DETECTION OF VIRUSES IN ENVIRONMENTAL SAMPLES USING ROTATING MEMBRANE ULTRAFILTRATION AND THE POLYMERASE CHAIN REACTION

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ABSTRACT

Currently, no method exists which can cost-effectively be used to detect a wide range of pathogens in environmental samples. Such a method is needed because a wide range of human pathogens can be transmitted by a water-borne route, leading to disease outbreaks. These outbreaks result in avoidable morbidity and mortality in the United States and worldwide. Commonly used indicator organisms do not provide a sensitive or accurate means of 'indicating' the presence of pathogens in the various water sources to which humankind is most commonly exposed, namely drinking water, shellfishing waters, bathing areas, or irrigation waters. Furthermore, a water-borne exposure pathway for many pathogens (e.g., *Helicobacter*) is not well characterized, because a method to detect low concentrations of a variety of pathogens is not available. Thus, a detection scheme that can be used to detect common human pathogens in order to assess exposure levels and exposure pathways in environmental water samples was developed.

The goal of this research was to concentrate and detect (RNA and DNA) model viruses in drinking water and non-drinking water environmental samples in order to demonstrate the feasibility of this detection scheme. Two technologies were used in conjunction in order to achieve this goal. First, a method to concentrate viruses efficiently by means of rotating membrane ultrafiltration (RMU) was developed. RMU provides a means for the concentration of bacterial and viral size particles from large volumes of water (10-20 L) to small volumes (50 mL) quickly and with a high recovery efficiency.

A crucial step in this concentration process was the secondary filtration and extraction step. The developed protocol was found to be sufficient in order to yield concentrated environmental samples that were amenable to molecular biological detection methods. These preparation procedures include a proteinase K digestion and a hexadecyltrimethylammonium bromide extraction, followed by a phenol:chloroform extraction and filtration using a series of molecular weight filters.

Second, the polymerase chain reaction (PCR) was used to detect multiple pathogens in concentrated environmental samples. In order to develop the technology, phi-X174 (a DNA coliphage) and MS2 (an RNA coliphage) were used in reconstruction experiments to determine the recovery efficiency and the sensitivity of detection of the developed methodology. Finally, PCR detection was performed on concentrated environmental water samples into which these organisms were spiked. For non-drinking water environmental samples, a double PCR using nested primers was required for detection of low numbers of spiked DNA virus, while RNA virus was not recoverable.

This methodology has been used to detect one or both of the target viruses in two environmental samples, Cambridge drinking water and Wachusett Reservoir water. In 10 liter samples of drinking water, 10 phi-X174 (DNA) per liter and 100 MS2 (RNA) per liter were detected. In 10 liters of freshwater, 1000 phi-X174 per liter were detected. This level of detection provides the sensitivity needed to monitor drinking water during a disease outbreak, to monitor source water for selected pathogens on a routine basis, to monitor a polluted environmental water source, or to assess a disinfection process.

Importantly, the method has been made so that additional pathogens can easily be added to the list of pathogens to be detected. This procedure provides a relatively quick (1 to 2 days), easy, and inexpensive method for pathogen detection from drinking water and environmental waters.

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I have worked in three laboratories on campus: the Thilly lab, the Fox lab, and the Parsons lab. During my residence at each lab, I learned a great deal and made many friends. I did most of my research in the Thilly and Fox labs, but I think it will be the lab get-togethers that I will remember most fondly. I consider the Parsons lab my home, and this is due in great part to Pat Dixon, Mrs. Harleman, and the people of the lab.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

"It is a difficult question, my friends, for any young [person] - that question I had to grapple with, and which thousands are weighing at the present moment in these uprising times - whether to follow uncritically the track [they] find [themselves] in, without considering [their] aptness for it, or to consider what [their] aptness or bent may be, and reshape [their] course accordingly. I tried to do the later, and I failed. But I don't admit that my failure proved my view to be the wrong one, or that my success would have made it a right one; though that's how we appraise such attempts nowadays - I mean, not by their essential soundness, but by their accidental outcomes. If I had ended by becoming like one of these gentlemen in red and black that we saw dropping in here by now, everybody would have said: 'See how wise that young man was, to follow the bent of his nature!' But having ended no better than I began they say: 'See what a fool that young fellow was in following the freak of his fancy!'"

Thomas Hardy Jude the Obscure, 1895

To my wife whom I love

1. INTRODUCTION

Many human pathogens are, or are suspected to be, spread via a water-borne route resulting in significant morbidity and mortality around the world. Conceptually, the goal of this research was to develop a detection scheme that can be used to detect common human pathogens in order to assess exposure levels and exposure pathways in environmental water samples. This goal was accomplished by concentrating and detecting RNA and DNA model viruses in environmental samples in order to demonstrate the feasibility of this detection scheme.

1.1 Statement of Problem

The ability to detect viruses and bacteria that are pathogenic to humans is important because it allows the determination of the level of exposure associated with ingestion of water which may contain these pathogens. Currently, indicator organisms are used to detect the "possible presence" of pathogens in water, because they are inexpensive and easy to use. Indicators, unfortunately, provide a poor means of safeguarding water supplies. This research focuses on the elimination of the need for indicator systems by directly detecting human pathogens. From a public health standpoint, direct pathogen detection from environmental samples provides a means to avoid exposure that is not too conservative and thus costly, while providing adequate protection from disease. Furthermore, water-borne transmission of many pathogenic microorganisms is not well described or, in some cases, is only suspected because the pathogens are difficult to detect in the environment. Many pathogens are difficult to isolate due to their low

concentration in a given water body or because the method of detection is not suited for environmental sampling. Direct detection of pathogens provides a means to assess these heretofore unknown avenues of exposure.

Other means of direct detection have critical short-comings, and these are discussed in detail in Chapters 3 and 4. Briefly, adsorption-elution filtration technology is the most common means in use today for concentrating viruses from water. Because the recovery efficiency of this process is dependent on water quality and virus characteristics, it is limited in its application, and thus its usefulness (in concentrating environmental samples), especially non-drinking water samples. Two means of detection are available for viruses that have been concentrated by adsorption-elution filtration: cell culture and gene probe technology. Cell culture requires the growth of pathogens of interest, which is not always possible. Moreover, cell culture detection is often limited by the toxicity of the concentrated sample in addition to being expensive and time consuming. Gene probe technology overcomes some of these problems, but the lack of sensitivity in detection and the use of radioactivity severely limits its use.

One of the major difficulties with direct detection methodologies is that many human pathogens exist which are thought to be transmitted through the ingestion of contaminated water. All of these organisms must be considered as possible sources of disease when developing a method to gauge the infectious potential of a water source. Currently, *no* technique is available which can quickly, easily, and inexpensively be used to detect *all* human pathogens in environmental samples. In fact, *few* techniques, which are quick, easy, and cheap, are available for the detection of *any* pathogens in environmental samples. Thus, as mentioned above, the presence of indicator

organisms in a water sample has been used to 'indicate' the possible presence of human pathogenic bacteria, viruses, and other organisms. Several indicator systems have been suggested in the literature and are discussed in detail in Chapter 2. In brief, the fecal coliform and *Escherichia coli* are by far the most commonly used indicator organisms, and the most generally accepted means of monitoring water quality. Unfortunately, they often do not have similar transport mechanisms or die-off patterns as viruses, and, not unexpectedly, they do not indicate the presence of viruses very well.

The tests performed in this research were designed so that additional pathogens could easily be included in the assay. In other words, the procedures used in testing water samples were designed so that detection of additional viral, bacterial, and protozoal pathogens could be accomplished with no change in methodology. Although a great deal of research has been carried out in related areas, namely concentration and detection of high levels of spiked virus in water that is relatively free of suspended solids and organic material, little has been published on detection schemes, especially the polymerase chain reaction (PCR), for environmental samples from non-potable environmental sources. In fact, not much is known about PCR amplification carried out in the heterogeneous reaction solutions that are encountered in environmental concentrates. Furthermore, the use of rotating membrane ultrafiltration (RMU) has not been well characterized for use in concentrating environmental samples for viral detection. Ultrafiltration, in general, has not been a popular method of concentrating viruses because, as will be discussed below, traditional ultrafiltration units could not achieve the flow rates necessary to be useful.

1.2 Prior Studies

A prior study entitled "Detection of the *Alu* Sequence Using the Polymerase Chain Reaction" was conducted under a grant from the Whitaker Health Sciences fund at MIT (June 1989 - July 1990) (Lewis, 1990). This study was concerned with the detection of a human-specific DNA sequence as an indicator of human fecal pollution in aquatic environments using the polymerase chain reaction (PCR). A human-specific indicator is an organism or substance whose presence can be used to differentiate between the source of human and animal fecal contamination. The source of fecal contamination is important for several reasons. First, the level of hazard associated with animal fecal contamination is lower because many pathogens, especially viruses, are often host specific. Additionally, engineering solutions to remove fecal contamination depend upon knowledge of the pollution source. As an example, a different pollution prevention procedure would be used for septic systems than for avian sources.

Human-specific DNA sequences are known to exist and their presence indicates the presence of human cells. The *Alu* sequence is a highly repetitive DNA sequence that is primate-specific. The function of the *Alu* sequence in the genome is not known; however, it is known to be conserved. Although *Alu*-family or *Alu*-equivalent sequences exist in lower animals, their DNA sequences vary enough to be differentiated by PCR. The *Alu* sequence was chosen as an indicator because it is repeated more than 10^5 times per human (or primate) cell, while each human excretes approximately 10^{11} cells per day. Thus, a large signal is provided for the recent release of human cells which can be detected, even if it is highly diluted, due to the sensitivity of PCR.

Primer sequences to be used in PCR amplification were chosen from the most highly conserved portions of the *Alu* sequence, but they were also selected to be different from other *Alu*-family sequences of lower animals. Three sets of primer sequences were chosen to be used in the amplification of the *Alu* sequence. The primers were then tested for possible amplification of non-human genomes, and the best set of primer sequences was retained. A variety of PCR reaction mixture conditions were tested for maximum yield of amplification product. Also, tests were performed involving the annealing temperature, the number of cycles of amplification, and initial concentrations of a variety of templates. The genomes of cows, rabbits, cats, and hamsters were shown not to be amplified by the chosen set of primer sequences, while human and monkey cells were amplified.

The method is believed to be useful in certain circumstances, but a background level of *Alu* sequence was found to exist due to the presence of humans in the laboratory. In other words, the *Alu* sequence provided such a large signal that it was difficult to avoid contamination of the samples during collection and processing. A detection limit of 10^4 *Alu* copies, equivalent to about one tenth of a human genome, was determined. Processes to decontaminate the reaction mixture were studied. Several procedures were examined including the exposure of components of the reaction mixture to ultraviolet light and heat. The most efficient means of contamination reduction was enzymatic treatment of the reaction mixture. Both restriction endonucleases (*ScrF.1* and *Mnl.1*) and exonucleases (Exonuclease III) proved effective. None of these enzymes attack single-stranded DNA, and thus could be used to degrade contaminative template DNA in the presence of primers

and *Taq* enzyme in the reaction mixture. Extensive testing was done with the PCR protocol to provide optimal conditions for amplification.

This research project has not been continued for two reasons. First, the problems concerning contamination of the samples during collection and processing and the rapid uptake of dissolved DNA in aquatic environments provide limitations to this test as a general indicator system. Contamination-free collection and handling of the samples provides a major difficulty. Moreover, due to the turnover time of the dissolved DNA in the environment, the presence and absence of *Alu* sequence would not be expected to correlate well with the vast array of pathogenic organisms which could be present. That is, this test could not be used as the sole means to judge the quality of a body of water; however, it can be used to provide insight into the source of pollution when used in conjunction with other indicators. Second, an indicator system is unnecessary if pathogens are directly detectable. Therefore, this research project concentrates on the elimination of the need for indicator systems and opts for direct detection of human pathogens. Because it was felt that research presented in this thesis was better suited for monitoring water quality, detection of the *Alu* sequence in environmental samples was not pursued (Lewis, 1990).

1.3 Statement of Goals

The goal of this research was to develop a detection scheme that can be used to detect common human pathogens in environmental water samples in order to assess human exposure levels and exposure pathways. More basically, this research provides a means of concentrating and detecting

microorganisms from environmental samples. In addition to this primary objective, several other characteristics must be present in the methodology in order to have a useful and innovative technology.

The methodology must be easy to use, quick to perform, and inexpensive, otherwise its use will be limited to the First World or those with access to laboratories with a highly-skilled technical staff. In other words, the technology should be designed to be available to the broadest possible range of users. This necessarily excludes the use of radioactivity, human cell culture, and expensive laboratory equipment such as an ultracentrifuge.

Also, the test must incorporate the ability to detect multiple pathogens or groups of pathogens in one sample (e.g., the enteroviruses instead of hepatitis A, poliovirus, coxsackievirus, echovirus, *etc.*). As the number of pathogens or groups of pathogens detected by this methodology becomes more comprehensive, the need for an indicator organism is eliminated. Ultimately, a variety of pathogens can be added to this 'library of pathogens' to be detected.

The combination of two technologies, rotating membrane ultrafiltration and the reverse transcriptase-polymerase chain reaction, are used to accomplish these goals. Furthermore, the development of a secondary filtration and extraction protocol to prepare the RMU concentrated sample for PCR was essential. These technologies are described in detail in Chapter 3 and 4.

A method for direct detection which can be expanded to include an indefinitely large number of human pathogens from environmental samples was developed. The method does not require the growth of the pathogen, nor

does it require individual procedures for the detection of each pathogen. The method is relatively inexpensive, many pathogens can be assayed in a single sampling, and it is fast (on the order of one day). This method was tested by detecting model DNA and RNA viruses in environmental samples that had been spiked with the viruses. The recovery efficiency of the filtration and extraction process and the sensitivity of detection were assessed by concentrating and detecting MS2 (RNA) and phi-X174 (DNA) viruses in drinking water and phi-X174 viruses in source water which had been spiked with these viruses. A bacterium, *Helicobacter pylori*, was also used in reconstruction experiments involving the secondary filtration and detection steps. These viruses have been chosen because they are the smallest viruses (20 nm) known to exist and are the same size as the picornaviruses, a group of water-borne human pathogens and significantly smaller than most important pathogens (see Table 1). Because the separation technology used in this research, RMU, is size-based, these model viruses provide a means of easily testing the system with the limiting case. Additionally, because the DNA and RNA molecule are the same (except for sequence) in all organisms, the detection scheme used in this research, PCR, provides a standard means of detecting the model viruses as well as other pathogens. All that is required are specific nucleic acid sequences for each pathogen or group of pathogens of interest.

A method such as the one described above is sorely needed. The indicator organisms in use today do not provide adequate protection from disease in some cases, while they are too conservative in others, resulting in unnecessary and costly closures of bathing and shellfishing areas (Hickey, 1989). On the other hand, with direct detection, a variety of indicator

organisms can be monitored in conjunction with pathogens, which would provide two levels of protection and a wealth of information that can be used to ensure the protection, but not overprotection, of water users. Thus, the detection of pathogens using RMU and PCR provides state-of-the-art protection for water supplies, while reducing the number of unnecessary restrictions on water use. A technique to monitor source water (water to be used as drinking water) that provides consistent recovery for multiple pathogens, regardless of water quality, is needed in the water industry, especially with regard to new monitoring requirements set forth in amendments made in 1986 to the Safe Drinking Water Act (surface water treatment rule and information collection rule 40 CFR Part 141). Also, the developed technology will become more valuable as water reuse becomes more prevalent. California, Florida, and Arizona, as well as Israel and many nations with arid climates, are already involved in water reuse programs. Reclaimed non-potable water can be used for irrigation and flush water (Metcalf and Eddy, 1991). Furthermore, the shellfishing industry (due to numerous shellfishing bed closures which have led to millions of dollars in lost revenue in Massachusetts alone) is actively seeking similar means for improved water testing techniques (Hickey, 1989). Each year, many clinical cases and many more sub-clinical cases of human disease have been reported that are suspected to be the result of water-borne transmission of viral, bacterial, and protozoal pathogens. It is the opinion of the author that many of these maladies could be eliminated, or at least their frequencies reduced, by the use of the developed technology.

PCR is a relatively new and powerful technology that is finding applications in a wide variety of fields. A host of both expected and

unexpected uses will be realized by encouraging research with PCR in environmental engineering. This research will help further the database for the use of PCR in environmental samples as well as provide a useful technology.

The finished product of this research is a system by which the selected viruses can be detected from large volume water samples without culturing or radioactivity, providing a relatively fast, inexpensive, and easy method for detecting viruses in the environment.

1.4 Overview of Thesis

This thesis will be concerned with the description of a novel method to detect human pathogens in environmental samples. Chapter 1 provides a conceptual framework for the purpose of this research, an overview of problems with current technology, and a brief description of the methods used in this research.

Chapter 2 is concerned with a description of common pathogens with regards to their significance in terms of morbidity and mortality and their prevalence in the environment. Also, indicator organisms are discussed with respect to their use and misuse in determining water quality. Finally, a dose-response model for common pathogens is presented, followed by a discussion of acceptable risk.

Chapter 3 describes an alternative methodology for the concentration of viruses, adsorption-elution filtration, with emphasis on its shortcomings in

recovering different virus types from non-drinking water environmental samples. The RMU technology is also described in detail.

Chapter 4 reports on two alternative virus detection schemes, cell culture and gene probe technology. Details are given regarding the limitation of cell culture due to the expense and difficulty of the method as well as the limitation of detection to culturable viruses. Discussion of gene probe technology focuses on the use of radioactivity and the high threshold of detection. Finally, PCR technology is defined.

In Chapter 5, the methods and materials used in experiments using RMU and PCR are given, followed by results obtained from this research. Initially, recovery efficiencies are determined in reconstruction experiments using RMU, followed by experiments designed to assess the detection sensitivity of the methodology. A secondary concentration process was developed which was essential for PCR detection. Then, the methodology was applied to the concentration and detection of model viruses in environmental samples.

In Chapter 6, conclusions based on the results of experimentation are made regarding the use of this methodology for environmental viral detection. Special consideration is given to applications of the developed technology.

2. BACKGROUND INFORMATION

"Acute gastroenteritis is second only to the common cold as the most frequent illness affecting the population of the United States" (Williams & Akin, 1986).

2.1 The Pathogens

2.1.1 Description of Common Pathogens

Many of the known pathogens which are thought to be transmitted by water are listed in Table 1. Human pathogenic viruses, though not all water-borne, come in all shapes and sizes (see Figure 1). Figure 2 shows pathways by which humankind is exposed to waterborne pathogens. All of these sources of water must be monitored in order to limit human exposure to pathogens. This extensive listing is given in order to present the variety of viruses that may be encountered, as well as to convey the fact that many different kinds of single- and double-stranded DNA and RNA viruses must be considered, both in terms of nucleic acid detection schemes and sorption characteristics). It should be noted that not all of the viruses listed in Table 2 are thought to be water-borne.

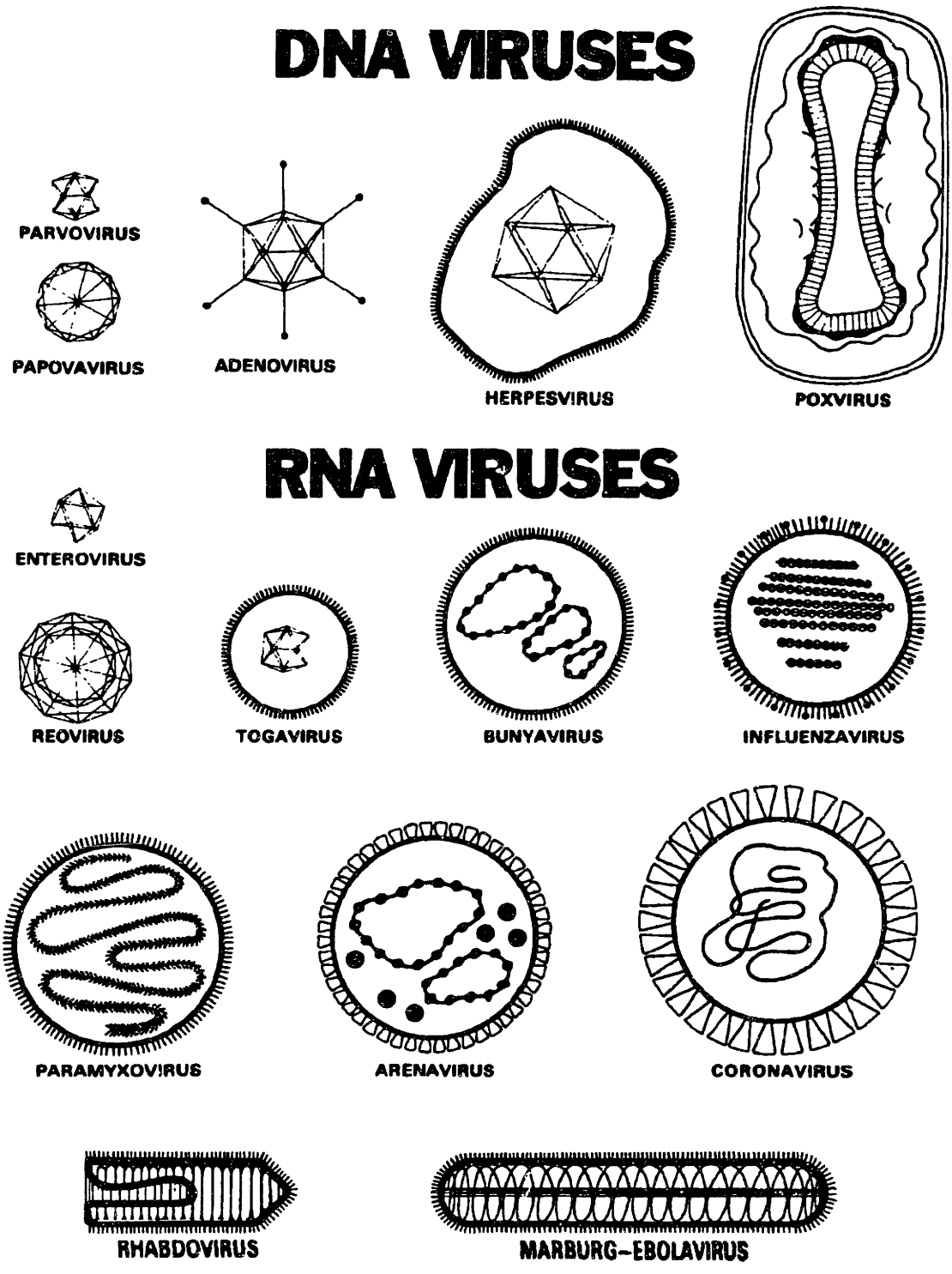
In Table 2, the symbols + and - represent positive- and negative-sense single stranded (ss) nucleic acid, respectively. The designation of positive-sense indicates the nucleic acid codes for a protein, whereas negative-sense nucleic acids are the complement of the coding sequence. Knowledge of the structure of the virus is important in determining the molecular weight of the filter to be used in RMU; this information is also used in classification and

Table 1: Partial Listing of Known and Possible Water-borne Human Pathogens

Bacteria	Viruses (serotypes)	Protozoa/Helminths
Campylobacter jejuni	Enteroviruses	*Balantium coli*
Campylobacter (spp)	Poliovirus (3)†	*Cryptosporidium*
Clostridium perfringens	Echovirus (34)	*Entamoeba histolytica*
E. coli (pathogenic)	Coxsackie A (24)	*Giardia lamblia*
Francisella	Coxsackie B (6)	*Ascaris lumbricoides*
Helicobacter (spp)	enterovirus 68-71(4)	*Enterobuis vericularis*
Legionella pneumophila	Hepatitis A (1)†	*Fasciola hepatica*
Leptospira (150 spp)	Calicivirus(2)	*Hymenolepis nana*
Pasteurella	Norwalk virus (1)†	*Necator americans*
Salmonella typhi	Rotavirus (4)†	*Shistosoma nansoni*
Salmonella (1700 spp)	Reovirus (3)†	*Taenia saginata*
Shigella (4 spp)	Astrovirus (5)	*Taenia solium*
Vibrio cholerae	Adenovirus (47)†	*Trichuris trichiura*
Vibrio (spp)	Parvovirus (2)†	
Yersinia enterocolitica	AAV (3)†	
opportunistic pathogens	Cytomegalovirus (1)†	
	Papovavirus (2)†	
	Coronavirus (1)	
	Hepatitis E	

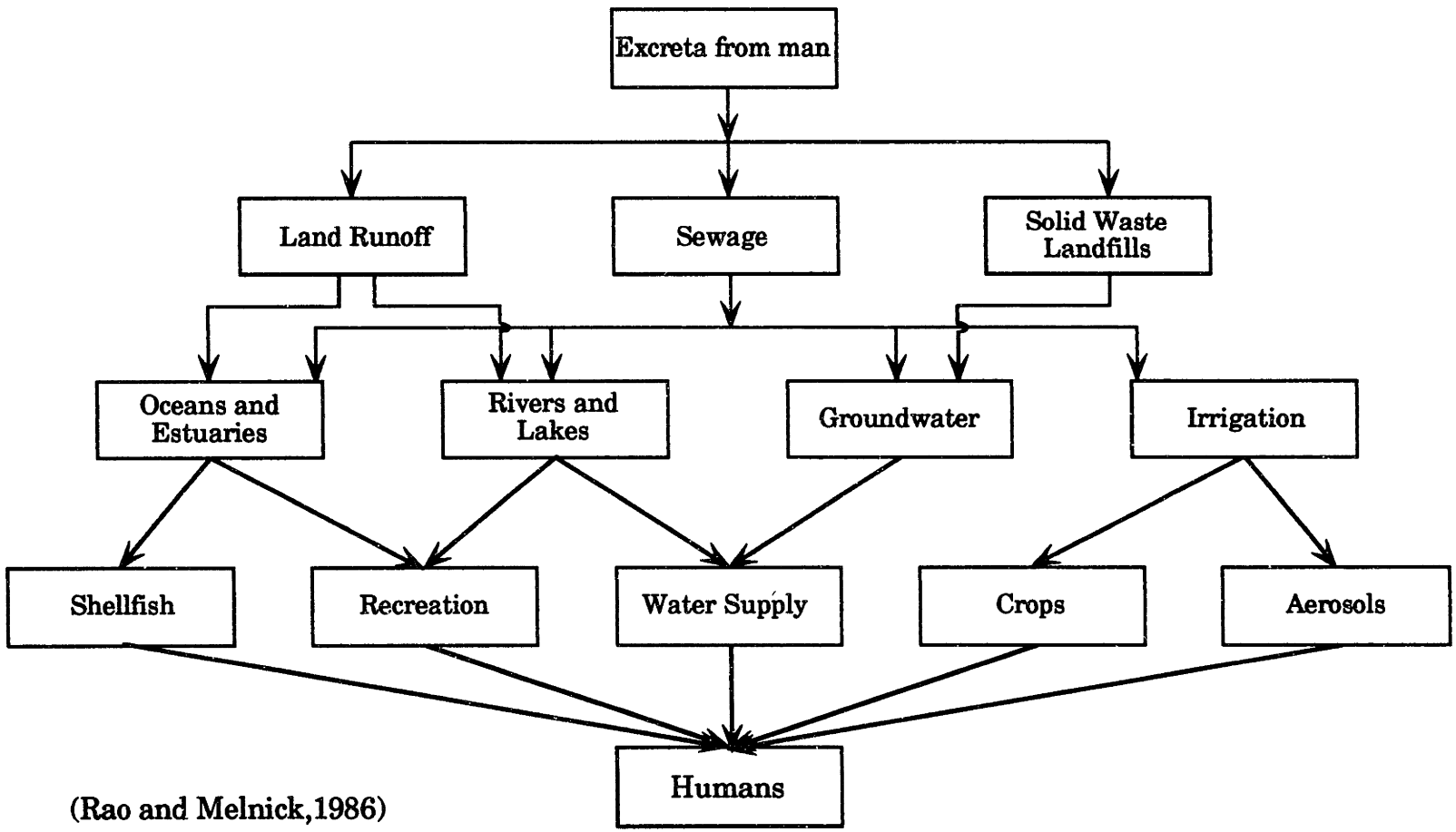
† genomic sequence known for some serotypes (GenBank & EMBL., 1985)
(CDC, 1990a; Emde, *et al.*, 1992; Hurst, *et al.*, 1989; Metcalf and Eddy, 1991; Rao & Melnick, 1986)

Figure 1: Features of Selected Viruses



(Borchardt, *et al.*, 1977)

Figure 2: Pathways of Exposure to Waterborne Pathogens



(Rao and Melnick,1986)

Table 2: Structure and Classification of Selected Mammalian Viruses

Virus families	Nucleic Acid (NA)	Structure	Envelope	Size (nm)	NA mw (x 10^6)
Caliciviridae	+ssRNA	spherical	-	30-40	2.8
Coronaviridae	+ssRNA	helical	+	60-1200	5-6
Picornaviridae †	+ssRNA	cubic	-	18-30	2-2.5
Togaviridae	+ssRNA	spherical	+	35-70	2-4
Toroviridae	+ssRNA	helical	+	35-170	6.5
Retroviridae	+RNA	spherical	+	80-100	3-12
Arenaviridae	-ssRNA	complex	+	50-300	1.1-3.2
Bunyaviridae	-ssRNA	complex	+	90-105	0.4-3.5
Filoviridae	-ssRNA	helical	+	80-1400	4.2
Orthomyxoviridae	-ssRNA	helical	+	80-120	0.2-2.5
Rhadboviridae	-ssRNA	helical	+	50-380	3.4-4
Paramyxoviridae	-/+ssRNA	helical	+	100-300	5-8
Reoviridae	dsRNA	cubic	-	54-80	10-20
Parvoviridae	+/-ssDNA	cubic	-	18-26	1.4-2
Hepadnaviridae	ss/dsDNA	cubic	+	22-42	1.6
Adenoviridae	dsDNA	cubic	-	70-90	20-30
Herpesviridae	dsDNA	cubic	+	110-200	40-150
Papovaviridae	dsDNA	cubic	-	40-55	2-5
Poxviridae	dsDNA	complex		170-450	85-240
Astrovirus (family?)	ssRNA	-		28-30	-

† includes enteroviruses

(Borchardt, *et al.*, 1977; CDC, 1990b; Hull, *et al.*, 1989; Palmer & Martin, 1982)

determining relatedness. The envelope of the virus, when it is present, is a membrane that surrounds the virus. It is present for protection and is also used in classifying the virus, and it would also affect the sorption characteristics of the virus. Finally, the molecular weight of a substance is "the ratio of the weight of a molecule of the compound [to] an atom of hydrogen" (Hull, *et al.*, 1989).

Human exposure to these pathogens results in morbidity and mortality around the world. More than 210,000 U.S. children are hospitalized (at a cost of $1 billion) and 4 to 10 million children worldwide die each year from gastroenteritis (CDC, 1990a). According to the Centers for Disease Control (CDC), there are three research priorities concerning enteric viruses: limited diagnostic capabilities are available for many agents, 50% of gastroenteritis cases are of unknown etiology, and modes of transmission and means of prevention are often unknown.

Many new viral agents have recently been discovered (e.g., enteric adenoviruses, calicivirus, astrovirus, and Norwalk virus) (Bridger, 1987; CIBA, 1987). Although previously unknown, these agents are thought to be major causes of water-associated infection. As an example, Norwalk virus (discovered in Norwalk, Ohio in 1968) is believed to be in the *Caliciviridae* family and has a diameter of 35 nm (Christensen, 1989). Norwalk virus has been reported as the leading cause of viral gastroenteritis in adults (CDC, 1990a). Prevalence studies have shown Norwalk virus "occurs in 50% to 70% of persons in developed countries, such as the USA and 80% to 100% of people from developing nations" (Johnson, *et al.*, 1990).

