for the differences in tempo and final volumes recorded in Tables 4-1 and 4-2.

TABLE 4-1 Recovery of phages V_1, MS-2 and B40-8 with the Pellicon casette system from 20L samples of seeded tap water.

Time (min)	Tempo (ml/min)	Volume		% Recovery		
		Start (L)	Final (ml)	V_1	MS-2	B40-8
20	900	20	186	32	3	ND
37	750	20	122	39	0,3	ND
36	600	20	230	20	0,1	ND
27	900	20	200	57	47	36
26	950	20	160	13	9	1
20	1100	20	200	41	0,01	26
84	300	20	2200	11	72	74
n				7	7	4
Range				11-41	0,01-72	1-74
Average				30	19	34
Median				32	3	36

ND = not done

TABLE 4-2 Recovery of phages V_1, MS-2 and B40-8 with the Pellicon casette system from 10L samples of seeded tap water.

Time (min)	Tempo (ml/min)	Volume		% Recovery		
		Start (L)	Final (ml)	V_1	MS-2	B40-8
30	300	10	100	4	30	22
29	520	10	176	ND	93	39
58	420	10	360	73	57	23
45	290	10	80	20	67	ND
40	280	10	110	75	63	ND
34	360	10	154	27	39	ND
n				5	6	3
Range				4-75	30-93	22-39
Average				40	58	28
Median				26	63	23

ND = not done

TABLE 4-3 Recovery of phages V_1, MS-2 and B40-8 with the positively charged cartridge filter from 20L samples of seeded tap water.

pH	Time (min)	% Recovery		
		V_1	MS-2	B40-8
5,6	20	0	0	0,1
5,7	25	2	5	0
5,7	20	30	24	10
5,7	15	14	36	83
5,6	48	26	48	38
5,5	12	49	26	83
5,6	16	5	0	3
5,6	28	20	0,1	ND
n		8	8	7
Range		0-49	0-48	0-83
Average		18	18	31
Median		20	24	10

ND = not done

TABLE 4-4 Recovery of phages V_1 and B40-8 from seeded tap water by adsorption to positively charged glass powder and elution with 1M NaCl - 3% beef extract buffer (pH 9,0).

Sample volume (L)	Elution time (min)	% Adsorption		% Recovery	
		V_1	B40-8	V_1	B40-8
10	15	94	100	38	17
10	15	100	93	35	38
10	15	97	99	19	13
10	15	99	98	9	22
10	30	96	100	18	17
10	15	95	97	0,1	2
n		6	6	6	6
Range		94-100	93-100	0,1-38	2-38
Average		97	98	20	18
Median		97	99	19	17

ND = not done
Charge on glass powder adjusted to positive by treatment with poly-ethylene-imine.

TABLE 4-5 Recovery of phages V_1 and B40-8 from seeded tap water by adsorption to positively charged glass powder and elution with 0,05M glycine buffer (pH 11,5).

Sample volume (L)	Elution time (min)	% Adsorption		% Recovery	
		V_1	B40-8	V_1	B40-8
10	10	99	ND	1	ND
10	10	95	84	6	1
10	10	86	71	0,008	0,2
10	10	99	83	0,02	0
n		4	3	4	3
Range		86-99	71-84	0,008-6	0-1
Average		95	79	2	0,5
Median		99	83	1	0,2

ND = not done
Charge on glass powder adjusted to positive by treatment with poly-ethylene-imine.

TABLE 4-6 Recovery of phages V_1 and B40-8 from seeded tap water by adsorption to positively charged glass powder and elution with 0,05M glycine - 3% beef extract buffer (pH 9,5).

Sample volume (L)	Elution time (min)	% Adsorption		% Recovery	
		V_1	B40-8	V_1	B40-8
7	10	100	ND	4	ND
10	10	97	92	3	6
10	10	99	100	15	14
10	10	100	97	17	3
1	5	96	96	5	3
1	5	93	ND	1	ND
1	10	97	ND	12	ND
1	10	95	97	2	3
1	10	99	ND	2	ND
n		9	5	9	5
Range		93-100	92-100	1-17	3-14
Average		97	97	6	6
Median		97	97	5	3

ND = not done
Charge on glass powder adjusted to positive by treatment with poly-ethylene-imine.

CHAPTER 5

PHAGES EXCRETED BY HUMANS AND ANIMALS

5.1 INTRODUCTION

In a search for phage indicators which would not be able to replicate in the environment and which may, in addition, be specific for human faecal pollution, Tartera and Jofre (1987) investigated the potential of *Bacteroides fragilis* phages. These phages were selected because the host bacteria are strict anaerobes and natural inhabitants of the gastro-intestinal tract of warm-blooded animals. In view of their highly specialised growth requirements, the possibility of *B fragilis* bacteria being able to grow or support the replication of phages in the environment are negligible, which implies that the phages would be specific indicators of faecal pollution. A variety of *B fragilis* host strains was evaluated for features such as phage susceptibility, human specificity and feasibility for application as a laboratory host strain. Eventually one specific strain designated *B fragilis* HSP40 was selected for further investigation. Studies included a survey of the presence of *B fragilis* HSP40 phages in 40 human, 40 cow, 50 pig, 21 rabbit, 28 mouse, 20 hen and 10 quail stool samples. A sensitive enrichment procedure was used for the detection of phages. Positive results were obtained only for 4(10%) of human stool samples, which suggested that *B fragilis* HSP40 phages are highly specific indicators of human faecal pollution. The attractive possibilities of this innovative discovery for application in a new concept for water quality assessment was immediately recognised and researchers world-wide embarked on studies in which the indicator value of *B fragilis* HSP40 phages was evaluated in more detail.

The objective of this study was to obtain more information on the specificity of *B fragilis* HSP40 phages for human faeces, and to compare in local conditions the incidence of *B fragilis* HSP40 phages in the stool of humans and a variety of animals with that of somatic and male-specific coliphages more commonly used as water quality indicators. The results should cast valuable light on the indicator value of all three of these groups of phages.

The incidence of somatic and male-specific coliphages in human and animal faeces has been investigated in a number of studies. Dhillon *et al* (1976) detected somatic coliphages in 78% of cow, 90% of pig and 77% of human faeces samples. In tests using *E coli* HfrH as host, no male-specific coliphages were detected in any of the samples.

Havelaar *et al* (1986) detected male-specific coliphages in appreciable numbers (10^2-10^6 PFU/g) only in the faeces from pigs, broiler chickens, sheep and calves, but not from dogs, cows, horses and humans. Somatic coliphages were detected in virtually all samples investigated. Numbers of male-specific and somatic coliphages were related, but phage counts were not related to counts of faecal coliforms, faecal streptococci or sulphite-reducing clostridia.

5.2 MATERIALS AND METHODS

5.2.1 Stool samples

Human stool samples (20) were obtained at two teaching hospitals from black and white male and female patients varying in age from 6 months to 85 years. Stool samples (69) from domestic animals (cattle, sheep, pigs, horses, dogs, cats, geese and rabbits) were obtained from various sources. Stool samples from baboons and vervet monkeys (24) were collected at the animal research centre of our Faculty. Stool samples (17) from higher primates (gorilla, orang-outang and chimpanzee) were kindly supplied by the National Zoological Gardens in Pretoria. Only freshly defaecated samples were used, and these were collected in sterile bottles. The samples were frozen at -20°C as soon as possible and kept at this temperature until examination. Before use the samples were thawed at 4°C.

5.2.2 Bacterial strains

The following host strains as described in Chapter 3 were used for the detection of bacteriophages in stool samples:

(a) *Escherichia coli* C (WG5) for the detection of somatic coliphages.
(b) *Escherichia coli* HS(pFamp)R and *Salmonella typhimurium* WG49 for the detection of male-specific coliphages.
(c) *Bacteroides fragilis* HSP40 for the detection of *B fragilis* phages.

5.2.3 Enrichment procedure

All stool samples were processed and tested for the presence of various phages by means of an enrichment procedure followed by plaque spot testing. Stool samples (1g wet mass) were incubated in 22ml volumes of growth medium inoculated individually with 3ml quantities of host cultures in the exponential growth phase, followed by a plaque spot test the next day.

5.2.4 Spot tests

Chloroform (0,4ml) was added to 1ml of the incubated enrichment culture, vortexed twice and centrifuged at 7 000rpm for 5min. The bacterial overlay in plates for spot tests was prepared by adding 0,3ml of a host culture in the logarithmic growth phase to 2,5ml of molten phage top agar. This suspension was mixed and poured onto phage bottom agar in a plate and evenly distributed. Excess surface moisture of the seeded overlay was permitted to dry, and a drop from each of the chloroform-treated enrichment cultures was placed on the plate, using a sterile inoculation loop. Not more than three evenly distributed spots were placed on each plate. After the spots had dried, the plates were incubated overnight at 37°C and then examined for bacterial lysis at each of the inoculated areas. Clear zones in the spotted areas were interpreted as phage lysis and reported as positive for isolation of phages to which the particular host strain is susceptible. If no clear zones formed, the samples were interpreted as negative. False-positive results for male-specific coliphages were ruled

out by testing the ability of the phages to infect the host bacteria at 28°C. Male-specific coliphages produce plaques only at about 37°C and not at 28°C, as described in Chapter 3.

5.2.5 Isolation of phages

Some somatic phage isolates of the higher primates were isolated and investigated by electron microscopy. Single plaques were removed by cutting the area around the plaque and lifting the agar from underneath with a sterile loop. The isolated plaques were transferred to 5ml nutrient broth. Cultures (2ml) of host bacteria in the logarithmic growth phase were added to each sample of isolated phages and incubated at 37°C for 2h. The bacteria with adsorb phages were recovered by centrifugation at 7 000rpm for 10min in a Sorvall centrifuge. The bacterial pellets were resuspended in distilled water and centrifuged in a Beckman ultracentrifuge at 35 000rpm for 90min. The supernatants were discarded and the pellets were resuspended in a small volume of distilled water.

5.2.6 Electron microscopy

The washed suspension of host bacteria with phages was negatively stained with 2% (W/V) phosphotungstic acid solution (pH 6,8) and placed on a 400 mesh Formvar carbon coated grid. The grid was dried and examined in a Philips 300 EM at 60kV as described by Grabow (1992). Electron microscopy was kindly carried out by Dr M B Taylor of our Department.

5.3 RESULTS

Results in Table 5-1 show that somatic coliphages were detected in 80% of human, 76% of domestic animal, 59% of monkey/baboon and 63% of higher primate stools. Male-specific coliphages were detected in 57% of human, 65% of domestic animal, 75% of monkey/baboon and 56% of higher primate stool samples when using the *E coli* host. Male-specific coliphages were detected in 40% of human, 45% of domestic animal, 54% of monkey/baboon and 44% of higher primate stool samples when using the *S typhimurium* host. *Bacteroides fragilis* phages using strain HSP40 as host were detected in 15% of human stools but in no animal stools except for one out of 19 samples of goose faeces.

Figure 5-1 shows a positive result of a spot test done on bacterial host *E coli* HS(pFamp)R as host. Clear spots are visible where lysis of the host bacteria occurred.

Figures 5-2, 5-3 and 5-4 show plaque assays of somatic coliphages isolated from higher primates.

Figures 5-5, 5-6 and 5-7 show electron micrographs of some of the somatic coliphages isolated from higher primates.

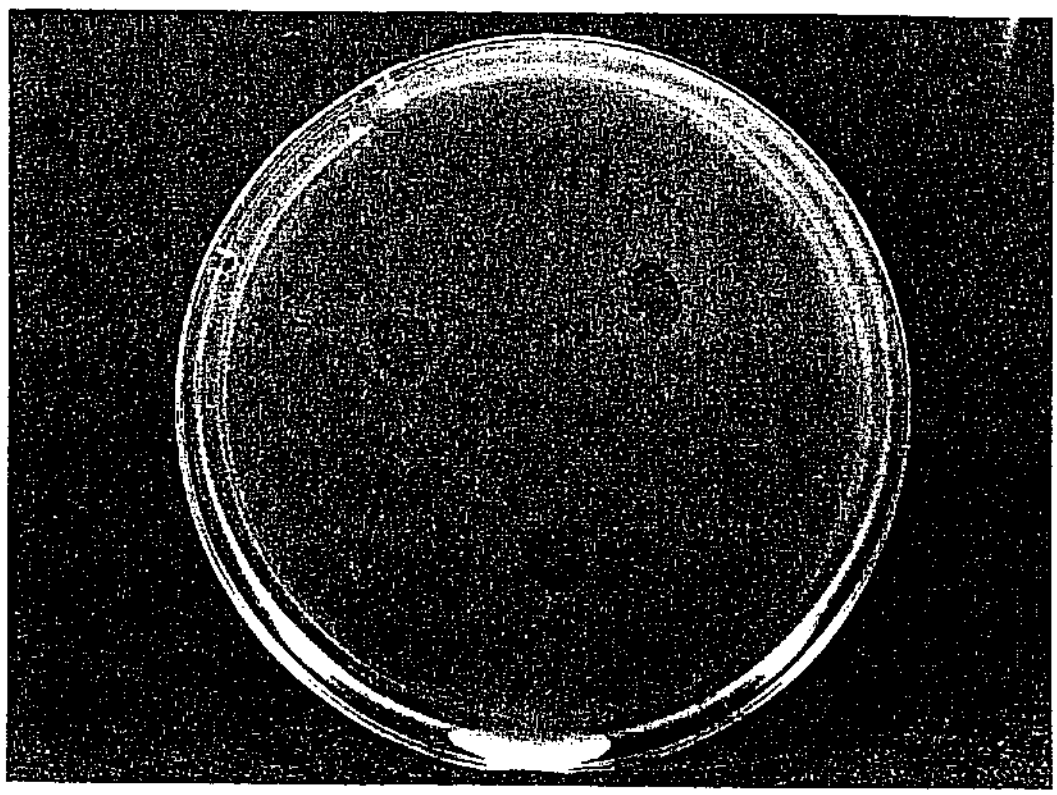


FIGURE 5-1 Spot test for male-specific coliphages using *E coli* HS(pFamp)R as host. Positive results are clearly visible in the form of complete lysis of host bacteria in the three spots.

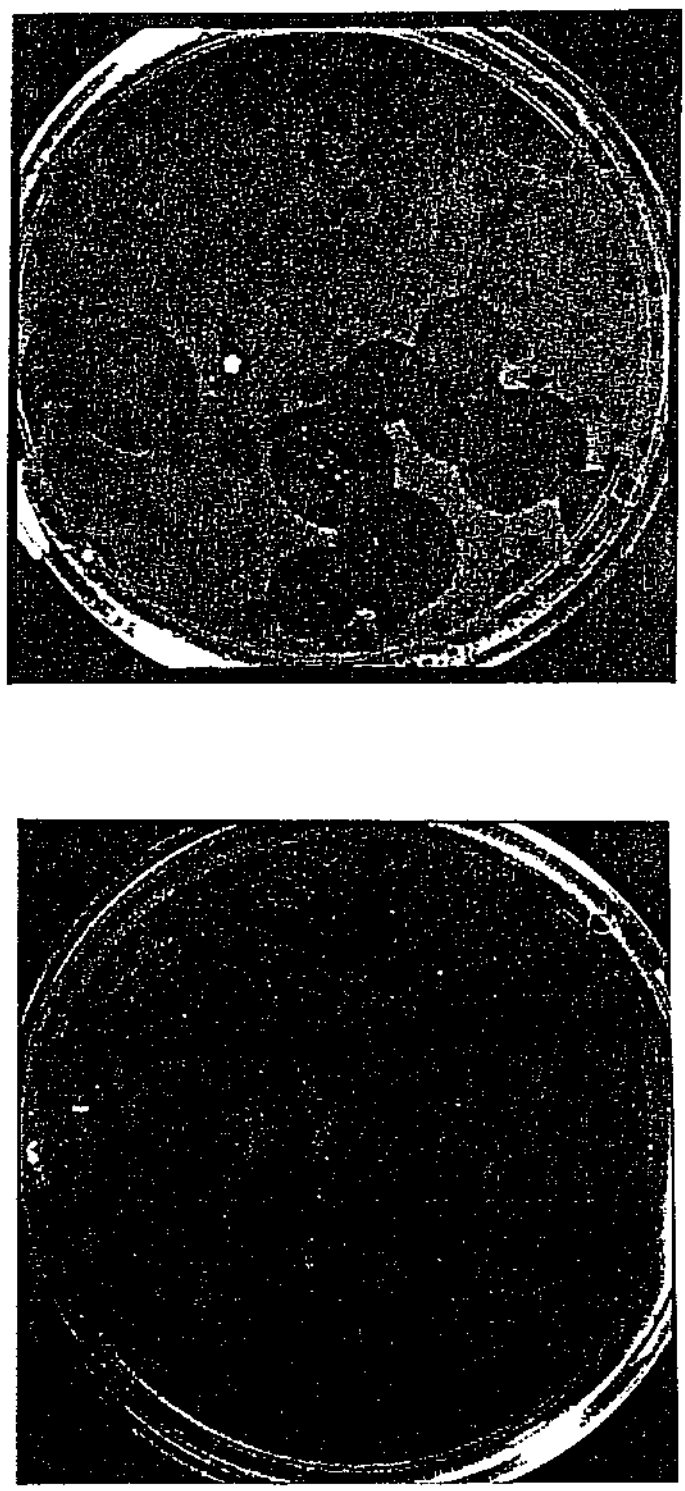


FIGURE 5-2 Plaque assay of somatic coliphages isolated from a chimpanzee stool sample.

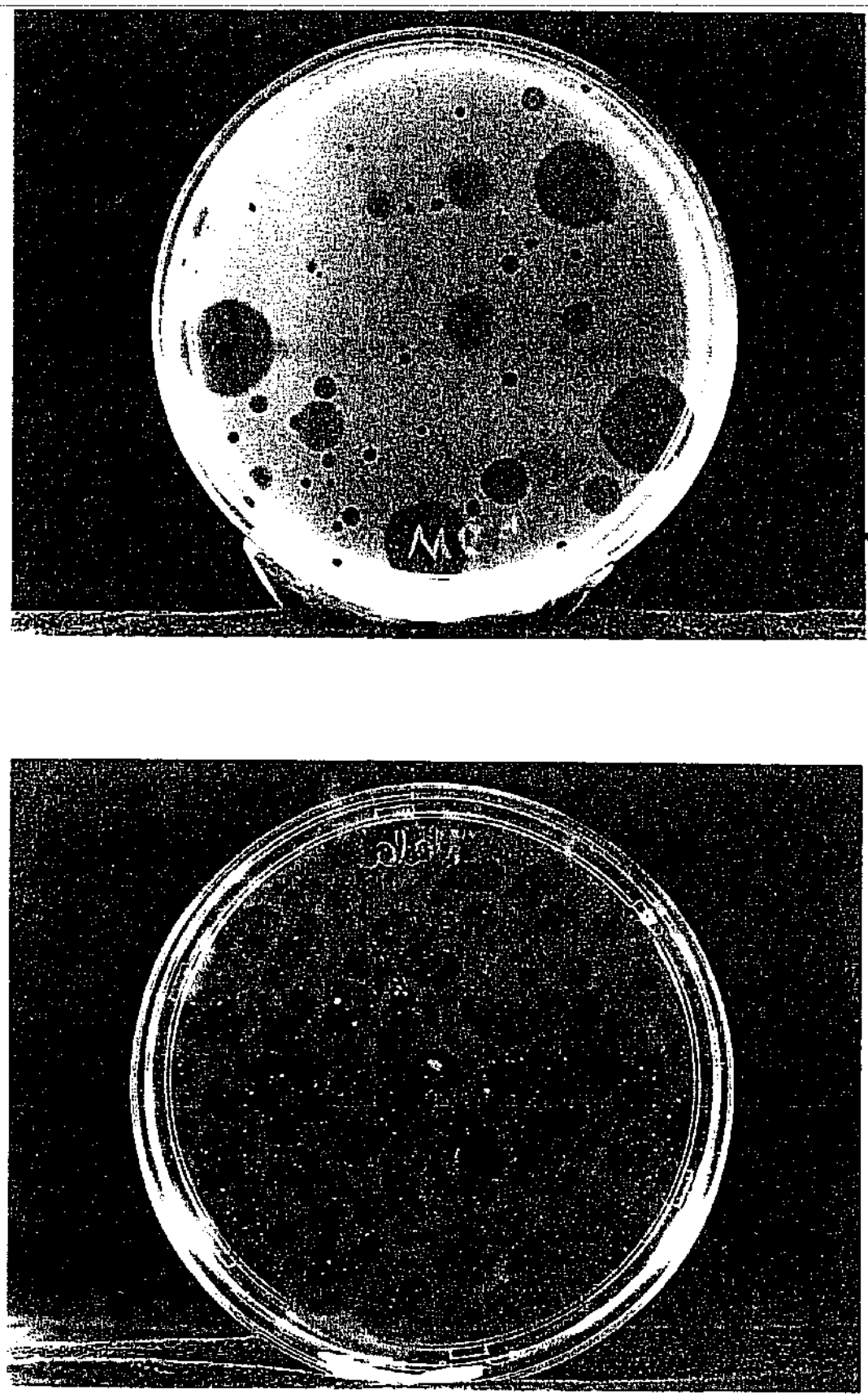


FIGURE 5-3 Plaque assays of somatic coliphages isolated from an orang-outang stool sample.