One reason for this prevalence may be Norwalk virus's resistivity to environmental stresses. Norwalk virus is resistant to chlorination; a 30 minute exposure to chlorine levels as high as 6.25 mg/L did not eliminate the infectivity of Norwalk virus (note: EPA guidelines call for residual and peak levels of chlorine in drinking water of ≥0.2 mg/L and 5 mg/L, respectively). Levels of chlorine as high as 10 mg/L were required for inactivation (CDC, 1990a).

One of the reasons Norwalk virus is so elusive is because it is difficult to detect and diagnose. Although Norwalk virus cannot be grown outside the human body, it was recently sequenced and PCR primer sequences exist (Lambden, *et al.*, 1993). Except for a recently developed commercial immunological test, PCR is one of the few means currently available for clinical diagnosis.

As shown in Table 2, rotaviruses (*Reoviridae* family), discovered 15 years ago, contain segmented double stranded RNA encapsidated in a double-layered icosahedral protein coat approximately 70 nm in diameter (Christensen, 1989). Group A human rotaviruses are the most common cause of diarrheal disease in children in the United States and the world (Kapikian, 1993). Rotavirus is responsible for 3.5 million cases of gastroenteritis per year in the United States and 140 million cases per year worldwide causing nearly 1 million deaths per year (CDC, 1990a). Rotavirus infections increase in the cooler months and are excreted at a rate of 1 trillion infectious particles per milliliter of stool by infected individuals.

Hepatitis A virus is a 27 nm enterovirus that causes an acute disease whose transmission is often associated with the consumption of contaminated

shellfish (Metcalf, 1978). It should be noted that the *Picornaviridae* family, of which hepatitis A virus is a member, also contains the smallest virions. Hepatitis A was the first and is one of the few viruses to have been shown conclusively to be transmitted in drinking water (Gerba, *et al.*, 1985).

Adenoviruses 40 and 41 (*Adenoviridae* family), calicivirus, and astrovirus generally affect children and may be responsible for 5% - 20% of hospitalizations due to gastritis. Although they are known to cause viral gastroenteritis, the importance of pestivirus, picobirnavirus, parvovirus, torovirus, and coronavirus is not currently known. Although the enteroviruses are, by far, the most commonly used viruses in concentration and detection experiments, they are not the major causes of disease. In fact, "an outbreak or case of gastroenteritis should not be attributed to an enterovirus merely because it was isolated in the stool of an affected person" (CDC, 1990a).

Between 1971 and 1988, 545 waterborne outbreaks of disease affecting 136,833 people have been reported to the CDC (CDC, 1990b). These figures obviously underestimate the number of water-borne illnesses because a causal link must be made between the water source and disease. Considering the average number of people affected per outbreak was around 250, low level exposure is not tracked in these statistics. The majority of these outbreaks were due to contaminated drinking water (90%), while a significant number resulted from exposure to recreational water (10%). In both cases, a small number of outbreaks affected a large number of people. Cases involving individual outbreaks are probably more common, but much more difficult to link to exposure to the ingestion of water. Exposure to the contaminated water was usually associated with cross-contamination, lack of

Table 3: Disease Outbreaks 1920-1988

Time Period	Disease	Number of Outbreaks	Number of Illness
1920-1940	Typhoid Fever	372	13,761
	Gastroenteritis	144	176,725
	Shigellosis	10	3,308
	Amebiasis	2	1,416
	Hepatitis A	1	28
	Chemical Poisoning	1	92
		530	195,330
1941-1960	Gastroenteritis	265	54,439
	Typhoid Fever	94	1,945
	Shigellosis	25	8,951
	Hepatitis A	23	930
	Salmonellosis	4	31
	Chemical Poisoning	4	44
	Para-Typhoid Fever	3	19
	Amebiasis	2	36
	Tularemia	2	6
	Leptospirosis	1	9
	Poliomyelitis	1	16
		424	
1961-1970	Gastroenteritis	39	26,546
	Hepatitis A	30	903
	Shigellosis	19	1,666
	Typhoid Fever	14	104
	Salmonellosis	9	16,706
	Chemical Poisoning	9	46
	Toxigenic *E. coli*	4	188
	Giardiasis	3	176
	Amebiasis	3	39
		130	46,374
1971-1988	Gastroenteritis	279	64,965
	Giardiasis	103	25,834
	Chemical Poisoning	55	3,877
	Shigellosis	40	8,806
	Viral Gastroenteritis	26	11,799
	Hepatitis A	23	737
	Salmonellosis	12	2,370
	Campylobacterosis	12	5,233
	Typhoid Fever	5	282
	Yersiniosis	2	103
	Cryptosporidosis	2	13,117
	Chronic Gastroenteritis	1	72
	Toxigenic *E. coli*	1	1,000
	Cholera	1	17
	Dermatitis	1	31
	Amebiasis	1	4
		564	138,247

(Craun, 1991)

treatment, or failure of treatment. *Shigella, Salmonella,* Norwalk virus, rotavirus, *Campylobacter*, *Yersinia*, *Giardia*, *Cryptosporidium,* and pathogenic *E. coli* were found to be the primary causes of outbreaks; however, over 50% of the outbreaks were of unknown etiology (Craun, 1991). Table 3 gives a breakdown of outbreaks occurring since 1920.

As can be seen from this table, many important bacterial and protozoal pathogens also exist. Although bacterial pathogens seem to be more prevalent in the past (typhoid fever), many of these pathogens are still important in the Third World (cholera). In addition, several bacterial pathogens remain at the top of the list of epidemiologically important pathogens. Bacterial pathogens remain an important cause of swimming-related illness (CDC, 1990b).

2.1.2 Minimum Infectious Dose

Little is known about the minimum dosage of most viruses necessary to cause infection. It should be noted that infection does not necessarily result in disease. That is, an individual can be infected without progression to disease. Infection refers only to propagation of the virus inside a host. Once infected, several outcomes may result: the infection can stimulate an immune response (and possibly immunity through infection), progress to sub-clinical disease, clinical disease, or death, each being less likely than the previous condition. Two conceptual models have been offered to describe the minimum infective dose:

1) Independent action: A single virus can be infective, depending on host defenses, virus-host interaction, *etc.*

2) Complete cooperation: Multiple viruses are required to work in concert in order for an infection to occur.

(Haas, 1983)

Models have been constructed using the first scenario. Because experimentation with low concentration of virus requires large study populations, as well as the need to infect human subjects with pathogens, little research exists with regard to the levels of virus which are considered epidemiologically important. Several models have been fitted to the paucity of data that does exist. Haas has suggested two models:

1) log-normal model (single hit exponential): $P = 1 - \exp(-rN)$ where P is the probability of infection, N is the average number of organisms ingested, and the r constant accounts for host-virus interactions, individual viral characteristics, host defenses, *etc.* This model assumes a single pathogen can cause infection.

2) beta-distributed model (single hit distributed r): $P = 1 - (1 + (N / \beta))^{-\alpha}$ where the constant r is replaced by a beta-distributed f(r) in order to describe a distribution of r values found in a population. Two new constants, α and β, now represent the factors formerly accounted for by the r constant. This model also assumes a single pathogen can cause infection.

(Haas, 1983)

Unfortunately, few data exist with which to test these models; however, some data are available and have been fitted with these models. As an example, Figure 3 shows the data and the fit for poliovirus. The β-distributed model was chosen because it was more conservative than the log-normal model. Neither model fits the data very well, although they were shown to be different from random (Haas, 1983). Furthermore, the models are used orders of magnitude away from the areas for which they were calibrated. Figure 4 gives the β-distributed model prediction for annual risk of infection from exposure to several pathogens assuming 2 L of water ingested per day.

The only information available regarding exposure to drinking water as a significant source of infection comes from a study by Payment, *et al.* In this study, approximately half of 600 Canadian households (1200 people) sharing a common water supply were given a reverse osmosis filter to produce drinking water, while the other half continued drinking regular tap water. The results of the study showed that for both families and children in a family ($p<0.01$ and $p<0.05$, respectively), there was a slight, but significant increase in gastroenteritis between those using filtered water and those drinking tap water. Furthermore, increased consumption of tap water led to increased incidence of disease. In these trails, the tap water was monitored for a range of indicators and pathogens, but none were detected. In this study, the tap water had been treated by pre-disinfection, alum flocculation, sand filtration, ozonation, and final chlorination to water quality levels similar to those required in the United States (Payment, *et al.*, 1991).

On the other hand, it has been suggested that low level exposure to pathogens could be a benefit against future infection at higher doses. As has been shown in animal studies, low level exposure to viruses can convey

Figure 3: Dose-Response Models for Attenuated Poliovirus 1

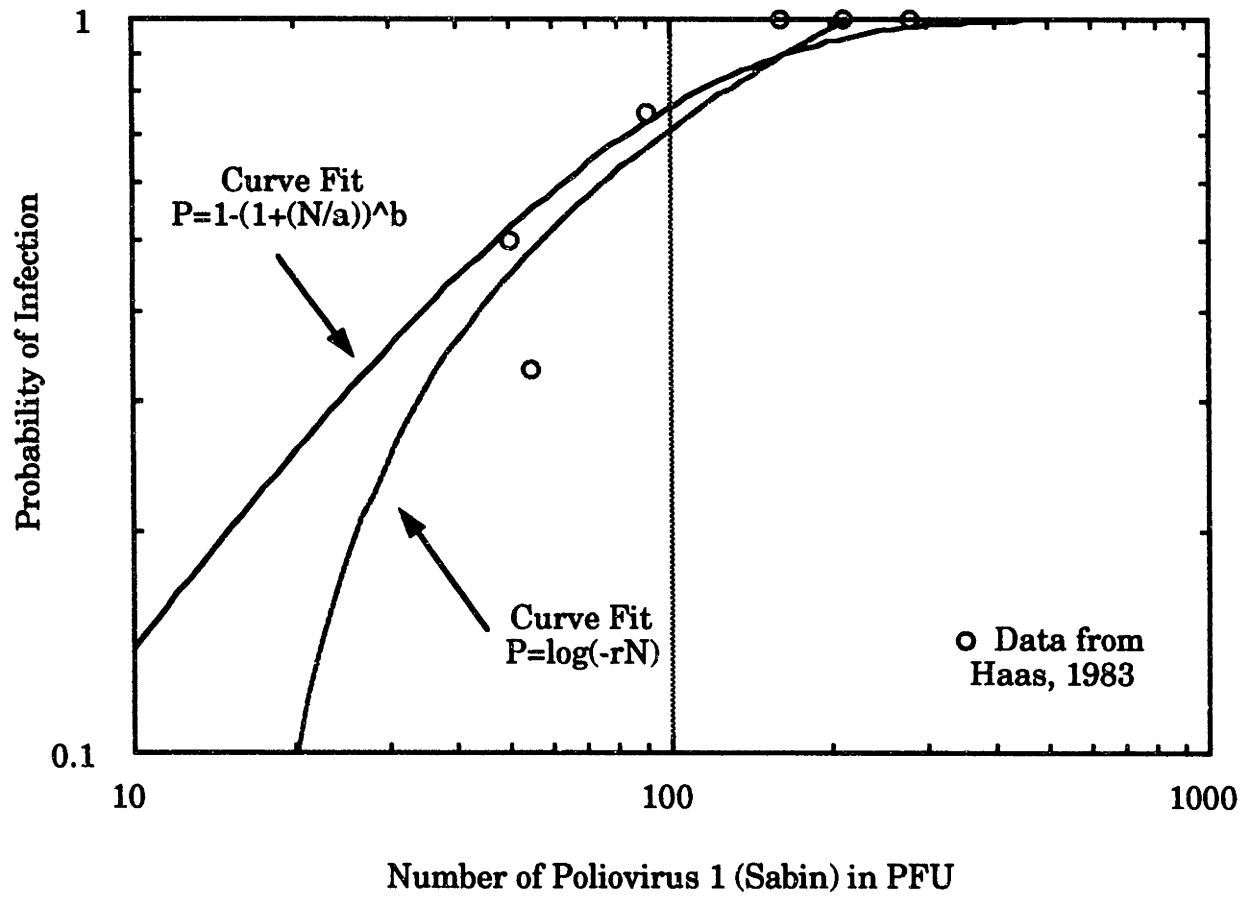
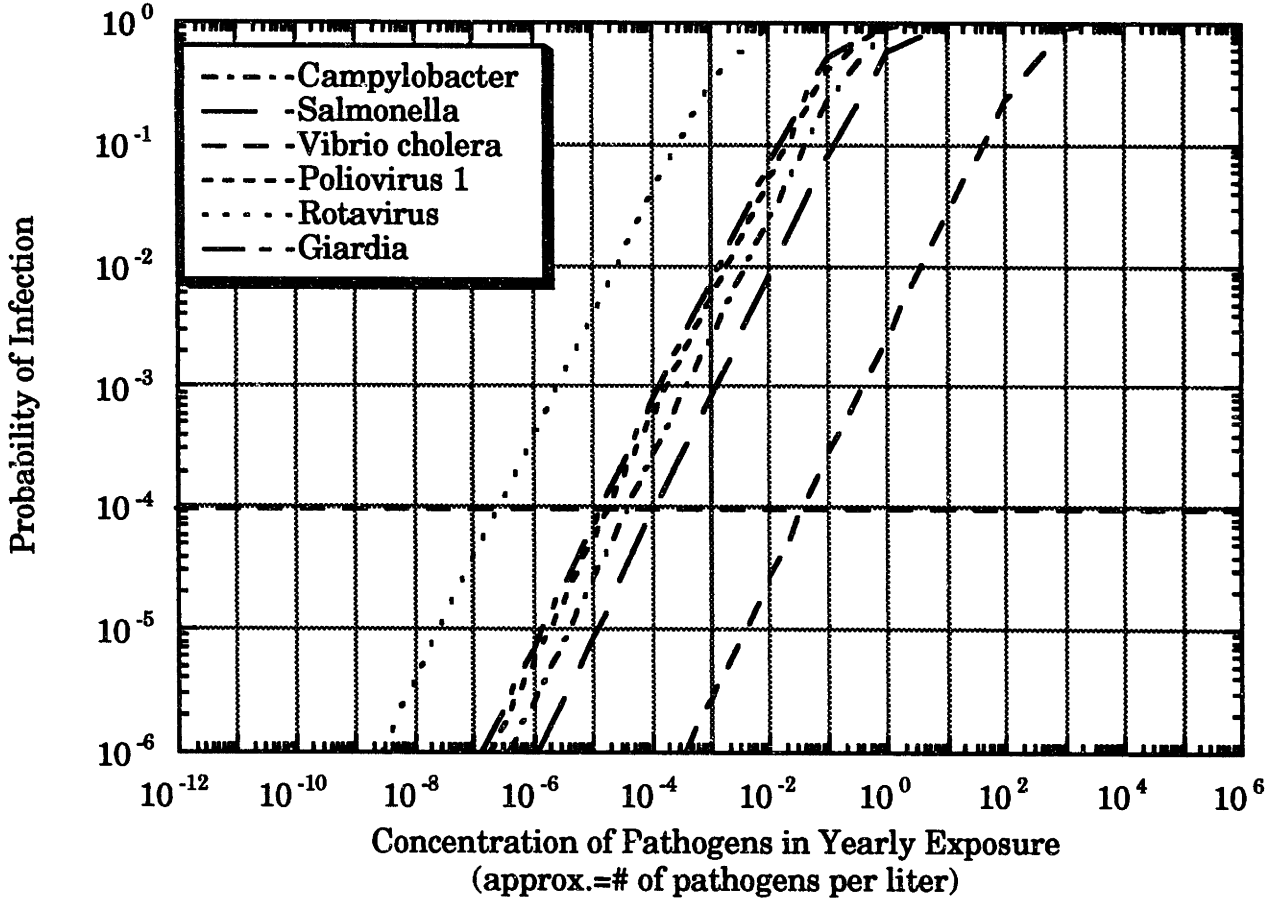


Figure 4: Annual Risk of Infection from Ingestion of Selected Pathogens



immunity through infection (Ward & Akin, 1984). This is the case for enteroviruses and rotaviruses for which vaccines are being developed, but is not the case for Norwalk virus, one of the most common viruses. For Norwalk virus, individuals having a preexisting antibody level show the highest risk of re-infection, unless the antibody levels are acutely elevated (CDC, 1990a).

This information, and the graphs presented above, indicate that modeling risk in order to arrive at an estimate of an epidemiologically important level of pathogens for various water types is currently not possible. In fact, reducing virus concentrations to extremely low levels may not even be desirable. One thing is clearly documented: the primary damage, both economically and in terms of human costs, is caused by exposure to the higher levels of human pathogens that occur during outbreaks of disease. These outbreaks primarily occur due to (1) the lack of monitoring in recreational waters and (2) the failure or absence of water treatment for drinking water. Thus, in order to develop a useful detection technique, it is necessary to determine levels of pathogens that are found in the environment in order to determine the detection sensitivity necessary for a good detection technology.

2.1.3 Levels of Pathogens

Levels of pathogens in the environment vary dramatically with the water source, level of treatment, and the surrounding environment. Also, due to the limitations, expense, and difficulties associated with current detection methods, limited information is available regarding pathogens in

environmental samples. When data are available, they usually involve a (1) positive/negative determination or (2) pathogen enumeration in polluted systems, using technologies for which recovery efficiencies and detection sensitivities are not known. Thus, although measurements of pathogen levels can be made, absence of detection does not indicate that no pathogens were present (e.g., the Payment study cited in the previous section). On the other hand, while a positive sample ensures the presence of the pathogen, the level of pathogen contamination cannot be ascertained with certainty unless a recovery efficiency has been determined, or the method used has been shown to give consistent recoveries regardless of water quality and pathogen type.

Because viruses and other pathogens are present at low concentrations in most natural water bodies and in drinking water, large volumes of water must be analysed in ordered to ensure the absence of pathogens. Average values for 'virus' concentration from different sources are on the order of 1 to 10 plaque forming units (PFU) per 100 mL in raw sewage, 1 to 10 PFU per 100 mL in primary effluent, 1 to 10 PFU per 10 L in secondary effluent, 1 to 10 PFU per 100 L in (Mississippi and Missouri) river water, 1 to 100 PFU per 1000 L in New York Bight, and 1 to 10 PFU per 10 L in Houston Ship Channel (Berg, 1978). 'Virus' in these experiments was operationally defined as anything that formed a plaque on the particular human cell culture used in that research; however, many human pathogenic viruses do not grow well in cell culture, most notably rotavirus and Norwalk virus (CDC, 1990a). These values have been summarized in Table 4. The numbers suggest that 10-100 liters may need to be tested in order to detect viruses in river water, while significantly lower volumes may be needed for sewage effluent. By examining large volumes of water, the background level of virus can be

determined; however, for specific water uses, the volume of water to be tested should be coordinated with the level of acceptable exposure. This level is given by the EPA as 1 infection in a population of 10,000 per year. These issues will be discussed in depth in Chapter 6.

Table 4: Levels of Pathogenic Virus in Environmental Waters (excluding Norwalk and other non-culturable viruses)

Swimming areas: †	1 PFU/ 40 L	2.5 viruses/ L
Drinking water: †	1 PFU/ 40-400 L	2.5-0.25 viruses/L
Urban River water: ††	100-10,000 PFU/ 100 L	100-10,000 viruses/L
Sewage (raw):††	100-10000 PFU/ 1 L	1000-1,000,000 viruses/L
Sewage (effluent):††	1-100 PFU/ 100 mL	100-100,000 viruses/L

† Suggested regulatory limit
†† Expected levels in First World countries.
††† The cell lines used in these experiments detect enterovirus, which typically contain 100 viruses per 1 PFU (Gerba, 1989)

2.2 Traditional Indicators of Water Quality

2.2.1 The Indicator System Defined

Indicators are substances or entities whose presence suggests the possible presence of another substance or entity that is too difficult or costly to measure directly. A variety of chemicals and organisms are used to indicate the quality of water. As mentioned in the Introduction, knowing the quality of a given body of water is important because exposure to the water may result in disease. Among other uses, the concentration of an indicator can be used as a design parameter to ensure that the disinfection system used in a water/wastewater treatment plant is adequate.

Many indicator systems have been developed over the last century. This is especially true of the last 30 years because, during this period, researchers have come to better understand the ecological interactions between indicators and the varying environments the indicators encounter. In addition, better techniques have been developed to aid in the isolation and enumeration of a variety of microorganisms, resulting in an increase in the number and type of organisms that are feasible for use as indicators (e.g., strict anaerobes and bacteriophage). The main groups that have been suggested as indicators consist of bacteria, chemicals, viruses, and yeasts. Only the current indicator system will be discussed here; however, an overview of alternative indicator organisms is given in Appendix I, and alternative indicators are listed in Table 5.

Table 5: Traditional Indicators of Pollution

Bacterial Indicators	Viral Indicators	Others
Total and fecal coliform	Coliphages (esp. MS-2)	coprostanol
Escherichia coli	F-specific coliphages	fecal sterols
Fecal Streptococcus	Bacteriophage of	*Candida albicans*
Enterococci	*Bacteroides fragilis*	sewage yeasts
Clostridium perfringens	*Bifidobacterium* spp	benthic organisms
fecal clostridia	Cyanophages	
Bifidobacterium		

2.2.2 Origins of the Current Indicator System

In the United States, total coliform and fecal coliform bacteria have been selected as the two groups of organisms that are best suited to be indicators of the possible presence of pathogens. In 1968, the first federal guidelines, which are the suggested limits, were set for recreational areas. The standards, which are the regulatory limits, have since been promulgated by the states. Currently thirteen states have standards involving total coliform and 54 states or U.S. territories have standards for fecal coliform levels (Cabelli, *et al.*, 1983). The EEC has standards for coliform and fecal streptococci, and many European countries have vastly different standards than those set in the U.S. (Volterra, *et al.*, 1984). It should be noted that drinking water and "interstate shellfish" area standards are set on the federal level, while recreational water quality and "intrastate shellfish" area standards are set by the state, usually in accordance with federal guidelines.

The accepted standards for fecal coliform (FC) and total coliform (TC) are based on a geometric mean of a minimum of 5 samples that must be taken each month. The fecal coliform and total coliform standards are the same for drinking water at <1 organism/ 100mL. In recreational areas, the standard is <200 FC/100mL or <1000 TC/100mL with not more than 10% of the samples for a given month exceeding 400 FC/100mL or 2000 TC/100mL (Cabelli, 1978a). For shellfishing areas, the limit is <14 FC/100mL or <70 TC/100mL with no more than 10% of the samples exceeding 43 FC/100mL or 230 TC/100mL (Cabelli, 1978a).

The recreational standard was based on epidemiological evidence from a study by Stevenson in the 1950's, and was promulgated as a guideline in

1968 by the Federal Water Pollution Control Administration (Cabelli, *et al.*, 1983). The Stevenson study found that a level of 2000 TC/100mL could be linked to "detectable health effects". Then, the 2000 TC value was extrapolated (from an assumed relationship between TC and FC) to 400 FC/100mL, which was divided in half for a safety factor, giving a value of 200 FC/ 100mL as the guideline (Cabelli, 1978a). The results of this study are considered by some investigators as inconclusive because of the methodology used; Cabelli stated the standards "were derived from barely detectable health effect rather than from exposure-response data" (Cabelli, *et al.*, 1983).

The shellfish area standard was developed during the 1920's in response to a typhoid epidemic (Duncanson & Saad, 1989), but was not adopted as a standard by the U.S. Public Health Service until 1946 (Cabelli, 1978a). In comparison, many European countries have vastly different guidelines and standards derived from different information and different assumptions. Overall, little epidemiological difference resulting from water consumption is found between the populations (Shuval, 1986). How and why these numbers have been chosen are strongly debated issues.

To find a good indicator, one may look to the human intestines, because this would be the reservoir for most water-borne human pathogens, especially those passed by the fecal-oral route. The facultative anaerobes are the most popular indicators, almost certainly because a century ago these organisms were the best studied and easiest with which to work. These organisms make up only about 0.1% of the total enteric bacterial population, and largely consist of *Escherichia coli, Klebsiella pneumoniae, Enterobacter aerogenes, Streptococcus faecalis,* and *Candida albicans* (Stanier *et al.* 1986, 599). The majority of the enteric bacteria is composed of the obligate anaerobes of the

genera *Bacteroides* [19%], *Bifidobacterium* [11%], *Peptostreptococcus* [9%], *Eubacterium* [7%], and *Rubinococcus* [3%] (Cabelli, 1978b). The following section gives a description of the coliform bacteria and deals with the positive and negative aspects associated with their use as indicator organisms.

2.2.3 Coliform and *Escherichia coli*

As mentioned above, the coliform bacteria have been used for almost a century as indicators of fecal pollution and the possible presence of human pathogens in both marine and fresh water environments. A coliform bacterium is operationally defined as any microorganism that ferments lactose with the production of acid and gas in less than 48 hours at 30°C to 37°C (Leclerc, *et al.*, 1977). A fecal coliform bacteria is a coliform bacteria that ferments lactose with acid and gas production at 44.5°C in 24 hours (Cabelli, 1978a). The name fecal coliform is a misnomer in that these organisms are not necessarily of fecal origin; they are simply thermotolerant coliform bacteria (den Blanken, 1985). The most probable number (MPN) and membrane filtration (MF) tests give a result, (+) or (-), for gas production and colored colony formation, respectively. In practice, little is done to further differentiate the coliform organisms and thus, false positives or false negatives are not easily identified.

The total coliform bacteria are aerobic or facultative anaerobic, non-sporing, Gram negative, lactose positive rods (Farmer & Brenner, 1977), mainly composed of microorganisms of species from the genera *Citrobacter, Enterobacter, Klebsiella,* and *Escherichia,* although others may be included (Mitchell & Chamberlin, 1978). Total coliform bacteria are a natural

inhabitant of the intestinal tract of all warm-blooded animals and they are excreted in large numbers on a daily basis. Total coliform bacteria are also naturally present in soil and water. Thus, a higher-than-natural background level of total coliform can be used as a fecal indicator. Fecal coliform are made up of species of *Escherichia* and *Klebsiella,* and in humans are composed mainly of *E. coli* and *K. pneumoniae.* These organisms are present in all warm-blooded animals (Holm, 1987), and have been found in cold-blooded animals as well (Freeman, 1985). *E. coli,* however, is almost certainly not a natural inhabitant of the gut of cold-blooded animals, but it can reproduce there if the ambient temperature is high enough (Buras, *et al.*, 1987).

The total coliform are usually more numerous than the fecal coliform, because they are naturally present in the environment. The use of total coliform as a recreational water indicator has been criticized because natural background levels of coliform can vary (with rainfall, temperature, *etc.*) and so they are difficult to use without additional historical information about the test site. In fact, Dufour states that some coliform are "seldom associated with fecal contamination" (Dufour, 1977). On the other hand, the total coliform are better accepted as indicators in drinking water. Because they have higher concentrations than, and include, the fecal coliform, they are considered a more conservative indicator (Mack, 1977).

Table 6: Traditional Criteria for an "Ideal Indicator"

The indicator should:

1) be a common enteric component of "healthy people"
2) be nonpathogenic
3) be solely of enteric origin (fecal)
4) be human specific
5) correlate well with the presence of pathogens
6) be more numerous than pathogens
7) mimic growth and/or die-off of pathogens outside the body
8) be as resilient in the environment as pathogens
9) be detected easily and have a cost-effective methodology for detection

(Feachem, *et al.*, 1983)

2.2.4 Problems with Indicators and Battery of Pathogens

In order to understand whether an indicator system is sufficient, it is necessary to define what makes a "good" water quality indicator. A list of generally accepted criteria for a good pathogen indicator is given in Table 6. Because some animals can transport human pathogens, human specificity is not necessarily an ideal characteristic. On the other hand, most viruses and many bacterial pathogens are host-specific or have a limited host range, and Kalter found that "from the information available, human disease as a result of waterborne transmission of non-human viruses has been inconsequential" (Kalter, 1986). This statement would imply that the use of an indicator that was human-specific may help to limit false positives (the indicator is present when human pathogens are not). Another important consideration is that some bacterial pathogens have the ability to grow in the environment. Therefore, the indicator should be able to mimic the growth, or lack of growth, of a given pathogen. This is no easy task as there are a variety of human pathogens (i.e., *Salmonella, Shigella, Vibrio,* pathogenic protozoans, spore and cyst formers) that do not have common survival and growth characteristics.

Indicators must reliably and consistently predict the presence of pathogens from a large number of sources under a broad range of conditions. Fecal pollution can come from (1) a point source, such as a sewage outfall or a septic tank, or from (2) non-point sources like road run-off, agricultural areas with domestic animals, or wild animals and birds. The pollution can be continuous or intermittent, and it can continue over the short term or the

long term. These scenarios are not a function of the pathogen, and yet they would all exert different and often conflicting demands on an indicator's ability to be present in sufficient number. Possibly the most difficult case involves pathogens that occur naturally in an environment. If an organism is to indicate this type of pathogen, it must also grow in the environment. On the other hand, it is an important concern that, in general, an indicator should not grow naturally in the environment.

Finally, in order to mimic pathogens, it is also necessary that the indicator be affected in much the same way as any given pathogen over a range of environmental conditions (high or low pH, temperature, salinity, sunlight intensity, nutrient and substrate levels, oxygen levels, adsorption to sediments, predation, toxics concentration, *etc.*) (Borrego, *et al.*, 1983). All these conditions must be mimicked or at least approximated, while the test for the organism remains inexpensive and efficient. Thus, it is not surprising that no known indicator matches the description given in the above-mentioned list. The discussion that follows will concern the ability of coliform bacteria to meet the criteria for a 'good indicator'.

The coliform bacteria meet several of the criteria set forth in Table 6 (numbers 1, 2, 6 and 9), but do not meet the remaining criteria. Because the fecal coliform are present in all warm-blooded animals (fails #4) and because members of this group (including *E. coli*) have been shown to replicate outside the body (fails #3), they are often present when no human pathogens are present (fails #5), resulting in the inappropriate closure of bathing and shellfishing areas. Although it is true that animals can carry human water-borne pathogens, the hazard associated with non-human fecal coliform is less because many pathogens are host specific. In fact, animals have yet to be

epidemiologically linked to the water-borne transmission of viral disease in humans (CDC, 1990a), although it is known that animals can transmit some bacterial and protozoan diseases. Also, many viruses and spore-forming bacteria are more resistant to environmental stresses and, therefore, are longer lived than coliform (fails #7 and #8), meaning that the absence of coliform does not ensure the absence of pathogens. The overwhelming reason for the inadequacy of fecal coliform and *E. coli* as indicators is that they are not good models for many pathogens (although they correlate reasonably well with *Salmonella* spp.) because they do not have similar survival capabilities or transport characteristics.

In the case of drinking water, total and fecal coliform are not conservative enough. Coliform bacteria showed 2.5 log reduction after 20 minutes at 3.2 mg/L of chlorine while coliphages showed 0.3 log reduction at the same level (compared with no loss of infectivity of Norwalk virus after 30 minutes at 6.25 mg/L) (CDC, 1990a).

A further problem with the coliform tests is that lactose positive members of *Aeromonas, Vibrio, and Pseudomonas,* which are not considered fecal coliform, can give false positives (Farmer & Brenner, 1977). Furthermore, the type of test that is done, MPN or MF, can give different results. Hendricks found the ratio of results from MPN and MF tests could range from 0.2 to 5 (Hendricks, 1978). Another factor that can cause results to vary is the temperature at which the test is performed. Dutka noted that at 44.5°C, recoveries of E. coli were 20% to 40% of the recoveries at lower temperatures (Dutka, 1979). Borrego, *et al.* wrote "*E. coli* does not conform to the concept of an indicator organism" because of its low environmental resistance (Borrego, *et al.*, 1983). It has also been found that at elevated

temperatures (Olson & Nagy, 1984), high levels of nutrients (Gerba & McLoed, 1976), and association with sediments (Struck, 1988), fecal coliform may have the ability to grow in the environment. *E. coli* is known to grow in water in tropical environments (Rivera, *et al.*, 1988). These same factors along with sunlight intensity, salinity, and predation also affect the die-off rate of coliform in more temperate climates (Milne, *et al.*, 1986). Struck found that fecal coliform levels could be 100 to 1000 times as concentrated in the sediments as in the water column (Struck, 1988). Rhodes and Kator found a three day growth phase of sediment bound coliform, followed by a concurrent rise in the microflagellate population and decline in the coliform population (Rhodes & Kator, 1988). Dufour felt that *E. coli* is the best indicator (Dufour, 1977), while Vlassoff felt that finding the fecal coliform *K. pneumoniae* was as significant as finding *E. coli* (Vlassoff, 1977).

The most common indicators of viral pathogens include the coliphages and bacteriophages of anaerobic intestinal bacteria discussed in Appendix I. The bacteriophages are viruses that infect bacteria, and the coliphages are bacteriophages of *E. coli*. No growth of anaerobic bacterial bacteriophages and F-specific coliphage can occur outside the intestinal tract. These are the most promising indicators for viruses because they meet criteria numbers 1, 2, 3, 6, and 9 from Table 6. Under varying environmental conditions, however, they fail criteria numbers 5, 7, and 8. Because there is such a wide range of pathogenic viruses, no single indicator could be expected to ensure that its presence or absence indicates the presence or absence of pathogens. The vast spectrum of pathogenic organisms, which are as different from each other as they are different from the indicators, ensures that no single indicator can adequately be used as a marker for the presence or absence of

pathogens. Thus, many of these systems do not conform to what is considered the definition of a good indicator.

The current situation might be acceptable if not for the growing number of shellfish area and bathing area closures. These closures have caused economic hardship, causing questions to arise about the nature of the coliform standards (Heufelder, 1989; Hickey, 1989). Concerns have been raised that coliform bacteria are naturally occurring and have been present throughout history, but have only been detected recently due to testing. On the other hand, outbreaks of disease have occurred and pathogens have been detected in waters meeting water quality standards. Analysis of field studies showed human pathogenic viruses were found in 44% of 'recreational area' sites and 35% of 'shellfishing area' sites when indicated to be safe by fecal coliform standards (Rao & Melnick, 1986).

No single indicator can comply with all of the requirements listed in Table 6. Under varying environmental conditions, many of the bacterial organisms listed in Table 5 have been shown to correlate poorly with bacterial pathogens, and often not at all with viral pathogens (Geldenhuys & Pretorius, 1989). Likewise, the viruses listed in Table 1 show some correlation with some pathogens under a limited set of conditions, but often show no correlation with the majority of pathogens over varying environmental conditions (i.e., pH, ionic strength, temperature, sorption, light intensity, toxic chemical concentrations, *etc.*). For this reason, the title 'indicator' is a misnomer. For lack of a better word, these organisms should be referred to as non-ideal indicators.

The question now becomes: which indicator system of those critiqued in the previous section and in Appendix I is the best? The answer is that each system has its benefits and its short-comings. In general, the best way to test a system for pollution is to use a battery of tests. In doing so, several indicators are utilized in order to get as much information as possible about a sampling site (Dutka, *et al.*, 1988). Currently, only coliform is in wide use. The reason the coliform indicator system is in use is best summed up by saying "we have an emotional attachment to coliform" because we have had a long history with it (Cabelli, 1978a). This system would provide a relatively conservative means of monitoring environmental waters, although it could not be used to guarantee the presence or absence of pathogens. Furthermore, it would be expensive and difficult to monitor multiple indicators. It would be better, if multiple organisms are to be monitored, to monitor pathogens directly.

2.2.5 Direct Detection

No indicator can consistently and absolutely be correlated to pathogens. In fact, researchers have shown under certain conditions that no correlation exists (Geldenhuys & Pretorius, 1989). To complete all the biochemical testing and culturing of individual bacterial and viral pathogens is out of the question, but to develop "molecular probes" for each is not.

Direct detection of pathogens eliminates the need for criteria numbers 1, 3, 4, 5, 6, 7, and 8 from Table 6. The major benefit of detecting the pathogens themselves is the knowledge of the presence or absence of pathogens, thereby eliminating the need for concern over relative survival and transport

characteristics. Furthermore, if the viruses are not cultured, then the handling of pathogenic material is minimized (eliminating criterion number 2). Only criterion number 9 remains, that being the need for a facile and cost-effective test. Such a test is available in the form of PCR, and is discussed in Chapter 4.

In the past, the reasons for using a non-ideal indicator system instead of simply detecting pathogens are (1) because the number of pathogens of interest is very large (i.e., the number of tests to detect them all would be too numerous), (2) because many of the pathogens cannot be cultured readily or are extremely difficult to culture, and (3) because the concentration of any given pathogen is probably very low. Direct detection of pathogens could be used to monitor water bodies with the same frequency as for non-ideal indicators with more useful results. On the other hand, it is also true that pathogens may be present sporadically, while non-ideal fecal indicators may be present consistently. At worst, the proposed technology provides a means of more easily detecting the relationship between non-ideal indicators and pathogens as well as giving an assessment of water quality at the time of sampling. Moreover, the proposed technology would be a powerful tool when used in conjunction with more traditional methods. In fact, traditional indicators can be monitored along with the pathogens by selecting "molecular probes" that detect any of a number of non-ideal indicators. PCR is not the only detection scheme available and, in fact, is not the most commonly used. Concentration and detection schemes for direct detection of pathogens are now discussed.

3. CONCENTRATION METHODOLOGIES

3.1 Introduction

Because pathogens are often present at levels in the environment which are far too low to be detectable directly, methods are needed to increase the pathogen concentration in the samples to detectable levels. In other words, water and other non-target materials must be removed, while retaining a significant portion of the pathogens of interest. Most currently developed technology uses either flocculation, centrifugation, filtration, or some combination of these physical separation processes to accomplish this goal.

For small water samples, traditional ultrafiltration, ultracentrifugation, cesium-gradient purification, and a variety of other similar methods have been used with some success. The sample volumes used in the techniques are usually less than one liter, and are not useful for regular monitoring of water samples, especially for non-potable environmental samples. These techniques are not widely used and will not be discussed here, although several reviews exist (Berg, 1987; Gerba & Goyal, 1982; Hurst, *et al.*, 1989).

Adsorption-elution technology is by far the most widely accepted means to concentrate viruses from environmental samples, especially drinking water samples. This technology has several short-comings, however, with regard to consistent recoveries of multiple pathogens from non-drinking water environmental samples. Rotating membrane ultrafiltration (RMU) is not widely used as a technique for pathogen concentration, but was found in this research to be an effective, efficient, and consistent technology for the

recovery of viruses from water. Both adsorption-elution and RMU are described in the following sections.

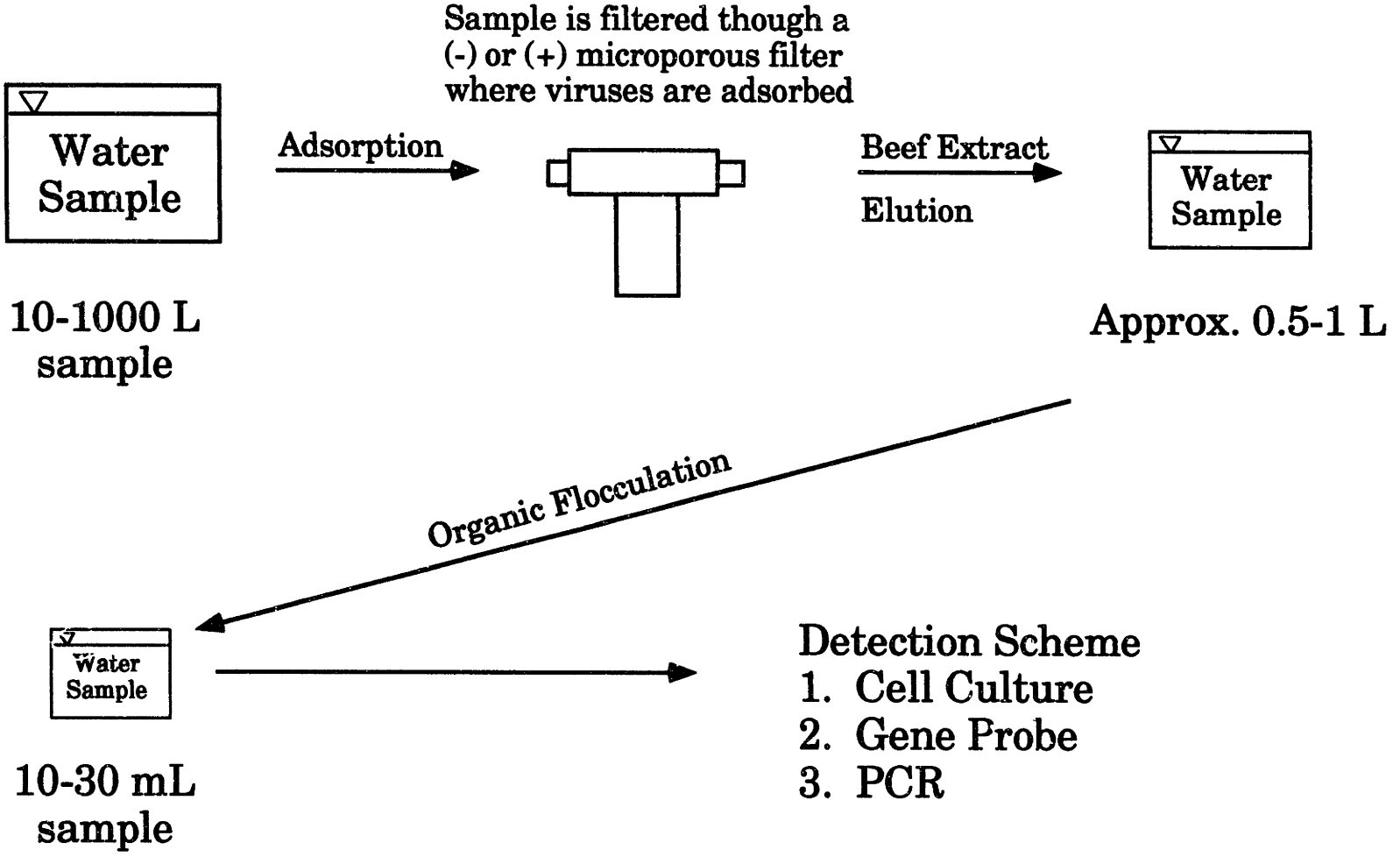
3.2 Adsorption-Elution Technology

3.2.1 Defined and Described

Sorption of viruses to glass powder and other surface-charged solids is well known, and provides the basis for adsorption-elution technology. In adsorption-elution technique, water is passed through a prefilter, followed by a microporous filter which carries an electronegative or electropositive charge, allowing the electrostatic sorption of positively- or negatively-charged particles, respectively (Rose, *et al.*, 1987). The pores in these filters are between 0.5 and 10 microns (Goyal, *et al.*, 1980), which are much larger than most viruses, but are small enough to be clogged by suspended solids. Thus, sorption characteristics (not size) determine the concentration potential of the microporous filters.

Water is passed through the filter under pressure. In the next step of the process, the charged particles that have been adsorbed to the filter during the adsorption process are eluted from the filter with a 1 liter volume of elutant containing a charge-neutralizing agent like beef extract. Finally, a secondary concentration step, usually organic flocculation, is used to reduce further the volume of the sample to about 30 mL. Figure 5 gives a pictorial view of the procedure.

Figure 5: Adsorption-Elution Methodology



As mentioned, adsorption-elution filters exist in two forms: electronegative filters and electropositive filters. Electronegative filters require that the pH of the unconcentrated water sample be near 3.5, and also require the addition of a divalent or trivalent metal salt to 50 mM or 0.5 mM, respectively. The optimum pH for viral adsorption is dependent on the isoelectric point of the virus to be sorbed. That is, viruses often carry a negative charge in the pH ranges found in nature, but at low pH they become positively charged. This positive charge is required in order for the negatively-charged filter to sorb the virus. The metal salt is thought to precipitate on the filter and provide additional binding sites for the virus. Although this is a tedious and expensive process, recovery efficiencies of pathogens in environmental samples are generally higher using electronegative filters. Because they sorb positively charged particles, and many particles in nature have a negatively charged surface (e.g., silicates, viruses), these filters are less affected by water quality. Humic material and positively-charged materials (at pH 3.5) are still a problem.

Electropositive filters do not require that the water sample be conditioned before filtration; however, they are more dramatically affected by water quality. Electropositive filters are the predominant filter type in use today. Data from the literature confirming these statements are given below.

3.2.2 Benefits of Adsorption-Elution Technology

There are several benefits to using the adsorption-elution technique. Adsorption-elution allows moderate to very large volumes (1 L to 1000 L) of

drinking water to be processed quickly (at 0.1 L/min. to 40 L/min.). Furthermore, this technique is well established and has been in use for well over a decade. It is precisely because it has been so well studied - much like the use of coliform bacteria as indicators - that it is the most commonly used concentration technique.

Viruses have been detected (with cell culture) in environmental sample concentrates using adsorption-elution under two scenarios. First, very large volume samples of polluted drinking water (100 L) were filtered and a positive/negative determination was made as to the presence of virus, but no attempt was made to gain information on recovery efficiency of the technique for the particular source of water. Furthermore, initial virus concentrations were not determined (Bloch, *et al.*, 1990).

Second, high levels of spiked virus (10^3 to 10^7 PFU/L of enterovirus) have been concentrated efficiently from large volume environmental samples (20 L) (Biziagos, *et al.*, 1989; Divizia, *et al.*, 1989; Farrah, *et al.*, 1991). A plaque forming unit, PFU, is the amount of virus required to form a plaque in cell culture (described in Chapter 4). Although it is not well known how many particles are in 1 PFU, it has been determined using electron microscopy to be approximately 100 particles for poliovirus 1, a typical enterovirus. The vast majority of research has been concerned with spiking large quantities of virus in small volumes of water to determine recovery efficiency using different viruses, different water sources, different filter types, and modifications to the protocol to increase recovery. Much less attention has been paid to detecting low levels of viruses and bacteria in environmental samples.

3.2.3 Problems with Adsorption-Elution Technology

There are several problems associated with the use of adsorption-elution. No reconstruction experiments have been performed on large water volumes and low concentrations of pathogens. No one has spiked a low number of different viruses into a large volume water sample and recovered them. More importantly, adsorption-elution has failed to recover different types of viruses in small volumes of various environmental waters in a consistent fashion. Also, non-drinking water samples have not been used in experiments to detect low concentrations of pathogens, and these water sources are useful for monitoring purposes.

Several studies have been performed to determine the effect of varying virus type and water quality on different kinds of microporous filters. It was found that adsorption-elution sorption characteristics are dramatically affected by water quality. Furthermore, filters vary greatly in their ability to sorb different virus types as well as bacteria and protozoa under varying environmental conditions.

Four different viruses were spiked into 1.3 liters of source water, and attempts were made to recover them using electropositive and electronegative filters. From the data shown in Table 7, it can be seen that reoviruses (cousins of rotaviruses) are poorly sorbed by either filter. Furthermore, overall recovery was low and highly variable. Many researchers (e.g., (Armon, *et al.*, 1988; Biziagos, *et al.*, 1989; Hurst, *et al.*, 1989) have reported a significant variation in recovery based on virus type. As a further example, Pinto *et al.* found 3% and 4% overall recovery of 10^5 MPN-CU (similar to

Table 7: Low Recovery of Certain Virus Types

Organism	Recovery % (SD)	
	(-) filter	(+)filter
poliovirus 1	47% (65)	36% (13)
echovirus 1	21% (51)	14% (16)
adenovirus SV11	25% (38)	22% (18)
reovirus 3	2% (8)	13% (14)

note: 10^7 PFU of poliovirus 1, echovirus 1, adenovirus SV11, and reovirus 3 were spiked into separate samples of 1.3 liters of source water. Half of the samples were adjusted to pH=3.5 and amended with 0.5 mM $MgCl_2$; the other half were adjusted to pH=7.5. Then, each set of samples was filtered though the appropriate Filtrite negatively-charged or Virusorb MDS positively-charged filter. Each experiment was repeated 3 - 5 times (Sobsey, *et al.*, 1984).

PFU) of viral hemorrhagic septicemia virus, a fish virus, spiked into 20 L of fresh (0.05 ntu) and ocean water (1.03 ntu), respectively, using (+) ZetaPlus filters (Pinto, *et al.*, 1993). Thus, these difficulties are well documented. In addition, electropositive filters are not recommended above pH = 8, which is a common pH found in ocean water (Rose, *et al.*, 1987).

Electropositive filters are also affected by pH variations (see Table 8). When 20 liter samples of drinking water were spiked with four types of virus, recovery was found to be dependent on pH and virus type. Furthermore, the research presented in Table 8 supports the concept that sorption characteristics of different viruses are affected to different extents by variations in water parameters. The pH of drinking water is variable depending on the treatment process, and the pH range in this experiment is not uncommon. Thus, the use of MDS electropositive filters will not provide consistent results even in drinking water, but especially in freshwater and ocean water, which often have dramatically different pH ranges.

In regard to pathogen detection in drinking water, both electronegative and electropositive filters lose ability to adsorb viruses in water containing humic material, organics, suspended solids, or varying pH and ionic strength (Bosch, *et al.*, 1991; Bosch, *et al.*, 1988; Hahn, *et al.*, 1989; Sobsey, *et al.*, 1984; Sobsey & Glass, 1984). As an example, Table 9 clearly shows the effect that humic material has on positively-charged and negatively-charged filters. As the level of humic material in the spiked tap water samples increases, recovery by adsorption-elution filtration decreases. It should be noted that a level of 3 mg/L of humic acid roughly corresponds to concentrations commonly found in drinking water. Likewise, 10 mg/L and 30 mg/L correspond to

Table 8: Low Recovery of Certain Virus Types at Various pH Levels

Organism	Recovery %	
	pH=6	pH=8
poliovirus 1	67%	26%
coxsackievirus B3	91%	1%
echovirus 1	6%	10%
coxsackievirus A9	3%	3%

note: 20 L of tap water containing $10^{3.3}$-$10^{7.3}$ PFU/L was filtered through a (+)MDS adsorption-elution filter (Joret, *et al.*, 1986).

Table 9: Low Recovery at Various Humic Acid Concentrations

Humic Acid	Recovery %	Filter Type
mg/L	mean (SD)	
0	24% (17)	-*
3	18% (12)	-*
10	11% (8)	-*
30	8% (1)	-*
0	60% (15)	(+)MDS**
3	38% (53)	(+)MDS**
10	11% (11)	(+)MDS**
30	5 %(2)	(+)MDS**

* water sample adjusted to pH = 3.5 and divalent salt added.

** water sample adjusted to pH = 7.5 and divalent salt added.

note: 0.3 L of tap water containing $10^{5.5}$ PFU/L poliovirus 1 was filtered through a (+)MDS or (-) adsorption-elution filter (Sobsey, *et al.*, 1984). Results confirmed by Hahn *et al.*, 1989 and others.

source water and 'dirty' river water (e.g., Charles River, Boston, MA). Both filter types show similar loss of recovery efficiency with an increase in humics concentration. Also note that only 300 mL of sample was used; as the sample volume increases, recoveries would be expected to decrease. In the same study, fulvic acid also adversely affected recoveries.

In a similar study, Hahn *et al.* showed that in 1 L samples spiked with 10,000 coliphage and filtered through an MDS positively-charged filter, recovery (shown in parentheses) was affected by substances in river water (50%) , nonionic detergents (25%), ionic detergents (10%), and humics (5%) (Hahn, *et al.*, 1989). Finally, in two separate studies, Bosch *et al.* found an effect due to water quality when 10^7 PFU of tissue-adapted rotaviruses were spiked into 20 L samples of distilled water (54±17%), tap water (46±14%), and sewage (5±2%), and recovered using glass powder negatively-charged filters (Bosch, *et al.*, 1988). Bosch *et al.* also found that when 10^5 PFU of hepatitis A were spiked into 10 L samples of tap water (10±2%), seawater (6±2%), freshwater (7±7%), and sewage (4±4%), and recovered using ZetaPlus positively-charged filters, recoveries were affected by sample type (Bosch, *et al.*, 1991).

Reasons for the failure of this technology with regard to water quality and virus type are not known. It is suspected that adsorption sites on the filter are blocked by other charged particles. Also, particles may be hindered from eluting from the filter due to interaction with other charged particles (i.e., they are irreversibly bound). Both hypotheses probably contribute to low recovery, in that both adsorption and elution efficiencies are reduced in the presence of turbidity, suspended solids, and humic material. It is not

surprising that recovery is dependent on virus type, in so far as individual viruses should be expected to have different sorption characteristics due to differences in surface charge, isoelectric point, *etc.* Because beef extract, which is used to elute the filter, has been shown to inhibit PCR, this technique is limited in terms of detection schemes that can be used after concentration (i.e., PCR cannot be used). Finally, the secondary concentration step involving organic flocculation has been shown to give low recovery efficiencies, depending on virus type (Bosch, *et al.*, 1991; Guttman-Bass, *et al.*, 1985).

The bottom line concerning the use of adsorption-elution technology is that widely varying results between different laboratories make it difficult to standardize the technique in terms of recovery efficiency. For various viruses in different types of environmental samples, recoveries have been reported that range from <1% to >100%. The technique cannot be optimized for all the variables (Jofre, *et al.*, 1989) and, therefore, the method cannot be standardized especially for non-drinking water samples. Sobsey stated that no one set of conditions allows optimum recovery for all pathogens, and that information from one virus cannot be used to extrapolate data for another (Sobsey & Glass, 1984). Little has changed in the last decade to improve this situation.

3.3 Rotating Membrane Ultrafiltration

3.3.1 Defined and Described

Rotating membrane ultrafiltration (RMU) uses size separation as the means of concentrating organisms from water samples. The RMU unit consists of two concentric cylinders, an internal rotating cylinder covered by a filter of a given molecular weight, and an external stationary cylinder (see Figure 6). Rotation of the internal cylinder causes Taylor vortices to be formed in fluid between the cylinders (shown in Figure 7) (Donnelly, 1991), which (unlike traditional tangential flow filtration) allows particles larger than the pores to be actively transported away from the filter surface, thereby diminishing the chance of clogging and reduced flow rates even in the presence of high solids (Kroner, *et al.*, 1987). RMU, unlike traditional ultrafiltration systems, has the unique and desirable quality that the shear rate is independent of the flow rate (Hallström & Lopez-Leiva, 1978), thereby allowing higher flows and much higher concentration potentials (e.g., yeast cells have been concentrated to 60%-80% solids concentration by this method) (Ofsthun, 1989).

Particulars of the RMU protocol used in this research are discussed in Chapter 5. Here, a more general description of the technology is given. A water sample is drawn into the volume between the cylinders using a peristaltic pump. Pressure is applied to the system and water is forced through the filter. The internal cylinder can rotate at a speed between 200 rpm and 2500 rpm to maintain the shear rate necessary to remove material from the filter surface. Pressure can vary between 0 psi and 100 psi, depending on the integrity of the couplings and tubing used. Two filter areas,

Figure 6: The Rotating Membrane Ultrafiltration Unit

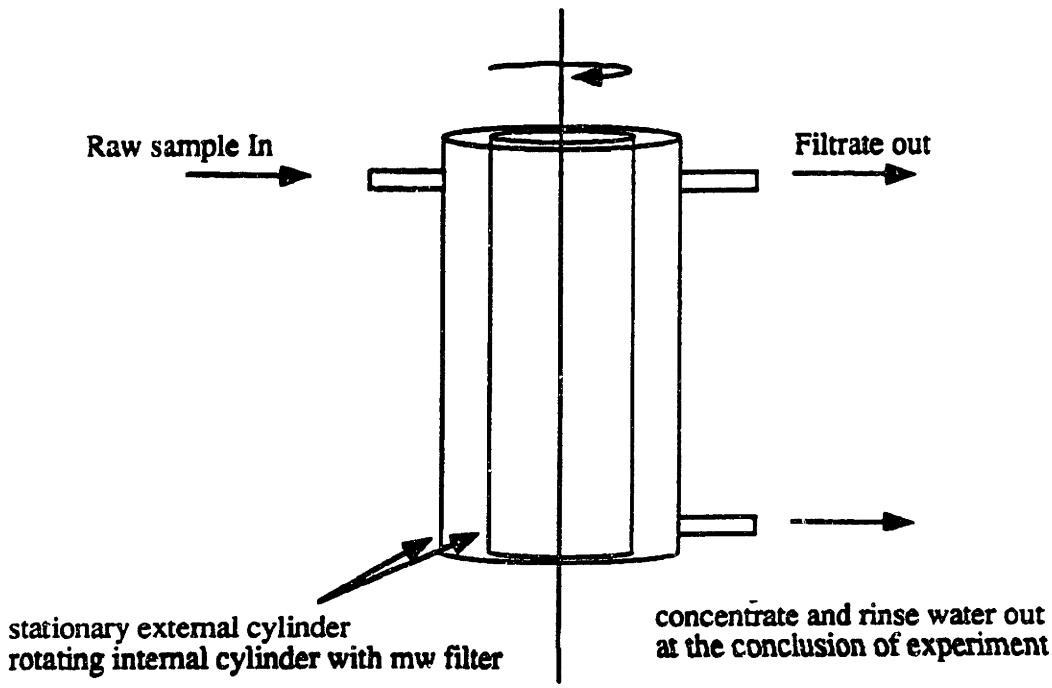


Figure 7: An Example of Taylor Vortices

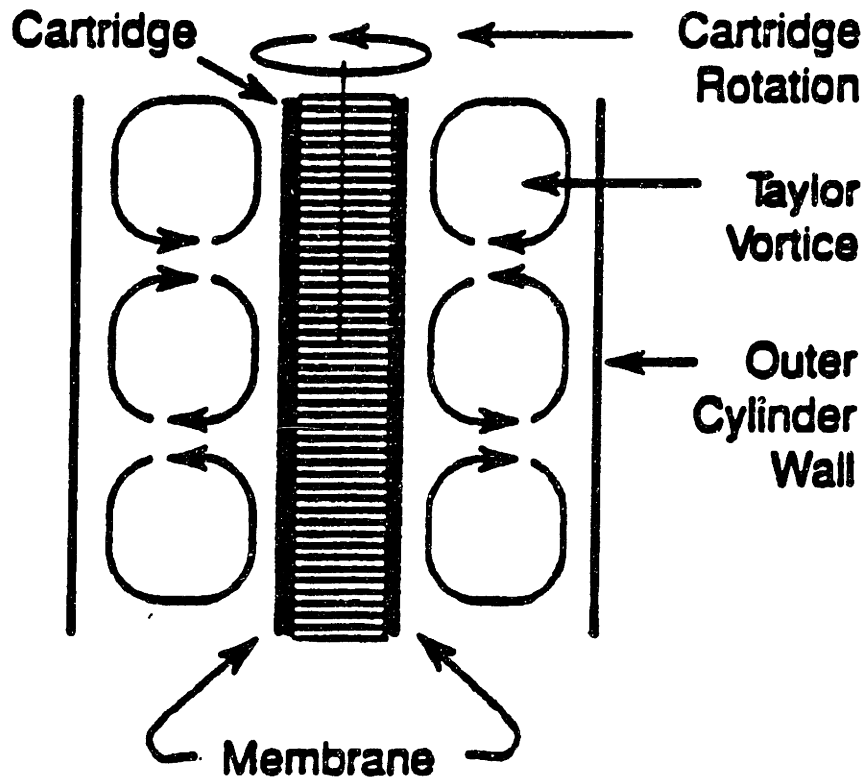
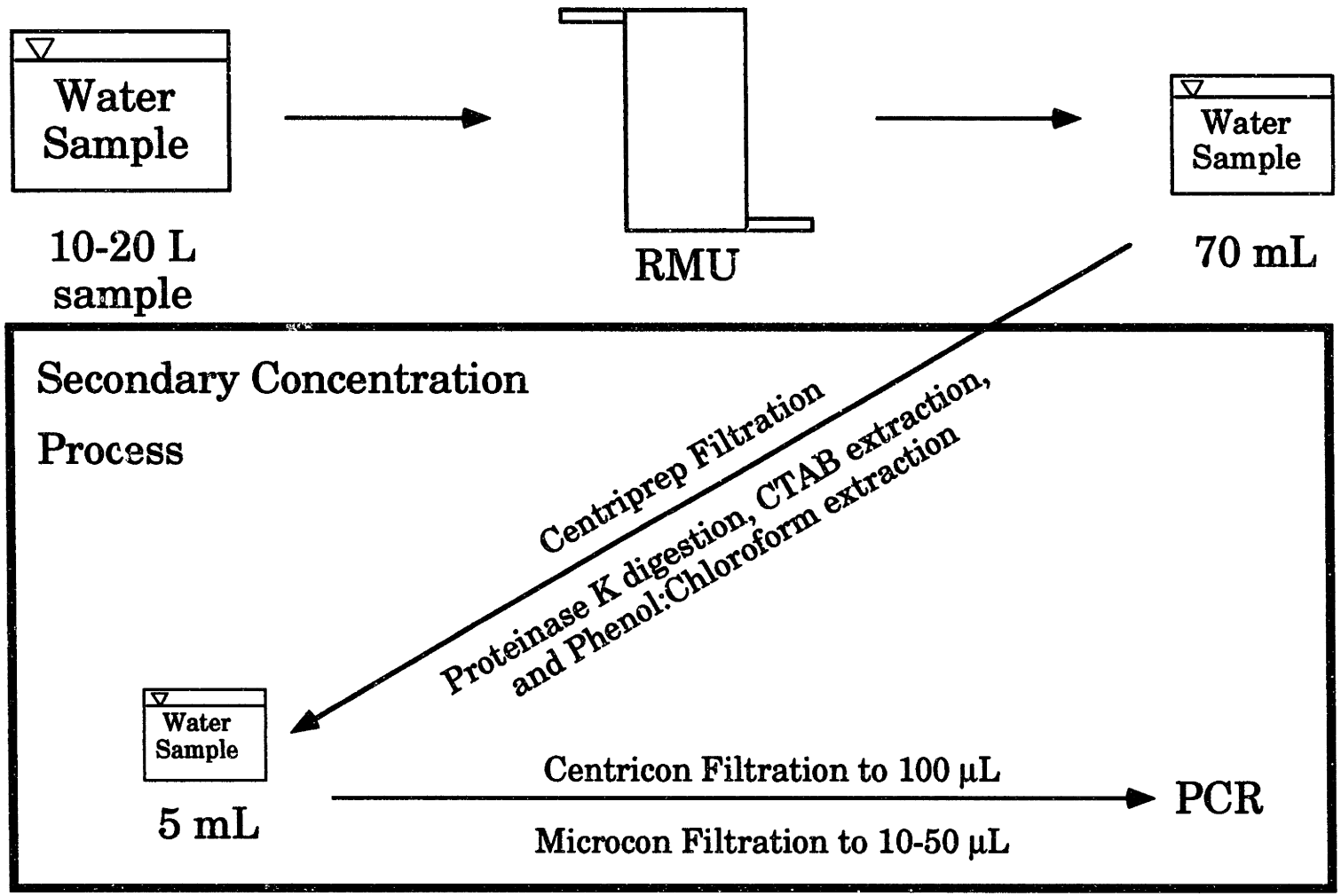


Figure 8: Rotating Membrane Ultrafiltration Methodology



200 cm^2 and 400 cm^2, are available for the UltraFilic filters with molecular weight cut-offs between 3 kDa and 100 kDa and for the polysulfonate filters with pore sizes between 0.2 μm and 0.45 μm. Flow rate is directly and linearly related to the pore size and surface area of the filter, as well as the system operating pressure.