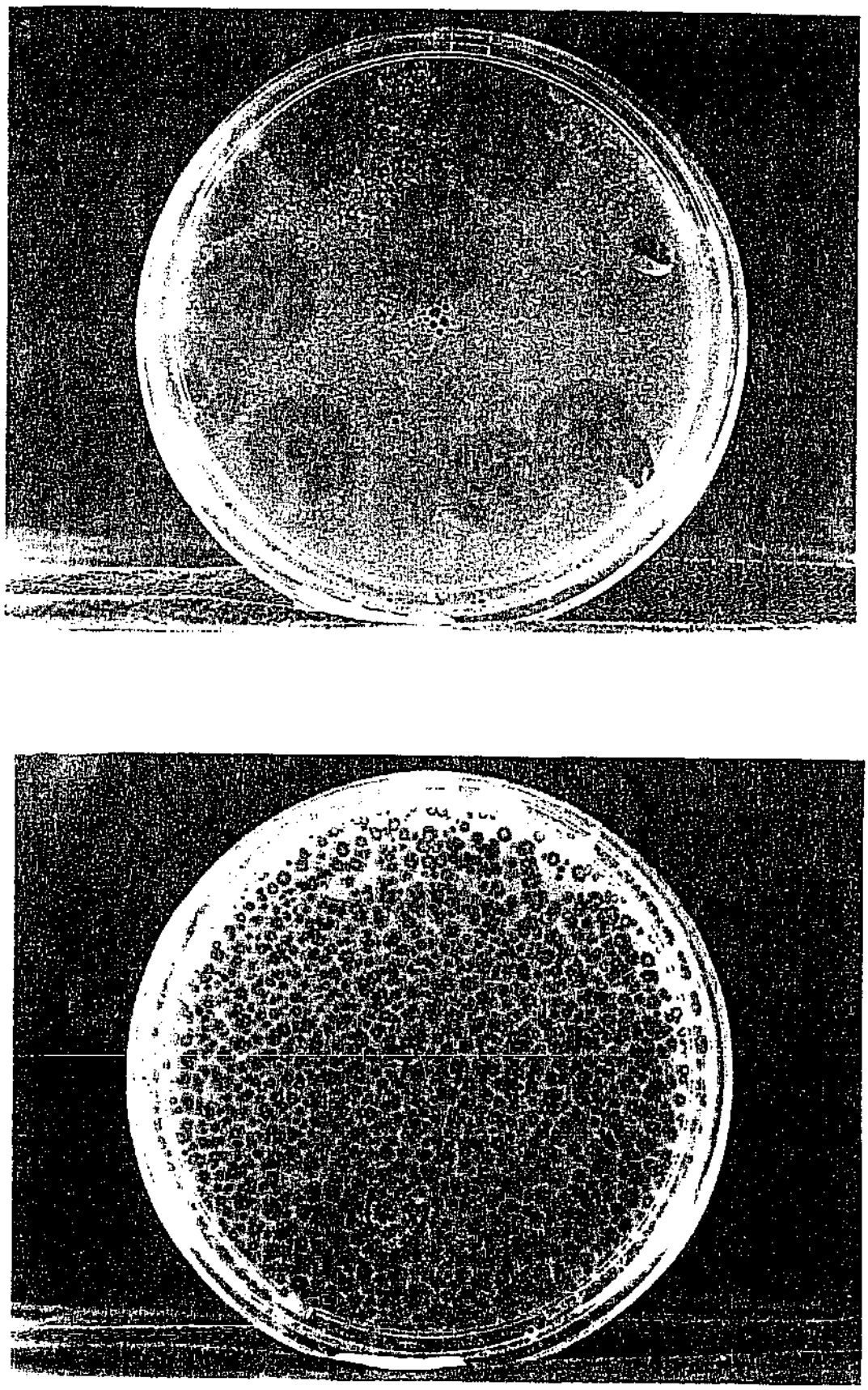


FIGURE 5-4 Plaque assays of somatic coliphages isolated from a gorilla stool sample.

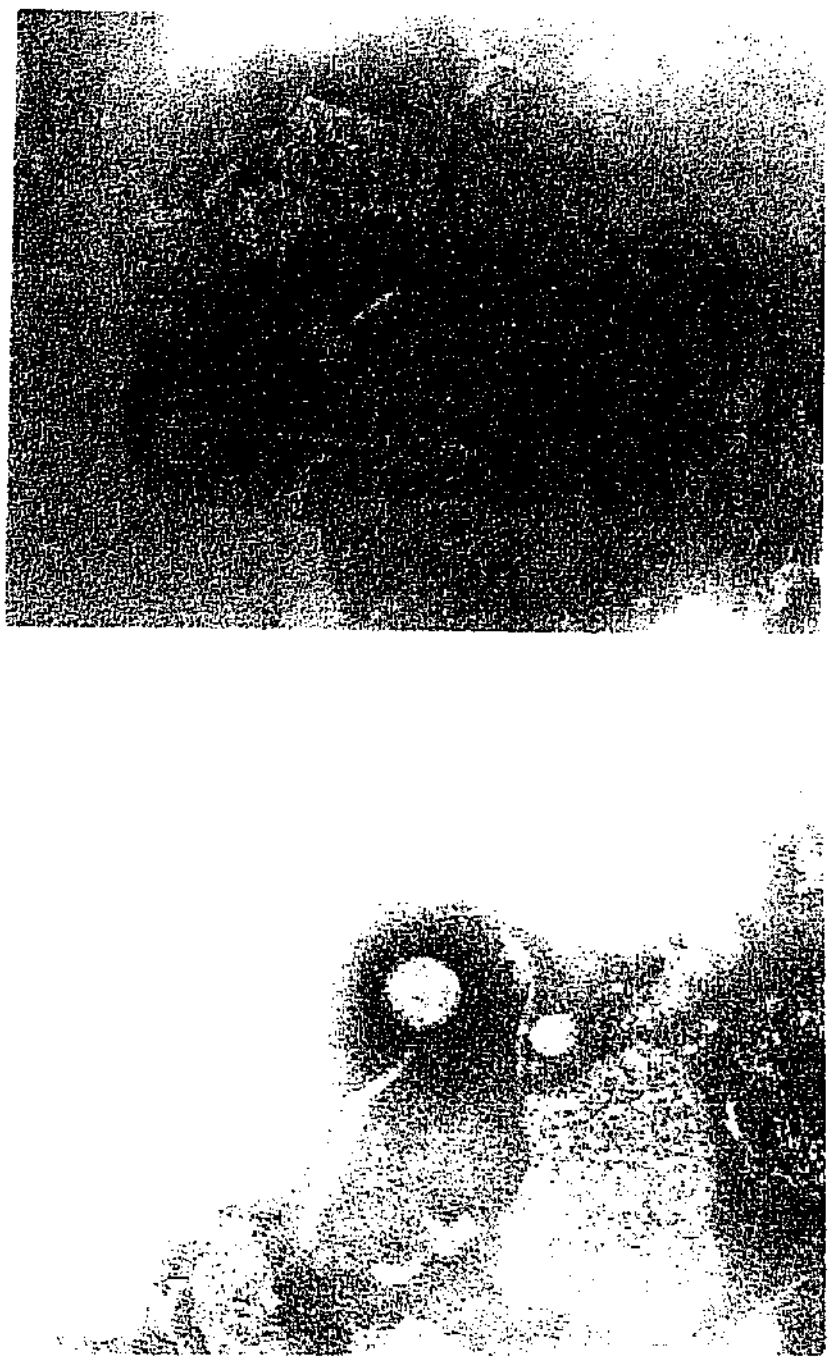


FIGURE 5-5 Electron micrographs of somatic coliphages isolated from small plaques of a gorilla stool sample.

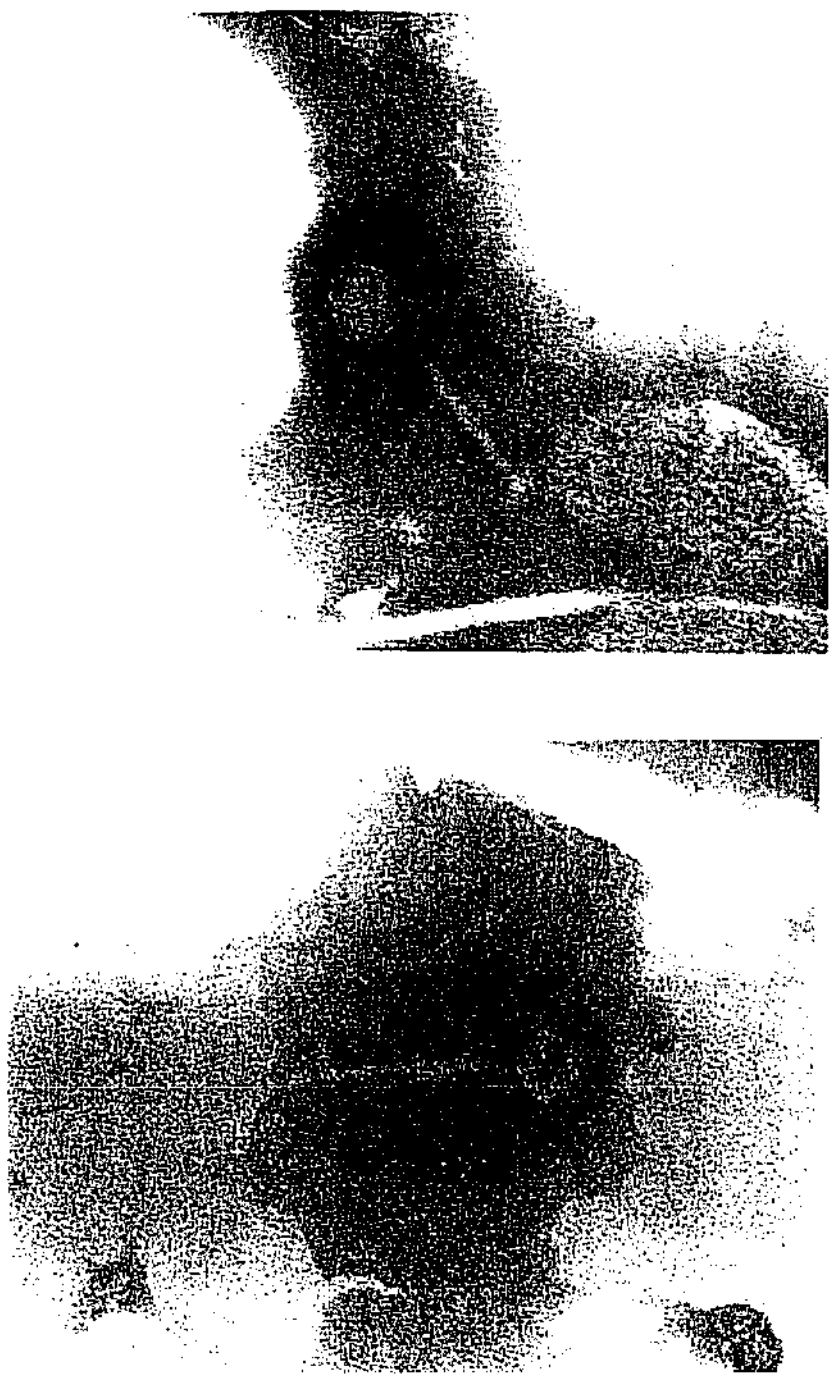


FIGURE 5-6 Electron micrographs of somatic coliphages isolated from large plaques of a gorilla stool sample.

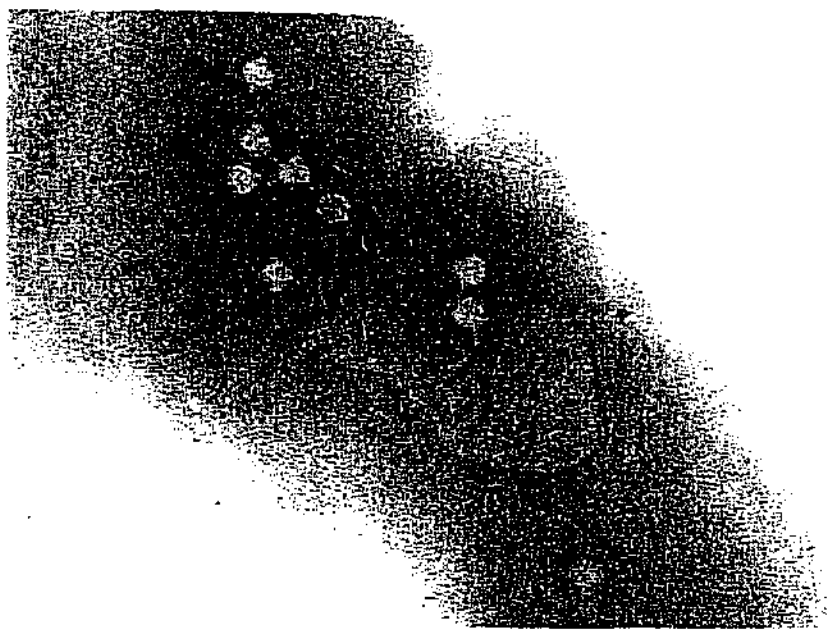


FIGURE 5-7 Electron micrograph of a somatic coliphage isolated from an orang-outang stool sample.

FIGURE 5-8 Electron micrograph of a somatic coliphage isolated from a chimpanzee stool sample.

TABLE 5-1 Incidence of somatic coliphages, male-specific coliphages and *Bacteroides fragilis* HSP40 phages in stool samples from hospitalised human patients.

Patient	Phages present (+) or absent (-)			
	Som	Male/E	Male/S	*Bact*
1. 90-11-07:09y/wM:Gastroenteritis	-	ND	-	-
2. 90-11-13:0,7y/wM:Gastroenteritis	+	ND	+	-
3. 90-11-13:1,3y/wF:Pneumoniae	+	ND	+	-
4. 90-11-22:1,8y/wF:Gastroenteritis	-	ND	-	-
5. 90-11-22:1,8y/wM:Gastroenteritis	+	ND	-	-
6. 90-11-22:0,9y/wF:Gastroenteritis	+	ND	+	-
7. 90-11-29:58y/wM:Pneumoniae	-	ND	+	+
8. 90-12-03:32y/wM:Gastroenteritis	+	ND	-	-
9. 90-12-20:51y/wF:Gastroenteritis	-	ND	-	-
10. 90-12-20:1,3y/bF:Gastroenteritis	+	ND	-	-
11. 91-01-10:33y/bF:Gastroenteritis	+	ND	+	-
12. 91-01-10:07y/wM:Fever + rash	+	ND	+	+
13. 91-01-10:1,6y/bM:Gastroenteritis	+	ND	+	+
14. 91-02-12:02y/wF:Gastroenteritis	+	-	ND	-
15. 91-02-12:24y/wM:Gastroenteritis	+	-	ND	-
16. 91-02-12:02y/wM:Gastroenteritis	+	+	ND	-
17. 91-02-12:04y/bF:Gastroenteritis	+	+	ND	-
18. 91-02-12:1,2y/wF:Gastroenteritis	+	+	ND	-
19. 91-11-07:85y/wF:Pneumoniae	+	-	-	-
20. 91-11-13:01y/bF:Gastroenteritis	+	+	+	-
Total positive % Positive	16 80%	4 57%	8 53%	3 15%

Som = somatic coliphages; Male/E = male-specific coliphages on *Escherichia coli* HS (pFamp)R; Male/S = male-specific coliphages on *Salmonella typhimurium* WG49; *Bact* = *Bacteroides fragilis* phages
y = year; w = white; b = black; M = male; F = female
ND = not done.

TABLE 5-2 Incidence of somatic coliphages, male-specific coliphages and *Bacteroides fragilis* HSP40 phages in stool samples from baboons (*Papio ursinus*) and monkeys (*Cercopithecus aethiops*).

Animal	Phages present (+) or absent (-)			
	Som	Male/E	Male/S	*Bact*
1. 91-07-09 : Baboon (Z)	-	-	-	-
2. 91-07-10 : Baboon (Z)	+	-	-	-
3. 91-07-15 : Baboon K1(ARC)	-	+	+	-
4. 91-07-15 : Baboon K2 (ARC)	+	+	+	-
5. 91-07-15 : Baboon K3 (ARC)	-	+	+	-
6. 91-07-15 : Baboon K4 (ARC)	+	+	+	-
7. 91-07-15 : Baboon K5 (ARC)	+	+	-	-
8. 91-07-15 : Baboon K6 (ARC)	-	-	-	-
9. 91-07-15 : Baboon K7 (ARC)	+	-	-	-
10. 91-07-15 : Baboon K8 (ARC)	+	+	+	-
11. 91-07-15 : Baboon K9 (ARC)	+	+	+	-
12. 91-07-15 : Baboon K10(ARC)	+	+	-	-
Total baboons positive	8 (67%)	8 (67%)	6 (50%)	0 (0%)
1. 91-07-09 : Monkey (Z)	+	-	-	-
2. 91-07-10 : Monkey (Z)	+	+	+	-
3. 91-07-15 : Monkey L1 (ARC)	+	+	+	-
4. 91-07-15 : Monkey L2 (ARC)	+	+	+	-
5. 91-07-15 : Monkey L3 (ARC)	+	+	-	-
6. 91-07-15 : Monkey L4 (ARC)	-	+	-	-
7. 91-07-15 : Monkey L5 (ARC)	+	+	+	-
8. 91-07-15 : Monkey L6 (ARC)	-	-	+	-
9. 91-07-15 : Monkey L7 (ARC)	-	+	+	-
10. 91-07-15 : Monkey L10 (ARC)	-	+	-	-
11. 91-07-15 : Monkey L11 (ARC)	-	+	+	-
12. 91-07-15 : Monkey L12 (ARC)	-	+	+	-
Total monkeys positive	6 (50%)	10 (83%)	8 (67%)	0 (0%)

Som = somatic coliphages; Male/E = male-specific coliphages on *Escherichia coli* HS (pFamp)R; Male/S = male-specific coliphages on *Salmonella typhimurium* WG49; *Bact* = *Bacteroides fragilis* phages. ND = not done

Samples obtained from animals at the H A Grové Animal Research Centre, University of Pretoria (ARC) and the National Zoological Gardens, Pretoria (Z).

TABLE 5-3 Incidence of somatic coliphages, male-specific coliphages and *Bacteroides fragilis* HSP40 phages in stool samples from higher primates.