The RMU used in this research is a benchtop model named Benchmark and is made by Membrex Corporation. This model is small and easily transportable, and when used in conjunction with a peristaltic pump and a power source could easily be used out of a van or similar vehicle. Another model, the Pacesetter, is also available which can achieve much higher flow rates. Figure 8 gives a pictorial view of the procedure using the Benchmark system. The membrane filter is also made by Membrex. Membrex describes the UltraFilic membrane, a proprietary product, as composed of a polyacrylonitrile matrix that has been chemically modified to maximize hydrophilicity.

In order to use ultrafiltration, in general, an appropriate molecular weight filter must be chosen. The filter size used in this research was chosen so that it would retain the smallest sized pathogens that may be encountered, which are the picornaviruses. These viruses are 20 nm spheres (see Table 2). Given $V=(4/3)\pi r^3$, specific weight of enteroviruses =1.3 g/cm^3 (Borchardt, *et al.*, 1977), and r=9 nm, the molecular weight of these viruses can be calculated to be 2.4×10^6. Thus, the 10^5 molecular weight filter used in this research provides a molecular weight cut-off which is an order of magnitude smaller than the smallest viruses. A 10^5 molecular weight filter should retain spherical particles down to 3 nm.

The presence of suspended solids affects the transport of viruses because viruses tend to sorb to particulate matter in the water column (Ohgaki, *et al.*, 1986). A benefit of the 10^5 molecular weight filter is that it easily removes these larger particles, while allowing the lower weight humic and fulvic acids to pass through it. The RMU filter used during this research was non-adsorbing, so that viruses were not lost to the filter itself.

After the entire water sample is filtered, the volume between the cylinders, the retentate (or concentrate), is collected. The filter can then be rinsed in order to obtain any material left on the filter by running water or buffer through the assembly. The final volume of concentrate can be reduced to a minimum volume of 35 mL, and is not dependent on the initial volume of the sample.

Before the sample can be used in a detection scheme, the volume of the RMU concentrate (usually 70 mL) must be reduced via a secondary concentration protocol. This means that the sample must be taken from 70 mL to between 5 µL and 100 µL. A 10^5 molecular weight Centriprep (Amicon) filtration, followed by a proteinase K digestion, a hexadecyltrimethylammonium bromide (CTAB) extraction, and multiple phenol:chloroform extractions and further filtration through a Centricon and Microcon (Amicon) filters were found to be necessary in order to have a sample of the appropriate volume and consistency to allow detection by PCR. This detection technology is discussed in the following chapter, while the results from RMU research are detailed in Chapter 5.

3.3.2 Benefits of Rotating Membrane Ultrafiltration

Traditionally, RMU has been used in protein separation technology, and has not received much use in the environmental field. There are, however, many benefits associated with the use of RMU technology as an environmental concentration technology, especially as compared to adsorption-elution technology. Concentration based on size separation is not dependent on virus type, but on the size of the particles being concentrated. Because recovery is solely based on size, consistent recovery efficiencies can be expected. Another researcher, who has used RMU to study the microbial ecology of naturally occurring bacteriophage in ocean water, found RMU recovery efficiencies to be between 70% and 80% (Paul, *et al.*, 1991). The results of this research, regarding high recovery efficiencies from RMU filtration regardless of virus type and water quality, will be presented in Chapter 5.

The bottom line with regard to RMU is that this technology provides a consistent means of concentrating viruses (and certainly larger organisms such as bacteria and protozoans) on the basis of size, and does not require the organisms to have a certain surface charge or other physical characteristics. As will be shown in Chapter 5, a consistent overall recovery efficiency has been achieved for use with environmental samples. This research has shown RMU to be an efficient means of concentrating the smallest model viruses to ensure recovery of the enteroviruses, which was heretofore untested.

3.3.3 Problems with Rotating Membrane Ultrafiltration

There are several areas in which adsorption-elution apparently outperforms RMU. Primarily, these issues have to do with the volume that can be concentrated and the rate at which it can be filtered. RMU is limited to a maximum of 30 L/hr for a 400 cm^2 filter at 40 psi and 2500 rpm, but, because RMU consistently obtains high recoveries from large volumes of water (10 L - 20 L), larger volumes (100 L) may not be necessary. In other words, a very large volume (100 L) can be filtered at 5% recovery (see above for adsorption-elution technology in non-drinking water environmental samples) or a large volume (10 L) at 50% recovery would give the same result (see Chapter 5 results on RMU). Furthermore, the quality of the sample, in terms of an inhibitory effect on the detection scheme, is also an important consideration. Whereas adsorption-elution adsorbs humic materials and other charged particles which can inhibit PCR, much of this material is passed through the RMU membrane because it has a lower molecular weight.

4. Detection Methodologies

4.1 Introduction

Several schemes are currently available for the direct detection of pathogens in the environment, although none of them are widely used. Detection methodologies can be differentiated by several general characteristics: 1) growth of the pathogen is required, 2) an individual test is required for each pathogen, and 3) a large number of pathogens are required for detection. The culture of pathogens requires the growth of pathogens. For viruses, different cell lines are used as hosts for different viruses. For other pathogens, selective media and growth conditions provide the means for detection. Although a different method is needed for each pathogen, often only a few organisms are needed for detection. Immunological techniques, electron microscopy, and gene probe technology do not require growth, but do require a large number of organisms for detection, unless the pathogens are grown before detection. Furthermore, these methods require a different test method to distinguish between organisms. PCR, on the other hand, does not require growth and can be used to detect multiple pathogens at low concentrations. Cell culture, gene probe technology, and PCR, which are the most common detection schemes used for environmental samples, will be described below.

Immunological tests, electron microscopy, and other common virological detection techniques will not be discussed in depth here because they have rarely been used on environmental samples. Briefly, immunological

techniques rely on recognition and binding of a pathogen's surface antigens by specific commercially-available antibodies in order to detect the presence of the pathogen. The antibodies are usually linked to something that is easily detectable such as a fluorescent probe, a radioactive probe, or an enzyme that can cause a colorometric reaction. These techniques do not detect viable pathogens unless growth of the pathogen is required before the detection scheme is used.

Several common techniques include immunofluorescence (IF), immunoperoxidase (IP), enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), biotin-avidin immunoassay (BAI), and radioimmunofocusassay (RIFA) (Rao and Melnick, 1986). These tests often require a large number (10^5 to 10^6) of target organisms for detection, and are not suited for use with environmental concentrates (Gerba, *et al.*, 1990). This high threshold of detection makes them inappropriate for use in environmental monitoring except as a means to confirm positives obtained in cell culture. In addition, specific antibodies against the pathogen of interest must be available. Finally, individual tests would be needed for each pathogen of interest, and a qualified lab technicians would be required to perform the testing.

Electron microscopy requires a similarly high threshold of detection. Although it does not require the growth of pathogens, it is impossible to detect and differentiate pathogens based on morphology, especially in concentrated environmental samples which may contain 'other' material that obscures results (Gerba & Goyal, 1982). Immunological techniques and electron microscopy provide an adequate means of viral detection in clinical specimens, but are not suited for environmental analysis.

Direct detection by the growth of microorganisms is accomplished differently for viruses and other microorganisms. In the case of bacterial or protozoal pathogens, growth on selective media under selective conditions is the primary means of detection. For example, *Clostridium perfringens,* an obligately anaerobic spore-former, must be grown in an anaerobic container with media specific for fecal clostridia. If a less restrictive media is used, a host of other anaerobic bacteria, yeasts, and other organisms, which are likely to be present in larger numbers, will overgrow and obscure the presence of *Clostridium perfringens*. After growth has occurred, a biochemical work-up, dot blot hybridization, immunological test, PCR, *etc.* must be used to confirm the identification of a recovered pathogen. Thus, an individual test is required for each organism of interest. The need for selectivity (to detect one organism from the many) precludes the use of an individual test for different organisms. The remaining discussion of cell culture applies only to viruses, but the use of gene probes and PCR applies equally to viruses, bacteria, and protozoans.

4.2 Cell Culture Technology

4.2.1 Defined and Described

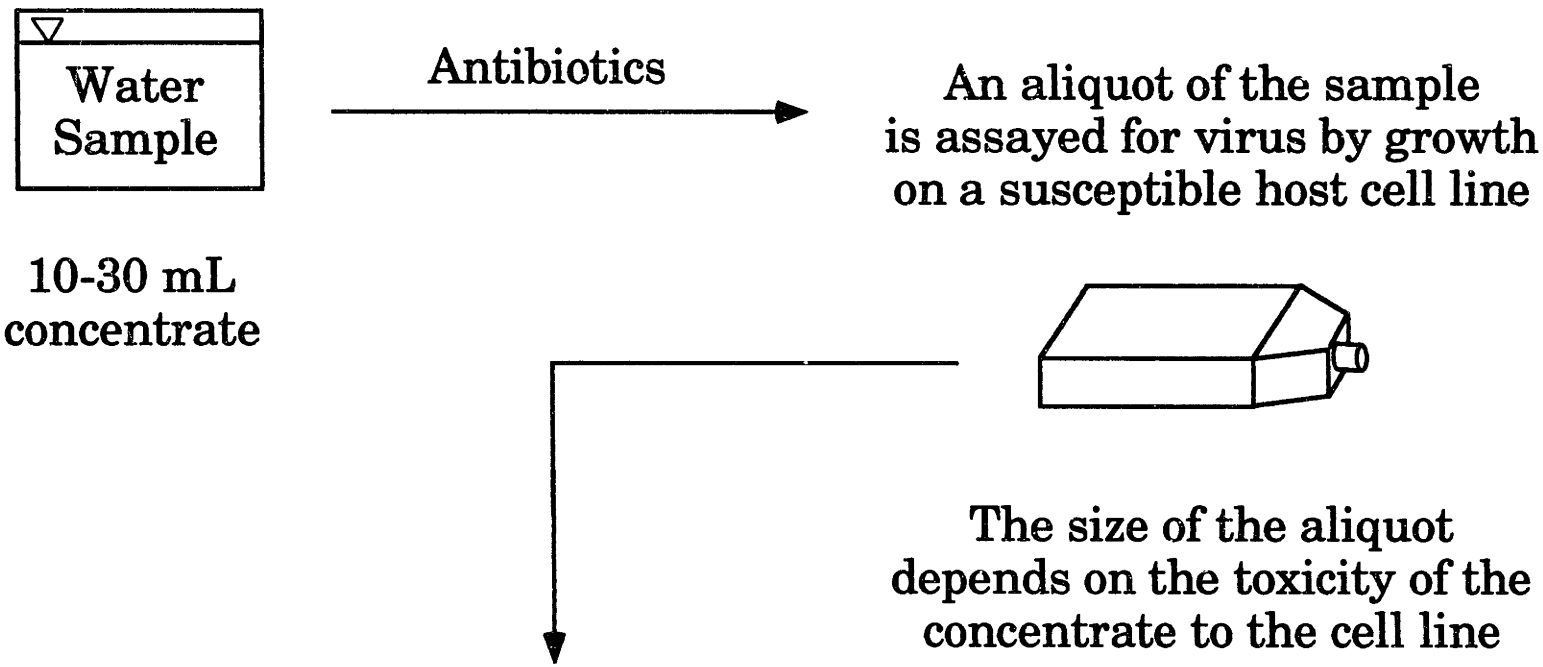
The most common methods of direct viral detection involve culturing pathogens on tissue cultures. First, a sample is concentrated using a method described in the previous chapter. The concentrates are treated with a variety of antibiotics to kill bacteria and other microorganisms that would obscure the growth of the viruses. The cell culture of viruses is accomplished by plating the concentrated samples onto cell lines on which the virus can

replicate. There are two common methods of cell culture. First, a monolayer of cells, usually human or other primate cells, is inoculated with the concentrate or some portion of the concentrate and incubated. Then, the samples are examined with a microscope for localized areas of infection, or a plaque, on the monolayer. This procedure is referred to as a plaque assay and the results are given in plaque forming units (PFU). One PFU is the amount of virus necessary to form 1 plaque on a monolayer of a particular cell line. The amount of virus in 1 PFU is not generally known, but it has been determined by electron microscopy for poliovirus 1 to be 100 virus particles (Gerba, *et al.*, 1989). It should be noted that this result was obtained using pure cultures of poliovirus and not poliovirus in environmental concentrates. The effect of the environmental concentrate, particularly a non-drinking water concentrate, on the number of viral particles per PFU is not known.

The second method used to quantify virus in cell culture is cytopathic effect (CPE), usually measured in units of tissue culture infectious dose, $TCID_{50}$. Briefly, a multi-well plate is used to grow host cells, and each well is inoculated with sample. If the cells in a well exhibit a cytopathic effect, then a positive result is recorded. One $TCID_{50}$ is the amount of virus necessary to cause a cytopathic effect in 50% of inoculated cultures.

The PFU and $TCID_{50}$ depend on the type of sample used, the sensitivity of the host cells to the virus, and the particular virus. The virus particle concentration in 1 $TCID_{50}$ is considered to be more than 1 PFU ($TCID_{50}$ > PFU > Viral Particles). The number of viruses needed for infection in humans, the minimum infective dose (MID), is not known. Thus, the significance of 1 PFU or 1 $TCID_{50}$ is not known. Although these methods are limited, they provide the only available means to quantify infectious virus.

Figure 9: Cell Culture Technology



For a full discussion of MID, see Chapter 2.1.2. Figure 9 gives a pictorial view of this procedure.

In addition to cell lines, other hosts are available for culturing viruses: embryonic eggs, animal models, and humans. These methods are not and cannot be used for regular monitoring.

4.2.2 Benefits of Cell Culture Detection

There are several benefits to the use of cell culture. Most importantly, a single tissue infective unit can be detected if the particular virus can grow in cell line in the presence of the concentrate. This is the least amount of virus that can be shown to be infective by traditional detection schemes (i.e., without attempting to infect a human). Thus, at least with regard to available detection schemes, cell culture is the only technique that can be used to show infectivity (though not human infectivity or human disease). Although this technique requires highly skilled technicians and a well equipped laboratory, the cell culture methodology is well defined and thoroughly tested. Cell culture is the standard methodology proposed by the EPA for use in an upcoming nationwide survey of viruses in drinking water called for by the monitoring requirements set forth in recent changes in the Safe Drinking Water Act (EPA, 1993).

4.2.3 Problems with Cell Culture Detection

One troublesome issue concerns the use of cell culture to detect wild-type viruses in environmental samples. Most studies involving the detection of pathogens in environmental samples use tissue-adapted strains of viruses on which wild-type strains may not be detected as easily. It is known that tissue culture-adapted strains often lose the ability to infect humans; the reverse of this situation may also occur. If this is the case, then the current cell lines used to detect viruses may not be suitable for detection of some wild-type strains of virus that are currently considered detectable.

A second problem is that cell culture is limited to 1 PFU if no toxicity exists in the concentrated sample. Because 1 PFU of a virus can contain 1 to 1000 viral particles (or more), fewer viral particles than are in 1 PFU may cause infection. The number of viral particles in 1 PFU has been shown to be dependent on the sensitivity of the cell line used and the type of virus (Ward & Akin, 1984). That is, the amount of virus in 1 PFU in one cell line could be 100 PFU in a more sensitive cell line. Furthermore, the effect of toxicity from concentrated samples on the number of viral particles needed for infection has not been discussed in the literature. Also, the effect of the toxicity of concentrated water samples is a major problem even in drinking water samples resulting in only a small aliquot of the sample being tested. Multiple passages of the sample thorough cell culture must be made in order to ensure against false negatives especially in the case of non-potable environmental samples (Gerba & Goyal, 1982). Chromatography columns, polyethylene glycol extractions, and various precipitations have all been used to reduce toxicity, but each step results in additional loss of virus and results have been variable as to their usefulness.

A third problem is that cell culture cannot detect nonculturable organisms. Epidemiologically speaking, the most important viruses do not grow in cell culture (e.g., Norwalk virus) and many viruses grow slowly even on special cell lines (e.g., rotavirus on MA104 cell line). As was described in Chapter 2, Norwalk virus, astrovirus, and many of the newly discovered "novel viruses" will not grow in cell culture or in other primates (Bridger, 1987; CIBA, 1987). The only source of virus and gauge of infectivity is a human being. Thus, in terms of providing safeguards to human exposure to pathogens in water, cell culture cannot be used to detect the most likely actors.

Moreover, many viruses and bacteria which commonly grow in cell culture can become viable but non-culturable. That is, many microorganisms can enter a viable (and infective) state, but lose the ability to be detected by means of traditional culture methods (Byrd, *et al.*, 1991). These pathogens would obviously by overlooked by culturing pathogens.

Another significant problem is that no single cell line can be used to detect all viruses. As an example, rotavirus grows (albeit slowly) on MA104 cells while enteroviruses grow on BGM cells. Neither will grow on the other cell line. This shortcoming is a major drawback to the use of this methodology. The maintenance of many cell types would be necessary in order to monitor all the different pathogens that may be present. This criterion is clearly too onerous for a monitoring system. The easiest viruses to grow are the enteroviruses (poliovirus, echovirus, coxsackievirus) which are not considered to be significant pathogens in terms of the number of people affected and the extent of the disease. Therefore, cell culture does not provide an adequate means of monitoring the quality of water, although it may be useful in

monitoring enteroviruses which could act as indicator organisms for pathogens which are more epidemiologically important. On the other hand, if enterovirus are to be used as indicators, bacteriophage provide about as much information and are easier and cheaper to use.

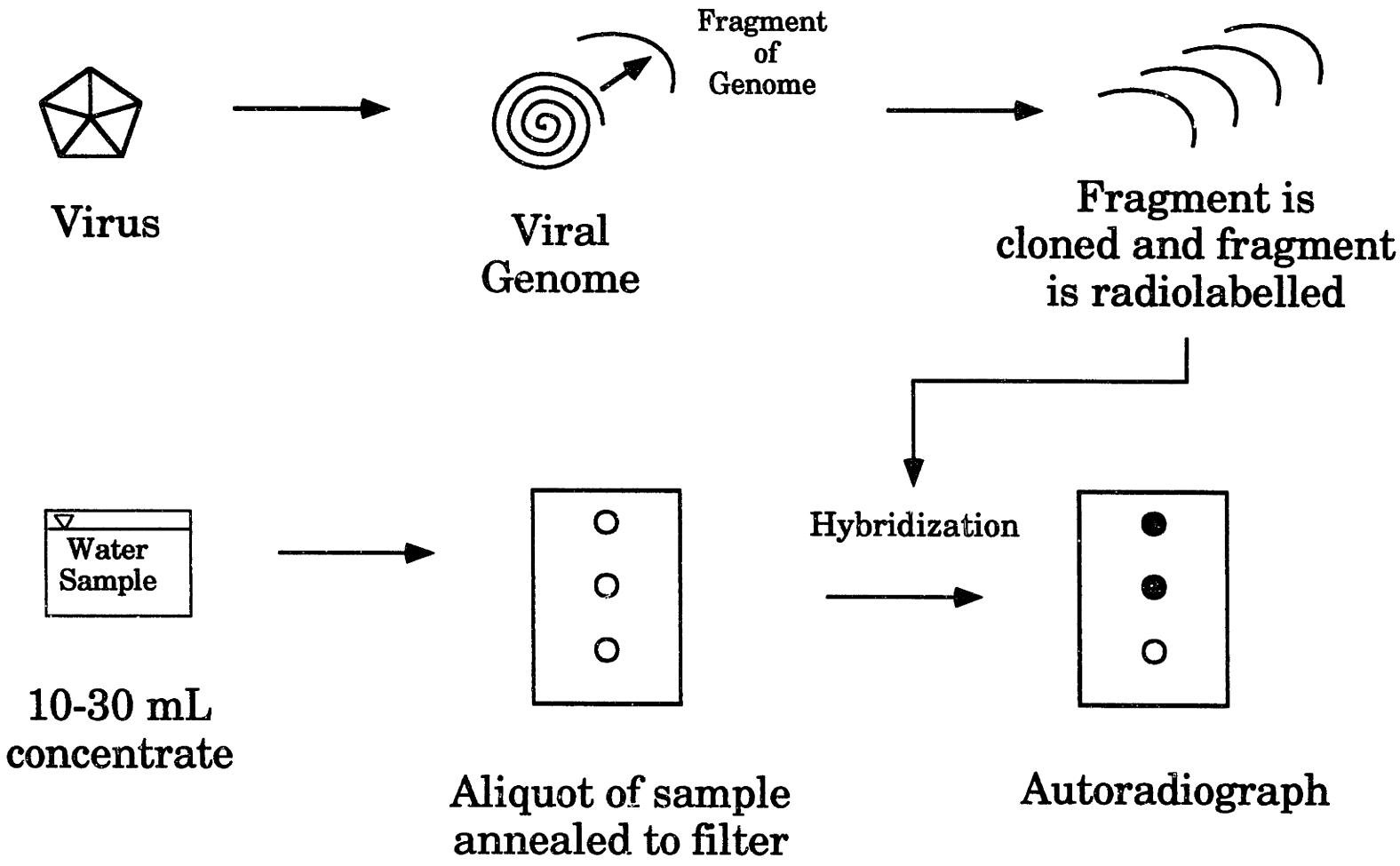
Finally, culturing pathogens is dangerous. In order to grow pathogens to detectable levels, a laboratory must be capable of dealing with concentrated pathogens. This method is expensive and slow, and is not suitable for routine testing.

4.3 Gene Probe Technology

4.3.1 Defined and Described

Gene probe technology uses a methodology that is almost identical to a dot blot hybridization. The nucleic acid in a sample is stuck to a filter and probed using a radioactive DNA fragment obtained from a gene of the target organism. In order to make a gene probe, a large conserved segment of a target organism's genome is inserted into a plasmid, and the plasmid is placed inside a host organism so that as the organism grows the number of copies of the plasmid increases. The plasmid is purified from the host organism, then the gene probe is cut out of the plasmid and labeled with radionuclides. Gene probes can also be made by using PCR to amplify the genome fragment to be used as the probe, and radioactivity can be incorporated as a part of the PCR process. This fragment is then used as a hybridization probe on a concentrated sample. Briefly, the concentrated sample is chemically extracted and a portion of the sample is denatured and

Figure 9: Cell Culture Technology



baked onto a nitrocellulose filter. The radioactive fragment is placed in solution around the nucleic acid on the filter. After 72 hours, the filter is rinsed, and if hybridization between the probe and sample has occurred, then a positive result is achieved. Figure 10 gives a pictorial view of the gene probe procedure.

4.3.2 Benefits of Gene Probe Detection

The major benefits of gene probe technology are in comparison with cell culture technology. It is relatively inexpensive; Gerba estimates about $50 per sample (Gerba, *et al.*, 1990) It is much faster and easier to use than cell culture because pathogens do not need to be cultured.

Gene probe technology has good sensitivity when the probe is highly radioactive. Highly radioactive probes are available that can detect as little as 1 PFU (100 copies) of poliovirus. These probes are made using a cloning process in which the probe is not cut out of the cloning vector, and both probe and cloning vector are labeled. This causes the probe to be very radioactive, but it also increases non-target hybridizations as the entire cloning vector also acts as a probe (Gerba, *et al.*, 1989).

In a study involving naturally contaminated drinking water, Margolin *et al.* used this highly radioactive probe to detect 2.6×10^3 PFU/L of poliovirus in adsorption-elution concentrated drinking water (remember that 1 PFU of poliovirus is 100 viral particles). On the other hand, gene probes failed to detect 80 PFU/L in the same experiment (Margolin, *et al.*, 1991; Margolin, *et al.*, 1989). The number of viruses present was also measured in cell culture,

but no recovery efficiency was determined so that the original contamination level in the water could not be calculated. In general, this level of sensitivity is not attainable, as will be described in the next section. Furthermore, it should be noted that the labeled cloning vector increases the background and the chance of false positives, especially in environmental samples.

4.3.3 Problems with Gene Probe Detection

There is a high threshold value for the absolute number of organisms needed for detection. Several investigators have reported limits of detection or threshold values for detection (see Table 10). These thresholds are the absolute number of copies of a gene necessary to be detectable with this technology, and they are not directly a function of the concentration of the organisms. From Table 10, it can be seen that 10^4 or more organisms are usually needed for detection. For environmental samples, these limits would be affected by the concentration of the organisms as compared to other non-target nucleic acids, as well as interference from other material that may be present in a sample. These limits hinder the use of gene probe technology significantly. Waters would either have to be highly contaminated, or very large volumes of water would have to be filtered, in order to detect this level of pathogens in a sample. Furthermore, only a single pathogen can be assayed at one time. Gene probes allow for the detection of families of organisms containing the same gene, but not for widely different organisms. Once a sample is consumed by gene probe analysis, no further testing can be performed. No product is formed in the assay as there is for cell culture

Table 10: Threshold Detection Limits for Gene Probes

Citation	Organism	Threshold
(De Leon & Gerba, 1991)	rotavirus SA11	2.5×10^4 particles
	hepatitis A	10^4 particles
	coxsackievirus B3	500-1000 PFU
(Dubrou, *et al.*, 1991)	poliovirus 1	10^2 RIFA
	poliovirus 3	10^3 RIFA
	coxsackievirus 6	10^4 RIFA
	echovirus 7	10^4 RIFA
(Margolin, *et al.*, 1991)	poliovirus 1	10^2 PFU
(Knight, *et al.*, 1991)*	salmonella	10^6 cells
	salmonella	3×10^5 cells/L
(Enriquez, *et al.*, 1993)**	poliovirus 1	10^5 PFU/L
(Tougianidou & Botzenhart, 1991)***	poliovirus 1	4×10^2 PFU/L

* in brackish water using a 0.22 µm filter.
** in well water and PBS.
*** in (-) adsorption-elution concentrated drinking water.

(living microorganisms) and PCR (amplified product) from which additional information can be drawn.

Although low levels of pathogens (100 virus particles) have been detected using highly radioactive gene probes, these probes have not been developed for other pathogens. More importantly, the use of highly radioactive material provides many other problems in the use of the methodology to regularly monitor a wide variety of water sources.

Other problems encountered with gene probes are that they require 72 hours for results, and there is no ability to detect multiple pathogens in one sample. This is a lengthy time scale, considering fecal coliform may indicate multiple pathogens and testing takes only one day. A gene probe test indicating the absence of one pathogen does not provide much of a safeguard. Gene probes would prove more effective in detecting pathogens in drinking water samples after an outbreak has occurred and the pathogen responsible is known.

In terms of experimental evidence for the usefulness of this technology, no one has detected low numbers of organisms by gene probe in reconstruction experiments. As described in the previous section, moderate numbers of poliovirus 1 have been detected in drinking water, but no recovery efficiencies were obtained. Thus, it is impossible to determine the original concentration of the pathogen.

Gene probes are sensitive to background interference and masking of positives, especially as a function of water quality. Gene probes have been found to be 100 times less sensitive when used on non-drinking water

environmental samples (Barkay & Sayler, 1988). Additionally, quantification of the concentration of pathogens in a sample is often difficult to determine for environmental samples (Martinez & Baquero, 1990). Finally, if a positive result is achieved with a gene probe, confirmation of the positive result is not possible. Once the test is run, there is no way to determine if non-target binding has occurred, and no nucleic acid can be recovered for further testing. With cell culture, more virus is available and with PCR, an amplified fragment is obtained.

Ultimately, the high threshold of detection for gene probes sets a severe limitation on their usefulness in detecting very low levels of organisms, unless very large volumes of water are filtered. In addition, this technology has not been proven to detect levels of virus that could be expected in the environment. Although viruses have been detected in adsorption-elution concentrated drinking water samples, no one has spiked low levels of organisms into large volumes of water and detected them.

4.4 Polymerase Chain Reaction

4.4.1 Defined and Described

The polymerase chain reaction (PCR) is a molecular biological tool with which a small amount of template DNA can be amplified enzymatically to quantities that are easily detected. Reverse transcriptase-polymerase chain reaction (RT-PCR) is a similar technique whereby a small number of copies of a specific RNA sequence can be changed into DNA enzymatically and then amplified by normal PCR to an easily detectable level. Most clinical and

environmental PCR is carried out using the thermostable DNA polymerase, *Taq* polymerase (Perkin-Elmer). A polymerase polymerizes individual bases (A, G, C, and T) into the DNA molecule. *Taq* enzyme is a tightly bound, thermostable protein recovered from the hot springs microorganism *Thermus aquaticus*. Traditionally, RT-PCR uses a thermolabile reverse transcriptase from Moloney murine leukemia virus (MMLV) or avian myelobastosis virus (AMV), followed by *Taq* polymerase amplification. In this research, RT-PCR uses a thermostable enzyme, *TetZ* (Amersham), which has two activities: reverse transcriptase and DNA polymerase. A reverse transcriptase uses an RNA template to make a complementary DNA template. *TetZ* enzyme is purified from another hot spring organism, *Thermus thermophilus.*

In RT-PCR, the RNA is specifically "reverse transcribed" by the RT enzyme into DNA in an initial incubation primed by short strands of DNA, called primers, which have been designed to be complementary to a region on the target organism's genome. Now all the template is in the form of DNA. PCR is a three step heat-cooling process: 1) DNA is heated to 94°C in order to separate, or melt, the double stranded molecule into two strands, 2) the DNA is cooled to a temperature (the annealing temperature, which varies based on primer sequence) at which the complementary primer sequences anneal to the template DNA, and 3) the temperature is raised to 72°C (which is the optimum temperature for the activity of the thermostable DNA polymerases) and each primer is extended. At the end of this three step process, there is about twice as much template DNA as was originally present. This process is repeated for a number of cycles in order to amplify the nucleic acid. For longer templates, the first and third steps should be longer, and for longer primers, the second step should be lengthened. At the

Figure 11: Reverse Transcription

The RT process begins by heating the reaction mixture to around 60°C (1) at which temperature the specific complementary primer sequence can anneal (2). Then, the RT enzyme can add DNA nucleotides (dNTP's) in a 5' to 3' direction that are complementary to the RNA template (3). This process is followed by normal PCR (Figure 12).

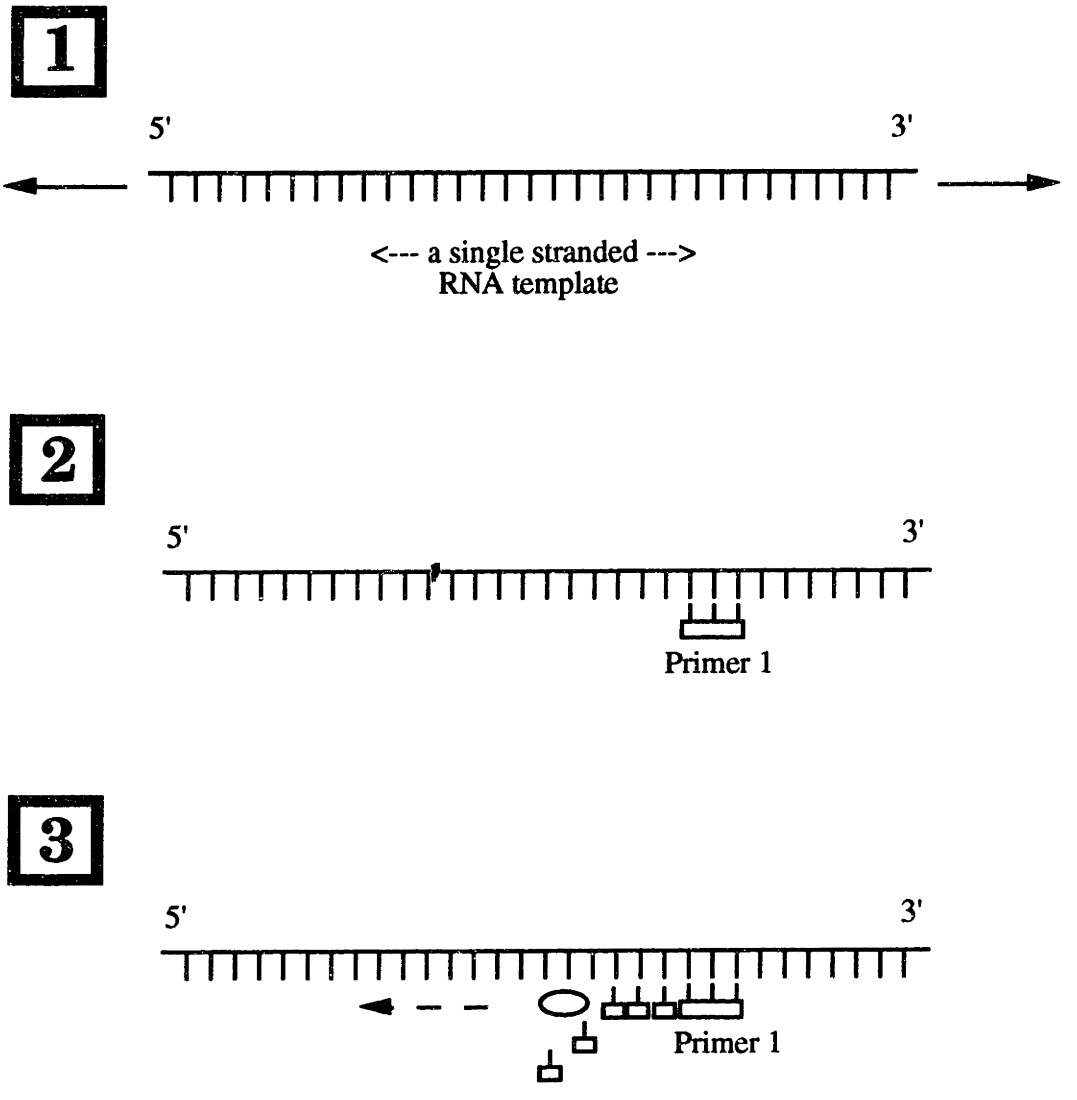
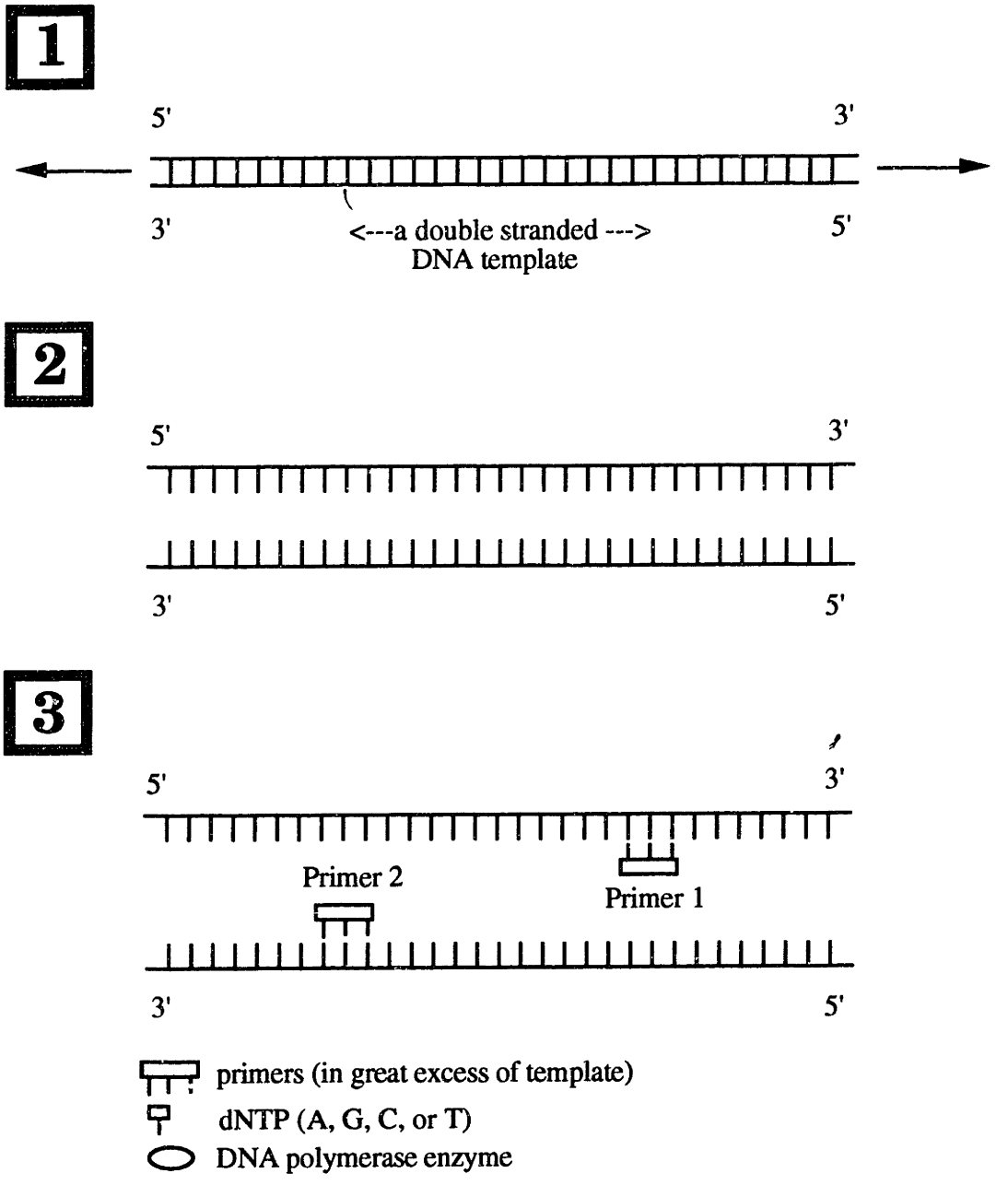
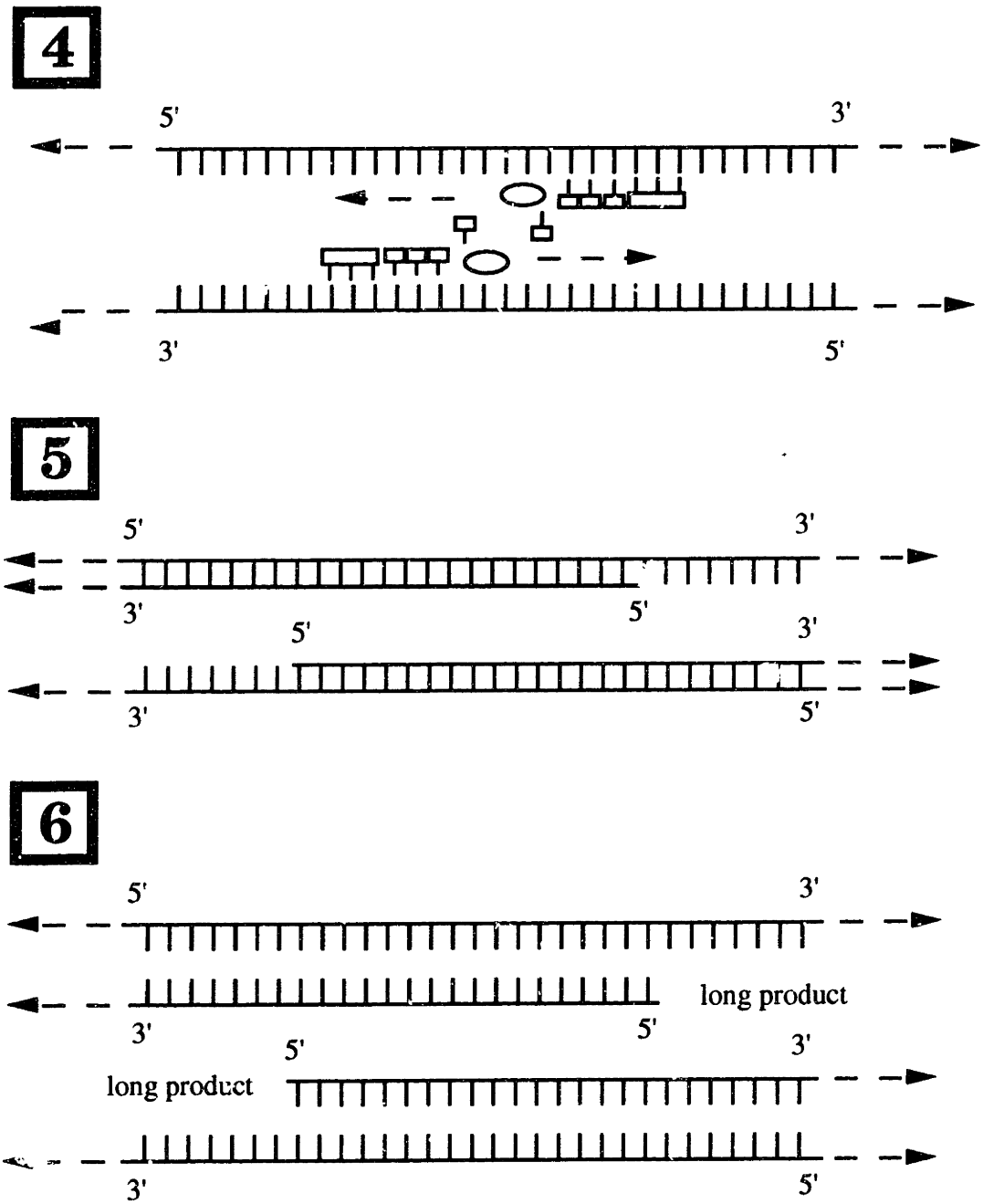


Figure 12: Polymerase Chain Reaction

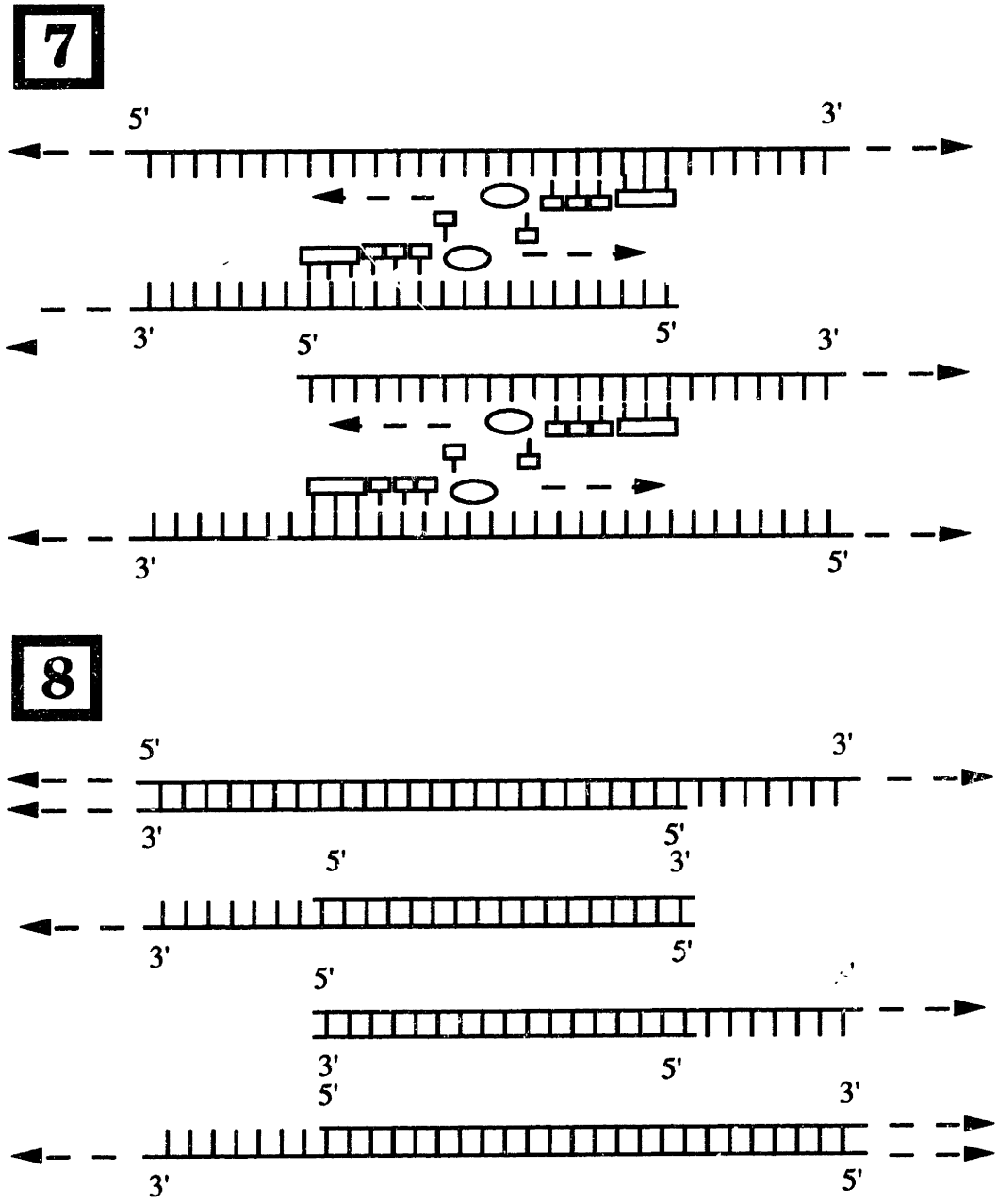
PCR can be carried out with either single or double stranded DNA. After the first round of PCR, ssDNA becomes dsDNA. A dsDNA template (1) is heated to 94°C (2). At this temperature DNA is denatured; that is, it separates from its double stranded, double helix form into two single complementary strands. Next, the reaction mixture is cooled to the annealing temperature (3); this temperature is highly dependent on the primers used. At this step the primers preferentially anneal to a complementary region on the template DNA.



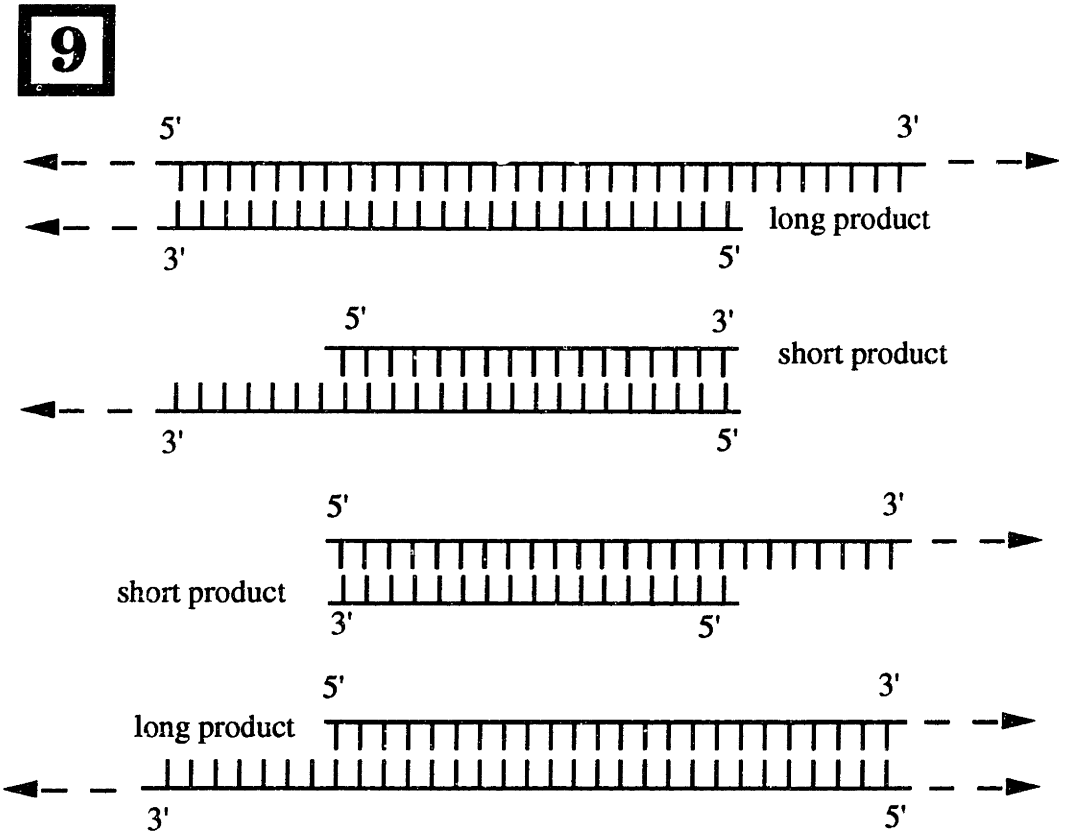
Finally, the reaction mixture is heated to about 72° C. At this temperature, the DNA polymerase enzyme is active, and DNA extension begins (4). At the end of the first cycle two stands of DNA are formed, each with one definite end. All of this product will be 'long product', because there is nothing to stop the DNA polymerase enzyme from replicating the template past the second primer position on the complimentary strand (5). At the beginning of the second cycle, the temperature is raised and the DNA strands are again denatured (6).



During the second round of amplification, the primers anneal both to the original template and the newly-made fragment, and extension occurs (7). In this instance, however, the extension can only proceed until the end of the new fragment is reached, resulting in short product formation. Thus, the fragment formed from the long product will be of a specific, finite length. The template, on the other hand, continues to produce long product (8).



As the next round of amplification occurs, both long and short product are present (9). The long product is produced arithmetically and will not be detected. The short product is amplified geometrically and can be detected easily after a number of cycles.



end of the PCR process, the samples are placed at 72°C for 10 minutes to allow any further extension to occur. These procedures are shown schematically in Figure 11 and Figure 12.

"Double PCR" was also used in this research. Double PCR refers to an initial round of PCR as described above, followed by a second round of PCR using nested primers in order to amplify nucleic acids in "gunky" samples where PCR efficiency is reduced. Nested primers are primers that are complementary to an internal region of the fragment amplified by the first primer set.

4.4.2 Benefits of Polymerase Chain Reaction Detection

PCR technology has associated with it many benefits; in fact, PCR is listed as an "ideal diagnostic technique" for viral detection in clinical samples (CDC, 1990a). Because all living things contain specific DNA or RNA molecules, the number of viruses that can be assayed in any given sample is limited only by the number of pathogen-specific primers that are available. Also, by choosing primers that produce different length fragments, the amplified pathogen DNA/RNA can be differentiated on the basis of length and visualized, without radioactivity, using a polyacrylamide gel electrophoresis (PAGE). In other words, each pathogen of interest will have associated with it primers which are specific to that pathogen and which amplify fragments that differ in length from other amplified fragments, and these fragments can be identified quickly and easily.

PCR requires about 4 hours to perform and is relatively inexpensive; it is much faster and easier to use than cell culture (weeks) and gene probes (3 days). PCR is also much more sensitive than cell culture or gene probes, which is shown in a comparative experiment in Table 11. In this experiment, a dilution series from high to low concentration of three organisms was performed, and an attempt was made to detect organisms in as large a dilution as possible. As can be seen in the table, RT-PCR was found to be 10^5 to 10^7 times more sensitive than gene probes, and 10 to 1000 times more sensitive than cell culture (Kopecka, *et al.*, 1993). PCR also has a greater specificity in detection than gene probes (Figueroa & Rasheed, 1991) because it not only relies on annealment of a specific primer sequence, but because the amplification product must be of a specific length.

In this research, as little as 1-10 copies of a DNA or RNA target nucleic acid sequence can be detected by PCR (see Chapter 5). Other researchers have also observed a PCR signal obtained from amplification of as few as 1 *E. coli* cell. This group has also multiplexed *Legionella* species proving that multiple pathogens can be detected and differentiated in a single sample (Bej, *et al.*, 1991a; Mahbubani, *et al.*, 1990). Unfortunately, these results were not achieved with environmental samples, or the sample size was small, 100 mL. PCR does not require radioactivity to achieve this level of sensitivity, nor does it require the growth of an organism.

Table 11: A Comparison of PCR, Gene Probes, and Cell Culture

Organism	Method	Amount of Sample Used*	Limit of Detection
			$TCID_{50}$
poliovirus 3	gene probe	25%	10^4
coxsackievirus 5			10^5
echovirus 7			10^6
poliovirus 3	cell culture	50%	1 to 10
coxsackievirus 5			1 to 10
echovirus 7			1 to 10
poliovirus 3	PCR	5%	0.1 to 0.01
coxsackievirus 5			0.1 to 0.01
echovirus 7			0.1 to 0.01

* Initial volume is 200 µL of mineral water

note: concentrated environmental samples did not PCR (e.g., 1000 $TCID_{50}$ spiked into 10 L of river water and concentrated by (+MDS) adsorption-elution gave no result).

(Kopecka, *et al.*, 1993)

Table 12: Problems with Current Methodologies

Method	Amount of Sample Used	Recovery
PCR	0.15 L	2 of 10 were +
Cell Culture	50%	2 of 10 were +
Gene Probe	3 L	1 of 10 was +

note: Ten 378-576 liter samples of groundwater were filtered through a (+) adsorption-elution filter followed by a Sephadex column. Then, they were assayed for enterovirus.
Abbaszadegan, *et al.*, 1993

Because PCR provides an amplified DNA fragment as a result of the assay, additional tests can be performed using this fragment. As an example, group-specific primers could be used to amplify related organisms or organisms with a common gene (e.g., all human rotaviruses or all enteroviruses). A second round of PCR using nested primers could be performed to amplify strain-specific sequences (e.g., different rotavirus strains) or virus type-specific sequences (e.g., a specific enterovirus). Thus, PCR provides a powerful means for monitoring groups of organisms, while also providing detailed information about the individual characteristics of the detected pathogens. Additionally, nested primers, Southern blot, and sequencing can be used to confirm a positive result.

4.4.3 Problems with Polymerase Chain Reaction Detection

In the past, some problems have been encountered in the use of PCR technology, but are overcome in this research (see Chapter 5). Other researchers have used adsorption-elution technology in conjunction with PCR, but have only been able to use a small volume of the concentrate in a PCR assay due to inhibition of amplification (see Table 12). In these experiments, no determination of recovery efficiency was made, and only a tiny portion of the concentrate could be used for PCR and for gene probe assay due to inhibition. From research presented here, RMU concentrates appear to be less susceptible to inhibition, perhaps because humic material and other small negatively charged particles pass through the RMU filter but are electrostatically sorbed to the adsorption-elution filters. In any event, for typical environmental samples, even those that appear to be free of

suspended solids and color, upon concentration become inhibitory. Clean-up of the sample to avoid inhibition of PCR is critical.

RMU technology used in conjunction with PCR has also been attempted (Tsai, *et al.*, 1993). Again, only a small volume of the concentrate was used in a PCR assay (2% of a 15 L sewage effluent/ocean water sample and 2% of a 100 mL sewage sample). In this research no extractions were performed and the sample was amplified directly; these samples must have been relatively free of solids and inhibitory substances. Furthermore, no low copy number spikes into these environmental samples were performed and no recovery efficiency was determined. As the only positives obtained in this research were at a wastewater effluent site, the level of virus in the water was probably high.

PCR can be inhibited by environmental contaminants, and the variation amongst water samples makes it impossible to ensure that all water samples will be amenable to clean-up. Thus, positive controls must be incorporated into the test. This is not an onerous task, as a "positive control" organism can be spiked into a sample of interest, concentrated in the sample, and detected along with the target organisms. The phi-X174 and MS2 viruses used in this research would be ideal for this task.

A limitation of PCR is that it detects viable, viable/non-culturable, and non-viable organisms, so that infectivity can not be determined. Recent papers, however, suggest that viability can be detected (Bej, *et al.*, 1991b). Moreover, Josephson, *et al.* found that environmental samples show rapid degradation of non-viable organisms (i.e., on the order of 2 days) (Josephson, *et al.*, 1993). Free nucleic acids are taken up relatively quickly in both

eutrophic and oligotrophic waters, and thus the nucleic acid from a damaged virus would probably disappear within 24 hours (Paul, *et al.*, 1989; Paul, *et al.*, 1987). These studies suggest non-infective "dead" pathogens will probably be destroyed relatively quickly in the environment. If the goal of the detection method is to safeguard public health, then viability is of lesser importance because the detection of a pathogen indicates recent pathogenic contamination. In this case, one would usually prefer to be conservative when making decisions regarding the possible pollution of a water body, so that PCR would not provide too sensitive a method of detection. Likewise, if the detection method is used in an epidemiological study of a pathogen after an outbreak, a more sensitive test would have a better chance of tracing the pathogen, which may be dying off.

Inhibition of PCR has been a major problem in the use of PCR technology on environmental samples. Inhibition has been described in the literature primarily for PCR on clinical specimens, namely feces, saliva, urine, blood, *etc.* The inhibiting substance is rarely isolated, but methods to remove inhibition have been noted (Ochert, *et al.*, 1994; Van Zwet, *et al.*, 1994). These methods include exclusion chromatography, affinity chromatography, and chemical extraction. Because large numbers of pathogens are usually present in clinical specimens, recovery efficiency is not a primary concern. Thus, it is not known if low numbers of organisms expected in environmental samples can be recovered using these methods.

Only a handful of papers have been published on PCR inhibition due to material in soils and sludges. In general, there is a paucity of reports on inhibition of PCR, perhaps because research is often published only after PCR inhibition has been overcome. In this research, the secondary

concentration steps to remove inhibitory substances were found to be crucial to providing a PCR amenable sample. In a review of environmental PCR (Steffan & Atlas, 1991), inhibition is mentioned; however, little is discussed concerning the clean-up of samples from various sources and the difficulties that have been reported by a number of researchers (Abbaszadegan, *et al.*, 1993; Straub, *et al.*, 1994; Tsai & Olson, 1992; Young, *et al.*, 1993). In these studies, humic material, metals (e.g., iron), magnesium scavengers, and other unknown inhibitors were postulated to be responsible for inhibition of PCR. Again, several methodologies, usually involving chromatography or extraction, were used to reduce or eliminate the inhibition, but no attempts were made to isolate target organisms from environmental samples containing a known concentration of pathogens. Usually, a sludge-amended sample was used, where the pathogen concentration was probably high, much as in the case of clinical samples. Another common scenario used samples that were spiked after concentration in order to test a method for removing inhibitors, but not for efficiently recovering pathogens. In either case, the recovery efficiency of the process was not important, because a large number of target organisms were present so that simply diluting the sample or using a small portion of the sample allowed amplification. In other words, the inhibitor was diluted out of the sample and the target organism had to be present at a higher concentration than the inhibitor. These methods do not provide an acceptable means for monitoring water sources or even for epidemiological studies because only a tiny portion of the sample can be assayed (see Table 12). Thus, very large samples must be collected and the samples must be heavily contaminated.

5. Experiments and Results

5.1 Description of Methodology

Rotating membrane ultrafiltration and the polymerase chain reaction were used to concentrate and detect two model viruses in environmental samples. General details concerning these technologies are given in Chapters 3 and 4, respectively. In initial experiments, RMU technology was used in reconstruction experiments using phi-X174 and MS2 viruses in order to determine the recovery efficiency from the RMU concentration process in buffered solutions, as well as environmental water samples. A secondary concentration procedure using filtration and extraction was developed. This process was found to be crucial to providing a sample that was amenable to PCR. Next, these viruses, as well as *Helicobacter pylori*, a bacterium, were used in reconstruction experiments to determine the recovery efficiency of this secondary concentration process.

Primer sequences for the viruses of interest were developed to amplify a specific portion of the phi-X174 (DNA) and MS2 (RNA) genomes using PCR and RT-PCR, respectively. *Helicobacter pylori* primer sequences were obtained from the literature. The sensitivity of detection for the model viruses and bacterium was assessed. Finally, a low concentration of the model viruses was spiked into large volume environmental samples, which were then concentrated by RMU, followed by a secondary filtration/extraction process, then detected by PCR.

5.2 Materials

5.2.1 General Reagents

General reagents used in this research are described in Appendix II.

5.2.2 Sampling Sites

Water samples were collected from the Charles River (CRW) at a single sampling site located east of the Harvard Bridge in Cambridge, MA. Cambridge drinking water (CDW), after treatment by alum coagulation and filtration, was collected from laboratory taps located in the laboratories of Drs. William Thilly and James Fox. CDW samples were dechlorinated using sodium thiosulfate according to *Standard Methods for the Examination of Water and Wastewater* (APHA, 1989). Source water was collected as untreated water from the Wachusett Reservoir (WRW) at the dam near Clinton, MA. All samples were stored at 4°C until they were processed.

5.2.3 Concentration and Detection Methodologies

5.2.3.1 RMU

In this research, a Benchmark GX RMU unit (Membrex, Fairfield, NJ) using a 400 cm^2, 100 kDa UltraFilic filter was employed. The UltraFilic membrane, a proprietary product, is composed of a polyacrylonitrile matrix that has been chemically modified to maximize hydrophilicity. During

filtration, the system was operated at 2200 rpm and 40 psi, unless otherwise noted.

Samples were placed into a clean plastic container and spiked with the appropriate organisms. The RMU can be used in several set ups. The two set ups used in this research were a recirculation mode for rinsing the filter and a dead stop mode for filtration (see Figure 13 and Figure 14). A peristaltic pump (Cole-Parmer Instruments, Chicago, IL) was used to pump the sample into the RMU and to maintain pressure during filtration. After the entire sample passed through the RMU unit, an additional 500 mL of TE buffer was passed through the filter in an effort to force any smaller molecular weight molecules that remained in the retentate through the filter. At this point, the volume between the cylinders was filled with the retentate, which should contain molecules greater than 100 kDa. The filtrate line was closed and the filter was then allowed to spin at 2200 rpm for 5 minutes in order to free any material from the filter surface. The filtrate line was opened, and the retentate volume was forced down to 35 mL at 10-20 psi and 1000 rpm, and then collected. The filter was then rinsed. In this process, a 200 mL volume of TE buffer was added to the RMU unit and spun at 2200 rpm and 10-20 psi for 5 minutes with the filtrate line closed. The filtrate line was then opened, and the rinse volume was forced down to 35 mL at 10-20 psi and 1000 rpm. This 'first rinse' was combined with the retentate, resulting in a final volume of 70 mL. This procedure, which was developed in this research, was used for all water sample sources and volumes unless otherwise noted.