Animal	Phages present (+) or absent (-)			
	Som	Male/E	Male/S	*Bact*
1. 91-02-15 : Chimpanzee	+	-	ND	-
2. 91-02-21 : Chimpanzee	+	-	ND	-
3. 91-05-15 : Chimpanzee	+	-	-	-
4. 91-05-23 : Chimpanzee	-	-	+	-
5. 91-05-23 : Chimpanzee	+	-	-	-
6. 91-05-27 : Chimpanzee	-	+	-	-
7. 91-06-05 : Chimpanzee	-	+	-	-
8. 91-07-10 : Chimpanzee	-	+	-	-
Total Chimpanzees	4 (50%)	3 (38%)	1 (16%)	0 (0%)
1. 91-02-15 : Gorilla	+	-	ND	-
2. 91-02-21 : Gorilla	+	+	ND	-
3. 91-05-15 : Gorilla	-	+	-	-
4. 91-05-23 : Gorilla	-	+	-	-
5. 91-05-27 : Gorilla	-	+	-	-
6. 91-06-04 : Gorilla	+	-	-	-
7. 91-07-09 : Gorilla	-	+	-	-
8. 91-07-10 : Gorilla	-	-	-	-
Total Gorilla	3 (38%)	5 (63%)	0 (0%)	0 (0%)
1. 91-02-15 : Orang-outang	+	+	ND	-
2. 91-02-21 : Orang-outang	+	+	ND	-
3. 91-05-23 : Orang-outang	+	+	-	-
4. 91-06-05 : Orang-outang	+	+	-	-
5. 91-07-09 : Orang-outang	+	-	+	-
6. 91-07-10 : Orang-outang	+	-	+	-
Total Orang-outang	6 (100%)	4 (67%)	2 (33%)	0 (0%)

Som = somatic coliphages; Male/E = male-specific coliphages on *Escherichia coli* HS (pFamp)R; Male/S = male-specific coliphages on *Salmonella typhimurium* WG49; *Bact* = *Bacteroides fragilis* phages; ND = not done
Samples obtained from the National Zoological Gardens, Pretoria

TABLE 5-4 Incidence of somatic coliphages, male-specific coliphages and *Bacteroides fragilis* HSP40 phages in stool samples from fowl.

Animal	Phages present (+) or absent (-)			
	Som	Male/E	Male/S	*Bact*
1. 91-05-20 : Turkey (F)	+	+	-	-
2. 91-05-06 : Goose (F)	+	-	-	-
3. 91-05-21 : Goose (C)	-	+	-	-
4. 91-05-21 : Goose (F)	-	+	-	-
5. 91-05-23 : Goose (F)	+	+	-	+
6. 91-05-23 : Goose (Z)	+	+	+	-
7. 91-05-27 : Goose (Z)	+	+	+	-
8. 91-05-29 : Goose (Z)	-	-	+	-
9. 91-06-03 : Goose (F)	-	-	-	-
10. 91-06-03 : Goose (F)	-	-	-	-
11. 91-06-03 : Goose (F)	-	-	-	-
12. 91-06-03 : Goose (F)	-	-	-	-
13. 91-06-03 : Goose (F)	-	-	-	-
14. 91-06-03 : Goose (F)	-	-	+	-
15. 91-06-03 : Goose (F)	-	-	-	-
16. 91-06-03 : Goose (F)	+	-	-	-
17. 91-06-03 : Goose (F)	-	-	-	-
18. 91-06-03 : Goose (F)	+	-	-	-
19. 91-06-04 : Goose (Z)	+	+	-	-
20. 91-06-05 : Goose (Z)	+	+	-	-
Total fowl positive	9 (45%)	8 (40%)	4 (20%)	1(0,5%)

Som = somatic coliphages; Male/E = male-specific coliphages on *Escherichia coli* HS (pFamp)R; Male/S = male-specific coliphages on *Salmonella typhimurium* WG49; *Bact* = *Bacteroides fragilis* phages. ND = not done.
Samples obtained from animals at a farm (F); the National Zoological Gardens (Z) and from residential stands in Pretoria (C).

TABLE 5-5 Incidence of somatic coliphages, male-specific coliphages and *Bacteroides fragilis* HSP40 phages in stool samples from small stock.

Animal	Phages present (+) or absent (-)			
	Som	Male/E	Male/S	*Bact*
1. 91-02-25 : Pig (F)	+	+	ND	-
2. 91-05-15 : Pig (Z)	+	+	-	-
3. 91-05-23 : Pig (F)	+	-	-	-
4. 91-05-23 : Pig (Z)	+	+	+	-
5. 91-05-27 : Pig (Z)	+	+	+	-
6. 91-05-29 : Pig (Z)	+	-	-	-
7. 91-06-05 : Pig (Z)	+	-	-	-
8. 91-06-04 : Pig (Z)	+	-	-	-
Total pigs positive	8 (100%)	4 (50%)	2 (29%)	0 (0%)
1. 91-02-25 : Sheep (F)	+	+	ND	-
2. 91-05-06 : Sheep (F)	-	+	-	-
3. 91-05-15 : Sheep (Z)	-	+	-	-
4. 91-05-21 : Sheep (F)	+	+	-	-
5. 91-05-23 : Sheep (F)	-	+	-	-
6. 91-05-23 : Sheep (Z)	-	-	-	-
7. 91-05-27 : Sheep (Z)	-	+	+	-
8. 91-05-29 : Sheep (Z)	+	-	-	-
9. 91-06-04 : Sheep (Z)	-	-	-	-
10. 91-06-05 : Sheep (Z)	+	-	-	-
Total sheep positive	4 (40%)	6 (60%)	1 (11%)	0 (0%)

Som = somatic coliphages; Male/E = male-specific coliphages on *Escherichia coli* HS (pFamp)R; Male/S = male-specific coliphages on *Salmonella typhimurium* WG49; *Bact* = *Bacteroides fragilis* phages. ND = not done
Samples obtained from animals at the National Zoological Gardens, Pretoria (Z) and at a farm (F).

TABLE 5-6 Incidence of somatic coliphages, male-specific coliphages and *Bacteroides fragilis* HSP40 phages in stool samples from large stock.

Animal	Phages present (+) or absent (-)			
	Som	Male/E	Male/S	*Bact*
1. 91-02-25 : Horse (F)	+	+	ND	-
2. 91-05-15 : Horse (Z)	+	-	-	-
3. 91-05-23 : Horse (Z)	+	+	-	-
4. 91-05-27 : Horse (Z)	+	-	-	-
5. 91-05-29 : Horse (Z)	+	-	+	-
6. 91-06-04 : Horse (Z)	+	-	-	-
7. 91-06-05 : Horse (Z)	-	-	-	-
Total horses positive	6 (86%)	2 (29%)	1 (17%)	0 (0%)
1. 91-02-25 : Cattle (F)	+	+	ND	-
2. 91-05-20 : Cattle (F)	+	+	-	-
3. 91-05-21 : Cattle (F)	-	+	-	-
4. 91-05-23 : Cattle (F)	+	+	-	-
5. 91-05-23 : Cattle (Z)	+	+	-	-
6. 91-05-27 : Cattle (Z)	-	+	+	-
7. 91-05-29 : Cattle (Z)	+	+	-	-
8. 91-06-04 : Cattle (Z)	+	-	-	-
9. 91-06-05 : Cattle (Z)	+	-	+	-
Total cattle positive	7 (78%)	7 (78%)	2 (25%)	0 (0%)

Som = somatic coliphages; Male/E = male-specific coliphages on *escherichia coli* HS (pFamp)R; Male/S = male-specific coliphages on *Salmonella typhimurium* WG49; *Bact* = *Bacteroides fragilis* phages. ND = not done
Samples obtained from the National Zoological Gardens, Pretoria (Z) and from a farm (F).

TABLE 5-7 Incidence of somatic coliphages, male-specific coliphages and *Bacteroides fragilis* HSP40 phages in stool samples from pets.

Animal	Phages present (+) or absent (-)			
	Som	Male/E	Male/S	*Bact*
1. 91-05-06 : Rabbit (F)	+	+	-	-
2. 91-05-20 : Rabbit (F)	-	+	-	-
3. 91-05-23 : Rabbit (F)	+	+	-	-
4. 91-05-23 : Rabbit (Z)	-	-	-	-
5. 91-05-27 : Rabbit (Z)	-	-	-	-
6. 91-05-29 : Rabbit (Z)	-	+	-	-
7. 91-06-04 : Rabbit (Z)	+	-	-	-
8. 91-06-05 : Rabbit (Z)	-	+	-	-
Total rabbits positive	3 (38%)	5 (63%)	0 (0%)	0 (0%)
1. 91-05-06 : Dog (F)	+	+	+	-
2. 91-05-20 : Dog (F)	+	+	-	-
3. 91-05-21 : Dog (F)	+	+	-	-
4. 92-03-04 : Dog (C)	-	-	-	-
Total dogs positive	3 (75%)	3 (75%)	1 (25%)	0 (0%)
1. 92-02-18 : Cat (C)	+	+	-	-
2. 92-02-18 : Cat (C)	+	-	-	-
3. 92-03-04 : Cat (C)	+	+	+	-
5. 92-03-07 : Cat (C)	+	+	+	-
Total cats positive	4 (100%)	3 (75%0	2 (50%)	0 (0%)

Som = somatic coliphages; Male/E = male-specific coliphages on *Escherichia coli* HS (pFamp)R; Male/S = male-specific coliphages on *Salmonella typhimurium* WG49; *Bact* = *Bacteroides fragilis* phages. ND = not done
Samples obtained from a farm (F) and residential stands in Pretoria (C).

5.4. 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 stool. The incidence of these phages in the excreta of some of the animals, notably primates (baboon and monkey) and higher primates (gorilla, chimpanzee and orang-outang) 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. More detailed analyses would fall beyond the objectives of this study.

The first important conclusion is that *B fragilis* HSP40 phages were detected only in the stool of humans and no animals (Tables 5-1 to 5-7). The only exception to this rule was a positive result for a sample of goose excreta. This exceptional case may be due to the goose having eaten human excreta or to accidental laboratory contamination, both of which may be possible with the highly sensitive enrichment procedure used for the detection of the phages.

The results on the specificity of *B fragilis* HSP40 phages for human excreta are in agreement with the findings of Tartera and Jofre (1987). One difference is that the latter authors found 10% of human stools to be positive and in this study 16% were positive (Table 5-1). The absence of *B fragilis* HSP40 phages even from the stool of higher primates (Table 5-3) is significant since these animals are closely related to humans. However, the possibility of very low numbers of animals other than man excreting phages which infect *B fragilis* HSP40 has not been ruled out.

Results on the incidence of phages in stool samples from man and animals obtained in different 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 (Tables 5-4 to 5-7) 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 stool of primate animals (baboons, monkeys, gorillas, chimpanzees, orang-outangs) would appear to be of the same order (Tables 5-2 and 5-3). Although only small numbers of samples were tested, it would appear that orang-outangs, pigs and cats have exceptionally high incidences of somatic coliphages in their stools (Tables 5-3, 5-5 and 5-7, respectively). The 80% incidence of somatic coliphages in human stool samples (Table 5-1) is similar to results reported by Dhillon *et al* (1976) and Havelaar *et al* (1986), but higher than the 24% reported by Osawa *et al* (1981) and the 1,6% to 14% of Furuse *et al* (1983).

The 0-75% incidence of male-specific coliphages in domestic animal stool samples (Tables 5-4 to 5-7) 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 male-specific coliphages in stool samples from the primates would seem to be of the same order as in domestic animals (Tables 5-2 and 5-3). The 53-57% incidence of male-specific coliphages in human stool samples is considerably higher than the 0% of Dhillon *et al* (1976), 0-33% of Osawa *et al* (1981) and the low incidence of Havelaar *et al* (1986). The sensitive enrichment procedure used in this study for the detection of male-specific coliphages may have played an important role in the higher incidences detected. The fact than only stools from hospitalised patients were investigated in this study may also play a role since Furuse *et al* (1983) found that the incidence of coliphages in stools from healthy individuals was only about 1,6% and 14% in stools from patients.

Results obtained in this study give no indication of the titres of phages in stool because phages were only detected by means of an enrichment procedure aimed at the qualitative detection of low numbers of phages. However, according to earlier studies (Dhillon *et al*, 1976; Havelaar *et al*, 1986) somatic and male-specific coliphages may be excreted in numbers of the order of 10^6 to 10^8 per gram of stool.

Data on the incidence of phages in the stool of man and animals reported in this and earlier studies, and on the numbers in which phages occur in stool, indicate that phages may occur in substantial numbers in sewage and sewage polluted water, which is in agreement with results of the analysis of sewage and various other water environments recorded in Chapter 8. The observation that somatic coliphages occur more frequently in stool samples (38-100%) than male-specific coliphages (0-83%) (Tables 5-1 to 5-7) is also in agreement with numbers in sewage and related water environments where somatic coliphages generally outnumber male-specific coliphages by a factor of about 10 (Chapter 8). The detection of *B fragilis* HSP40 phages only in 15% of human stool samples is in agreement with observations that generally these phages occur in wastewater in numbers well below those of male-specific coliphages (Chapter 8).

The *E coli* HS(pFamp)R host strain (Debartolomeis and Cabelli, 1991) consistently yielded higher numbers of positive results for male-specific coliphages than the *S typhimurium* WG49 host strain (Havelaar and Hogeboom, 1984) (Tables 5-1 to 5-7). Such a direct comparison of these two hosts for male-specific coliphages has not previously been reported. The reasons for the difference have not been investigated, but may be due to differences in susceptibility or possibly even false-positive results. Investigation of the latter possibility by characterisation of phage isolates was not attempted but would seem to be justified in follow-up studies.

Major findings and conclusions from this investigation may be summarised as follows:

1. Methods for detection of the phages concerned have successfully been applied to a variety of stool samples.
2. The qualitative enrichment procedure proved successful for the sensitive detection of phages in stool samples. The procedure may, therefore, prove equally successful for the detection of small numbers of these phages in water and food.
3. *Bacteroides fragilis* HSP40 phages are highly specific for human faecal pollution. These phages may, therefore, serve as a useful indicator for distinguishing between faecal pollution of human and animal origin.
4. Valuable information has been obtained on the incidence of the phages concerned in the stool of humans and animals, which confirms their validity as indicators of faecal pollution.

Results of this study, including the assessment of phage detection methods in practice and the details gathered on the incidence of phages in human and animal faeces, are of fundamental importance for further research on the indicator value of the phages concerned.

CHAPTER 6

INCIDENCE OF PHAGES, BACTERIAL INDICATORS AND HUMAN VIRUSES IN SELECTED FRESHWATER ENVIRONMENTS

6.1 INTRODUCTION

Information on the numbers of phages, commonly used indicator bacteria and human viruses in various water environments and treatment processes is required to assess the indicator value of phages, particularly with regard to the following requirements of indicators (see Chapter 2):

1. Indicators have to be present in polluted water environments at all times in at least the same numbers as pathogens.
2. Indicators should survive in all polluted water environments or treatment processes for at least as long as pathogens.

In addition to information which has already been reviewed in Chapter 2, details in the literature which are of fundamental importance to work covered by this Chapter may be summarised as follows:

Domestic sewage in Hong Kong has been found to contain between 36 and 15 900 plaque forming units (PFU) of somatic coliphages per ml, with sewage from urban areas generally having higher counts than sewage from rural areas (Dhillon *et al*, 1970). Similar levels have been reported for wastewater in other parts of the world, with counts generally running in the order of 10^3-10^4 PFU/ml (Ignazitto *et al*, 1980; Furuse *et al*, 1981; Havelaar *et al*, 1984; Kfir *et al*, 1991). Similar counts of somatic coliphages have also been reported for slaughterhouse and hospital wastewaters (Osawa et al, 1981; Havelaar, 1986).

Male-specific coliphages have consistently been detected in sewage, with counts in mixed domestic/industrial wastewater in the order of 10^3-10^4 PFU/ml (IAWPRC, 1991). Similar counts have been reported for wastewater from hospitals, pig slaughtering houses and poultry processing plants (IAWPRC, 1991). Although the above findings indicate that male-specific phages may in certain water environments reach counts similar to those of somatic coliphages, other results show that generally somatic coliphages outnumber male-specific coliphages by a factor of at least 10 (Grabow *et al*, 1984; Kfir *et al*, 1991).

Although the incidence in wastewater of phages which infect certain strains of *B fragilis* has been reported some time ago (Nacescu *et al*, 1972; Booth *et al*, 1979; Cooper *et al*, 1979), the first data on the incidence of *B fragilis* HSP40 phages in water environments were only published by Tartera and Jofre in 1987. In the latter study the phages were found to reach counts as high as 10^3/ml in Barcelona sewage, and up to 10/ml in sewage-polluted seawater. *Bacteroides fragilis* HSP40 phages were also regularly detected in sewage-polluted river water, marine sediments, and even in groundwater when using an enrichment procedure for detection. In 1989 Tartera *et al* reported average *B fragilis* HSP40 counts of about 50/ml in city sewage. In slaughterhouse wastewater with faecal coliform counts of the order of 10^4/ml, these phages were detected in only 2 of 20 samples using an enrichment procedure the

results of which indicate counts of less than 2/100ml. Water and sediments from a game reserve contained counts of faecal coliforms and coliphages similar to those of other polluted environmental waters, but no *B fragilis* HSP40 phages were detected. The results for slaughterhouse effluents and water environments in a game reserve confirm the specificity of *B fragilis* HSP40 phages for human faecal pollution described in Chapter 5.

Relative ratios of various phages, commonly used bacterial indicators and human viruses may vary quite extensively for different water environments. A number of studies do, however, give sound indications at least of certain trends and basic data which confirm the indicator value of different groups of indicators. In various water environments in or near Barcelona, faecal coliforms outnumbered somatic coliphages by a factor of about 2 to 100, *B fragilis* HSP40 phages by a factor of about 10^2 to 10^4, and human viruses by a factor of about 10^5 (Tartera *et al*, 1989). The ratio of faecal streptococci and *Clostridium perfringens* to somatic coliphages, *B fragilis* HSP40 phages and human virus was generally lower than that of faecal coliforms by a factor of about 10 and 100, respectively. *Bacteroides fragilis* HSP40 phages outnumbered human viruses by a factor of 20 to 1000. As mentioned earlier, the ratio of bacterial indicators of faecal pollution and coliphages to *B fragilis* HSP40 phages was much higher in slaughterhouse wastewater and water environments in a game reserve. Fundamentally important observations in various studies are that in all waters studied *B fragilis* HSP40 phages consistently outnumbered human viruses, and like human viruses these phages were not able to replicate in any of the water environments (Jofre *et al*, 1989; Tartera *et al*, 1989).

In some southern African water environments standard plate counts were higher than somatic coliphage counts by a factor of 10^3-10^5 (Grabow *et al*, 1984). In the same waters counts of total coliforms, faecal coliforms and faecal streptococci tended to run sometimes higher and sometimes lower than those of somatic coliphages by a factor of up to 100 both ways. Total coliforms outnumbered faecal coliforms by a factor of up to 10 and sometimes even more. Counts of faecal coliforms were generally slightly higher than those of faecal streptococci. Apart from rare exceptions, counts of somatic coliphages outnumbered those of human viruses by a factor of 10^3 or more. According to Kfir *et al* (1991) counts of somatic coliphages reached levels of 2×10^4/ml in settled sewage, 5×10^3/ml in polluted seawater, 400/ml in secondary treated wastewater, and about 60/ml in polluted river water. Somatic coliphages generally outnumbered male-specific coliphages by a factor of 10 or more.