Between uses, the filter was kept moist in 0.2% sodium azide. Before usage, it was rinsed in distilled water and placed into the RMU unit. The filter was further cleaned by flow through the unit with 2 L solution of

Figure 13: RMU Filtration Set-Up

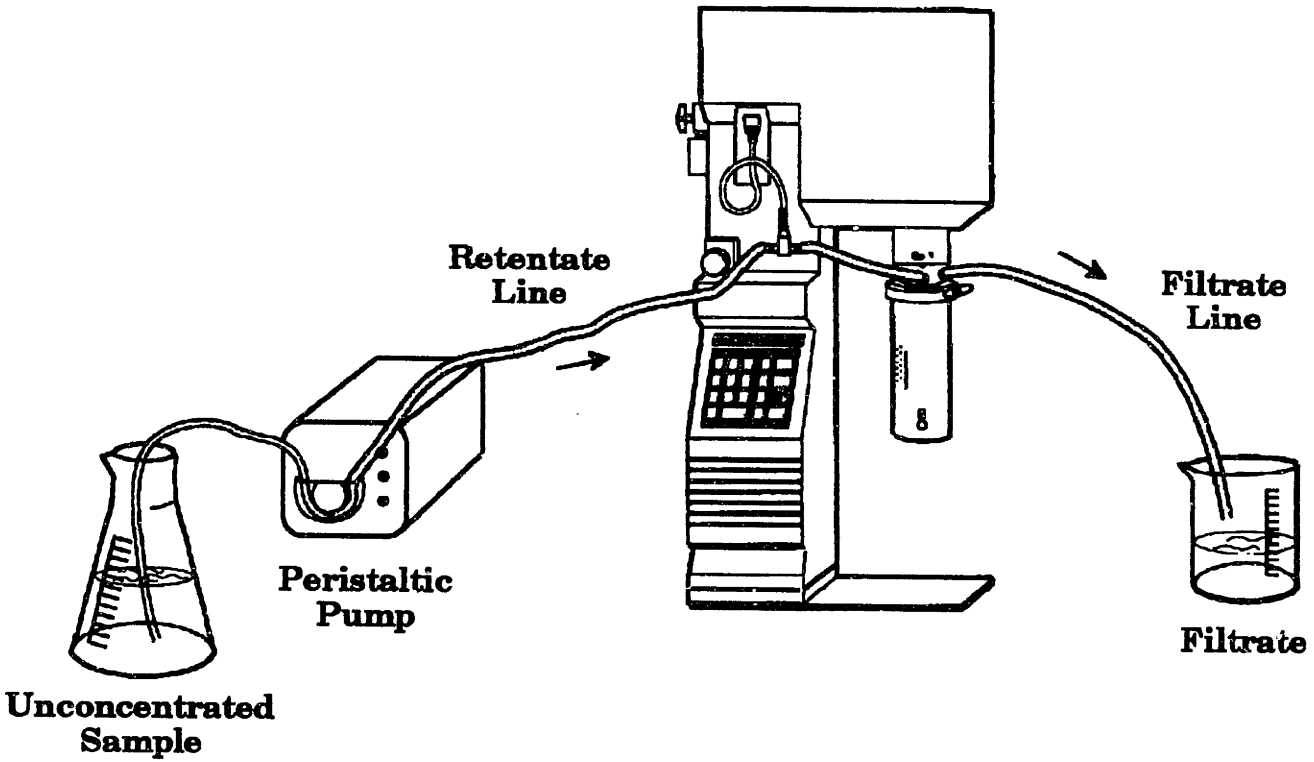
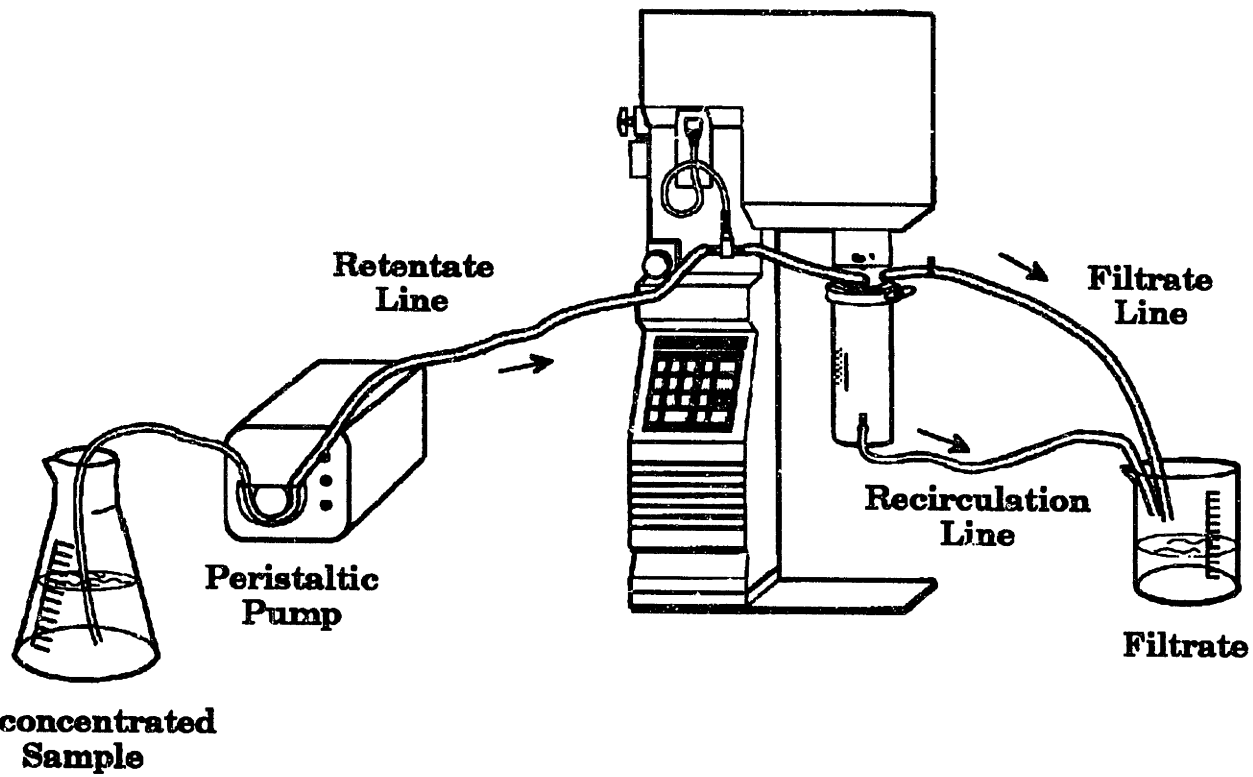


Figure 14: RMU Rinse Set-Up



sodium hydroxide (NaOH) pH=12.4 in distilled water for 5 minutes with both the recirculation line and filtrate line open, and again for 5 minutes with the filtrate line closed. This cleaning was followed by a similar flow through the filter of 4 L of distilled water. This regiment was also performed after each water sample was filtered in order to prevent cross contamination and to disinfect the RMU unit. Finally, the filter was cleaned before storage.

5.2.3.2 Secondary Concentration

In the secondary concentration procedures, the 70 mL sample was spin-concentrated at 500x g to 2-5 mL using a Centriprep (Amicon, Beverly, MA) 100 kDa filter that had been through a passivation process. Briefly, the filter was allowed to soak overnight in a 1% bovine serum albumin (BSA) solution in order to block sorption sites at the filter/filter holder junction. The discovery of the need for this process will be described in detail in Section 5.4. All Amicon filters are composed of a low adsorption YM membrane.

For samples containing RNA, 500 units of human placental RNasin was added to the sample, which was incubated at 37°C for 10 minutes. To the concentrated sample, sodium dodecylsulfate (SDS) was added to 0.5% final concentration followed by the addition of a proteinase K to 0.1 mg/mL. This mixture was incubated at 37°C for 30 minutes. SDS is a detergent that disrupts the cell membranes. Proteinase K is an enzyme that digests a wide variety of proteins. Then, sodium chloride (NaCl) was added to 0.7 M final concentration after which hexadecyl trimethyl ammonium bromide (CTAB) was added to 1% final concentration. This mixture was incubated at 65°C for

10 minutes. The solution must be above 0.5 M NaCl because CTAB binds nucleic acid at lower ionic strength. At high ionic strength, CTAB binds polysaccharides so they can be removed from solution. An equal volume of 24:1 chloroform:isoamyl alcohol was added, mixed vigorously, and centrifuged at 10,000x g. The supernatant was collected. The interface was also collected in a 2 mL Eppendorf tube, vortexed, and re-spun at 14,000x g, after which the "interface" supernatant was combined with the original supernatant in order to maximize recovery. The pooled supernatants were then vigorously mixed with an equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol, and spun at 10,000x g. Phenol:chloroform extractions were performed until no material was present at the aqueous phase/solvent interface. For drinking water samples, only one extraction was necessary, while source water required three extractions. Phenol:chloroform extractions remove proteinaceous material and other hydrophobic substances. A final chloroform extraction was performed to remove any phenol. Phenol was found to degrade the filters used in the next step of the protocol. Because chloroform has low solubility in water, the Amicon filters showed no adverse effects from exposure to the extracted concentrate. Next, the sample was filtered through a Centricon (Amicon, Beverly, MA) 100 kDa filter to 200 μL . For drinking water samples, an additional filtration through a Microcon (Amicon, Beverly, MA) 100 kDa filter to 5-10 μL was performed. At each stage after filtration, the filter was rinsed in a small volume of TE buffer and the rinse was pooled with the rest of the retentate. An equal volume of distilled water was added to the final concentrated sample to dilute salts, and the sample was refiltered through the Microcon filter.

5.2.3.3 PCR

A Gene Machine II (U.S.A./Scientific Plastics, Ocala, FL) was used for all nucleic acid amplifications. All samples were heat spiked to 94°C. For RT-PCR, an initial incubation at 60°C for 30 minutes was followed by PCR. The PCR cycle used was as follows: 94°C for 1 minute, the annealing temperature for 1 minute, and 72°C for 1 minute for 40 cycles (any variation in methodology is noted in the particular experiment) followed by 72°C for 10 minutes and storage at 4°C. The amplified sample was visualized by polyacrylamide gel electrophoresis (PAGE).

5.2.4 Microorganisms

In this research, the bacteriophage phi-X174 and MS2 were used because they are easy to culture and their morphology is similar to that of the picornaviruses (see Figure 2). Phi-x174 phage (Microvirus type species) is an unenveloped, 27 nm, cubic virus that infects the Enterobacteria. This virus is composed of a protein coat encapsidating a 5386 base circular ssDNA strand. Phi-X174 has a molecular weight of $6x10^6$ (Frankel-Conrat, 1985; GenBank & EMBL., 1935). The phi-X174 used in this research was obtained from the American Type Culture Collection, ATCC 13706-B1. The *E. coli* used as a host for phi-X174 was obtained from New England Biolabs (strain HF 4717). MS2 phage (Levivirus type species) is an unenveloped, 23 nm, cubic virus that infects the Enterobacteria. It also consists of a protein capsid encasing a 3569 base +ssRNA strand, and has a 3.6×10^6 molecular weight (Hull, *et al.*, 1989). The MS2 phage and its host *E. coli* were both obtained from the

American Type Culture Collection, ATCC 15597-B1 and ATCC 15597, respectively.

Because size separation was used for the concentration of water samples, these viruses provide a limiting case as they are similar in size and shape to the smallest viruses. Furthermore, these model viruses were chosen to reduce the difficulty and hazard of using pathogens in the testing procedure. Because PCR was used for detection, no difference in protocol is required for the model viruses and pathogens, except that different primers would be used.

Viruses were prepared by an amended method suggested by Benbow *et al.* (Benbow, *et al.*, 1971). Briefly, 200 mL of tryptic soy broth (TSB) containing 1 mM calcium chloride was mixed with 2 mL of an overnight culture of host *E. coli* and 1 fresh plaque of the phage, and incubated for 3 hours at 37°C. Then, the mixture was spun at 10,000x g for 20 minutes and the pellet was recovered and dissolved into 10 mL of distilled water. Virus titer was usually between 10^8 to 10^{10} viruses/mL. Stock phage could be stored at 4°C for several months with little reduction in titer. For everyday usage, stock phage were diluted in TE buffer.

E. coli was prepared from a method suggested in *Standard Methods* (APHA, 1989). Bacteria were grown in a medium containing TSB and 10% w/v glycerol at pH=7.2 to 0.5 optical density at 520 nm. The bacteria were stored in 15 mL tubes at -20°C for up to 6 months. Methods for plating phage in recovery efficiency experiments were also derived from protocols described in *Standard Methods*. First, 5.5 mL of tryptic soy agar (TSA) at 48°C, 80 µL of 5% triphenyl tetrazolium chloride (TPTZ) in ethanol, 1 mL of stock *E. coli,*

and up to 5 mL of sample or a dilution of the sample were mixed in 50 mL tubes and poured into petri dishes. If samples had a volume of less than 5 mL, distilled water was used to bring the volume to 5 mL. The plates were incubated overnight at 37°C, and counted the following day. TPTZ is enzymatically converted from colorless to a dark red by *E. coli.* Hence, clear plaques, or areas where the E. coli had been lysed by phage, were counted in order to enumerate phage.

Helicobacter pylori was obtained from the laboratory of Dr. James Fox, and was kindly prepared by Ms. Nancy Taylor. The bacteria were counted using optical density at 660 nm and a standard curve. These bacteria were frozen at -20°C in the media in which they were grown. For everyday usage, dilution of *Helicobacter* in TE buffer were maintained.

5.2.5 Primer Sequences

Primer sequences are short strands of DNA that are complementary to the portions of the template fragment. The primers are the elements which allow the PCR process to amplify only specific templates. The specificity of nucleic acid amplification is dependent upon the primers annealing (ideally) only to the template DNA. The specificity of the primer sequences depends on proper selection from conserved, unique regions of the pathogens' genome and on the stringency of temperature and reagent conditions used during the amplification process. Once the primers have annealed, a new DNA strand can be generated, and through multiple cycles of annealing and extension, amplification occurs. Importantly, this amplified template has associated with it a fixed length which is known *a priori.* Therefore, it can be detected

by nucleic acid size separation by polyacrylamide gel electrophoresis (PAGE). Thus, not only must the primers be capable of annealing to complementary regions of the template nucleic acid, the amplification product must also be of the expected length (meaning the primers must have annealed in the correct orientation at the proper distance apart). This characteristic adds to the specificity of PCR over nucleic acid probes.

Hopefully, the number of mismatches between the primers and non-target environmental DNA and RNA will result in "other" nucleic acid 1) not being amplified, 2) being amplified with low efficiency so that it is not detected, or 3) providing a fragment that is a different length from the length of the expected fragment. After an initial number of cycles, the relationship between the amount of nucleic acid, PCR efficiency, and number of PCR cycles is given by the equation:

$$\text{Final \# of copies} = \text{Initial \# of copies} \times (1 + \text{efficiency})^{\text{\# of cycles}}$$

Thus, differences in PCR efficiency can dramatically affect the amount of product formation, even if the initial number of copies of two sequences are different.

As mentioned above, primer sequences for PCR must be chosen from conserved, unique portions of the target organism's genome. Two sets of 20 base primers were developed for both phi-X174 and MS2. From the two sets of primers developed, all four possible combinations allow proper amplification of the target sequences for each phage. The *Helicobacter pylori* primers were obtained from the literature (Ho, *et al.*, 1991). These primers were chosen from a portion of the genome coding for the 16S rRNA. All primers are shown in Table 13.

Table 13: Phi-X714, MS2, and Helicobacter Primers

Phi-X174 primers

Name	5'-3' Sequence	GC Content	Melting Temp
PA1	TGT CTC ATC ATG GAA GGC GC	55%	62°C
PA2	CAT TAC ATC ACT CCT TCC GC	50%	60°C
PB1	GCT CGT CGC TGC GTT GAG GC	70%	68°C
PB2	TGT TTC CTG CGC GTA CAC GC	60%	64°C

MS2 primers

Name	5'-3' Sequence	GC Content	Melting Temp
MSA1	GGA AAC CCG ATT CCC TCA GC	60%	64°C
MSA2	CGT CTA TTA GTA GAT GCC GG	60%	64°C
MSB1	CGC TAC GAA TTC CGA CTG CG	50%	60°C
MSB2	GTC ACT GTG CGG ATC ACC GC	65%	66°C

***Helicobacter pylori* primers**

Name	5'-3' Sequence	GC Content	Melting Temp
Hp1	CTG GAG AGA CTA AGC CCT CC	60%	64°C
U3*	CAG CAG CCG CGG TAA TAC	61.1%	58°C

* All primers are 20 bases long except U3 which is 18 bases.

Figure 15: Genes D and E of Phi-X174

1
GAGTCCGATG CTGTTCAACC ACTAATAGGT AAGAAATCAT GAGTCAAGTT

51
ACTGAACAAT CCGTACGTTT CCAGACCGCT TTGGCCTCTA TTAAGCTCAT

101
TCAGGCTTCT GCCGTTTTGG ATTTAACCGA AGATGATTTC GATTTTCTGA

151 PB1--->
CGAGTAACAA AGTTTGGATT GCTACTGACC GCTCTCGTGC TCGTCGCTGC

201
GTTGAGGCTT GCGTTTATGG TACGCTGGAC TTTGTGGGAT ACCCTCGCTT

251
TCCTGCTCCT GTTGAGTTTA TTGCTGCCGT CATTGCTTAT TATGTTCATC

301 PA1--->
CCGTCAACAT TCAAACGGCC TGTCTCATCA TGGAAGGCGC TGAATTTACG

351
GAAAACATTA TTAATGGCGT CGAGCGTCCG GTTAAAGCCG CTGAATTGTT

401 <---PB2
CGCGTTTACC TTGCGTGTAC GCGCAGGAAA CACTGACGTT CTTACTGACG

451 <---PA2
CAGAAGAAAA CGTGCGTCAA AAATTACGTG CGGAAGGAGT GATGTAATGT

501
CTAAAGGTAA AAAACGTTCT G

Primer Pair	Amplified Fragment Length
PA1 PA2	180 bp
PA1 PB2	113 bp
PB1 PA2	312 bp
PB1 PB2	245 bp

*Gene E is underlined, and the primers are in bold

Figure 16: MS2 Lysis Gene

1601 MSB1--->
GGAACTAACC ATTCCAATTT **TCGCTACGAA TTCCGACTGC** GAGCTTATTG

1651 MSA1--->
TTAAGGCAAT GCAAGGTCTC CTAAAAG**ATG GAAACCCGAT TCCCTCAGCA**

1701 <---MSA2
ATCGCAGCAA **ACTCCGGCAT CTACTAATAG ACG**CCGGCCA TTCAAACATG

1751
AGGATTACCC ATGTCGAAGA CAACAAAGAA GTTCAACTCT TTATGTATTG

1801
ATCTTCCTCG CGATCTTTCT CTCGAAATTT ACCAATCAAT TGCTTCTGTC

1851 <---MSB2
GCTACTGGAA **GCGGTGATCC GCACAGTGAC** GACTTTACAG CAATTGCTTA

1901
CTTAAGGGAC GAATTGCTCA CAAAGCATCC GACCTTAGGT TCTGGTAATG

Primer Pair	Amplified Fragment Length
MSA1 MSA2	54 bp
MSA1 MSB2	201 bp
MSB1 MSA2	112 bp
MSB1 MSB2	259 bp

*the MS2 lysis gene is underlined, and the primers are in bold

A special consideration in primer selection was a high GC content. The GC content is the amount of guanidine and cytosine as compared to the total bases. G-C base pairs have three hydrogen bonds holding them together, while A-T base pairs have only two. By choosing primers that contain a high GC content, the annealing temperature in the PCR process can be increased because there are more hydrogen bonds binding the primer to the template. Because the vast majority of nucleic acids that exist in the environment have not been sequenced, and because a higher GC content would allow a higher annealing temperatures, primers were chosen with a high GC content in the hope that a high annealing temperature would reduce the number of non-target primer interactions.

An equation, given by Ruano (Ruano, 1989), is often used to calculate the proper annealing temperature. Melting temperatures calculated using this formula are shown in Table 13.

T_d = 4°C (#G + #C) + 2°C (#A + #T) for 20 base primers

Annealing temperatures calculated from this formula and a melting map for two sets of primers for phi-X174 (PA1PA2 and PB1PB2) are in reasonable agreement, although the melting map temperatures are slightly higher in general. This is not unexpected, as Ruano suggests using slightly higher-than-calculated annealing temperatures for best results (Ruano, 1989).

The phi-X174 primers were developed from a region of overlap between lysis gene E and a coat protein gene D. The lysis gene of phi-X174 is somewhat unique in that it is a single protein that allows lysis of the *E. coli* cell, unlike most other phage that have multiple proteins (Young, *et al.*,

1990). Furthermore, the region of overlap between the coat protein and the lysis gene should be well conserved because a mutation would affect both genes. The phi-X174 DNA sequence is shown along with the primer sequence location and fragment length information in Figure 15. Each of these sets of primers amplifies a specific length of DNA. Note that because the primer's attachment sites are a specific length apart on the template DNA, amplified fragments can easily be differentiated from each other on the basis of length. After primers were selected, they were screened against other sequences in GenBank to ensure that they were unique. No identical matches were found for any of the primers. These primer sequences mismatched at least 8 out of 20 bases with the closely-related G4 bacteriophage, which also produces a single lysis protein (Witte, *et al.*, 1990). This number of mismatched bases will not be expected to anneal to the G4 template, and thus should not give a signal if the divalent salt concentration in the reaction mixture is not too high and the annealing temperature in the PCR process is not too low.

The phi-X174 primers were also screened for amplification of Charles River water isolates. Phage that attacked the phi-X174 host *E. coli* strain were found to be present in CRW at a concentration of 10^3 phage per liter. Eleven plaques were isolated from the Charles River water on the phi-X174 host *E. coli*. The phage were grown as described above, and subjected to PCR using the 4 sets of primers. No phage showed amplification using any combination of primers. Although these results are not conclusive, they give some confidence in the selectivity of the primer.

Similar care was taken in the selection of primers for MS2. The lysis gene was again chosen as the site for primer selection. A segment of the MS2 genome (as DNA) is shown in Figure 16 along with the locations of the primer

sequences. The primers were checked against GenBank, and no perfect matches were found. In fact, no close matches (more than 8 mismatches in 20 bases) were found. When the MS2 host *E. coli* was used to recover phage from CRW samples, no plaques were observed (less than 200 per liter). Thus, little interference from indigenous MS2 phage is expected in environmental concentrates.

5.2.6 Reaction Mixture, Enzymes, and PCR Conditions

The reaction mixture (RM) is the solution in which amplification takes place. It contains a pH buffer, monovalent and divalent salts, primers, the DNA nucleotide triphosphates (adenosine dATP, guanosine dGTP, cytidine dCTP, and thymidine dTTP), enzyme, and the template. Gelatin, glycerol, BSA, DMSO, or detergents are often included to facilitate the reaction. In this research, only gelatin was used in PCR buffers. The RT buffers were supplied by the company that manufactured the enzyme.

The optimal concentration of each of the reagents in the reaction mixture is very important. Salt concentrations that are too high or too low can cause the PCR process to fail. High salt concentrations have been observed to increase the likelihood of non-target interactions. This is suspected to occur when sites on the template which are normally coulombically blocked due to mismatches with the primer are able to anneal to the primer as a result of electrostatic masking from the high ionic strength. In other words, the electrical interactions with the fluid mask the repulsion from base pair mismatches. Low salts can cause the primers not to anneal or may inactivate the enzyme.

Table 14: RT-PCR Reaction Mixtures

Reverse Transcription Reaction Mixture

Reagent	Stock Concentration	*TetZ*	*Tth*
RT Buffer	10x stock	3 µL	3 µL
dNTP's	5 mM	1.2 µL	1.2 µL
$MnCl_2$	50 mM	-	0.54 µL
primer 1	5 µM	4.5 µL	4.5 µL
RT enzyme	5 U/µL	0.5 µL	1 µL
RNasin	50 U/µL	0.5 µL	1 µL
dH_2O/sample	-	to 30 µL	to 30 µL

DNA Reaction Mixture

Reagent	Stock Concentration	*TetZ*	*Tth*
DNA Buffer	10x stock	7 µL	7 µL
$MgCl_2$	10 mM	-	10.5 µL
primer 2	5 µM	2.1 µL	2.1 µL
dH_2O/sample	-	to 70 µL	to 70 µL

Table 15: *Taq* Polymerase Reaction Mixtures

***Taq* Buffers**

10x stock buffer	Buffer A	Buffer B	Buffer C	Buffer C'
$MgCl_2$	7.5 mM	15 mM	22.5 mM	22.5 mM
KCl	500 mM	500 mM	500 mM	500 mM
Tris pH 8.4	100 mM	100 mM	100 mM	-
Tris pH 7.4	-	-	-	100 mM
Gelatin	0.1%	0.1%	0.1%	0.1%

***Taq* Reaction Mixture**

Reagent	Stock Concentration	*Taq*
Buffer	10x stock	10 µL
dNTP's	5 mM	4 µL
primer 1 & 2	5 µM	2 µL each
Taq enzyme	5 U/µL	0.3 µL
dH_2O/sample	-	to 100 µL

The PCR process can also be sensitive to the concentrations of other reagents, such as the enzyme concentration and the primer to template ratio. The reaction mixture chosen in these experiments was developed as a matter of trial and error (see later in this chapter), although the protocol set forth Ruano was used as a basis for the reaction mixture design (Ruano, 1989). An optimized RM is crucial so that the highest possible efficiency of amplification is obtained allowing product formation in the fewest possible cycles. This is desirable because non-target sequences have less of an opportunity to obscure results. The final RT reagent concentrations in the RM are given in Table 14, while PCR buffers and reagent concentrations in the RM are given in Table 15.

5.2.7 Polyacrylamide Gel Electrophoresis

The method used to detect the amplified DNA involved 'running' an aliquot of sample through a polyacrylamide gel (PAGE). The gel consists of a network of cross-linked polymers (acrylamide and *bis*-acrylamide) through which the DNA must move. The gel is placed in an electric field, and because the DNA is negatively charged, it moves unidirectionally through the field (i.e., down the gel). The speed with which the DNA moves through the gel, and thus the distance moved in a given time, is a function of the length of the strands of DNA. Thus, the longer strands move slowly whereas the shorter strands move more quickly. In these experiments, a 6% polyacrylamide gel was used. The 6% gels were prepared by making a solution that contained 1x TBE and 6% polyacrylamide in 30 mL of distilled water. The polymerization

was initiated by the addition of TEMED and stock ammonium persulfate, both to a final concentration of 0.12%.

In general, a 10μL sample size mixed with 2μL of 6x loading dye was loaded onto the gel. An electric potential of 300 volts was placed across the gel. The field was maintained for about 45 minutes. Then, the gel was soaked in 500 mL of a 20 μg/L solution of ethidium bromide in distilled water for three minutes, followed by a brief destaining in distilled water. Ethidium bromide was a DNA intercalating agent and causes the DNA to fluoresce under ultraviolet light. Photographs were then taken of the uv-exposed gel as a permanent record.

5.3 Reconstruction Experiments Using Rotating Membrane Ultrafiltration

Reconstruction experiments were performed to determine the recovery efficiency of the ultrafiltration unit used to concentrate viruses from water samples. An initial attempt was made using a five liter sample (concentrated by a factor of 70) and an eleven liter sample (concentrated by a factor of 300) of Charles River water. Each was filtered through a Benchmark GX ultrafiltration unit (at 2000 rpm and 40 psi) with no recoverable coliphage in the filtrate. The Charles River samples contained approximately 1300±900 (μ±SD) indigenous coliphage per liter that attacked the host *E. coli* strain for phi-X174. Phage were measured in the unconcentrated water sample and the filtrate. No phage was detected in the filtrate that was tested, giving a value of less than 4 PFU/L in the total filtrate. Thus, less than 0.2% of the indigenous phage got through the filter.

More comprehensive testing has been performed. In a series of experiments, phage were spiked into several buffers and several environmental samples in order to gauge recovery efficiency. Phage plaque assays were performed on aliquots collected from: (1) the unspiked sample, (2) the spiked sample, (3) the filtrate, (4) first and/or second filter rinse water, and (5) the retentate. (Note that the terms retentate and concentrate can be used interchangeably, although here retentate is used to emphasize the volume retained by a filter and concentrate is used to denote the concentrated sample throughout the process). A filter rinse is defined as follows: after removing the retentate, 200 mL of TE buffer was spun in the filter at 2200 rpm for 10 minutes at 20 psi. In the first filter rinse, the rinse water was further concentrated to 35 mL after the spin time, and combined with the retentate.

As an example, two liter volumes of PBS, 5% glycerol, and 10 mM sodium tetraborate ($Na_2B_4O_7$) were spiked with 10^6 phi-X174 and concentrated as described in Chapter 5.2.3.1 A concentration factor of 40 was achieved for each of the three sample types. The three buffers were chosen because they are commonly used to store viruses, and no significant virus die-off would be expected simply from exposure to them. No phage were detected in the unspiked, unconcentrated buffers (<200 phage/L detection limit). The mean concentration of phage in the spiked, unconcentrated sample was taken as 100%. Less than 1% of the spiked phage was found in the filtrate (PBS = 0.4%, glycerol = 0.2±0.02%, and $Na_2B_4O_7$ = 0.7±0.2% where the results are given as mean ± standard deviation). Less than 4% of the phage could be recovered in a rinse of the filter (PBS = 4%, glycerol = 3±1%, and $Na_2B_4O_7$ = 3±0.2%). This phage is recoverable because the rinse is mixed with the

retentate before proceeding to the secondary concentration. The majority of the phage was recovered in the concentrate (PBS = 84%, glycerol = 118±34%, and $Na_2B_4O_7$ = 68±2%). All samples are the result of three replicates except in the case of PBS.

In another experiment using two spike concentrations, two 2 liter samples of 10 mM $Na_2B_4O_7$ were spiked with 10^4 and 10^6 phi-X174 and concentrated. No phage were detected in the unspiked, unconcentrated sample (<200 phage/L). The filtrates contained 5% and 2±0.1%, respectively, of the spiked phage. This is a higher level of phage in the filtrate than is often noted, and may be due to the filter's age. Because the level of phage did not account for a significant portion of the spiked phage, the matter was not pursued. The retentate/first rinse contained the majority of the spiked phage (10^4 spike, 77±7%; 10^6 spike, 78±4%). Examination of samples from the first rinse, before being combined with the retentate, showed that an additional 10% of the virus can be recovered (10^4 spike, 9±2%; 10^6 spike, 10%) by performing a first rinse. The second rinse contained 0.1±0.2% and 0.5±0.01%, respectively, of the spiked phage suggesting that additional filter rinses do not increase recovery significantly.

In an experiment using MS2 phage, 10^4 MS2 phage were spiked into a 5 liter sample of PBS. The sample was concentrated as described in Chapter 5.2.3.1. In this experiment, plaque assays were performed on the spiked, unconcentrated sample and the retentate/first rinse. The retentate was found to contain 76±8% of the spiked phage in the spiked, unconcentrated sample. At least three replicates were used in deriving these results. It can be seen that MS2 phage is also concentrated with high recovery efficiency.

Table 16: Recovery of MS2 and Phi-X174 using RMU

Sample Source	Volume L	Spiked Phage PFU		Recovery in Conc./Rinse % (SD)	Recovery in Filtrate % (SD)
5% Glycerol	2	ϕX174	10^6	118% (34)	0.2% (0.02)
PBS	2	ϕX174	10^6	84%	0.4%
10 mM $Na_2B_4O_7$	2	ϕX174	10^6	68% (2)	0.7% (0.2)
10 mM	2	ϕX174	10^4	77% (7)	5%
$Na_2B_4O_7$	2	ϕX174	10^6	78% (4)	2% (0.1)
PBS	5	MS2	10^4	76% (8)	-

The purpose of these experiments was to determine a recovery efficiency for RMU technology. Most of the phage was recovered in the retentate/concentrate and the first rinse. The results presented above are summarized in Table 16. Little virus was lost in the filtrate or to the filter as gauged by the second rinse. These experiments illustrate that RMU can be used to recover even the smallest viruses with high efficiency. RMU also provides a relatively quick means of concentrating microorganisms. Flow rates in these buffered solutions (as well as clear water flow rates) consistently ranged from 28 to 30 L/hr when the system was operated at 40 psi.