Data on relative survival rates suggest that generally speaking phages survive for at least as long as most commonly used bacterial indicators and human viruses (IAWPRC, 1991). More specifically, Jofre *et al* (1989) found that the ratio of *B fragilis* HSP40 phages to enterovirus and rotaviruses was similar in wastewater and in sediments of seawater into which the wastewater had been discharged, which suggests that the survival time for the phages closely resembled that of the human viruses. Similar observations have been reported for inland wastewaters (Grabow *et al*, 1984), and somatic coliphages proved at least as resistant to all treatment stages in advanced wastewater reclamation systems than commonly used bacterial indicators and human viruses (Grabow, 1990). In controlled laboratory experiments male-specific coliphages did not survive as long as hepatitis A, polio and rotaviruses in seeded seawater (Chung and Sobsey, 1992). However, *B fragilis* phages (not *B*

fragilis HSP40) survived for at least as long as the human viruses in seeded seawater or sediments. All viruses and phages survived significantly longer in seawater or sediments at 5°C than at 25°C. In similar experiments on river water, Springthorpe *et al* (1992) found that the survival of male-specific phage MS-2 was comparable to that of hepatitis A and polioviruses. The survival of male-specific coliphages closely resembled that of hepatitis A and polioviruses also in landfills and landfill leachates (Gray *et al*, 1992), which adds to evidence suggesting that phages may serve as reliable indicators of human viruses in a wide variety of environments (IAWPRC, 1991).

Comparison of data on the relative incidence and survival of phages, indicator bacteria and human viruses reported from various laboratories in different parts of the world has major shortcomings because different detection methods are being used which generally implies that completely different populations of indicators and viruses are being studied. However, all these data are important because they contribute to a better understanding of the complete picture and the potential value of various indicators.

In view of the above, the purpose of this study was to compare the incidence and survival of phages, commonly used indicator bacteria and human viruses in selected South African water environments. This work is new in so far as that *B fragilis* HSP40 phages have not previously been studied in this country, a new host for the detection of male-specific phages was used, and tests were done on waters not previously investigated.

6.2 MATERIALS AND METHODS

6.2.1 Water samples

6.2.1.1 City sewage

Samples of settled and treated wastewater were collected at the Daspoort purification plant in Pretoria. This plant has two intakes, one from the sewerage system of parts of the city which consist primarily of residential areas (domestic wastewater), and the second intake which receives a mixture of domestic and industrial wastewater. The following samples were collected:

6.2.1.1.1 Daspoort settled sewage (Domestic)
6.2.1.1.2 Daspoort settled sewage (Domestic and industrial)
6.2.1.1.3 Daspoort activated sludge effluent
6.2.1.1.4 Daspoort chlorinated sandfilter effluent (Daspoort biofilter effluent chlorinated after sandfiltration)

6.2.1.2 Polluted river water

Samples were collected from water in the Apies River which flows through Pretoria at a point some 500m downstream of the inflow of the purified wastewater effluent from the Daspoort plant.

6.2.1.3 Hospital wastewater

Samples were collected from two sites in the sewerage system of the H F Verwoerd Hospital. This teaching hospital of our Faculty has 900 beds and offers a comprehensive range of medical and public health services to patients of all races and socio-economic levels. The two sampling sites represent wastewater from different parts of the hospital.

6.2.1.4 Slaughterhouse effluent

Samples were collected at the Rooiwal Slaughterhouse near Pretoria which processes mainly cattle and pigs. Samples were collected early in the morning, which was the peak slaughtering time, from process water as well as the effluent of an aeration pond.

6.2.2 Collection and processing of samples

Samples were collected from August 1991 to September 1992. Samples were collected in sterile nalgene bottles, transported at ambient temperature and processed within 5h of collection. Samples were homogenised by means of a Model T25 Ultra-Turrax. In tests for somatic and male-specific coliphages interference by contaminant organisms was controlled by using drug-resistant host strains. In the case of tests for *B fragilis* HSP40 phages drug-resistance of the host was not sufficient and samples were decontaminated by chloroform-treatment (Tartera and Jofre, 1987). As necessary tenfold dilutions of homogenised samples were prepared in sterile pH 7,2 phosphate buffered saline (PBS).

6.2.3 Bacteriophage counts

Phages were enumerated by means of plaque assays using the following host strains as described in Chapter 3:

6.2.3.1 *Escherichia coli* C (WG4) and its nalidixic acid-resistant strain WG5 for the detection of somatic coliphages

6.2.3.2 *Escherichia coli* HS(pFamp)R and *Salmonella typhimurium* WG49 for the detection of male-specific coliphages

6.2.3.3 *Bacteroides fragilis* HSP40 for the detection of *B fragilis* phages

Plaque assays were basically carried out by mixing 1,0ml of test sample with 0,3ml of an overnight broth culture of the host (logarithmic growth phase) in 2,5ml of soft agar at 45°C. This mixture was poured over a bottom agar layer in a 9cm Petri dish, allowed to solidify, and incubated at 37°C. Plaques were counted 18 h later. All tests were carried out in triplicate and results expressed as numerical averages (geometric means) of plaque-forming units (PFU)/ml.

6.2.4 Counts of bacteria

6.2.4.1 Faecal coliforms

Membrane filtration using M-FC Agar (Difco) without rosolic acid and incubation at 44,5°C/24h. Typical blue colonies were counted without further characterisation (Grabow *et al*, 1981).

6.2.4.2 Faecal streptococci

Membrane filtration using M-Enterococcus Agar (Difco) and incubation at 37°C/48h. Typical red colonies were counted without further characterisation (Grabow, 1986).

6.2.5 Enumeration of human enteric viruses

Enumeration of human enteric viruses was based on the detection of a cytopathogenic effect (CPE) in the BGM monkey kidney cell line using 96-well microtitre plates for most probable number (MPN) assays on five wells for each tenfold dilution. Cells were cultured in Minimal Essential Medium with Earl Salts (Gibco) supplemented with antibiotics and 2% foetal calf serum. Plates were incubated for at least 10 days at 37°C in a 5% CO_2 atmosphere. Details of general cell culture procedures used in our laboratory have been described elsewhere (Grabow *et al*, 1982, 1992).

6.3 RESULTS

6.3.1 Daspoort settled sewage (Domestic)

Average counts of somatic coliphages per ml were $1,5x10^4$ using *E coli* C (WG4) and $1,4x10^4$ using *E coli* C (WG5) as hosts (Table 6-1). Average counts of male-specific coliphages per ml were $2,6x10^3$ using the *E coli* host strain and $2,1x10^3$ using the *S typhimurium* WG49 host strain (Table 6-2). Average counts of faecal coliforms and faecal streptococci per ml were $3,8x10^5$ and $3,9x10^5$, respectively (Table 6-3). Average counts of *B fragilis* HSP40 phages and human viruses per ml were 218 and 64, respectively (Table 6-4).

6.3.2 Daspoort settled sewage (Domestic and industrial)

Average counts of somatic coliphages per ml were $1,7x10^4$ using *E coli* C (WG4) and $1,6x10^4$ using *E coli* C (WG5) as hosts (Table 6-1). Average counts of male-specific coliphages per ml were $1,1x10^4$ using the *E coli* host strain and $5,6x10^3$ using the *S typhimurium* WG49 host strain (Table 6-2). Average counts of faecal coliforms and faecal streptococci per ml were $6,3x10^5$ and $5,1x10^4$, respectively (Table 6-3). Average counts of *B fragilis* HSP40 phages and human viruses per ml were 120 and 69, respectively (Table 6-4).

6.3.3 Activated sludge effluent

Average counts of somatic coliphages per ml were 37 using *E coli* C (WG4) and 29 using *E coli* C (WG5) as hosts (Table 6-1). Average counts of male-specific coliphages per ml were 6 using the *E coli* host strain and 16 using the *S typhimurium* WG49 host strain (Table 6-2). Average counts of faecal coliforms and faecal streptococci per ml were 814 and 50, respectively (Table 6-3). No *B fragilis* HSP40 phages were detected and tests for human viruses were not carried out (Table 6-4).

6.3.4 Chlorinated sandfilter effluent

Average counts of somatic coliphages per ml were 32 using *E coli* C (WG4) and 20 using *E coli* C (WG5) as hosts (Table 6-1). Average counts of male-specific coliphages per ml were 8 using the *E coli* host strain and 10 using the *S typhimurium* WG49 host strain (Table 6-2). Average counts of faecal coliforms and faecal streptococci per ml were 523 and 46, respectively (Table 6-3). No *B fragilis* HSP40 phages were detected and tests for human viruses were not carried out (Table 6-4).

6.3.5 Apies River water

Average counts of somatic coliphages per ml were 53 using *E coli* C (WG4) and 58 using *E coli* C (WG5) as hosts (Table 6-1). Average counts of male-specific coliphages per ml were 5 using the *E coli* host strain and 15 using the *S typhimurium* WG49 host strain (Table 6-2). Average counts of faecal coliforms and faecal streptococci per ml were 442 and 102, respectively (Table 6-3). No *B fragilis* HSP40 phages were detected and tests for human viruses were not carried out (Table 6-4).

6.3.6 Hospital wastewater

Average counts of somatic coliphages per ml were 3210 using *E coli* C (WG4) and 1910 using *E coli* C (WG5) as hosts (Table 6-5). Average counts of male-specific coliphages per ml were 5650 using the *E coli* host strain and 3440 using the *S typhimurium* WG49 host strain (Table 6-6). Average counts of faecal coliforms and faecal streptococci per ml were $5,6x10^4$ and $2,8x10^3$, respectively (Table 6-7). Average counts of *B fragilis* HSP40 phages and human viruses per ml were 133 and 8, respectively (Table 6-8).

6.3.7 Slaughterhouse wastewater - process water

Average counts of somatic coliphages per ml were $3,7x10^4$ using *E coli* C (WG4) and $2,8x10^4$ using *E coli* C (WG5) as hosts (Table 6-9). Average counts of male-specific coliphages per ml were $1,2x10^3$ using the *E coli* host strain and $3,2x10^3$ using the *S typhimurium* WG49 host strain (Table 6-10). Average counts of faecal coliforms and faecal streptococci per ml were $3,3x10^5$ and $3,8x10^4$, respectively (Table 6-11). No *B fragilis* HSP40 phages and human viruses were detected (Table 6-12).

6.3.8 Slaughterhouse wastewater - aeration pond effluent

Average counts of somatic coliphages per ml were 9900 using *E coli* C (WG4) and 6750 using *E coli* C (WG5) as hosts (Table 6-9). Average counts of male-specific coliphages per ml were 64 using the *E coli* host strain and 93 using the *S typhimurium* WG49 host strain (Table 6-10). Average counts of faecal coliforms and faecal streptococci per ml were $9,9x10^3$ and $2,0x10^3$, respectively (Table 6-11). No *B fragilis* HSP40 phages and human viruses were detected (Table 6-12).

In order to facilitate the comparison and interpretation of the above results for the 108 samples which have been analysed for various phages, indicator bacteria and human viruses, the results have also been summarised in Tables 6-13 to 6-15.

TABLE 6-1 Counts of somatic coliphages in selected waters.

Sample	Host *E coli* WG5			Host *E coli* WG4		
	n	Range (mean) PFU/ml	Positive samples (%)	n	Range (mean) PFU/ml	Positive samples (%)
1	12	$4,7x10^3$ - $4,7x10^4$ ($1,4x10^4$)	100	12	$8,3x10^3$ - $3,1x10^4$ ($1,5x10^4$)	100
2	12	$4,0x10^3$ - $7,0x10^4$ ($1,6x10^4$)	100	12	$3,4x10^3$ - $3,3x10^4$ ($1,7x10^4$)	100
3	12	7 - 68 (29)	100	12	18 - 74 (37)	100
4	12	0 - 39 (20)	92	12	0 - 63 (32)	92
5	12	20 - 135 (58)	100	12	11 - 150 (53)	100

n = number of samples

Sample 1 = settled sewage (domestic)WG4 = Nalidixic acid sensitive
Sample 2 = settled sewage (domestic + industrial)WG5 = Nalidixic acid resistant
Sample 3 = activated sludge effluent
Sample 4 = chlorinated sandfilter effluent
Sample 5 = Apies River

TABLE 6-2 Counts of male-specific coliphages in selected waters.

Sample	Host *E coli* HS(pFamp)R			Host *S typhimurium* WG49		
	n	Range (mean) PFU/ml	pos samples (%)	n	Range (mean) PFU/ml	pos samples (%)
1	12	$6,3x10^2$ - $6,0x10^3$ ($2,6x10^3$)	100	12	$7,0x10^2$ - $4,1x10^3$ ($2,1x10^3$)	100
2	12	$3,5x10^2$ - $4,3x10^4$ ($1,1x10^4$)	100	12	$4,0x10^2$ - $1,2x10^4$ ($5,6x10^3$)	100
3	12	2 - 18 (6)	100	12	1 - 36 (16)	100
4	12	0 - 19 (8)	92	12	0 - 13 (10)	92
5	12	0 - 18 (5)	92	12	2 - 44 (15)	100

n = number of samples

Sample 1 = settled sewage (domestic)
Sample 2 = settled sewage (domestic + industrial)
Sample 3 = activated sludge effluent
Sample 4 = chlorinated sandfilter effluent
Sample 5 = Apies River

TABLE 6-3 Counts of faecal coliforms and faecal streptococci in selected waters.

Sample	Faecal coliforms (FC)			Faecal streptococci (FS)		
	n	Range (mean) CFU/ml	pos samples (%)	n	Range (mean) CFU/ml	pos samples (%)
1	12	$5,3x10^4$ - $7,0x10^5$ ($3,8x10^5$)	100	12	$2,4x10^3$ - $7,6x10^4$ ($3,9x10^4$)	100
2	12	$2,3x10^5$ - $1,4x10^6$ ($6,3x10^5$)	100	12	$2,4x10^4$ - $7,6x10^4$ ($5,1x10^4$)	100
3	12	49 - $3,0x10^3$ (814)	100	12	1 - $1,7x10^2$ (50)	100
4	12	0 - $2,1x10^3$ (523)	92	12	0 - 148 (46)	83
5	12	50 - $2,1x10^3$ (442)	100	12	10 - 570 (102)	100

n = number of samples

Sample 1 = settled sewage (domestic)
Sample 2 = settled sewage (domestic + industrial)
Sample 3 = activated sludge effluent
Sample 4 = chlorinated sandfilter effluent
Sample 5 = Apies River

TABLE 6-4 Counts of *Bacteroides fragilis* HSP40 phages and human viruses in selected waters.

Sample	*B fragilis* HSP40 phages			Human viruses		
	n	Range (mean) PFU/ml	Pos samples (%)	n	Range (mean) MPN/ml	Pos samples (%)
1	12	136 - 270 (218)	100	7	0 - 107 (64)	71
2	12	70 - 147 (120)	100	7	0 - 110 (69)	71
3	12	0	0	ND	ND	ND
4	12	0	0	ND	ND	ND
5	12	0	0	ND	ND	ND

n = number of samples
ND = not done

Sample 1 = settled sewage (domestic)
Sample 2 = settled sewage (domestic + industrial)
Sample 3 = activated sludge effluent
Sample 4 = chlorinated sandfilter effluent
Sample 5 = Apies River

TABLE 6-5 Counts of somatic coliphages in hospital wastewater.

Samples	Host *E coli* WG5			Host *E coli* WG4		
	n	Range (mean) PFU/ml	pos samples (%)	n	Range (mean) PFU/ml	pos samples (%)
1	12	30 - 510 (191)	100	12	87 - 435 (321)	100
2	12	2 - 180 (138)	100	12	25 - 500 (263)	100

n = number

1 = Sampling site 1
2 = Sampling site 2

WG4 = Nalidixic acid sensitive
WG5 = Nalidixic acid resistant

TABLE 6-6 Counts of male-specific coliphages in hospital wastewater.

Samples	Host *E coli* HS(pFamp)R			Host *S typhimurium* WG49		
	n	Range (mean) PFU/ml	pos samples (%)	n	Range (mean) PFU/ml	pos samples (%)
1	12	12 - $1,1x10^3$ (565)	100	12	12 - $1,8x10^3$ (344)	100
2	12	42 - 955 (378)	100	12	99 - $1,0x10^3$ (625)	100

n = number of samples

1 = Sampling site 1
2 = Sampling site 2

TABLE 6-7 Counts of faecal coliforms and faecal streptococci in hospital wastewater

Sample	Faecal coliforms			Faecal streptococci		
	n	Range (mean) CFU/ml	pos samples (%)	n	Range (mean) CFU/ml	pos samples (%)
1	12	$1,6x10^4$ - $8,3x10^4$ ($5,6x10^4$)	100	12	50 - $1,2x10^4$ ($2,8x10^3$)	100
2	12	$1,5x10^4$ - $3,1x10^5$ ($1,6x10^5$)	100	12	$1,5x10^3$ - $9,0x10^3$ ($4,1x10^3$)	100

n = number of samples

1 = Sampling site 1
2 = Sampling site 2

TABLE 6-8 Counts of *B fragilis* HSP40 phages and human viruses in hospital wastewater.

Sample	*B fragilis* HSP40 phages			Human viruses		
	n	Range (mean) PFU/ml	pos samples (%)	n	Range (mean) MPN/ml	pos samples (%)
1	12	0 - 325 (133)	42	12	0 - 12 (8)	50
2	12	0 - 147 (81)	33	12	0 - 12 (10)	50

n = number of samples

1 = Sampling site 1
2 = Sampling site 2

TABLE 6-9 Counts of somatic coliphages in slaughterhouse wastewater.

Samples	Host *E coli* WG5			Host *E coli* WG4		
	n	Range (mean) PFU/ml	pos samples (%)	n	Range (mean) PFU/ml	pos samples (%)
1	12	$2,5x10^4$- $3,3x10^4$ ($2,8x10^4$)	100	12	$2,8x10^4$x$4,0x10^4$ ($3,7x10^4$)	100
2	12	67 - $15,5x10^2$ (675)	100	12	45 - $23,3x10^2$ (990)	100

n = number of samples

1 = process water
2 = aeration pond effluent

TABLE 6-10 **Counts of male-specific coliphages in slaughterhouse wastewater.**

Samples	Host *E coli* HS(pFamp)R			Host *S typhimurium* WG49		
	n	Range (mean) PFU/ml	pos samples (%)	n	Range (mean) PFU/ml	pos samples (%)
1	12	$8,8x10^2$ - $1,4x10^3$ ($1,2x10^3$)	100	12	$2,3x10^3$ - $4,9x10^3$ ($3,2x10^3$)	100
2	12	19 - 187 (64)	100	12	15 - 207 (93)	100

n = number of samples

1 = process water
2 = aeration pond effluent

TABLE 6-11 **Counts of faecal coliforms and faecal streptococci in slaughterhouse wastewater.**

Sample	Faecal coliforms			Faecal streptococci		
	n	Range (mean) CFU/ml	pos samples (%)	n	Range (mean) CFU/ml	pos samples (%)
1	12	$7,0x10^4$ - $8,0x10^5$ ($3,3x10^5$)	100	12	$1,0x10^3$ - $4,5x10^4$ ($3,8x10^4$)	100
2	12	$4,0x10^3$ - $10,0x10^3$ ($9,9x10^3$)	100	12	$11,0x10^2$ - $4,0x10^3$ ($2,0x10^3$)	100

n = number of samples

1 = process water
2 = aeration pond effluent

TABLE 6-12 **Counts of *Bacteroides fragilis* HSP40 phages and human viruses in slaughterhouse wastewater.**

Sample	*B fragilis* HSP40 phages			Human viruses		
	n	Range (mean) PFU/ml	pos samples (%)	n	Range (mean) MPN/ml	pos samples (%)
1	12	0	0	7	0	0
2	12	0	0	7	0	0

n = number of samples

1 = process water
2 = aeration pond effluent

TABLE 6-13 Counts of phages, indicator bacteria and human viruses per ml in selected waters.