Because size separation is used and the smallest viruses were tested, these results prove that RMU provides good recoveries, and that larger viruses and bacteria should be concentrated with similar or higher recovery. In order to ensure that materials in environmental samples did not interfere with the concentration process, both Cambridge drinking water and Charles River water were tested. Because of the poor water quality, high solids, and high levels of humic materials, the CRW sample provided a worst case scenario for waters that would be tested. Few people ingest CRW because water quality is perceptibly poor; thus, exposure is limited and the need for monitoring large water volumes is also limited.

To examine the effect of environmental samples on virus recovery, 10^5 and 10^6 phi-X174 were each spiked into separate 2 L volumes of CRW and distilled water. The samples were filtered, and a plaque assay was performed on an aliquot taken from the unconcentrated, spiked samples and from the retentate. Because phi-X174 and other phage that attack the host *E. coli*

may be found naturally in water, the background concentration of the phage was also measured. Background levels of phage in unspiked, unconcentrated CRW were $5.6x10^2 \pm 5.6x10^2$ phage per liter ($\mu \pm SD$) that attacked the stock *E. coli* host strain. This background level of virus is much lower than the level of virus spiked into the sample, and cannot interfere with results. No phage was found in the unspiked, unconcentrated distilled water. In the concentrates, high recoveries were observed (CRW spike = 10^5, 94±41%; CRW 10^6, 70±4%; distilled water 10^5, 80±12%; distilled water 10^6, 106±22%). All samples are the result of three replicates. These results suggest that little difference in recovery efficiency exists between vastly different water types.

Further testing was performed using MS2 phage. A 5 liter sample of CRW was spiked with 10^5 MS2, and aliquots for plaque assays were taken as described above. Interestingly, although viruses that attack the MS-2 host *E. coli* strain might be expected to be found naturally in water, upon testing, no such phage were found in unspiked, unconcentrated CRW samples (<0.1% of spiked phage or <400 phage/L). In the filtrate, 2±1% of spiked phage was detected. The concentrate and first rinse contained 68±6% of the spiked phage. A second rinse revealed 0.3±0.05% of spiked phage. All the results are derived from at least three replicates. MS2 was recovered with similar efficiency to that for phi-X174.

Phi-X174 (10^5 phage) was also spiked into 5 L of CDW. Prior to spiking the phage, the water was brought to 50 mg/L final concentration of sodium thiosulfate ($Na_2S_2O_3$) in order to dechlorinate the water. After a half an hour, the sample was spiked then filtered and aliquots from the different portions of the filtered sample were plated. As might be expected, no phage

were detected in the unspiked, unconcentrated sample. The filtrate contained 0.8±0.6% of the spiked phage, while the retentate contained 31±4% of the spiked phage. A first rinse allowed the recovery of an additional 3±0.5% of the spiked phage. Although this recovery is not unacceptably low, it is noticeably lower than recoveries from other experiments. Furthermore, the low recovery is seen in each portion of the samples from this source (e.g., retentate, rinse, *etc.*). In order to explain this result, phi-X174 was spiked into dechlorinated CDW, allowed to sit for 10 minutes, and then plated. The recovery with no concentration was 57±10%, suggesting that CDW itself is toxic to phage. If this factor is taken into account, a recovery of 55% is calculated for the experiment presented above. It is not known whether residual chlorine or some other factor was responsible for the die-off. Again, although these phage may not be culturable, they are still detectable by PCR.

Flow rates for CDW consistently averaged between 27 and 30 L/hr. This rate is similar to that for the buffered solutions and the clear water flux, indicating that this water is relatively free of particulate matter which could reduce the flow rate. CRW and WRW, on the other hand, averaged 17 L/hr for the first three liters and dropped to 11 L/hr average for the last three liters of a 10 L sample using the RMU at 40 psi. Volumes of 20 liters of CRW have been filtered with the flow rate for the final liter approaching 9 L/hr. CRW and WRW contain more material which reduces the flow rate. Likely, because CDW has been treated by flocculation, many small particles have been removed.

Table 17: Recovery of MS2 and Phi-X174 using RMU from Environmental Samples

Sample Source	Volume L	Spiked Phage PFU		Recovery in Conc./Rinse % (SD)	Recovery in Filtrate % (SD)
CRW	2	ϕX174	10^5	94% (41)	-
	2	ϕX174	10^6	70% (4)	-
distilled	2	ϕX174	10^5	80% (12)	-
water	2	ϕX174	10^6	106% (22)	-
CRW	5	MS2	10^5	68% (6)	2% (1)
CDW	5	ϕX174	10^5	31% (4)*	0.8% (0.6)

nd = none detected

* Phi-X174 was added to dechlorinated CDW for 1 hour, and without RMU concentration 56% could be recovered. This gives 55% recovery. Furthermore, phage that die-off during concentration would still be detectable by PCR.

From these results, RMU has been consistently shown to allow greater than 60% recovery of spiked organisms and is not dependent on the type of virus or the source of the water sample. These results are all summarized in Table 17. One of the major benefits of PCR is that even if viruses are injured or killed in the concentration process, they are still detectable. Thus, the recovery efficiencies derived here are the lower limits of recovery when PCR is used as the means of detection instead of plaque assay. Although the virus recoveries obtained in these experiments were good, a high overall recovery followed by PCR detection is the best test of the methodology. Overall recovery refers to RMU concentration as well as secondary extraction and filtration. These experiments are discussed below.

To summarize these results, no difference was seen in the recovery efficiency between MS2 and phi-X174. Furthermore, on the order of 1% of spiked organisms was found in the filtrate, and less than 1% was detected in a secondary rinse of the filter. It was also determined that on the order of 10% increases in recovery could be obtained if the filter was rinsed and this "first rinse" was combined with the retentate. Water quality was also shown not to affect recovery efficiency. These findings are important because they indicate that the technique can be standardized: meaning an average recovery can be determined, and factors such as water quality and virus type do not have to be considered. Importantly, the consistent recovery efficiencies described above allow quantification.

The only downside to the use of RMU is that water samples from untreated sources have a flow rate of 11-17 L/hr, whereas treated drinking water has a flow rate of 27-30 L/hr. This low flow rate limits the volume of

sample that can be filtered in a reasonable amount of time. One way around this problem is the use of a larger RMU model, the Pacesetter (Membrex), which has been reported to filter at a rate of 100 L/hr. Alternatively, while 40 psi was the limit of pressure that could be used in this research, the use of high pressure tubing and stainless steel connectors would allow higher flow rates which should increase linearly with pressure.

Another consideration is that with the higher recovery efficiencies achieved with RMU, lower sample volumes are required. If, as was discussed in Chapter 4, 300 L of water is filtered, but only 3 L can be used in PCR detection due to inhibitory effects of the concentrate, then this methodology provides better results.

5.4 Secondary Concentration Recoveries

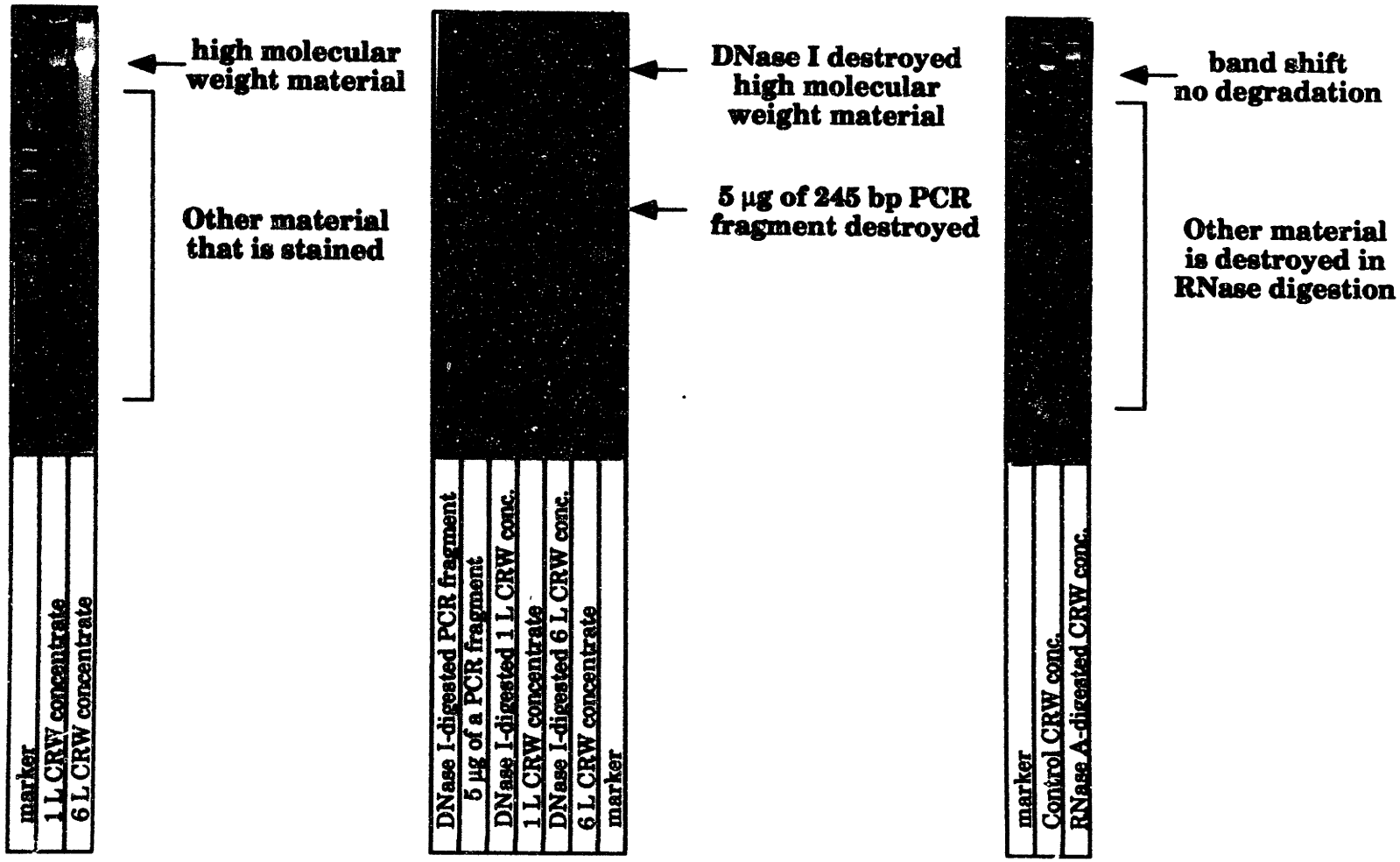
As mentioned above, the secondary concentration process involves taking the RMU concentrate from 70 mL to 100 µL or less. In addition to volume reduction, several extractions are used to remove extraneous material from the sample to facilitate volume reduction and remove substances that might prove inhibitory to PCR amplification. This secondary concentration involves a Centriprep filtration, a proteinase K digestion, a CTAB extraction, phenol:chloroform extractions, a Centricon filtration, and a Microcon filtration. These processes are described in more detail in Chapter 5.2.3.

Although RMU could be used to concentrate any type of water sample quickly and efficiently without pre-treatment of the sample, the secondary concentration process could not. Initially, samples were concentrated without

extractions using Centriprep and Centricon filters, but both the CDW and WRW samples contained too many solids to pass through the filter even after extensive filtration times at great than 500x g. Not surprisingly, these partially concentrated samples could not be used with PCR due to inhibition. Early experiments used phenol:chloroform extractions to remove material that hindered filtration and inhibited PCR.

When non-drinking water samples are concentrated and run on a PAGE gel, high molecular weight material is trapped at the top of the gel. A 6 liter and 1 liter sample of CRW was concentrated to 300 μL and 120 μL ($2x10^4$ and $8x10^3$ times concentration), respectively, as described in Chapter 5.2.3 (except that no proteinase K digestion and no CTAB extraction were performed). A picture of this high molecular material from 10 μL of each of the 6 L and 1 L concentrate is shown in Figure 17. This material was tested in order to determine its composition. A 5 liter CRW sample was concentrated to 130 μL ($4x10^4$ times concentration) as described in Chapter 5.2.3 (except that no proteinase K digestion and no CTAB extraction were performed). A 10 μL and 1 μL sample (about 10% and 1% of the sample, respectively) of this concentrate were each digested with DNase I. Identical samples without DNase I digestion were also run as controls. Additionally, two samples containing approximately 5 ng of amplified phi-X174 DNA (PB1PB2) were also used; one sample was digested with DNase I while the other was run as a control. Briefly, the reaction mixture contained a final concentration of 1x DNase I buffer, 50 μg of DNase I, sample, and sterile, distilled water to 50 μL. The samples were placed at 37°C for 60 minutes, followed by running 10 μL aliquots of the sample on a PAGE gel. It can be seen from Figure 17 that treatment with DNase I completely destroys the band.

Figure 17: Characterization of the Concentrate



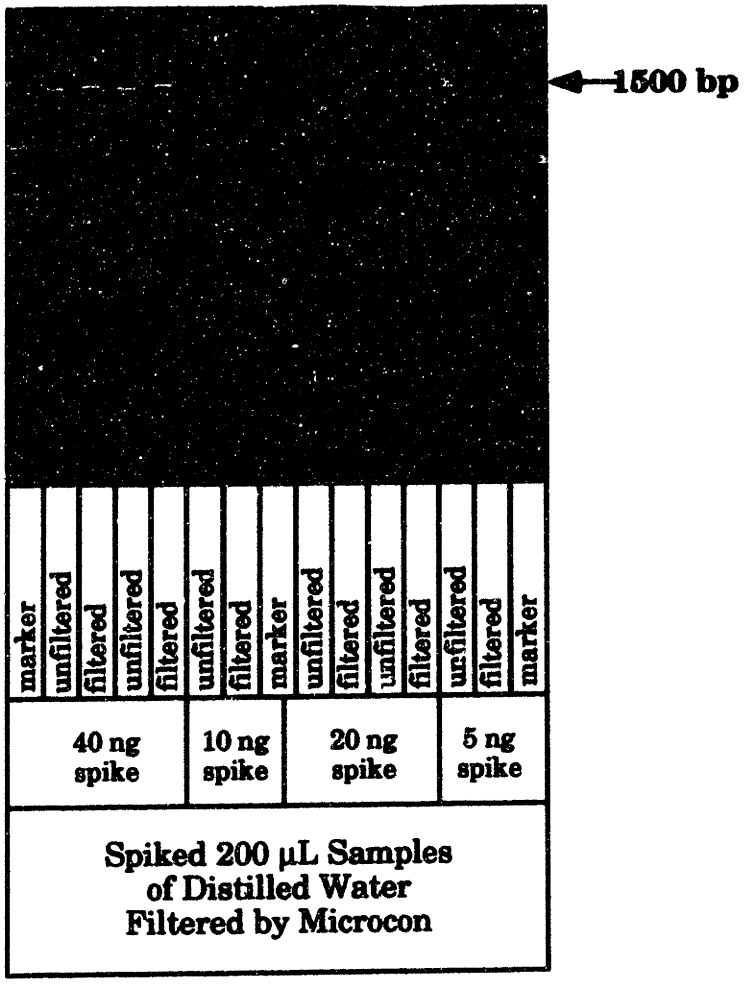
Next, an RNase treatment of the band was performed. Ten microliters of the 6 liter concentrate mentioned above was mixed with EDTA and RNase A and incubated at 37°C for 1 hour. Then, the digested sample was run alongside an identical undigested sample on a PAGE gel and on agarose. RNase A does not remove the high molecular weight material at the top of the gel, but it can be seen to remove material that has smeared down the lane (see Figure 17). Interestingly, a band shift was observed in the RNase A digestion. The high molecular weight material runs higher on the gel after treatment with RNase A, most probably indicating DNA-binding activity by the RNase A protein. Thus, the material that appears on the gel is mostly composed of DNA, but RNA is also present. Similar bands were observed in CRW and WRW concentrates and other environmental samples (e.g., ocean water and sewage). Thus, a characteristic of untreated environmental samples is that a significant amount of DNA and RNA is present.

Humic material (Aldrich) was also tested. Humic material appears as a light smear on a PAGE gel that does not form a band. The humic material appeared to fluoresce with a multicolored sheen that can be distinguished from nucleic acid. A 10 μg and a 1 μg sample of humic material along with a 10 μL of the 5 L CRW concentrate were digested with DNase I in an identical experiment to that described above. The CRW concentrate was digested, while the humic material was unchanged after digestion. The DNase I-digested CRW concentrate did not contain a similar fluorescence, indicating that the substance responsible for the color in the CRW concentrate is either <1 μg/10μL or that it is not humic material. These results help to characterize the high molecular weight material observed on the gel as DNA and the smear down the gel as RNA. The next step in characterizing the

methodology was to determine the recovery efficiency of the secondary process.

Because the secondary concentration was to be performed after extractions, any nucleic acid would be free of a cell wall or capsid. Therefore, a PCR fragment, which was amplified using primers for the flaA (flagellin) gene of *Helicobacter pylori*, was used (Akopyanz, *et al.*, 1992). This fragment is 1500 bp in length, and provides a conservative model for 3000 bp nucleic acid of the smallest viruses. Although the manufacturers provide recovery data for nucleic acid recovery from their products of >90%, these experiments were meant as a check on that work. The fragment was amplified for 30 cycles in RM's containing either Buffers B, C, or C' with a PCR cycle of 94°C for 1 minute, 54°C for 1.5 minutes, and 72°C for 2 minutes in order to assess the best reaction mixture conditions. Buffers B and C provided similar results, while Buffer C' had a lower efficiency of amplification. These fragments were used to test recovery from Microcon filtration. A 10 µL and a 20 µL portion of amplified fragment were taken from each of the three RM's (B and C contained about 2 ng/µL and C' contained about 0.5 ng/µL), added to 200 µL of sterile, distilled water, and put through a Microcon at 500x g until 10 µL remained. This retentate was run on a gel alongside 10 µL or 20 µL of the corresponding control sample. As can be seen from Figure 18, the amount of amplified product between the filtered and unfiltered samples is not distinguishable. This is most apparent for the Buffer C' fragment. Due to the lower amplification efficiency, less product was formed and the faint bands formed on the gel provide the opportunity to see any minor differences in recovery.

Figure 18: Microcon Recovery



In order to test the recovery from the Centriprep and Centricon filters, the PCR fragment was amplified using Buffer C, except that 2μL of radioactive dATP (α-labeled with ^{32}P) was used in the reaction mixture to make the fragment more easy to track in dilute solutions. The fragment was subjected to 30 cycles of PCR, and then run on a PAGE prep gel. The fragment was cut out of the gel and electroeluted from the gel slice. Briefly, one well of a two welled electroelution container was covered with dialysis tubing, while the other well was covered with Sartorius membrane that had be soaked in water. The gel slices were loaded into the well covered by dialysis tubing, and a solution containing 0.1% TBE and 0.005% SDS was used to fill the electroelution container. The container was placed into an electroelution tray containing 2x TBE and a potential difference of 75 volts was placed across the tray for two hours. After that time, the DNA fragment was collected from above the Sartorius membrane. The gel-purified fragment was then spiked into 75 mL of distilled water and concentrated using a Centriprep at 500x g until approximately 5 mL of concentrate remained. Only a small portion of the fragment could be detected in the retentate as determined by Geiger counter. Upon examination of the Centriprep filter, the majority of the radioactivity appeared to be on the filter. After the filter was removed using a razor blade, the filter was found to be devoid of radioactivity. The filter holder/filter interface, however, was highly radioactive.

It was determined that the "glue" that held the filter to the filter holder was binding the DNA. Several blocking agents were chosen to passivate the membrane: tRNA, SDS, PEG, Triton X, Tween 20, and BSA. Eight 15 mL samples of sterile, distilled water were spiked with 10 μL of the stock gel-purified radioactive fragment (approximately 6500 cpm) and concentrated to

2 mL. The samples were then concentrated using a blocked Centriprep. Blocking was accomplished by adding 2-5 mL of the appropriate blocking agent to the Centriprep and allowing it to sit overnight. The blocking agent was rinsed from the Centriprep with sterile, distilled water and the sample containing the radiolabeled PCR fragment was added. Radioactivity was measured using a scintillation counter. Briefly, 5 mL of scintillation cocktail and the sample were mixed and placed in the counter. Recoveries of radioactivity in the concentrates were as follows: 10 μg/mL tRNA (69%), 100 μg/mL tRNA (85%), 500 μg/mL tRNA (94%), 5% SDS (71%), 5% PEG (64%), 5% Triton X100 (95%), 5% Tween 20 (76%), 1% BSA in PBS (90%). In these experiments, radioactivity in the filtrates was indistinguishable from the background radiation. After these initial results, duplicate experiments were run on the tRNA and BSA blocking agents, and the average recoveries were as follows: 100 μg/mL tRNA (84±2%), 500 μg/mL tRNA (89±8%), 1% BSA in PBS (84±9%). BSA was chosen because of the high recovery efficiency and because residual amounts left after concentration are known not to interfere with PCR and may even increase efficiency.

The Centricon and Microcon filters were then tested. Two 5 mL samples of sterile, distilled water were spiked with 10 μL of fresh gel-purified radioactive fragment (12,000 cpm). One of the samples was filtered through a Centricon while the other was phenol:chloroform extracted and filtered through a Centricon. In addition, 400 μL of sterile distilled water was spiked with 10 μL of the same radioactive fragment and concentrated through a Microcon filter. Radioactivity was measured in the concentrate and the filtrate. Recoveries were 67% for the Centricon filtration alone, 73% for the Centricon filtration and extraction, and 83% for the Microcon filtration.

Radioactivity was also measured in the filtrates, and accounted for about 1% of spiked radioactivity for each filter.

In the next phase of experimentation, all the individual filtrations were performed in conjunction. Each of three 50 mL samples of PBS were spiked with one of the following 1000, 100, or 0 MS2 phage. The samples were concentrated to 1.5 mL using a BSA-blocked Centriprep, then to 250 μL using a Centricon, and finally to 100 μL using a Microcon. The overall recovery of phage was 81% and 75% for the 1000 and 100 spiked phage, respectively, and no phage were found in the unspiked sample.

A final set of overall experiments were performed in order to insure good recovery even when the recovery volume was as low as 5 μL. Four 50 mL samples of PBS were spiked with 100 MS2 phage. Each was concentrated by successive filtration using Centriprep, Centricon, and Microcon filters to a final volume of 5 μL. The average recovery in the retentate of the 4 experiments was 65 ±11% (μ±SD). A PBS rinse was performed at each stage of concentration, and the rinse was combined with the concentrate (except in the final rinse of the Microcon). A 200 μL PBS rinse of the Microcon was plated separately for each of the 4 samples. The recovery in the rinse was 13±4%, bringing the average recovery to 78% for the entire process.

All of these results are presented in summary form in Table 18. These experiments give an indication of recoveries that can be expected in the secondary concentration process. These experiments were performed in order to detect any "weak links" in the recovery process in order to find an acceptable recovery methodology.

Table 18: Recovery Efficiency of Secondary Concentration Process

Organism	Sample Source	Conc. Factor	Initial Spike	Recovery	Method
			PFU	% (SD)	
DNA	distilled.	15	**	84%(9)	Cp
		50	**	73%	p:c/Cc
		50	**	67%	Cc
		80	**	83%	Mc
MS2	PBS	500	1000	81%	Cp/Cc/Mc
			100	75%	
			0	0	
MS2	PBS	10,000	100	65%(11)	Cp/Cc/Mc
				13% (4)	rinse

** 2000 bp radiolabelled DNA
p:c = phenol:chloroform, Cp = Centriprep, Cc = Centricon, Mc = Microcon
nd = none detected

In order to determine the overall recovery of the process by PCR, two sets of three 50 mL samples of sterile, distilled water were spiked with one of the following: 10 phi-X174, 1 phi-X174, or none. Half the samples were subjected to the following: Centriprep filtration, hot (65°C) phenol:chloroform extraction, Centricon filtration, and Microcon filtration resulting in a final volume of 40 μL. The other three samples were filtered in a similar manner except that no phenol:chloroform extraction was performed. The two sets of concentrated samples and a positive control (consisting of 10, 1, and 0 phi-X174) which had not been filtered were subjected to 40 cycles of PCR using PB1PB2 primers at 65°C annealing temperature. All samples containing 10 phage gave a positive for amplification; the 1 PFU and blank samples did not (Figure 19 and Table 19). That is, 10 phage were detected by PCR whether or not the filtration and/or extraction had been performed. Thus, if 10 phage are present in a concentrated sample, they can be detected by PCR after the secondary concentration process. These results confirm the recoveries demonstrated by the experiments discussed earlier in this section. A similar experiment was performed in the Fox laboratory using *Helicobacter pylori*. In their experiment, 100, 10, and 0 bacteria were spiked into 50 mL of water and filtered as described above. The sample containing 100 and 10 bacteria both gave product upon amplification.

The effect on recovery of "material" present in the environmental sample was also considered. Initially, when environmental samples were concentrated, PCR periodically failed to yield an amplification product. This result could be the result of two factors: inhibition due to co-concentrated material or low recovery at some step in the concentration process due to the presence of material in the concentrated sample. These hypotheses were

tested in an experiment where four 5 liter samples of dechlorinated CDW were concentrated using RMU to 70 mL, followed by Centriprep filtration to 5 mL. The samples were extracted using a phenol:chloroform extraction and further filtered to 40 μL using the Centricon and Microcon filters. Each of the 5 liter samples was spiked with 1000 phi-X174 at a different point in the concentration process. Spike 1 occurred prior to RMU filtration. Spike 2 occurred after RMU filtration, but before Centriprep filtration. Spike 3 occurred after Centriprep filtration, but before extraction. Finally, spike 4 occurred after extraction, but before Centricon and Microcon filtration (see Figure 20). All samples were subjected to 35 cycles of PCR using 65°C annealing temperature and the PB1PB2 primers.

From these results, it can be seen that the phenol:chloroform extraction step results in a loss of signal (see Figure 21 and Table 20). The concentrate allows the amplification of 10 phage (as indicated by positives from spike 4), so that inhibition due to materials in the concentrate could be ruled out. Thus, the extraction step, which was shown to allow good recovery in distilled water, is obstructed by environmental samples.

During the phenol:chloroform extraction of CDW, a thick brown interface was formed. This interface is the result of denatured proteins and other materials present in CDW. Periodically, but not consistently, a signal could be obtained from 1000 phi-X174 in 10 L CDW concentrates that had been hot phenol:chloroform extracted. Likely, the viruses were trapped in the aqueous/organic phase interface by their association with protein. As well, phenol:chloroform extractions alone may not remove all of the material that could be inhibitory to the PCR of low numbers of phage. A proteinase K

Figure 19: Relative Recovery of Phi-X174 During Secondary Filtration

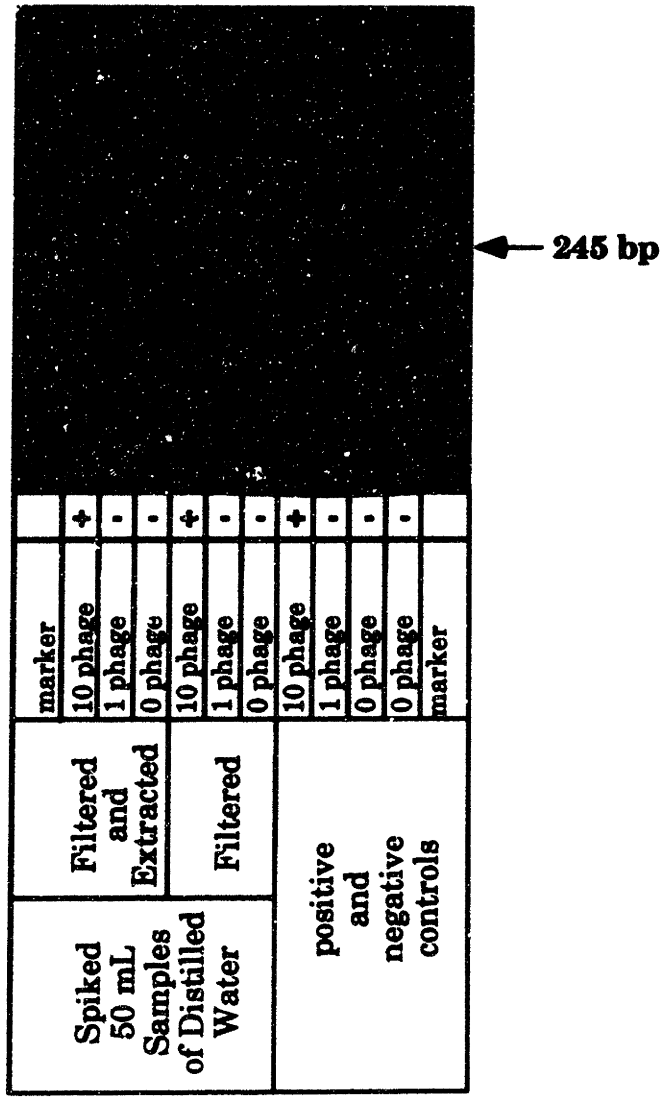


Table 19: Relative Recovery of Phi-X174 During Secondary Filtration

Initial Spike into 50 mL of dH_2O	% of Sample used**	Sample Prep Method	Band?	Recovery
10 PFU	100%	A	+	200 PFU/L
1 PFU	100%	A	-	
0 PFU	100%	A	-	
10 PFU	100%	B	+	200 PFU/L
1 PFU	100%	B	-	
0 PFU	100%	B	-	
10 PFU	pos. control		+	
1 PFU	pos. control		-	
0 PFU	pos. control		-	
	neg. control		-	

A = RMU to Centriprep to Centricon to Microcon to PCR (40 cycles)
B = RMU to Centriprep to phenol:chloroform extraction to Centricon to Microcon to PCR (40 cycles)
**1,250 times concentration 50 mL to approximately 40 μL

Figure 20: Flow Chart of Experiment

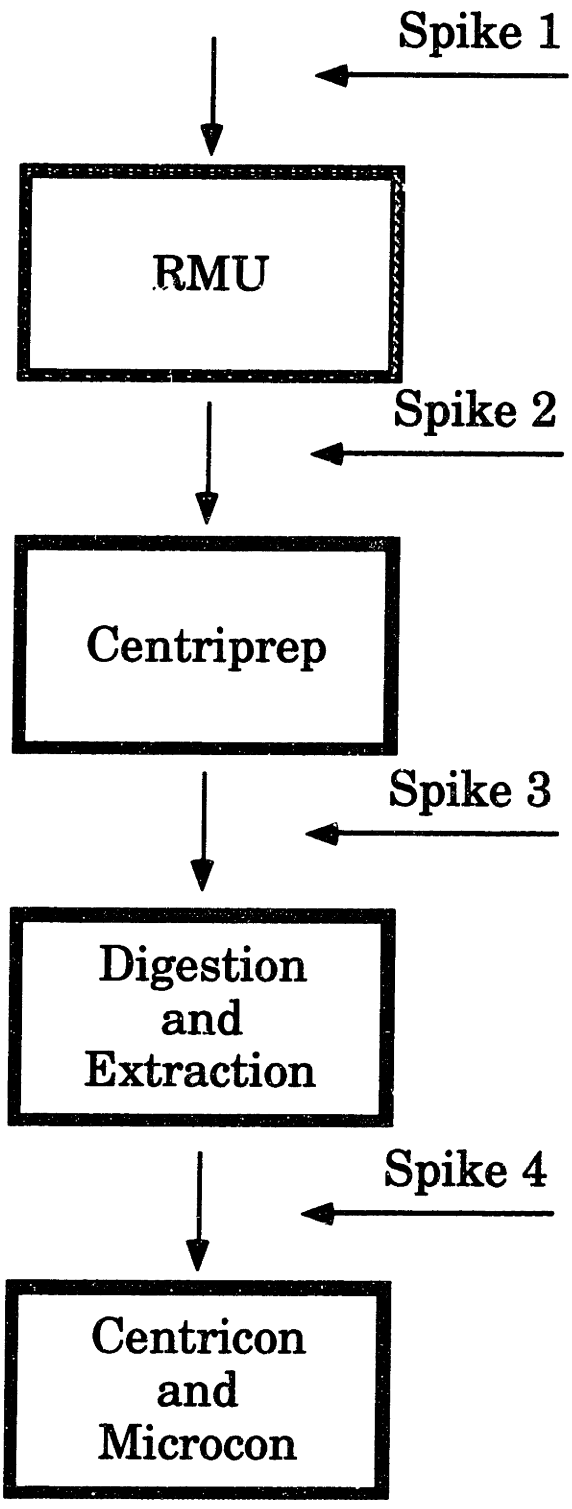


Figure 21: Examination of Secondary Concentration Process

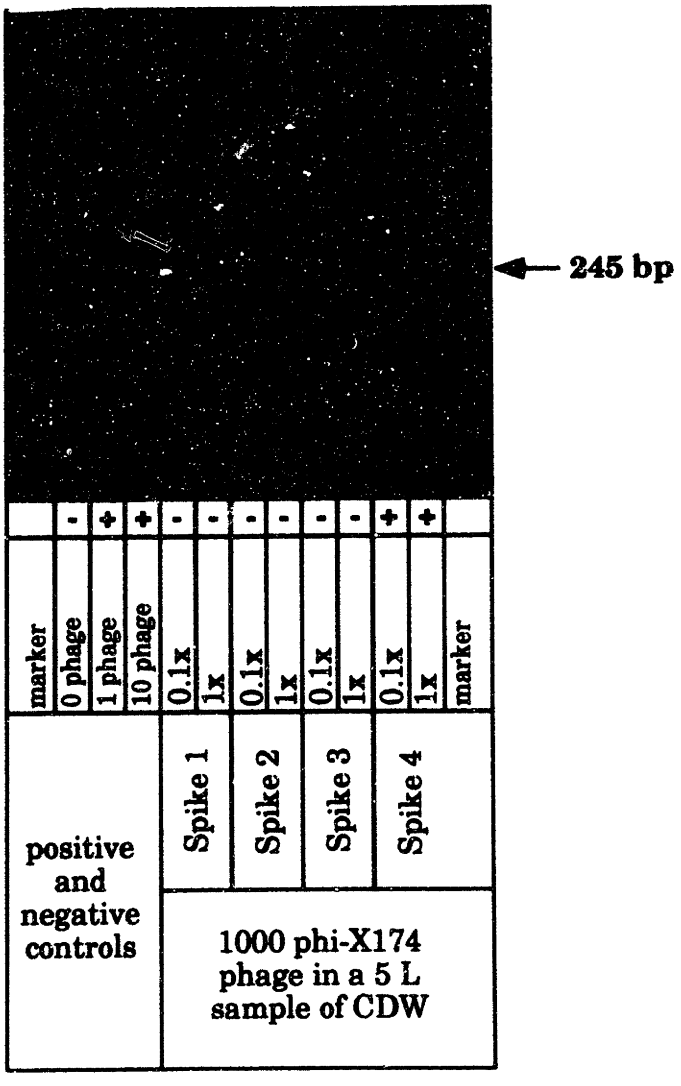


Table 20: Examination of Secondary Concentration Process

Initial Spike into 5 L of CDW*	% of Sample used**	Band?	Spike Occurred Between	Volume after Process
1000 PFU	90%	-	CDW and RMU	5 L to 70 mL
	10%	-		
1000 PFU	90%	-	RMU and Centriprep	70 to 5 mL
	10%	-		
1000 PFU	90%	-	Centriprep and p:c extraction	5 mL
	10%	-		
1000 PFU	90%	+	p:c extraction and Centricon/Microcon PCR	5 mL to 40 µL
	10%	+		
	pos. control	+		
	neg. control	-		

**11,000 times concentration 5 L to approximately 45 µL
RMU to Centriprep to phenol:chloroform extraction to Centricon to Microcon to PCR (40 cycles)

digestion followed by CTAB extraction was used to "free up" the phage so that they could be properly extracted.