SOURCE		SP(WG4)	SP(WG5)	MS(E)	MS(S)	BP	EV	FC	FS
Settled sewage domestic	No. samples	12	12	12	12	12	7	12	12
	Minimum	$8,3x10^3$	$4,7x10^3$	630	700	136	12	$5,3x10^4$	$2,4x10^3$
	Maximum	$3,1x10^4$	$4,7x10^4$	$6,0x10^3$	$4,1x10^3$	270	107	$7,0x10^5$	$7,6x10^4$
	Geometric mean	$1,5x10^4$	$1,4x10^4$	$2,6x10^3$	$2,1x10^3$	218	64	$3,8x10^5$	$3,9x10^4$
Settled sewage domestic + industrial	No. samples	12	12	12	12	12	7	12	12
	Minimum	$3,4x10^3$	$4,0x10^3$	345	400	70	9	$2,3x10^5$	$2,4x10^4$
	Maximum	$3,3x10^4$	$7,0x10^4$	$4,3x10^4$	$1,2x10^4$	147	110	$1,4x10^6$	$7,6x10^4$
	Geometric mean	$1,7x10^4$	$1,6x10^4$	$1,1x10^4$	$5,6x10^3$	120	69	$6,3x10^5$	$5,1x10^4$
Activated sludge effluent	No. samples	12	12	12	12	12	ND	12	12
	Minimum	18	7	2	0	0	ND	49	0
	Maximum	74	68	19	36	0	ND	$3,0x10^3$	165
	Geometric mean	37	29	6	16	0	ND	814	50
Chlorinated sandfilter effluent	No. samples	12	12	12	12	12	ND	12	12
	Minimum	0	0	0	0	0	ND	0	0
	Maximum	63	39	19	13	0	ND	$2,1x10^3$	148
	Geometric mean	32	20	8	10	0	ND	523	46
Aplas River water	No. samples	12	12	12	12	12	ND	12	12
	Minimum	11	20	0	2	0	ND	50	10
	Maximum	150	135	18	44	0	ND	$2,1x10^3$	570
	Geometric mean	53	58	5	15	0	ND	442	102

TABLE 6-14 Counts of phages, indicator bacteria and human viruses per ml in hospital wastewater.

SOURCE		SP(WG4)	SP(WG5)	MS(E)	MS(S)	BP	EV	FC	FS
Hospital site 1	No. samples	12	12	12	12	12	12	12	12
	Minimum	87	30	12	12	0	0	$1,6x10^4$	50
	Maximum	435	510	$1,1x10^3$	$18x10^3$	325	12	$8,3x10^4$	$1,2x10^4$
	Geometric mean	321	191	565	344	133	8	$5,6x10^4$	$2,8x10^3$
Hospital site 2	No. samples	12	12	12	12	12	12	12	12
	Minimum	25	2	42	99	0	0	$1,5x10^4$	$1,5x10^3$
	Maximum	$5,0x10^2$	$1,8x10^2$	$10x10^2$	$1,0x10^3$	147	12	$3,1x10^5$	$9,0x10^3$
	Geometric mean	263	138	378	625	81	10	$1,6x10^5$	$4,1x10^3$

TABLE 6-15 Counts of phages, indicator bacteria and human viruses per ml in slaughterhouse wastewater.

SOURCE		SP(WG4)	SP(WG5)	MS(E)	MS(S)	BP	EV	FC	FS
Slaughterhouse process water	No. samples	12	12	12	12	12	7	12	12
	Minimum	$2,8x10^4$	$2,5x10^4$	$8,8x10^2$	$2,3x10^3$	0	0	$7,0x10^4$	$1,0x10^3$
	Maximum	$4,0x10^4$	$3,3x10^4$	$1,4x10^3$	$4,9x10^3$	0	0	$8,0x10^5$	$4,5x10^4$
	Geometric mean	$3,7x10^4$	$2,8x10^4$	$1,2x10^3$	$3,2x10^3$	0	0	$3,3x10^5$	$3,8x10^4$
Slaughterhouse aeration pond effluent	No. samples	12	12	12	12	12	7	12	12
	Minimum	45	67	19	15	0	0	$4,0x10^3$	$11,0x10^2$
	Maximum	$23,3x10^3$	$15,5x10^2$	187	207	0	0	$10,0x10^3$	$4,0x10^3$
	Geometric mean	990	675	64	93	0	0	$9,9x10^3$	$2,0x10^3$

SP = somatic coliphages; MS(E) = male-specific coliphages on *E coli*; MS(S) = male-specific coliphages on *S typhimurium*; BP = *Bacteroides fragilis* HSP40 phages; EV = human viruses; FC = faecal coliforms; FS = faecal streptococci

6.4 DISCUSSION

The observation that somatic coliphages outnumber male-specific coliphages in most waters tested by a factor of about 5-10 (Tables 6-13 to 6-15) is in agreement with earlier findings (IAWPRC, 1991; Kfir *et al*, 1991). Grabow *et al* (1984) reported smaller differences, but they used different host strains. In hospital wastewater at both sampling sites male-specific coliphages outnumbered somatic coliphages by a factor of up to 2. This reversed ratio would seem to suggest that humans may excrete a higher ratio of male-specific to somatic coliphages than animals. However, a higher ratio of male-specific to somatic coliphages has also been reported for certain environmental waters which would not necessarily seem to consist predominantly of human excreta (Kfir *et al*, 1991). The generally higher ratio of somatic to male-specific coliphages may also, at least in part, be due to the ability of coliform bacteria to replicate somatic phages, but not male-specific phages, in environmental waters under optimal conditions (Grabow *et al*, 1978; IAWPRC, 1991). The lower ratio of somatic to male-specific coliphages in activated sludge effluent and chlorinated sandfilter effluent than in settled sewage at the Daspoort purification plant may indicate that male-specific coliphages are more resistant than somatic coliphages to the treatment processes concerned. The levels of somatic and coliphages in the settled sewage samples are very similar to those reported earlier for wastewater in South Africa (Grabow *et al*, 1984, Kfir *et al*, 1991) and other parts of the world (IAWPRC, 1991).

The average ratio of faecal coliforms to faecal streptococci was 20:1 and 39:1 in hospital wastewater, and 5:1 and 9:1 in slaughterhouse wastewater (Tables 6-14 and 6-15). These results are in agreement with earlier observations that the ratio of faecal coliforms to faecal streptococci is higher in human than in animal faeces, and support suggestions that this ratio may be used to distinguish between faecal pollution of human and animal origin (Clausen *et al*, 1977). In the samples of settled sewage, treated effluents and the river water, faecal coliforms outnumbered faecal streptococci by a factor of about 5-10. This ratio, and the counts of the organisms, are in agreement with counts of these organisms reported for wastewaters in many parts of the world (Grabow *et al*, 1978, 1984). In most waters tested, counts of somatic coliphages were about 10 times lower than those of faecal coliforms, which implies that they were at about the same level as those of faecal streptococci (Tables 6-13 to 6-15), with counts of male-specific coliphages about ten times lower.

In settled sewage and hospital wastewater average counts of *B fragilis* HSP40 phages were lower than those of male-specific coliphages by a factor of about 2-10 (Tables 6-13 to 6-15). Counts of *B fragilis* HSP40 phages were closer to those of human viruses than any of the other indicators tested, but on average *B fragilis* HSP40 phages still outnumbered human viruses by a factor of about 2-10. The counts of *B fragilis* HSP40 phages in settled sewage (Table 6-13) are remarkably similar to those reported by Tartera *et al* (1989) for comparable wastewaters in and around Barcelona. The absence of *B fragilis* HSP40 phages from slaughterhouse wastewaters (Table 6-15) confirms the specificity of these phages for human excreta (see Chapter 5), is in agreement with results reported by Tartera et al (1989) for slaughterhouse wastewater near Barcelona, and reflects an additional indicator advantage because the possibility of animal enteric viruses causing infection in humans is small (see Chapter 2).

Based on the above results and data published in the literature the relative numbers of the organisms concerned could be about as follows in typical urban wastewater:

Human viruses	= 1
Bacteroides fragilis HSP40 phages	= 5
Male-specific coliphages	= 100
Somatic coliphages	= 500
Faecal streptococci	= 500
Faecal coliforms	= 5000

However, as indicated in this and other studies, the above ratio may vary considerably, particularly in effluents such as those from hospitals and slaughterhouses. In addition, the cell culture methods used in this study can only detect a small proportion of human enteric viruses which may be present in wastewater (see Chapter 2), and may only represent the tip of the iceberg of numbers of human viruses in sewage-polluted water environments. This implies that although the results obtained in the present and earlier studies show valuable indicator features for *B fragilis* HSP40 phages and the other indicators investigated, more information is required on the incidence of human viruses in sewage and sewage-polluted environments to reliably assess the value of various indicators for monitoring the virological safety of water.

Results obtained in this study demonstrate the following indicator features of *B fragilis* HSP40 phages:

1. The phages consistently outnumber enteric viruses, at least those detectable by cell culture propagation, in a variety of water environments.
2. The phages are specific for human faecal pollution, which implies that they can be used to distinguish between faecal pollution of human and animal origin.
3. Their specificity for human excreta implies that the presence of these phages is closely related to human viruses, which is a valuable indicator feature because enteric viruses of animal origin do not constitute a meaningful health risk to humans.
4. From the limited information obtained on treated waters it would not appear that the phages are less resistant to unfavourable environmental conditions than human viruses.

A disadvantage of the relatively low numbers in which *B fragilis* HSP40 phages occur in marginally polluted water is that they are not readily detectable by direct plaque assays. However, this practical shortcoming can be overcome by a variety of practical and inexpensive methods for increasing the sensitivity of detection techniques. Among these are enrichment procedures for qualitative presence/absence tests or quantitative most probable number methods (see Chapter 3), or the application of any of a number of methods for the recovery of the phages from water (see Chapter 4).

Additional useful information obtained in this study include the findings that the nalidixic acid-resistant *E coli* C host strain WG5 yields only marginally lower counts of somatic coliphages than the drug-sensitive strain WG4 (Tables 6-13 to 6-15). On average the differences were insignificant. These results confirm earlier observations

(Havelaar and Hogeboom, 1983; Grabow *et al*, 1984). The drug-resistant strain has valuable advantages for the enumeration of somatic coliphages in heavily polluted water, where interference by contaminant bacteria can be suppressed by the addition of nalidixic acid to plates for plaque assays.

Another valuable finding is that on average there was no meaningful difference in the numbers of male-specific coliphages detected by means of the *E coli* host strain of Debartolomeis and Cabelli (1991) and the *S typhimurium* host strain constructed by Havelaar and co-workers (IAWPRC, 1991). Even though the phages detected by the two hosts have not been characterised, the results are meaningful. As far as can be established, these two hosts have not previously been directly compared with regard to numbers of phages detected in various water environments.

CHAPTER 7

INCIDENCE OF PHAGES, BACTERIAL INDICATORS AND HUMAN VIRUSES IN SEAWATER AND SHELLFISH

7.1 INTRODUCTION

The incidence and behaviour of *B fragilis* HSP40 phages in polluted seawater and shellfish, has not yet been extensively investigated, particularly in terms of correlations to other phages, indicator bacteria and human viruses. Jofre *et al* (1989) reported that in sediments at beaches exposed to wastewater pollution near Barcelona *B fragilis* HSP40 phages consistently outnumbered enteric viruses (viruses detectable by a cytopathogenic effect on the BGM monkey kidney cell line) and rotaviruses by a factor of more than 10. The ratio of *B fragilis* HSP40 phages to enteric viruses and rotaviruses in the marine sediments was similar to that in sewage, which suggests that survival of the phages in the marine environment resembled that of the human viruses. Statistical analysis indicated that numbers of enteric viruses and rotaviruses in the sediments correlated significantly with those of *B fragilis* HSP40 phages but not with those of somatic coliphages.

In studies on selected sites along the South African coastline, (Grabow *et al*, 1989) found that total coliforms, faecal coliforms, faecal streptococci and somatic coliphages generally outnumbered enteric viruses (as detected by cytopathogenic effect in primary vervet kidney cells and the PLC/PRF/5 human liver cell line) in seawater and shellfish at heavily polluted sites. However, in three samples of seawater collected at these sites enteric viruses were detected but no faecal coliforms, and in another sample enteric viruses were detected in seawater in the absence of faecal streptococci. The latter comparison of incidences refers to 10 litre ultrafiltration concentrates tested for viruses, and 100ml samples tested for bacteria by membrane filtration. In studies on seawater and shellfish at marginally polluted sites, enteric viruses were never detected in seawater in the absence of the indicators. However, in shellfish collected at these sites, enteric viruses were detected in one sample which yielded negative results for faecal streptococci and three samples which yielded negative results for somatic coliphages. These results show that at least conventional tests for faecal coliforms, faecal streptococci and somatic coliphages may all have shortcomings as indicators for the presence of enteric viruses in seawater and shellfish.

As far as could be established, the incidence and behaviour of *B fragilis* HSP40 phages in shellfish has not previously been investigated. This Chapter describes an attempt to obtain some information required for assessment of the value of *B fragilis* HSP40 phages as indicators, particularly with regard to human viruses, in wastewater polluted seawater and shellfish in these environments. The study basically consisted of the analysis of seawater and mussels collected at selected sites for various phages, bacterial indicators and enteric viruses. Another objective was to determine the extent to which techniques developed for the detection of *B fragilis* HSP40 phages in freshwater (Chapters 3 to 6) can be applied to seawater and shellfish meat.

7.2 MATERIALS AND METHODS

7.2.1 Shellfish and seawater

Black mussels (*Mytilus galloprovincialis*) were obtained from a site in the immediate vicinity of the Paapenkuil marine wastewater discharge at Port Elizabeth which contains domestic and industrial effluent, and from Bloubergstrand, a well known recreational beach near Cape Town. Seawater was collected from the same location as the mussels. The mussels and seawater were transported at 4-10^{0}C and analysed within 24h of collection. In the laboratory they were scrubbed vigorously to remove detritus and fouling organisms. The mussels were pooled to give 50g samples of mussel meat. The mussel meat was homogenised in 100ml buffered isotonic peptone water which contained 10g peptone (Difco), 9g Na_2HPO_4 (Merck), 5g NaCl (Holpro) and 1,5g KH_2PO_4 (Merck) per litre. Tenfold dilutions of the mussel meat and seawater samples were made in sterile PBS (pH 7,2) and plaque assays for phages were carried out using the techniques described in Chapter 3.

7.2.2 Methods of enumeration

Phages and enteric viruses were enumerated as described in Chapter 6. Total coliforms, faecal coliforms and faecal streptococci (enterococci) were enumerated by a spread-plate procedure using m-Endo Agar, m-FC Agar and m-Enterococus Agar (all from Difco), respectively (Grabow *et al*, 1992).

7.3 RESULTS

Results for mussels and seawater obtained from Paapenkuil are shown in Tables 7-1 and 7-2. Somatic coliphages using *E coli* C (WG4) as host were detected at an average count of 3504/g in mussels and 256/ml in seawater. Male-specific coliphages using *E coli* HS(pFamp)R as host were detected at average counts of 124/g in mussels and 16/ml in seawater. Phages of *B fragilis* HSP40 were detected at average counts of 31/g in mussels and 0,4/ml in seawater. Total coliforms were detected at average counts of $7,1x10^4$/g in mussels and $2,9x10^4$/ml in seawater. Faecal coliforms were detected at average counts of $2,6x10^4$/g in mussels and $7,8x10^3$/ml in seawater. Enterococci were detected at average counts of $2,7x10^3$/g in mussels and $7,7x10^2$ /ml in seawater. No enteric viruses were detected in the mussel samples tested.

Results for mussels and seawater obtained from Bloubergstrand are shown in Tables 7-3 and 7-4. Somatic coliphages using *E coli* C (WG4) as host were detected at average counts of 2/g in mussels. Male-specific coliphages using *E coli* HS(pFamp)R as host were detected at average counts of 0,6/g in mussels. Phages of *B fragilis* were not detected in any of the mussel samples. None of the phages were detected in any of the seawater samples. Total coliforms were detected at average counts of 420/g in the mussels and 199/ml in seawater. Faecal coliforms were detected at average counts of 2/g in mussels and 10/ml in seawater. Enterococci were detected at average counts of 24/g in mussels and 3/ml in seawater.

TABLE 7-1 Counts of phages, bacterial indicators and enteric viruses per gram in mussels collected from sewage polluted seawater at Paapenkuil

Date	Som	MS	*Bact*	EV	TC	FC	FS
90-01-29	1 860	40	-	-	22 000	2 400	1 760
90-02-26	99	18	-	-	9 000	1 700	580
90-03-12	122	0	-	0	5 000	500	250
90-03-26	830	16	-	-	8 000	1 300	3 000
90-04-17	360	37	-	-	7 000	500	143
90-04-25	307	3	-	0	24 000	1 100	1 500
90-05-29	6 130	103	-	-	28 000	17 000	1 400
90-06-25	11 000	60	0	0	160 000	30 000	9 330
90-07-23	6 433	687	0	0	170 000	30 000	6 660
90-08-07	6 720	123	10	-	300 000	5 000	4 200
90-08-20	5 133	280	113	-	50 000	1 400	1 000
Average	3 504	124	31	0	71 181	26 181	2 711

- = not done
Som = somatic coliphages(*E coli* C WG4)
MS = male-specific coliphages(*E coli* host)
Bact = *Bacteroides fragilis* HSP40 phages
EV = enteric viruses
TC = total
FC = faecal coliforms
FS = streptococci/enterococci

TABLE 7-2 Counts of phages, bacterial indicators and enteric viruses per ml in sewage polluted seawater at Paapenkuil

Date	Som	MS	*Bact*	TC	FC	FS
90-01-29	160	2	-	12 150	5 100	830
90-02-26	45	5	-	2 500	430	16
90-03-12	116	0,3	-	2 000	1 300	73
90-03-26	34	6	-	760	28	24
90-04-17	100	3	-	15 000	900	80
90-05-29	83	7	-	20 600	15 000	416
90-06-25	1 700	11	0	150 000	6 000	1 670
90-07-23	250	117	0	193 000	43 000	4 060
90-08-07	23	0,3	0	3 000	310	30
90-08-20	49	9	2	27 000	5 700	500
Average	256	16	0,4	29 101	7 777	770

- = not done
Som = somatic coliphages(*E coli* C WG4)
MS = male-specific coliphages(*E coli* host)
Bact = *Bacteroides fragilis* HSP40 phages
TC = total coliforms
FC = faecal coliforms
FS = streptococci/enterococci

TABLE 7-3 Counts of phages and bacterial indicators per gram in mussels collected at Bloubergstrand

Date	Som	MS	*Bact*	TC	FC	FS
90-01-22	0	0	-	1 700	4	13
90-02-04	12	0,3	-	170	2	37
90-02-20	0	0	-	23	0	7
90-03-12	3	0	-	130	0	23
90-03-19	6	3	-	2 400	4	40
90-03-26	0	0	-	80	2	100
90-04-17	0	0	0	30	0	3
90-05-07	0,3	0	-	50	0	0
90-05-28	0	0	-	30	2	3
90-06-11	0	0	-	0	0	33
90-06-25	0	0	0	400	7	23
Average:	2	0,6	0	420	2	24

- = not done
Som = somatic coliphages(*E coli* C WG4)
MS = male-specific coliphages(*E coli* host)
Bact = *Bacteroides fragilis* HSP40 phages

TC = total coliforms
FC = faecal coliforms
FS = streptococci/enterococci

TABLE 7-4 Counts of phages and bacterial indicators per ml in seawater at Bloubergstrand

Date	Som	MS	*Bact*	TC	FC	FS
90-02-04	0	0	-	50	33	20
90-02-20	0	0	-	0	0	1
90-03-12	0	0	-	56	46	0
90-03-19	0	0	-	1 300	0	0
90-03-26	0	0	-	850	0	6
90-04-17	0	0	0	33	0	0
90-05-07	0	0	-	13	3	0
90-05-28	0	0	-	2	0	1
90-06-11	0	0	-	20	2	0
90-06-25	0	0	-	7	3	3
90-07-11	0	0	0	48	37	7
Average:	0	0	0	199	10	3

- = not done
Som = somatic coliphages(*E coli* C WG4)
MS = male-specific coliphages(*E coli* host)
Bact = *Bacteroides fragilis* HSP40 phages
TC = total coliforms
FC = faecal coliforms
FS = streptococci/enterococci

7.4 DISCUSSION

In seawater heavily polluted with wastewater at the Paapenkuil sampling site the ratio in average counts of *B fragilis* HSP40 phages, male-specific coliphages, somatic coliphages, faecal streptococci and faecal coliforms (Table 7-1) was similar to that in inland wastewaters recorded in Chapter 6. Numbers and ratios of faecal coliforms, faecal streptococci and somatic coliphages were similar to those reported earlier for comparable waters (Grabow *et al*, 1989). The total coliform to faecal coliform ratio of 3:1 is also similar to that reported by Grabow *et al* (1989).

Ratios of *Bacteroides fragilis* phages to somatic coliphages, male-specific coliphages, faecal coliforms and faecal streptococci were, however, similar to those in inland wastewaters recorded in Chapter 6. This would be expected because samples of seawater were collected at Paapenkuil very near to the point of wastewater discharge.

In the marginally polluted seawater at Bloubergstrand numbers of all phages were below the detection limits of the direct plaque assays used (Table 7-4). The ratio in average counts of faecal coliforms to faecal streptococci (3:1) was lower than in water at Paapenkuil (10:1), which suggests longer survival of the faecal streptococci in remotely or marginally polluted seawater, or a higher content of animal faecal pollution (see Chapters 5 and 6). The higher ratio of total coliforms to faecal coliforms at Bloubergstrand (20:1) than at Paapenkuil (4:1) may be due to multiplication of total coliforms in the Bloubergstrand environment. In view of the obviously low level of pollution at Bloubergstrand, no attempt was made to detect enteric viruses in seawater or shellfish at this site.

Counts of phages and bacterial indicators in the flesh of mussels collected at the Paapenkuil site clearly illustrate how these filterfeeders accumulate organisms from water in their environment. Counts of somatic coliphages and male-specific coliphages in mussel flesh outnumbered those in the surrounding seawater by a factor of about 10. In the case of *B fragilis* HSP40 phages the factor of accumulation would appear to be even higher, namely of the order of 78. The accumulation of *B fragilis* HSP40 phages by shellfish has not previously been reported. Although only four samples were tested for each, and only two of these were the same samples, the results do suggest that on average *B fragilis* HSP40 phages outnumbered enteric viruses by a factor of at least 30, which is more than the average ratio of about 5:1 in inland wastewaters (Chapter 6) and is in agreement with ratios reported for marine sediments by Jofre *et al* (1989). The seemingly efficient accumulation of *B fragilis* HSP40 phages by mussels, and significantly higher numbers than human viruses in mussel flesh, would count in favour of these phages as indicators for human viruses in shellfish. These observations should be substantiated by tests on larger numbers of samples. The small number of samples tested for *B fragilis* HSP40 phages is because this part of the study was carried out at an early stage when the method for the detection of the phages had not been satisfactorily established.

The finding that total coliforms, faecal coliforms and faecal streptococci in mussel flesh would not appear to exceed those in the surrounding seawater by the same factor as phages (Tables 7-1 to 7-4), has previously been noted (Grabow *et al*, 1989)

and may indicate that bacteria are less efficiently accumulated by mussels, more easily released by the mussels, or less resistant to conditions in the digestive tract of the mussels.

Counts of all phages and bacterial indicators in seawater and mussels at Bloubergstrand were too low to make meaningful conclusions about their indicator features. The results do, however, show that the quality of seawater at Bloubergstrand was well within limits generally accepted fit for recreational purposes, and that the quality of the mussels was likewise well within limits considered acceptable for human consumption (Grabow *et al*, 1989).

Although for the purposes of this particular study only a limited number of samples has been analysed, the following conclusions may be drawn from the results:

7.4.1. Methods developed for the detection of somatic coliphages, male-specific coliphages and *B fragilis* HSP40 phages in freshwater can successfully be applied to seawater and shellfish meat.

7.4.2 *Bacteroides fragilis* HSP40 phages would seem to outnumber human viruses in shellfish meat by a factor of at least 30, which counts in favour of the potential use of these phages as indicators for human viruses in shellfish meat.

7.4.3 Even though the bacterial indicators generally outnumber phages quite substantially in both seawater and mussels, mussels would appear to accumulate phages more efficiently than the bacteria, which may count in favour of phages as indicators of viruses in shellfish meat.

This study also reveals that counts of *B fragilis* HSP40 phages, and even male-specific coliphages and somatic coliphages, tend to be too low for detection by direct plaque assays in the quality evaluation of many water environments and samples of shellfish meat. This implies that the sensitivity of detection methods would have to be increased by using enrichment procedures or recovery techniques as described in Chapters 4 and 5. Even with the inclusion of procedures for increased sensitivity, the phage methods would still be much faster, less expensive, less complex and less labour intensive than tests for viruses.

Although this study has been successful in obtaining valuable basic information on indicator features of *B fragilis* HSP40 phages relative to coliphages and bacterial indicators, more detailed studies are required for the meaningful formulation of indicator concepts. Additional information required would include more accurate data on the discharge of indicators and pathogens, notably human viruses, to marine environments, their survival in seawater and marine sediments under varying conditions, as well as their uptake and release by various shellfish, and their survival inside the shellfish.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

The literature reviewed in Chapter 2 presents evidence that infectious waterborne diseases have major public health implications world-wide, and constitute one of the most important challenges to the water industry and public health services. Epidemiological data on many outbreaks of waterborne diseases confirm that viruses feature prominently among the large variety of pathogens that may directly or indirectly be transmitted by water. Evidence has also been presented from the literature that bacterial indicators of faecal pollution generally used for convenient, rapid, inexpensive and simple assessment of the safety of water or the efficiency of treatment processes, have meaningful shortcomings with regard to many pathogens, particularly human viruses. In the review of literature special attention has been given to the recent innovative discovery that phages which infect *Bacteroides fragilis* strain HSP40 have attractive indicator features, at least for certain purposes.
Experimental research endeavours of this study were primarly aimed at an evaluation of the potential value of *B fragilis* HSP40 phages as practical indicators for assessment of water quality and the efficiency of treatment systems, particularly with regard to human viruses.

Techniques which have been established proved satisfactory for the detection of *B fragilis* HSP40 phages, male-specific phages and somatic coliphages in a variety of samples, including human and animal stool, raw and treated city sewage, hospital and slaughterhouse wastewater, river water, seawater and suspensions of shellfish meat. The phage host strains used were obtained from the scientists who originally described them, and they were applied, maintained and stored strictly according to their instructions, which implies that results obtained are internationally comparable and acceptable. Findings that the naladixic acid sensitive and resistant strains of *Escherichia coli* C (WG4 and WG5, respectively) yield counts of somatic coliphages which do not differ significantly (Chapter 6), implies that WG5 can reliably be used for the detection of these phages in samples which contain high numbers of potentially interfering organisms. These results confirm those obtained by Grabow *et al* (1984) and Havelaar and Hogeboom (1984) in tests on different waters. However, since WG4 generally tends to yield slightly higher counts, this would be the host of choice in tests on water, such as drinking water supplies, which do not contain interfering organisms.

Observations that the *E coli* host strain of Debartolomeis and Cabelli (1991) consistently yielded more positive results for male-specific coliphages in stools from humans and animals than the *Salmonella typhimurium* host strain (WG49) of Havelaar and Hogeboom (1984) (Chapter 5), but that counts of male-specific phages in various water environments obtained by these two host strains did not differ significantly (Chapter 6), are interesting and have not previously been reported. The reasons for these results are not clear and may warrant further investigation. Important, however, is that the two strains would appear to be equally suitable for the screening of male-specific coliphages in water environments, and that differences in male-specific

coliphages detected would require characterisation of isolates. In our hands the two strains were equally convenient to use, and under correct experimental conditions both gave readily distinguishable plaques (Chapter 3).

Generally speaking all the phages studied were detectable by techniques which are much less complex, much less expensive in terms of materials and facilities, and much faster than procedures for the detection of human viruses. The detection of somatic coliphages was particularly simple and can easily be carried out by any microbiological laboratory. The detection of male-specific coliphages requires a little more skill and know-how, particularly in terms of getting host strains correctly into the logarithmic growth phase for plaque assays. The detection of *B fragilis* HSP40 phages proved the most difficult of the phage techniques. A substantial level of expertise and appropriate facilities are required for successful maintenance of the host cultures, maintenance of the strict anaerobic conditions required for growth of the host strain and the development of plaques, and for controlling contaminants which interfere with cultivation of the host strain and plaque assays.

Results of the survey for somatic coliphages, male-specific coliphages and *B fragilis* HSP40 phages in stools from humans and a variety of animals, including many which have not previously been tested, contribute valuable information to assessment of the indicator features of these phages (Chapter 5). The finding that somatic and male-specific coliphages are excreted in variable but probably higher frequency by all warm-blooded animals than previously thought, confirms their value as indicators of faecal pollution. Especially important is the extended confirmation of previous observations that *B fragilis* HSP40 phages are highly specific for human stool, and that these phages were excreted by the patients included in this study at higher frequency than humans tested in previous studies (Chapter 5). These findings confirm that *B fragilis* HSP40 phages can indeed be used to distinguish between faecal pollution of human and animal origin.

Data obtained on the relative numbers of phages, bacterial indicators and human viruses in a variety of water environments (Chapters 6 and 7), add valuable details to information required for assessment of the indicator value of phages. Results obtained in these investigations, and data published in the literature from other parts of the world, indicate that generally speaking the relative numbers of the indicators concerned may approximately be as follows in most sewage polluted water environments:

Human viruses	= 1
Bacteroides fragilis HSP40 phages	= 10
Male-specific coliphages	= 100
Somatic coliphages	= 500
Faecal streptococci	= 500
Faecal coliforms	= 5 000
Total coliforms	= 100 000

This is a slight modification to the ratios suggested in Chapter 6 in so far as that results obtained in Chapter 7 and data published by Jofre *et al* (1989) would seem to indicate that the ratio of *B fragilis* HSP40 phages to human viruses detected by conventional cell culture techniques may generally be closer to 10:1 than 5:1. It must, however, be kept in mind that these ratios may vary extensively for different

water environments, as illustrated in Chapters 6 and 7. One important variable is that phages would seem to be fairly consistently excreted by a relatively constant percentage of people, while viruses are excreted only by infected individuals and numbers in wastewaters are subject to variables such as epidemics of infections, vaccination programs and seasonal fluctuations.

The above ratios imply that numbers of *B fragilis* HSP40 phages may more closely resemble those of enteric viruses than any of the other indicators. This has the disadvantage that numbers of the phages are often too low for detection by direct plaque assays. In addition, data obtained in this and most other studies on numbers of human viruses in water environments are limited to viruses detectable by conventional cell culture techniques, which may represent only the tip of the iceberg of the total number of viruses in sewage polluted environments (see Chapter 2).

In this study direct plaque assays were used to investigate the incidence of various phages in water environments (Chapters 6 and 7). In tests on marginally or remotely polluted waters these assays proved not sensitive enough to detect low numbers of phages, particularly male-specific coliphages and *B fragilis* HSP40 phages. However, the results of qualitative enrichment procedures (Chapters 3 and 5) and evaluation of the efficiency of techniques for the quantitative recovery of small numbers of viruses from large volumes of water (Chapter 4), show that the sensitivity of phage techniques can substantially be increased. The more sensitive methods would still be much faster, easier and less expensive than tests for human viruses.

No indication has been obtained in this study that any of the phages failed to survive for as long as bacterial indicators or human viruses in the waters studied. However, this has not been investigated in detail and conclusions must be drawn with caution. Although also not investigated in any detail, the results gave no indication of any possibility of *B fragilis* HSP40 phages being able to multiply in water environments. This would be expected in view of the strict anaerobic growth conditions and the growth factors required by the host, and has been confirmed by Tartera and Jofre (1987).

Compared with the other groups of phages, *B fragilis* HSP40 phages and male-specific phages share the advantage of not replicating in water environments, which resembles the behaviour of human viruses. Somatic coliphages may under favourable conditions replicate in water environments (see Chapter 6). The major advantage of *B fragilis* HSP40 phages is that they are highly specific indicators of human faecal pollution and human viruses, while male-specific coliphages only indicate faecal pollution by humans or warm-blooded animals, and somatic coliphages may not even be specific for faecal pollution since the phages may replicate in water environments. Coliphages, and particularly somatic coliphages, have the advantage that they occur in larger numbers in water environments than *B fragilis* HSP40 phages, and that they are detectable by easier techniques than the *B fragilis* HSP40 phages.

In conclusion results obtained in this study supply evidence of the following indicator features of *B fragilis* HSP40 phages:

1. The phages are present whenever human viruses are present.
2. The phages generally outnumber human viruses by a factor of of about 10.
3. Like human viruses, the phages cannot multiply in the environment.

4. The phages are at least as resistant as human viruses to unfavourable conditions in the environment and to water treatment processes.
5. The phages are highly specific for human faecal pollution and human viruses.
6. Numbers of phages more closely resemble those of human viruses than many other indicators.
7. The phages are detectable by relatively simple, inexpensive and rapid techniques.
8. The phages and their host constitute no health risk and are safe to work with.

Although valuable and fundamentally significant results have been obtained, some additional details which were beyond the objectives of this study are required for further assessment of the value of *B fragilis* HSP40 phages as indicators of human viruses. Follow-up research should, therefore, include studies on aspects of *B fragilis* HSP40 phages such as:

1. Relative survival of the phages and human viruses in various water environments.
2. Optimisation, evaluation and implementation of more sensitive techniques for detection of the phages.
3. More detailed investigation of the relative incidence of the phages and human viruses in various water environments.
4. More detailed investigation of the relative resistance of the phages and human viruses to water treatment and disinfection processes.
5. Evaluation and optimisation of techniques for the decontamination of test samples by, for instance, membrane filtration or chloroform treatment.
6. Characterisation of phages in terms of morphology, structure and composition in order to obtain information on possible variation in properties and behaviour in water environments.
7. Investigation of the variability of numbers of the phages in water environments as a result of factors such as seasonal variation and variation in the excretion by humans.
8. Search for alternative *B fragilis* hosts which may detect higher numbers of phages specific for human faecal pollution.
9. In order to maintain the highest level of quality control and to ensure that results obtained are internationally comparable and acceptable, it would be essential to participate in international work on the standardisation of techniques and quality control programs.
10. Formulation of practical and reliable techniques and limits for the inclusion of *B fragilis* HSP40 phages in water quality monitoring programs.

REFERENCES

Abeles, A L, K M Snyder and D K Chattoraj. 1984. P1 plasmid replication: replicon structure. J. Mol. Biol. 17 : 307 - 324.

Ackermann, H W. 1969. Bacteriophages proprietes et premieres etapes d'une classification. Path-Biol. 17 : 1003 - 1024.

Ackermann, H W and A Eisenstark. 1974. The present state of phage taxonomy. Intervirology 3 : 201 - 219.

Ackermann, H W. 1975. La classification des bacteriophages des cocci gram-positifs: Micrococcus, Streptococcus et Staphyllococcus. Path-Biol. 23 : 247 - 253.

Ackermann, H W. Cubic, filamentous and pleomorphic bacteriophages. In CRC Handbook of Microbiology. Vol II 2nd edn. Laskin, A.I. and Lechevalier, H A pp 673 - 695. Cleveland, Ohio : CRC Press.

Ackermann, H W and T M Nguyen. 1983. Sewage coliphages studied by electron microscopy. Appl. Environ. Microbiol. 45 : 1049 - 1059.

Adams, M H. 1953. In Bacteriophages. New York: Interscience Publishers, Inc.

Adams, M H. 1953. Criteria for a biological classification of bacterial viruses. Ann. N. Y. Acad. Sci. 56 : 442 - 459.

Adams, M H. 1959. Bacteriophages. Interscience, New York.

Anderson, E S. 1957. The relations of bacteriophages to bacterial ecology. Seventh Symp. of the Soc. for Gen. Microbiol, Royal Institution, London.

APHA. 1989. Standard methods for the examination of water and wastewater. 17th Edn. Washington, D.C., American Public Health association.

Atkins, J F, J A Steitz, C W Anderson and P Model. 1979. Binding of mammalian Ribosomes to MS2 phage RNA Reveals an overlapping Gene Encoding a Lysis Function. Cell 18 : 247 - 256.

Ayres, P A. 1970. Coliphages in sewage and the marine environment. p 275 - 298. In F A Skinner and J M Shewan, Aquatic Microbiology, Academic Press, London.

Balluz, S A, H H Jones and M Butler. 1977. The persistence of poliovirus in activated sludge treatment. J. Hyg. 78 : 187 - 192.

Balows, A, W J Hausler, K L Herrmann, H D Isenberg and H J Shadomy. 1991. In Manual of Clinical Microbiology. 5th Edn. p 538. American Society for Microbiology, Washington, D.C.

Bell, R G. 1976. The limitation of the ratio of fecal coliform to total coliphage as a water pollution index. Wat. Res. 10 : 745 - 748.

Beremand, M N and T Blumenthal. 1979. Overlapping genes in RNA Phage: a New Protein Implicated in Lysis. Cell 18 : 257 - 266.

Berg, G, R B Dean and D R Dahling. 1968. Removal of Poliovirus 1 from secondary effluents by lime flocculation and sand filtration. J. Am. Wat. Wks. Ass. 60 : 193 - 198.

Berg, G. 1969. The fate of viruses in a marine environment. In Advances in Water Pollution Research, Proceedings of the 4th International Conference, Prague Ed: S H Jenkins. pp 833 - 834. Pergamon Press, New York.

Berg, G, D R Dahling and D Berman. 1971. Recovery of small quantities of viruses from clean waters on cellulose nitrate membrane filters. Appl. Microbiol. 22 : 608 - 614.

Berg, G. 1973. Reassessment of the virus problem in sewage and in surface and renovated waters. In Progress in water technology. Ed: S H Jenkins. pp 87 - 94. Pergamon Press, New York.

Berg, G, D R Dahling, G A Brown and D Berman. 1978. Validity of faecal coliforms, total coliforms, and faecal streptococci as indicators of viruses in chlorinated primary sewage effluents. Appl. Environ. Microbiol. 36 : 880 - 884.

Bergh, O, K Y Borsheim, G Bratbak and M Heldal. 1989. High abundance of viruses found in aqautic environments. Nature 340 : 467 - 469.

Berman, D, M Rohr and R S Safferman. 1980. Concentration of Poliovirus in Water by Molecular Filtration. Appl. Environ. Microbiol. 40 : 426 - 428.

Berry, S A and B G Noton. 1976. Survival of bacteriophages in seawater. Wat. Res. 10 : 323 - 327.

Birge, E A. 1981. Bacterial and bacteriophage genetics. Springer Verlag, New York.

Bitton, G, L T Chang, S R Farrah, and K Clifford. 1981. Recovery of coliphages from wastewater effluents and polluted lake water by the magnetic-organic flocculation method. Appl. Environ. Microbiol. 41 : 93 - 96.

Bitton, G. 1987. Fate of bacteriophages in water and wastewater treatment plants. In Phage Ecology. Ed: Goyal, S M, C P Gerba, G Bitton. pp 181 - 195. Whiley, New York.

Bixby, R L, and D J O'Brien. 1979. Influence of fulvic acid on bacteriophage adsorption and complexation in soil. Appl. Environ. Microbiol. 38 : 840 - 845.

Bloom, H H, W N Mack, B J Krueger and W L Mallman. 1959. Identification of enteroviruses in sewage. J. Infect. Dis. 195 : 61 - 68.

Booth, S J, R L Van Tassel, J L Johnson, and T D Wilkens. 1979. Bacteriophages of Bacteroides. Rev. Infect. Dis. 1 : 325 - 334.

Borrego, J J, F Arrabal, A de Vicente, L F Gomez and P Romero. 1983. Study of microbial inactivation in the marine environment. J. Wat. Pollut. Control Fed. 55 : 297 - 302.

Borrego, J J and P Romero. 1985. Coliphage survival in seawater. Wat. Res. 19 : 557 - 562.

Borrego, J J, M A Morinigo, A de Vicente, R Cornax and P Romero. 1987. Coliphages as an indicator of faecal pollution in water, its relationship with indicator and pathogenic micro-organisms. Wat. Res. 21 : 1473 - 1480.

Borrego, J J, R Cornax, M A Morinigo, E Martinez-Manzares and P Romero. 1990. Coliphages as an indicator of faecal pollution in water. Their survival and productive infectivity in natural aquatic environment. Wat. Res. 24 : 111 - 116.

Borrego, J J, R Cornax, D R Preston, S R Farrah, B McElhaney and G Bitton. 1991. Development and Application of New Positively Charged Filters for Recovery of Bacteriophages from Water. Appl. Environ. Microbiol. 57 : 1 218 - 1 222.

Bosch, A, R M Pinto, A R Blanch, and J T Jofre. 1988. Detection of human rotavirus in sewage through two concentration procedures. Wat. Res. 22 : 343 - 348.

Bosch, A, R Gajardo, F X Abad, J M Diez and J Jofre. 1991. Concentration of Hepatitis A virus in environmental samples. Wat. Sci. Technol. 24 : 229 - 234.

Bradley, D E. 1967. Ultrastructure of bacteriophages and bacteriocins. Bact. Rev. 31 : 230 - 314.

Brock, T D and M T Madigan. 1988. Biology of microorganisms. 5 th Edn. Prentice Hall International, INC.

Brown, A C. 1987. Marine pollution and health in South Africa. S. Afr. Med. J. 71 : 244 - 248.

Buchanan, R E, N E Gibbons, S T Cowan, J G Holt, J Liston, R G E Murray, C F Niven, A W Ravin, R Y Stanier. 1974. In Bergey's Manual of Determinative Bacteriology. 8th Edn. The Williams and Wilkins Company, Baltimore. p 385.

Bulmer, M. 1989. Codon usage and secondary structure of MS-2 phage RNA. Nuc. Acids Res. 17 : 1 839 - 1 843.

Burge, W D, D Colacicco and W N Cramer. 1981. Criteria for achieving pathogen destruction during composting. J. Wat. Pollut. Control Fed. 53 : 1 683 - 1 690.

Burkhardt, W, W D Watkins and S R Rippey. 1992. Survival and Replication of Male-Specific Bacteriophages in Molluscan Shellfish. Appl. Environ. Microbiol. 58 : 1 371 - 1 373.

Burns, R W and O J Sproul. 1967. Viricidal effects of chlorine in wastewater. J. Wat. Pollut. Control Fed. 39 : 1 834 - 1 841.

Bush, A F and J D Isherwood. 1966. Virus removal in sewage treatment. J. San. Engrs. Div., Proc. Am. Soc. Civ. Engrs. 92 : 99 - 107.

Cabelli, V J. 1983. Waterborne viral infections. In Viruses and disinfection of water and wastewater. Ed: M Butler, A R Medlen and R Morris. University of Surrey Press, Guilford, England. pp 107 - 130.

Cabelli, V. 1989. Swimming-associated illness and recreational water quality criteria. Wat. Sci. Technol. 21 : 13 - 21.

Chang, L T, S R Farrah and G Bitton. 1981. Positively charged filters for virus recovery from wastewater treatment plant effluents. Appl. Environ. Microbiol. 42 : 921 - 924.

Clarke, N A, R E Stevenson, S L Chang and P W Kabler. 1961. Removal of enteric viruses from sewage by activated sludge treatment. Am. J. Pub. Hlth. 51 : 1 118 - 1 129.

Clausen, E M, B L Green and W Litsky. 1977. Fecal streptococci: Indicators of Pollution. In Bacterial Indicators/Health Hazards Associated With Water. Ed: A W Hoadly and B J Dutka. American Society for Testing and Materials. pp 247 - 264.

Cliver, D O. 1971. Viruses in water and wastewater: effects of some treatment methods. In Proceedings of the 13th Water Quality Conference. pp 149 - 159. University of Illinois, Urbana.

Coetzee, O J. 1962. Bakteriophagen als Indikator fakaler Wasserverunreinigung. Gesundheits-Ingenieur 12 : 371 - 372.

Coetzee, O J. 1966. Bacteriophage as index of faecal pollution. Public Health p45.

Cooper, S W, E G Szymczak, N V Jacobus and F P Tally. 1984. Differentiation of Bacteroides ovatus and Bacteroides thataiotaomiron by means of bacteriophage. J. Clin. Microbiol. 20 : 1 122 - 1 125.

Cornax, R, M A Morinigo, M C Balebona, D Castro and J J Borrego. 1991. Significance of several bacteriophage groups as indicators of sewage pollution in marine waters. Wat. Res. 25 : 673 - 678.

Curtis, A, A Suttle, M Chan and M T Cottrell. 1990. Nature 347 : 467 - 468.

Dahling, D R, G Berg and D Merna. 1974. BGM a continous cell line more sensitive than primary Rhesus and African Green Monkey kidney cells for recovery of viruses from water. Health Lab. Sci. 11 : 275 - 282.

Dahling, D R and B A Wright. 1986. Recovery of viruses from water by a modified flocculation procedure for second-step concentration. Appl. Environ. Microbiol. 51 : 1 326 - 1 331.

Davis, B D, R Dulbecco, H N Eisen and H S Ginsberg. 1990. Microbiology. 4 th Edn. J B Lippincott Company, Philadelphia.

Debartolomeis, J and V J Cabelli. 1991. Evaluation of an Escherichia coli Host strain for Enumeration of F Male-specific Bacteriophages. Appl. Eviron. Microbiol. 57 : 1 301 - 1 305.

Deetz, T R, E R Smith, S M Goyal, C P Gerba, J V Vallet, H L Tsai, H L Dupont and B H Heswick. 1984. Occurence of rota and enteroviruses in drinking and environmental waters in a developing nation. Wat. Res. 18 : 572 - 577.

Deibel, R H. 1964. The group D streptococci. Bact. Rev. 28 : 330 - 366.

DeMichele, E. 1974. Water reuse, virus removal and public health. In Virus Survival in Water and Wastewater Systems. Ed: J F Malina Jr and B P Sagik. pp 45 - 56. Center for Research in Water Resources, University of Texas, Austin.

Dhillon, T S, Y S Chan, S M Sun, and W S Chau. 1970. Distribution of coliphages in Hong Kong sewage. Appl. Microbiol. 20 : 187 - 191.

Dhillon, E K S and T S Dhillon. 1974. Synthesis of indicator strains and density of ribonucleic acid-containing coliphages in sewage. Appl. Microbiol. 27 : 640 - 647.

Dhillon, T S, E K S Dhillon, H C Chau, W K Li, and H C Tsang. 1976. Studies on bacteriophage distribution: virulent and temperate bacteriophage content of mammalian feces. Appl. Environ. Microbiol. 32 : 68 - 74.

Dias, F F and J V Bhat. 1965. Microbial ecology of activated sludge. II. Bacteriophages, coliforms and other organisms. Appl. Microbiol. 13 : 257 - 261.

Divizia, M, A L Santi and A Pana. 1989. Ultrafiltration: an efficient second step for hepatitis A virus and poliovirus concentration. J. Virol. Methods 23 : 55 - 62.

Drury, D F and D C Wheeler. 1982. Applications of a Serratia marcescens bacteriophage as a new microbial tracer of aqueous environments. J. Appl. Bact. 53 : 137 - 142.

Dufour, A P. 1977. Escherichia coli, the fecal coliform. In Bacterial Indicators / Health Hazards Associated with Water. Ed: A W Hoadley and B J Dutka. American Society for Testing and Materials, Philadelphia, Pa.

Durham, D and H W Wolf. 1973. Wastewater chlorination: Panacea or placebo? Water Sewage Works 120 : 67 - 69.

Dutka, B J. 1973. Coliforms are an inadequate index of water quality. J. Environ. Hlth. 36 : 39 - 46.

Engelbrecht, R S and E O Greening. 1978. Chlorine resistant indicators. In Indicators of viruses in water and food. Ed: G Berg. pp 243 - 266. Ann Arbor Science Publishers, Inc., Ann Arbor, Mich.

Ewert, D L and M J B Paynter. 1980. Enumeration of bacteriophages and host bacteria in sewage and the activated sludge treatment process. Appl. Environ. Microbiol. 39 : 576 - 583.

Farrah, S R, C P Gerba, C Wallis and J L Melnick. 1976. Concentration of viruses from large volumes of tap water using pleated membrane filters. Appl. Environ. Microbiol. 31 : 221 - 226.

Farrah, S R, S M Goyal, C P Gerba, C Wallis and J L Melnick. 1977. Concentration of enteroviruses from estuarine water. Appl. Environ. Microbiol. 33 : 1 192 - 1 196.

Farrah, S R, S M Goyal, C P Gerba, C Wallis and J L Melnick. 1978. Concentration of poliovirus from tap water onto embrane filters with aluminium chloride at ambient pH levels. Appl. Environ. Microbiol. 35 : 624 - 626.

Farrah, S R and G Bitton. 1979. Low molecular weight substitutes for beef extract as eluants for poliovirus adsorbed to membrane filters. Can. J. Microbiol. 25 : 1 045 - 1 051.

Farrah, S R, D O Shah and L O Ingram. 1981. Effects of chaotropic and antichaotropic agents on elution of poliovirus adsorbed to membrane filters. Proc. Natl. Acad. Sci. U.S.A. 78 : 1 229 - 1 232.

Fattal, B and M Nishmi. 1977. Enterovirus types in Israel sewage. Wat. Res. 11 : 393 - 397.

Feachem, R. 1975. An improved role for faecal coliform to faecal streptococci ratios in the differentiation between human and non-human pollution sources. Wat. Res. 9 : 689 - 690.

Fields, H A and T G Metcalf. 1975. Concentration of adenovirus from seawater. Wat. Res. 9 : 357 - 364.

Fiers, W. 1979. In Comprehensive Virology. Ed: H Fraenkel-Conrat and R R Wagner, Plenum, New York. pp 69 - 204.

Freifelder, D. 1987. In Molecular Biology. Second Edition. Ed: Jones and Bartlett Publishers, Inc. Boston, Portola Valley. pp 551 - 593.

Funderburg, S W and C A Sorber. 1985. Coliphages as indicators of enteric viruses in activated sludge. Wat. Res. 5 : 547 - 555.

Furuse, K, T Adoi, T Shiba, T Sakurai, T Miyake and I Watanabe. 1973. Isolation and grouping of RNA phages. IV. A survey in Japan. J. Keio Med. Soc. 50 : 363 - 376.

Furuse, K, S Osawa, J Kawashiro, R Tanaka, Z Ozawa, S Sawamura, Y Yanagawa, T Nagao, and I Watanabe. 1983. Bacteriophage distribution in human faeces: continuous survey of healthy subjects and patients with internal and leukemic diseases. J. Gen. Virol. 64 : 2039 - 2043.

Gajardo, R, J M Diez, J Jofre and A Bosch. 1991. Adsorption-elution with negatively and positively-charged glass powder for the concentration of hepatitis A virus from water. J. Virol. Methods 31 : 345 - 352.

Gangemi, J D, E V Connell, B G Mahlandt and G A Eddy. 1977. Arenavirus concentration by molecular filtration. Appl. Environ. Microbiol. 34 : 330 - 332.

Garelick, H and J E Scutt. 1981. Concentration of virus from tap water at ambient salt and pH levels using positively charged filter media. Wat. Res. 15 : 815 - 816.

Geldenhuys, J C and P D Pretorius. 1989. The occurrence of enteric viruses in polluted water, correlation to indicator organisms and factors influencing their numbers. Wat. Sci. Technol. 21 : 105 - 109.

Geldreich, EE and B A Kenner. 1969. Concepts of faecal streptococci in stream pollution. J. Wat. Pollut. Control Fed. 41 : R336 - R352.

Geldreich, E E. 1978. Bacterial populations and indicator concepts in feces, sewage, stormwater and solid wastes. In Indicators of viruses in water and food. Ed: G Berg. Ann Arbor Science Publishers, Inc., Ann Arbor, Mich. pp 51 - 97.

Gerba, C P, C Wallis and J L Melnick. 1975. Viruses in water: the problem, some solutions. Environ. Sci. Technol. 9 : 1 122 - 1 126.

Gerba, C P, C H Stagg and M G Abadie. 1978. Characterization of sewage solid-associated viruses and behavior in natural waters. Wat. Res. 12 : 805 - 812.

Gerba, C P, S R Farrah, S M Goyal, C Wallis, and J L Melnick. 1978. Concentration of enteroviruses from large volumes of tapwater, treated sewage, and seawater. Appl. Environ. Microbiol. 35 : 540 - 548.

Gerba, C P. 1987. Phage as indicators of faecal pollution. In Phage Ecology. Ed: Goyal, S M, C P Gerba, G Bitton. Whiley, New York. pp 197 - 209.

Glass, J S and R T O'Brien. 1980. Enterovirus and coliphage inactivation during activated sludge treatment. Wat. Res. 14 : 877 - 882.

Goyal, S M. 1980. Indicators of Viruses. In Viral Pollution of the Environment. Ed: G Berg. CRC Press, Boca Raton, Florida.

Goyal, S M, K S Zerda, and C P Gerba. 1980. Concentration of coliphages from large volumes of water and wastewater. Appl. Environ. Microbiol. 39 : 85 - 91.

Goyal, S M and C P Gerba. 1982. Concentration of coliphages from water by membrane filters. In Methods in Environmental Virology. Marcel Dekker, New York. pp 59 - 116.

Goyal, S M, and C P Gerba. 1983. Viradel method for detection of rotavirus from seawater. J. Virol. Methods 7 : 279 - 285.

Goyal, S M, C P Gerba, and G Bitton. 1987. In Phage Ecology. Ed: John Wiley and Sons, New York. pp 45 - 74.

Grabow, W O K. 1968. The virology of wastewater treatment. Wat. Res. 2 : 675 - 701.

Grabow, W O K, B W Bateman and J S Burger. 1978. Microbiological quality indicators for routine monitoring of wastewater reclamation systems. Prog. Wat. Technol. 10 : 317 - 327.

Grabow, W O K, J S Burger and E M Nupen. 1980. Evaluation of acid-fast bacteria, Candida albicans, enteric viruses and conventional indicators for monitoring wastewater reclamation systems. Prog. Wat. Technol. 12 : 803 - 817.

Grabow, W O K, C A Hilner and P Coubrough. 1981. Evaluation of standard and modified M-Fc, MacConkey, and Teepol media for membrane filtration counting of fecal coliforms in water. Appl. Environ. Microbiol. 42 : 192 - 199.

Grabow, W O K, W C du Randt, O W Prozesky and W E Scott. 1982. Microcystis aeruginosa toxin: Cell culture toxicity, hemolysis and mutagenicity assays. Appl. Environ. Microbiol. 43 : 1 425 - 1 433.

Grabow, W O K, V Gauss-Muller, O W Prozesky and F Deinhardt. 1983. Inactivation of Hepatitis A virus and indicator organisms in water by free chlorine residuals. Appl. Environ. Microbiol. 46 : 619 - 624.

Grabow, W O K, P Coubrough, E M Nupen and B W Bateman. 1984. Evaluation of coliphages as indicators of the virological quality of sewage-polluted water. Water S.A. 10 : 7 - 14.

Grabow, W O K. 1986. Water quality assessment and control in South Africa. S.A. J. Sci. 82 : 342 - 346.

Grabow, W O K. 1986. Indicator systems for assessment of the virological safety of treated drinking water. Wat. Sci. Technol. 18 : 159 165.

Grabow, W O K and P Coubrough. 1986. A practical direct plaque assay for coliphages in 100 ml samples of drinking water. Appl. Environ. Microbiol. 52 : 430 - 433.

Grabow, W O K, G K Idema, P Coubrough and B W Bateman. 1989. Selection of indicator systems for human viruses in polluted seawater and shellfish. Wat. Sci. Tecnol. 21 : 111 - 117.

Grabow, W O K. 1990. Microbiology of drinking water treatment: reclaimed wastewater. In Drinking Water Microbiology - Progress and Recent Developments. Ed: G A McFetters. Springer Verlag, New York. pp 185 - 203.

Grabow, W O K, D L Puttergill and A Bosch. 1992. Propagation of adenovirus types 40 and 41 in the PLC/PRF/5 primary liver carcinoma cell line. J. Virol. Methods 37 : 201 - 208.

Grabow, W O K, J C de Villiers and C Schildhauer. 1992. Comparison of selected methods for the enumeration of fecal coliforms and Escherichia coli in shellfish. Appl. Environ. Microbiol. 58 : 3 203 - 3 204.

Gray, M, R DeLeon and M D Sobsey. 1992. May 1992 Washington DC IAWPRC Symposium on Health-Related Water Microbiology.

Guelin, A. 1948. Etude quantitative de bactreiophage de la mer. Ann. Inst. Pasteur. 74 : 104 - 112.

Harm, W. 1980. Biological effects of Ultraviolet Radiation. Cambridge University Press, Cambridge, Mass.

Havelaar, A H and W M Hogeboom. 1983. Factors affecting the enumeration of coliphages in sewage and sewage polluted waters. Antonie van Leeuwenhoek 49 : 387 - 397.

Havelaar, A H and W M Hogeboom. 1984. A method for the enumeration of male-specific bacteriophages in sewage. J. Appl. Bacteriol. 56 : 439 - 447.

Havelaar, A H, W M Hogeboom and R Pot. 1984. F-specific RNA bacteriophages in sewage; methodology and occurence. Wat. Sci. Technol. 17 : 645 - 655.

Havelaar, A H and Nieuwstad. 1985. Bacteriophages and faecal bacteria as indicators of chlorination efficiency of biologically treated wastewater. J. WPCF 57 : 1 084 - 1 088.

Havelaar, A H, K Furuse and W H Hogeboom. 1986. Bacteriophages and indicator bacteria in human and animal faeces. J. Appl. Bacteriol. 60 : 255 - 262.

Havelaar, A H and W M Hogeboom. 1988. A method for the enumeration of male-specific bacteriophages in sewage. Appl. Bacteriol. 56 : 439 - 447.

Havelaar, A H and W M Pot-Hogeboom. 1988. F-specific RNA-bacteriophages as model viruses in water hygiene : ecological aspects. Wat. Sci. Technol. 20 : 399 - 407.

Havelaar, A H, W M Pot-Hogeboom, K Furuse, R Pot and N P Hormann. 1990. F-specific RNA bacteriophages and sensitive host strains in faeces and wastewater of human and animal origin. J. Appl. Bacteriol. 69 : 30 - 37.

Havelaar, A H, M Butler, S R Farrah, J Jofre, E Marques, A Ketratanakul, MT Martins, S Ohgaki, M D Sobsey and U Zaiss. 1991. Bacteriophages as model viruses in water quality control. Wat. Res. 25 : 529 - 545.

Hayes, W. 1968. In The Genetics of Bacteria and their Viruses. 2nd Ed. Blackwell scientificpublications, Oxford and Edinburgh.

Hejkal, T W, F M Wellings, A L Lewis, and P A LaRock. 1981. Distribution of viruses associated with particles in wastewater. Appl. Environ. Microbiol. 41 : 628 - 634.

Hill, W F, E W Akin and W H Benton. 1971. Detection of viruses in water: a review of methods and application. Wat. Res. 5 : 967 - 995.

Hilton, M C and G Stotzky. 1973. Use of coliphages as indicators of water pollution. Can. J. Microbiol. 19 : 747 - 751.

IAWPRC Study Group on Water Virology. 1983. The health significance of viruses in water. Wat. Res. 17 : 121 - 132.

IAWPRC Study Group on Health Related Water Microbiology. 1991. Bacteriophages as model viruses in water quality control. Wat. Res. 25 : 529 - 545.

Ibister, J D, J A Simmons, W M Scott and J F Kitchens. 1983. A simplified method for coliphage detection in natural waters. Acta Microbiol. 32 : 197 - 206.

Idema, G K, B W Bateman, R Kfir and W O K Grabow. 1991. A comparison of methods for the isolation of a wide range of viruses from shellfish. Wat. Sci. Technol. 24 : 427 - 430.

Ignazitto, G, L Volterra, F A Aulicino, and A M D'Angelo. 1980. Coliphages as indicators in a treatment plant. Wat., Air, Soil Pollut. 13 : 391 - 398.

Jansons, J and M R Buccens. 1986. Virus detection in water by ultrafiltration. Wat. Res. 20 : 1 603 - 1 608.

Jofre, J, A Bosch, F Lucena, R Girones, and C Tartera. 1986. Evaluation of Bacteroides fragilis bacteriophages as indicators of the virological quality of water. Wat. Sci Technol. 18 : 167 - 173.

Jofre, J, M Blasi, A Bosch and F Lucena. 1989. Occurrence of bacteriophages infecting Bacteroides fragilis and other viruses in polluted marine sediments. Wat. Sci. Technol. 21 : 15 - 19.

Johnson, K M. 1979. Sewage-based shellfish culture and the public health : virological aspects. University of California, Berkeley, Ph.D. thesis, p 127.

Jothikumar, N, A Dwarkadas and P Khanna. 1988. An effective approach for the reconcentraion of viruses from water samples with magnesium compound. Abstract paper presented in First Asia Pacific Congress of Medical Virology, Singapore.

Jothikumar, N, A Dwarkadas and P Khanna. 1990. A simple elution and reconcentration technique for viruses concentated on membrane filters from drinking water samples. Wat. Res. 24 : 367 - 372.

Kai, S, S Watanabe, K Furuse and A Osawa. 1985. Bacteroides bacteriophages isolated from human faeces. Microbiol. Immunol. 29 : 895 - 899.

Kapuscinski, R R and R Mitchell. 1983. Sunlight-induced mortality of viruses and Escherichia coli in coastal seawater. Environ. Sci. Technol. 17 : 1 - 6.

Katzenelson, E, B fattal anf T Hostovsky. 1976. Organic Flocculation: an Efficient Second-Step Concentration Method for the Detection of Viruses in Tap Water. Appl. Environ. Microbiol. 32 : 638 - 639.

Keller, R and N Traub. 1974. The characterisation of a Bacteroides fragilis bacteriophage recovered from animal sera: Observations on the nature of Bacteroides bacteriophage carrier culture. J. Gen. Virol. 24 : 895 - 899.

Kenard, R P and R S Valentine. 1974. Rapid determination of the presence of enteric bacteria in water. Appl. Microbiol. 27 : 484 - 487.

Kennedy, Jr, J J, G Bitton, and J L Oblinger. 1985. Comparison of selective media for assay of coliphages in sewage effluent and lake water. Appl. Environ. Microbiol. 49 : 33 - 36.

Kessik, M A and R A Wagner. 1978. Electrophoretic mobilities of virus adsorbing filter materials. Wat. Res. 12 : 263 - 268.

Keswick, B H. 1982. Monitoring disinfection of enteric viruses with bacteriophages. In Viruses and Disinfection of Water and Wastewater. Ed: M Butler, A R Medlen and R Morris. University of Surrey Press, England. pp 298 - 305.

Kfir, R, P Coubrough and W O K Grabow. 1991. The occurrence of male-specific and somatic bacteriophages in polluted South African waters. Wat. Sci. Technol. 24 : 251 - 254.

Kollins, S A. 1966. The presence of human enteric viruses in sewage and their removal by conventional sewage treatment methods. Adv. Appl. Microbiol. 8 : 145 - 193.

Kott, Y. 1966. Estimation of low numbers of Escherichia coli bacteriophages by use of the most probable number method. Appl. Microbiol. 14 : 141 - 144.

Kott, Y. 1966. Survival of T bacteriophages and coliform bacteria in sea water. Bull. Inst. Mar. Sci. 11 : 1 - 6.

Kott, Y, N Roze, S Sperber and N Betzer. 1974. Bacteriophages as viral pollution indicators. Wat. Res. 8 : 165 - 171.

Kott, Y, H Ben-Ari and L Vinokur. 1978. Coliphages survival as viral indicator in various wastewater quality effluents. Prog. Wat. Technol. 10 : 337 - 346.

Kott, Y. 1981. Viruses and bacteriophages. Sci. Total Environ. 18 : 13 - 23.

Kott, Y. 1984. Coliphages as reliable enteric virus indicators. In Viruses in Water. Ed: J L Melnick. Karger, Basel. pp171 - 174.

Krahn, P M, R J O'Callaghan and W Paranchych. 1972. Stages in Phage R17 infection. Virology 47 : 628 - 637.

Kruse, C W. 1965. Mode of Action of Halogens on Bacteria and Viruses and Protozoa in Water Systems. The Johns Hopkins University, Baltimore Maryland.

Landry, E F, J M Thomas and T J Vicale. 1978. Efficiency of beef extract for the recovery of poliovirus from wastewater effluents. Appl. Environ. Microbiol. 36 : 544 - 548.

Logan, K B, G E Rees, N D Seeley, and S B Primrose. 1980. Rapid concentration of bacteriophages from large volumes of freshwater: evaluation of positively charged, microporous filters. J. Virol. Methods 1 : 87 - 97.

Luria, S E. 1953. In General Virology. John Wiley and Sons, Inc., New York.

Malina, J, K Ranganathan, B Sagik and B Moore. 1975. Poliovirus inactivation in activated sludge. J. Wat. Pollut. Control Fed. 47 : 2 178 - 2 183.

Marzouk, Y , S M Goyal and C P Gerba. 1980. Relationship of viruses and indicator bacteria in water and wastewater of Israel. Wat. Res. 14 : 1 585 - 1 590.

Matthews, R E F. 1982. Classification and nomenclature of viruses. Fourth Report of the International Committee on Taxonomy of Viruses. Interviral. 17 : 1 - 199.

McFetters, G A, G K Bissonnette, J J Jezeski, C A Thomson and D G Stuart. 1974. Comparitive survival of indicator bacteria and enteric pathogens in well waters. Appl. Environ. Microbiol. 27 : 823 - 829.

Mesquita, M M F. de. 1988. Bacterial and bacteriophage investigations using the mussel Mytilus edulis. University of Newcastle upon Tyne, Ph.D. thesis, p 244.

Mesquita, M M F, L M Evison and P A West. 1991. Removal of faecal indicator bacteria and bacteriophages from the common mussel (Mytilus edulis) under artificial depuration conditions. J. Appl. Bacteriol. 70 : 495 - 501.

Metcalf, T D, C Wallis and J L Melnick. 1974. Environmental factors influencing isolation of enterovruses from polluted surface waters. Appl. Microbiol. 27 : 920 - 926.

Metcalf, T G. 1978. Indicators for viruses in natural waters. In Water Pollution Microbiology. Ed: R Mitchel. Vol 2 Wiley-Interscience, New York.

Mix, T W. 1974. The physical chemistry of membrane-virus interaction. Dev. Ind. Microbiol. 15 : 136 - 142.

Montgomery, J M. 1982. Evaluation of treatment effectiveness for reducing thihalomethanes in drinking water. Final Report U.S. Environmental Protection Agency, EPA-68-01-6292, Cincinnati, Ohio.

Moore, B E, B P Sagik, and J F Malina, Jr. 1975. Viral association with suspended solids. Wat. Res. 9 : 197 - 203.

Morinigo, M A, D Wheeler, C Berry, C Jones, M A Munoz, R Cornax and J J Borrego. 1992. Evaluation of different bacteriophage groups as faecal indicators in contaminated natural waters in Southern England. Wat. Res. 26 : 267 - 271.

Nacescu, N, H Brandis and H Werner. 1972. Isolation of two Bacteroides fragilis phages from sewage and detection of lysogenic strains. Zbl. Bak. Hyg. Abt. Orig. A 219 : 522 - 529.

Niemi, M. 1976. Survival of Escherichia coli phage T7 in different water types. Wat. Res. 10 : 751 - 755.

Nupen, E M, N C Basson and W O K Grabow. 1980. Efficiency of ultrafiltration for the isolation of enteric viruses and coliphages from large volumes of water in studies on wastewater reclamation. Prog. Wat. Technol. 12 : 851 -863.

Omura, T, H K Shin and A Ketratanakul. 1985. Behaviour of coliphages in oxidation ponds. Wat. Sci. Technol. 17 : 219 - 227.

Osawa, S, K Furuse, and I Watanabe. 1981. Distribution of ribonucleic acid coliphages in animals. Appl. Environ. Microbiol. 41 : 164 - 168.

Pancorba, O C, G Bitton, S R Farrah, G F Gifford and N R Overman. 1988. Poliovirus retention in soil columns after application of chemical and polyelectrolyte conditioned dewatered sludges. Appl. Environ. Microbiol. 54 : 118 - 123.

Parker, W F. 1981. A note on the use of membrane faecal coliform medium for enhancing resolution and accuracy when enumerating a small plaquing coliphage. J. Appl. Bacteriol. 51 : 81 - 84.

Payment, P, M Trudel and R Plante. 1985. Elimination of Viruses and Indicator Bacteria at Each Step of treatment during Preparation of Drinking Water at Seven Water Treatment Plants. Appl. Environ. Microbiol. 49 : 1 418 - 1 428.

Payment, P, A Berube, D Perreault, R Armon and M Trudel. 1989. Concentration of Giardia lamblia cysts, Legionella pneumophilla, Clostridium perfringens, human enteric viruses, and coliphages from large volumes of drinking water, using a single filtration. Can. J. Microbiol. 35 : 932 - 935.

Pelczar, Jr M J, E C S Chan, and N R Krieg. 1988. In Microbiology. Fith Edition. Ed: McGraw-Hill Book Co, Singapore. p416.

Preston, D R, T V Vasudeven, G Bitton, S R Farrah and J L Morel. 1988. Novel approach for Modifying Microporous Filters for Virus Concentration form Water. Appl. Environ. Microbiol. 54 : 1 325 - 1 329.

Primrose, S B and M Day. 1977. Rapid concentration of bacteriophages from aquatic habitats. J. Appl. Bacteriol. 42 : 417 - 421.

Primrose, S B, N D Seeley, and K Logan. 1981. The recovery of viruses from water: methods and applications. In Viruses and Wastewater. Ed: M Goddard and M Butler, Treatment, Pergamon, Oxford. pp 211 - 231.

Primrose, S B, N D Seeley, K B Logan and J W Nicolson. 1982. Methods for studying aquatic bacteriophage ecology. Appl. Environ. Microbiol. 43 : 694 - 701.

Raibaud, P, M Caulet, J V Galpin and G Mocquot. 1961. Studies on the bacterial flora of the alimentary tract of pigs: 11 streptococci: selective enumeration and differentiation of the dominant group. J. Appl. Bacteriol. 24 : 285 - 306.

Rao, N U and N A Labzoffsky. 1969. A simple method for the detection of low concentrations of viruses in large volumes of water by the membrane technique. Can. J. Microbiol. 15 : 399 - 403.

Rose, J B, S N Singh, C P Gerba and L M Kelley. 1984. Comparison of microporous filters for concentration of viruses from wastewater. Appl. Environ. Microbiol. 47 : 989 - 992.

Safferman, R S and M E Morris. 1976. Assessment of virus removal by a multistage activated sludge process. Wat. Res. 10 : 413 - 420.

Salyers, A A. 1984. Bacteroides of the human lower intestinal tract. Ann. Rev. Microbiol. 38 : 293 - 313.

Sarrette, B A, C D Danglot and R Vilagines. 1977. A new and simple method for recuperation of enteroviruses from water. Wat. Res. 11 : 355 - 358.

Scarpino, P V. 1978. Bacteriophage indicators. In Indicators of viruses in water and food. Ed: G Berg, Ann Arbor, Science Publishers, Ann Arbor, Mich.

Schaub, S A and B P Sagik. 1975. Association of enteroviruses with natural and artificially introduced colloidal solids in water and infectivity of solid-associated virus. Appl. Environ. Microbiol. 30 : 212 - 222.

Scheuerman, P R, S R Farrah and G Bitton. 1987. Reduction of microbiological indicators and viruses in a cypress strand. Wat. Sci. Technol. 19 : 539 - 546.

Schwartzbrod, L and F Lucena. 1978. Concentration des enterovirus par adsortion-elution sur poudre de verre. Proposition d'un apparaeillage simlifie. Microbia 4 : 55 - 68.

Seeley, N D, G Hallard and S B Primrose. 1979. A portable device for concentrating bacteriophages from large volumes of freshwater. J. Appl. Bacteriol. 47 : 145 - 152.

Seeley, N D and S B Primrose. 1980. The effect of temperature on the ecology of aquatic bacteriophages. J. Gen. Virol. 46 : 87 - 95.

Seeley, N D and S B Primrose. 1980. Concentration of bacteriophages from natural waters. J. Appl. Bacteriol. 46 : 103 - 116.

Seeley, N D and S B Primrose. 1982. The isolation of bacteriophages from the environment. J. Appl. Bacteriol. 53 : 1 - 17.

Selna, M W and R P Miele. 1977. Virus sampling in wastewater-field experiments. J. Environ. Eng. Div. ASCE. 103 : 693 - 705.

Shah, P C and J McCamish. 1972. Relative chlorine resistance of poliovirus I and coliphages f2 and T2 in water. Appl. Microbiol. 24 : 658 - 659.

Shields, P A, S A Berenfeld and S R Farrah. 1985. Modified membrane-filter procedure for concentration of enteroviruses from tap water. Appl. Environ. Microbiol. 49 : 453 - 455.

Shields, P A and S R Farrah. 1986. Concentration of viruses in beef extract by flocculation with ammonium sulfate. Appl. Environ. Microbiol. 51 : 211 -213.

Shields, P A, T F Ling, V Tjatha, D O Shah and S R Farrah. 1986. Comparison of positively charged membrane filters and their use in concentrating bacteriophages in water. Wat. Res. 20 : 145 - 151.

Silverman, P M and R C Valentine. 1969. The RNA Injection Step of Bacteriophage f2 Infection. J. Gen. Virol. 4 : 111 - 124.

Simkova, A and J Cervenka. 1981. Coliphages as ecological indicators of enteroviruses in various water systems. Bull. World Health Org. 59 : 611 - 618.

Singh, S N and C P Gerba. 1983. Concentration of coliphage from water and sewage with charge-modified filter aid. Appl. Environ. Microbiol. 45 : 232 - 237.

Smith, E M, C P Gerba and J L Melnick. 1978. Role of sediment in the persistence of enteroviruses in the marine environment. Appl. Environ. Microbiol. 35 : 685 - 689.

Snedd, M C V P Olivieri, K Kawata and C W Kruse. 1980. The effectiveness of chlorine residuals in inactivation of bacteria and viruses introduced by post-treatment contamination. Wat. Res. 14 : 403 - 408.

Sobsey, M D, C Wallis, M Henderson and J L Melnick. 1973. Concentration of enteroviruses from large volumes of water. Appl. Environ. Microbiol. 26 : 529 - 534.

Sobsey, M D and B L Jones. 1979. Concentration of poliovirus from tapwater using positively charged microporous filters. Appl. Environ. Microbiol. 37 : 588 - 595.

Sobsey, M D and J S Glass. 1980. Poliovirus concentration from tapwater with electro-positive adsorbent filters. Appl. Environ. Microbiol. 40 : 201 - 210.

Sobsey, M D and A R Hickey. 1985. Effects of humic and fulvic acids on poliovirus concentration from water by microporous filtration. Appl. Environ. Microbiol. 49 : 259 - 264.

Sockett, P N, P A West and M Jacob. 1985. Shellfish and public health. PHLS Microbiol Digest. 2 : 29 - 35.

Spencer, R. 1955. A marine bacteriophage. Nature 175 : 690.

Springthorpe, V S, C L Loh, W J Robertson and S A Sattar. 1992. In situ survival of indicator bacteria, MS-2 phage and human pathogenic viruses in river water. Washington DC IAWPRC Symposium on Health-Related Water Microbiology.

Sproul, O J, R T Thorup, D F Wentworth and J S Atwell. 1970. Salt and virus inactivation by chlorine and high pH. In Proceedings of the Speciality Conference on Disinfection. Am. Soc. Civ. Engrs, New York.

Stagg, C E, C Wallis and C P Gerba. 1978. Chlorination of solids-associated coliphages. Prog. Wat. Technol. 10 : 381 - 387.

Steitz, J A. 1968. Identification of the A Protein as a Structural Component of Bacteriophage R17. J. Molec. Biol. 33 : 923 - 936.

Stetler, R E. 1984. Coliphages as indicators of enteroviruses. Appl. Environ. Microbiol. 48 : 668 - 670.

Strauss, J H and R L Sinsheimer. 1963. Purification and properties of bacteriophage MS-2 and of its ribonucleic acid. J. Molec. Biol. 7 : 43 - 54.

Strohmaier, K. 1967. Virus concentration by ultrafiltration. In Methods in Virology. Ed: Academic Press, New York. pp 245 - 274.

Sturdza, S A and M Russo-Pendelesco. 1967. Recherches ecologiques phago-bacteriennes dans le milieu enterieur. Arch. Roum. Pathol. Exp. Microbiol. 26 : 125 - 154.

Sugiyama, T and D J Nakada. 1970. Translational Control of Bacteriophage MS2 RNA Cistrons by MS2 coat protein : Affinity and specificity of the Interaction of MS2 Coat Protein with MS2 RNA. Molec. Biol. 48 : 349 - 355.

Sweet, B A, R D Ellender and J L Leong. 1974. Recovery and removal of viruses from water utilising membrane techniques. Develop. Ind. Microbiol. 15 : 142 - 159.

Tartera, C and J Jofre. 1987. Bacteriophages active against Bacteroides fragilis in sewage-polluted waters. Appl. Environ. Microbiol. 53 : 1 632 - 1 637.

Tartera, C, A Bosch and J Jofre. 1988. The inactivation of bacteriophages infecting Bacteroides fragilis by chlorine treatment and U.V.-irradiation. FEMS Microbiol. Lett. 56 : 313.

Tartera, C, F Lucena and J Jofre. 1989. Human origin of Bacteroides fragilis Bacteriophages Present in the Environment. Appl. Environ. Microbiol. 55 : 2 696 - 2 701.

Valentine, R C and A C Allison. 1959. virus particle adsortion. I. Theory of adsorption and experiments on the attachment of paricles to non-biological surfaces. Biochim. Biophys. Acta. 34 : 10 - 23.

Vaughn, J M and T G Metcalf. 1975. Coliphages as indicators of enteric viruses in shellfish and shellfish raising estaurine waters. Wat. Res. 9 : 613 - 616.

Velz, C J. 1970. In Applied stream sanitation. Wiley-Interscience, New York. p 47.

Vilagines, R, B Sarrette and C Danglot. 1978. Quantitative recovery of enteroviruses from water by glass powder in dynamic suspension. Abstracts of the 4th International Congress for Virology. The Hague, Netherlands, 30 August - 6 September 1978, p 150.

Wallis, C and J L Melnick. 1967. Concentration of enteroviruses on membrane filters. J. Virol. 1 : 472 - 477.

Wallis, C J L Melnick and C P Gerba. 1979. Concentration of viruses from water by membrane chromatography. Annu. Rev. Microbiol. 33 : 413 - 437.

Wallis, C M Henderson and J L Melnick. 1972. Enterovirus concentration on cellulose membranes. Appl. Environ. Microbiol. 23 : 476 - 480.

Wellings, F M, A L Lewis and C W Mountain. 1976. Demonstration of solids-associated virus in wastewater and sludge. Appl. Environ. Microbiol. 31 : 354 - 358.

Wentsel, R S, P E O'Neil and J F Kitchens. 1983. Evaluation of coliphage detection as a rapid indicator of water quality. Appl. Environ. Microbiol. 43 : 430 - 434.

West, P A. 1989. Human pathogens and public health indicator organisms in shellfish. In Methods for the Microbiological Examination of Fish and Shellfish. Ed: B Austin and D A Austin. Chichester: Ellis Horwood. pp 273 - 308.

Yates, M V, C P Gerba and L M Kelley. 1985. Virus persistence in groundwater. Appl. Environ. Microbiol. 49 : 778 - 781.