5.5 Detection of Viruses Using the Polymerase Chain Reaction

Initial work with PCR involved the determination of the detection limit for the viruses and bacterium of interest. The detection limit is the minimum, or threshold, value in number of organisms required for detection. Primer-template interaction, reaction mixture conditions, and annealing temperature are all critical to the ability to detect low copy number. In initial experiments using phi-X174, Buffer A (see Table 15) was shown not to support amplification even at high copy number. Buffers B, Buffer C, and Buffer C' were all found to allow amplification, but Buffer C' provided more product in 30 cycles using primers PB1 and PB2 whether using 60°C or 65°C as the annealing temperature. The buffers differ in $MgCl_2$ concentration with A containing the least and C the most. The prime in C' indicates a lower pH buffer. The different primer sets (PA1PA2, PA1PB2, PB1PA2, and PB1PB2) were tested for their ability to detect phi-X174 using Buffer C' in the reaction mixture. Primers PA1PB2 were chosen as they provided the most product in 30 cycles.

Tests using the Helicobacter primers, Hp1 and U3, were also conducted. Attempts were made to amplify 10^6 Helicobacter under a variety of conditions. Buffer C, Buffer C', Buffer B, Buffer B' (pH=7.4) were used in separate RM's and amplified using the annealing temperatures 55°C, 60°C, and 65°C for 35 PCR cycles. Only the high pH buffers (Buffer B and C)

allowed amplification at 55°C with Buffer B producing significantly less product than Buffer C. Buffer C allowed approximately equal amplification product formation at 55°C and 60°C. No amplification product was formed in any buffer at 65°C. Again, these tests were not performed with an eye on quantification, but to find reaction mixture conditions that significantly increased product formation. Thus, Buffer C at an annealing temperature of 60°C was determined to be the best choice for reaction mixture condition. This was fortuitous because phi-X174 and, as will be shown below, MS2 can be amplified optimally at an annealing temperature of 60°C.

Next, the sensitivity of detection for phi-X174 and Helicobacter was determined. Separate dilution series of the virus and the bacterium ranging from 10^3, 10^2, 10^1, 10^0, and 10^{-1} copies were subjected to 30 cycles of PCR using the PB1PB2 phi-X174 primers in a RM containing Buffer C' and the Hp1U3 Helicobacter primers in a RM containing Buffer C using a 60°C annealing temperature. A signal was detected for phi-X174 at as few as 1 copy, and a signal was detected down to 10 copies for Helicobacter (see Figure 22 and Table 21). These results have been duplicated and often act as a positive control in experiments. It should be noted that 1 copy of phi-X174 does not always give signal; in other experiments the signal ends at 10 phi-X174 in the dilution series. This result is not unexpected because when the average number of viruses in a sample is one, one or more viruses will be present 63% of the time based on a Poisson distribution of viruses in the sample.

MMLV/*Taq*, *Tth*, and *TetZ* were examined for their use in RT-PCR. Initial experiments using the manufacturer's recommendations for the reaction mixture did not give an amplification product. When the reverse

of the two sets of six aliquots were run on a PAGE gel, the gel was dried, and the gel was exposed to a phospho-imager plate for 1 hour after which the plate was counted. A linear regression of a log plot of cpm versus cycles revealed a PCR efficiency of 89±4%.

MMLV/*Taq*, *Tth*, and *TetZ* were examined for their use in RT-PCR. Initial experiments using the manufacturer's recommendations for the reaction mixture did not give an amplification product. When the reverse transcription reaction mixture volume was increased to 30 µL from the recommended 20 µL, an amplification product was observed for both *Tth* and *TetZ* in 40 cycles using the all four possible primer sets. MMLV/*Taq* RT-PCR never produced an amplification product when amplified under the manufacturer's recommended protocol, as well as under amendments that allowed amplification with the other enzymes. Obviously, the optimal reaction mixture conditions for this enzyme were not determined. Because *Tth* and *TetZ* provide a means to use one thermostable enzyme and a higher annealing and extension temperature (60°C versus 37°C), the use of MMLV, which is not thermostable, was discontinued.

In addition to the increase in RT-RM volume, it was determined that the amount of each of the constituents in the reaction mixture (excluding the buffer) should not be proportionally increased with the increase in RM volume. That is, the amount of each constituent remained the same between the 20 µL and 30 µL RM's, and so the concentration of each of the constituents in the reaction mixture (excluding the buffer) actually went down. In fact, the enzyme was used at half the original concentration with no loss product formation. Next, a dilution series of MS2 phage ranging from 10^3, 10^2, 10^1, 10^0, and 10^{-1} was subjected to RT-PCR using *Tth* and *TetZ*.

Figure 22: Sensitivity of PCR Detection of Phi-X174 and Helicobacter

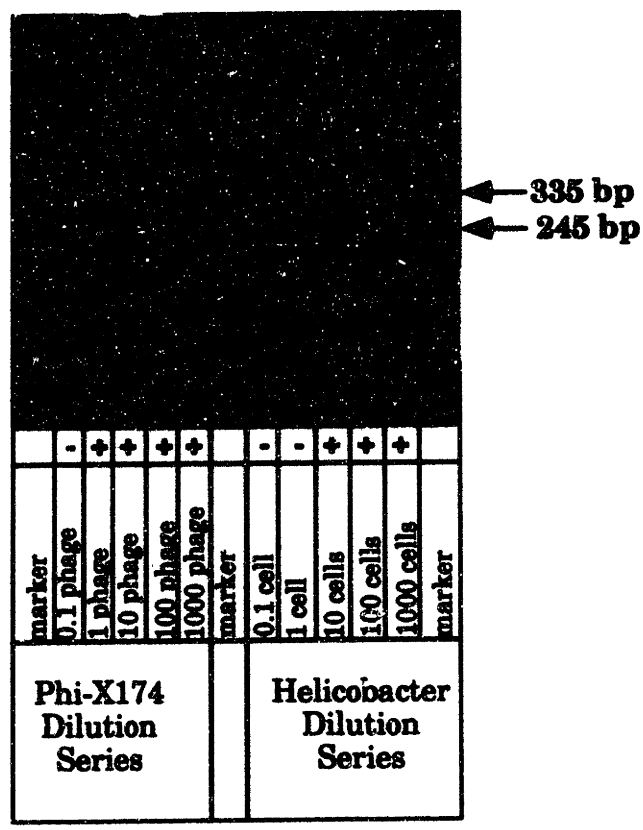


Figure 23: Sensitivity of RT-PCR Detection of MS2

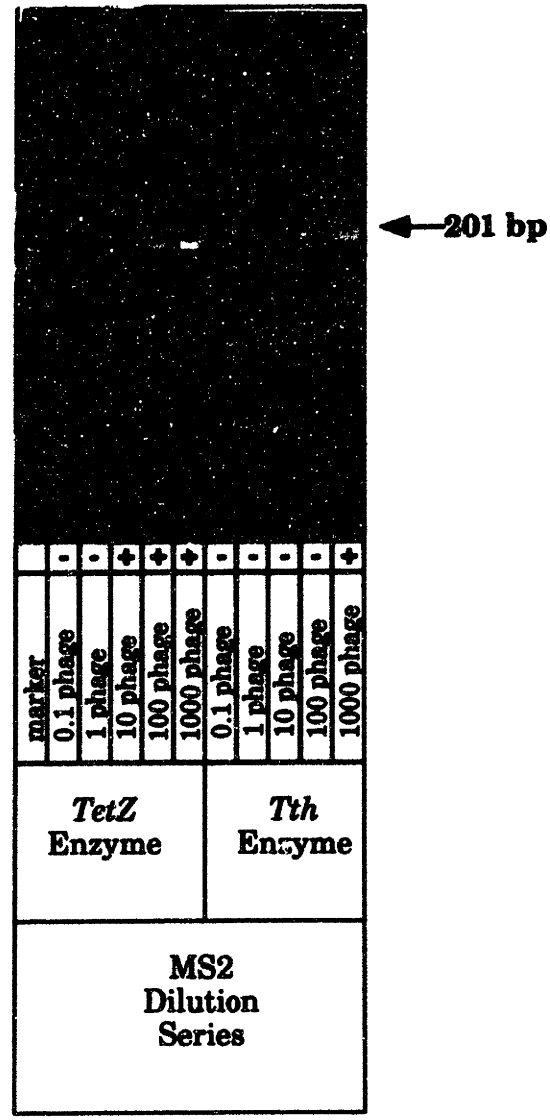


Table 21: Sensitivity of PCR and RT-PCR Detection

Organism	Nucleic Acid	Initial Spike	Band?
phi-X174	DNA	1000 PFU	+
		100 PFU	+
		10 PFU	+
		1 PFU	+
		0.1 PFU	-
H. pylori	DNA	1000 cells	+
		100 cells	+
		10 cells	+
		1 cell	-
		0.1 cells	-
MS2	RNA	1000 PFU	+
		100 PFU	+
		10 PFU	+
		1 PFU	-
		0.1 PFU	-

Using *TetZ*, a PCR signal was detected down to 10 MS2 phage in the dilution series after 40 PCR cycles using the MSA1MSB2 primer set. In tests using the *Tth* enzyme, 1000 copies of MS2 was the minimum detection limit under the same conditions (see Figure 23 and Table 21). Although *TetZ* and *Tth* are manufactured by different companies, they are purified from the same organism. Perhaps the reason for the difference in amplification was a difference in the buffers supplied by the manufacturer. In any event, *TetZ* provides the sensitivity necessary for detection of at least 10 organisms. These results have been duplicated.

As a culmination of this research, the model viruses were spiked into large volumes (10 liters) of two important environmental water sources: Cambridge drinking water (CDW) and Wachusett Reservoir water (WRW). A drinking water sample was selected because of the importance of monitoring drinking water in order to reduce disease outbreaks. A source water sample was chosen because monitoring pathogens in source water provides an important means of safeguarding drinking water (where viruses are often too dilute to be detected). Because disinfection processes are required to reduce the amount of pathogens in drinking water by four orders of magnitude or more, higher concentrations of pathogens in source water can indicate lower levels in drinking water.

pH, turbidity, and color data were collected for both CDW and WRW. These common water quality parameters were used to further characterize the water samples. Eight 10 L samples were taken from each source, corresponding to a sample for a DNA virus/blank, an RNA virus/blank, and two duplicates of those experiments. There was little variation in the

turbidity and color between the duplicate samples of CDW, 0.55±0.08 ntu and 16±0.8 color units, respectively. Although there was some slight variation, little difference could be seen between the duplicate source water samples, 0.46±0.1 ntu and 18±2 color units. The pH of all the samples was between 6.5 and 7. These numbers are similar to those obtained by Susan Murcott in her study of the reservoir a year earlier. These samples were very different, however, in their ability to inhibit PCR. The most noticeable difference was the flow rate of the water through the filter, indicating that many small particles were present in the reservoir water which had been removed by the drinking water treatment process. Furthermore, when visualized on a PAGE gel much more DNA and RNA are present in the reservoir water (compare Figure 26 and Figure 27) than in the drinking water samples. Again, this difference can likely be attributed to removal of the microorganisms in the treatment process.

First, two 10 liter samples of CDW were collected and dechlorinated. Then, 1000 phi-X174 were spiked into one of the samples. The samples were concentrated using the complete procedure described in Chapter 5.2.3. The final volume of the samples was 40 µL (250,000x concentration factor). Then 30%, 10%, and 1% of both the blank and spiked sample were subjected to 40 cycles of PCR as described in Chapter 5.2.3 using an annealing temperature of 60°C and the PB1PB2 primer set. No signal was detected in the concentrated, unspiked sample, while both the 30% and 10% dilution of the concentrated, spiked sample provided signal. Thus, a 100 DNA viruses per liter detection limit has been achieved (see Figure 24 and Table 22).

In order to test further the limits of detection, four 10 liter samples of CDW were seeded as follows: 0, 0, 100, and 1000 phi-X174. Again, these

samples were filtered and extracted based on the developed protocol. The final volume of the concentrated samples was approximately 50 μL (200,000x concentration factor). A dilution series of each concentrated sample (1x, 0.1x, and 0.01x) was subjected to 40 cycles of PCR as described above. Neither of the two blanks produced amplified product. All three of the dilutions from the 1000 phage spike produced the appropriate fragment upon amplification. The 100 phage spiked produced a band only in the 10 times dilution. The detection limit was thus 10 DNA viruses per liter of drinking water (Figure 25 and Table 23). The signal from 10 phi-X174 is not as strong as it is for 100 phi-X174 per liter even at 10 times dilution. This suggests that even though the same number of copies of a sequence are present (i.e., in a 0.1 dilution of 1000 phage per liter or the full sample containing 100 phage per liter) the concentration of the environmental sample is an important factor. Sampling 10% or 20% of a sample is acceptable because the sample can be divided between several PCR tube to test the entire sample. When only 1% of the sample is used this is not possible.

Next, MS2 phage was tested in a similar fashion. Two 10 liter samples of CDW were collected and dechlorinated. Then, 1000 MS2 were spiked into one of the samples. The samples were concentrated using the complete procedure described in Chapter 5.2.3. The final volume of the samples was 25 μL (400,000x concentration factor). Then, 70%, 20%, 10%, and 1% of both the blank and spiked sample were subjected to 40 cycles of PCR as described in Chapter 5.2.3 using 60°C annealing temperature and the MSA1MSB2 primers. No signal was detected in the blank. The 70% dilution and 1% dilution of the spiked sample did not provide signal, while both the 20% and 10% dilution of the spiked sample did provide signal. This provides a 100

RNA viruses per liter of drinking water detection limit. Again, the full strength sample (70%) does not allow amplification likely because of the same reasons discussed for DNA detection. Attempts to detect 10 RNA viruses per liter have failed. Perhaps this is the result of RNA degradation in the concentrated sample, or simply that the RT enzyme has a lower activity in the more concentrated sample (see Figure 26 and Table 24) In this research, the reverse transcriptase enzyme was found to be more susceptible to inhibition than *Taq*. This is not surprising in that RT enzymes are known to be sensitive to salt and buffer conditions in the reaction mixture.

Initial attempts to detect DNA and RNA viruses in source water failed. In several attempts to spike 1000 phi-X174 and 1000 MS2 into the same 10 liter samples of WRW, no amplification was detected after 40 PCR cycles. The concentrates were found not to support PCR even when diluted by three orders of magnitude. Two 10 liter samples of Wachusett Reservoir water (WRW) were obtained. One of the samples was spiked with 10^4 phi-X174 and 10^4 MS2 and the other was used as a blank. The samples were concentrated as described in Chapter 5.2.3 to 250 μL (40,000 times concentration factor). For phi-X174, 16%, 2%, and 0.2% and for MS2, 6%, 2%, and 0.2% of the concentrated sample were subjected to 40 cycles of PCR using primer set PB1PA2 for phi-X174 and MSA1MSB2 for MS2 using a 60°C annealing temperature. No amplification product was observed for any of the dilutions for either phage or for the blank.

It was hypothesized that a "double PCR" using nested primers might be able to detect virus in the concentrated samples if amplification had not been completely inhibited, but instead occurred at low efficiency. Nested primers

would provide a means to reduce interference from non-target sequences that may have been co-amplified in the first PCR amplification.

Therefore, 2 μL of each sample were added to a fresh reaction mixture tube containing nested primers, and amplified for an additional 40 cycles. Primer set PA1PB2 in Buffer C' was used for phi-X174 and MSA1MSA2 in Buffer C was used for MS2. After this amplification, all the dilutions of the spiked 10 L sample became positive with the appropriate amplification product from phi-X174, while the phi-X174 blanks remained negative (see Figure 27 and Table 25). All of the dilutions of the MS2-spiked samples also became positive, but so did the MS2 blanks (see Figure 28 and Table 26). Thus, either a relative of MS2 phage exists naturally in the environment, or the MS2 primers are not selective enough. As well, a higher molecular weight non-target sequence was also amplified in the MS2 blank and spiked sample.

Figure 24: Detection of Spiked Phi-X174 in Cambridge Drinking Water

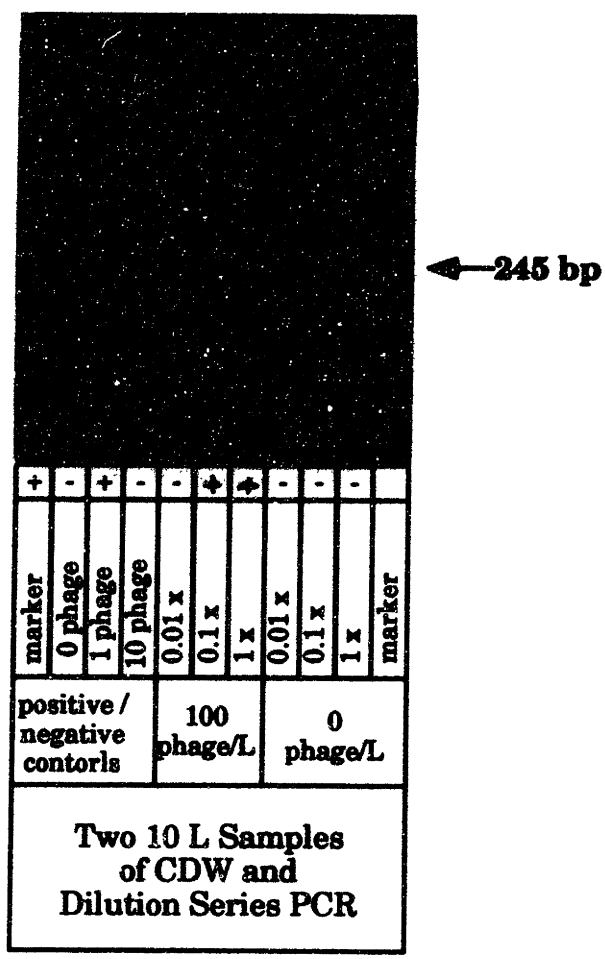


Table 22: Detection of Spiked Phi-X174 in Cambridge Drinking Water

Initial Spike into 10 L of CDW*	% of Sample used**	# of Phage in PCR Tube †	Band?	Recovery
1000 PFU	30%	150	+	**100 PFU/L**
	10%	50	+	**100 PFU/L**
	1%	5	-	
0 PFU	30%	0	-	
	10%	0	-	
	1%	0	-	
	pos. control	10	+	
	neg. control	0	-	

* CDW = Cambridge drinking water
**200,000 times concentration 10 L to approximately 50 µL
RMU to Centriprep to extractions to Centricon to Microcon to PCR (40 cycles)
† assumes 50% recovery efficiency

Figure 25: Detection of Spiked Phi-X174 in Cambridge Drinking Water

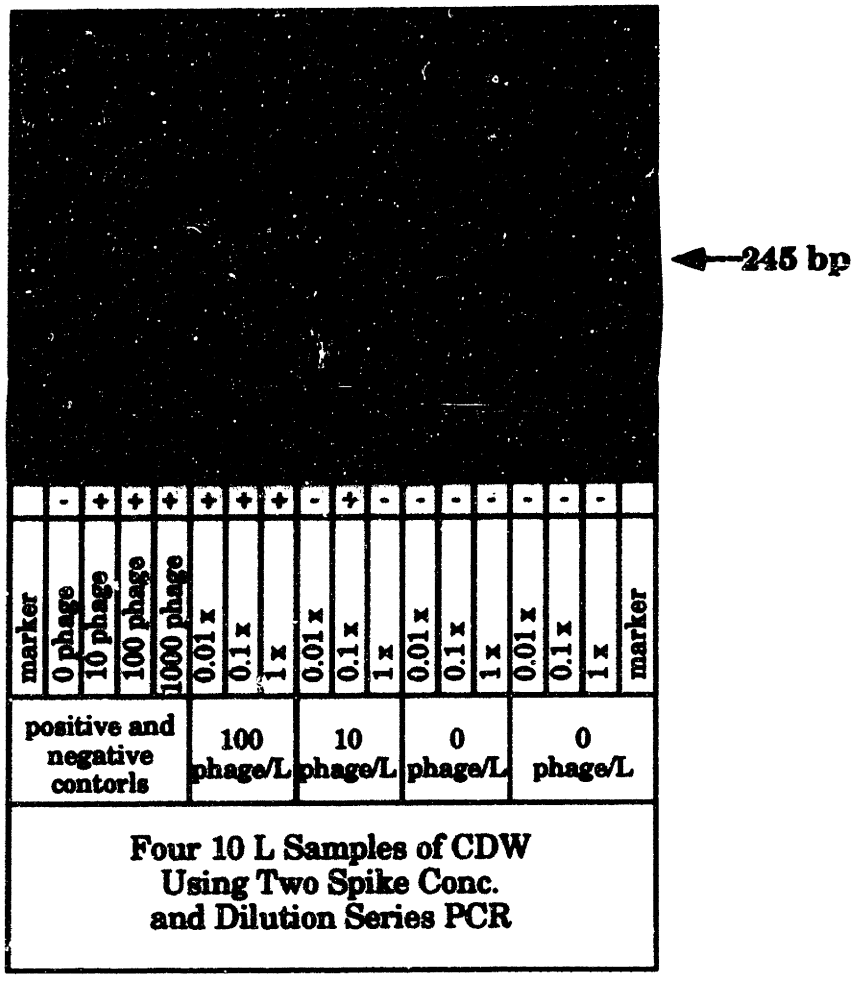


Table 23: Detection of Spiked Phi-X174 in Cambridge Drinking Water

Initial Spike into 10 L of CDW*	% of Sample used**	# of Phage in PCR Tube †	Band?	Recovery
1000 PFU	90%	450	+	**100 PFU/L**
	10%	50	+	**100 PFU/L**
	1%	5	+	**100 PFU/L**
100 PFU	90%	45	-	
	10%	5	+	**10 PFU/L**
	1%	0.5	-	
0 PFU	90%	0	-	
	10%	0	-	
	1%	0	-	
0 PFU	90%	0	-	
	10%	0	-	
	1%	0	-	
	pos. control	10	+	
	neg. control	0	-	

* CDW = Cambridge drinking water
**200,000 times concentration 10 L to approximately 50 µL
RMU to Centriprep to extractions to Centricon to Microcon to PCR (40 cycles)
† assumes 50% recovery efficiency

Figure 26: Detection of Spiked MS2 in Cambridge Drinking Water

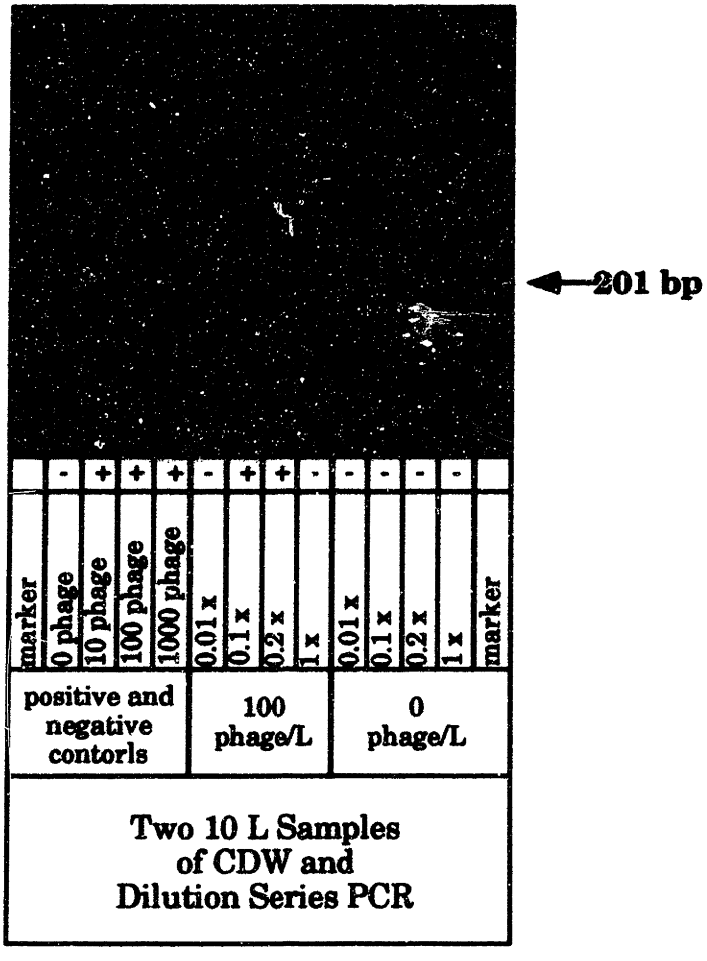
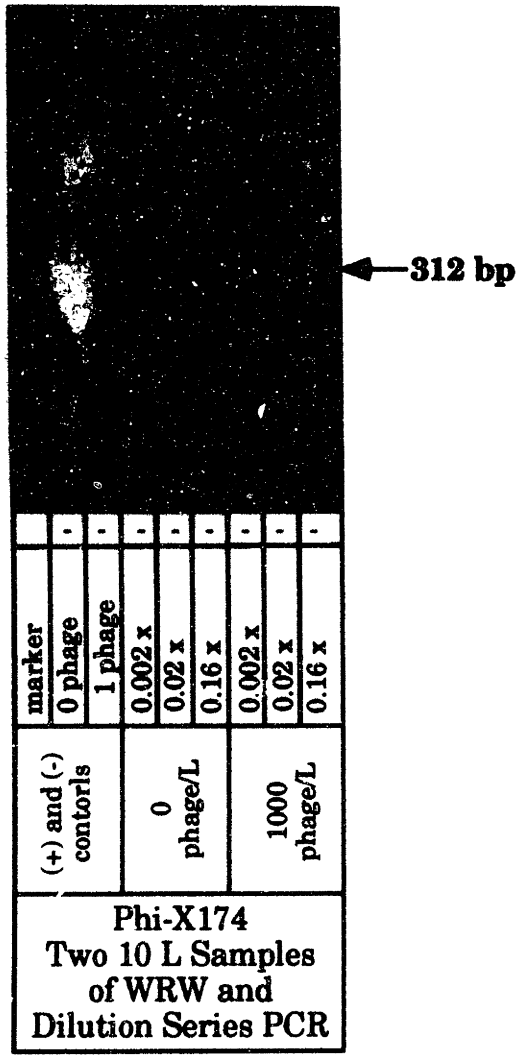


Table 24: Detection of MS2 in Cambridge Drinking Water

Initial Spike into 10 L of CDW*	% of Sample used**	# of Phage in PCR Tube †	Band?	Recovery
1000 PFU	68%	340	-	
	20%	100	+	**100 PFU/L**
	10%	50	+	**100 PFU/L**
	1%	5	-	
0 PFU	68%	0	-	
	20%	0	-	
	10%	0	-	
	1%	0	-	
	pos. control	10	+	
	neg. control	0	-	

* CDW = Cambridge drinking water
**200,000 times concentration 10 L to approximately 50 µL
RMU to Centriprep to extractions to Centricon to Microcon to PCR (40 cycles)
† assumes 50% recovery efficiency

Figure 27: Detection of Spiked Phi-X174 in Wachusett Reservoir Water



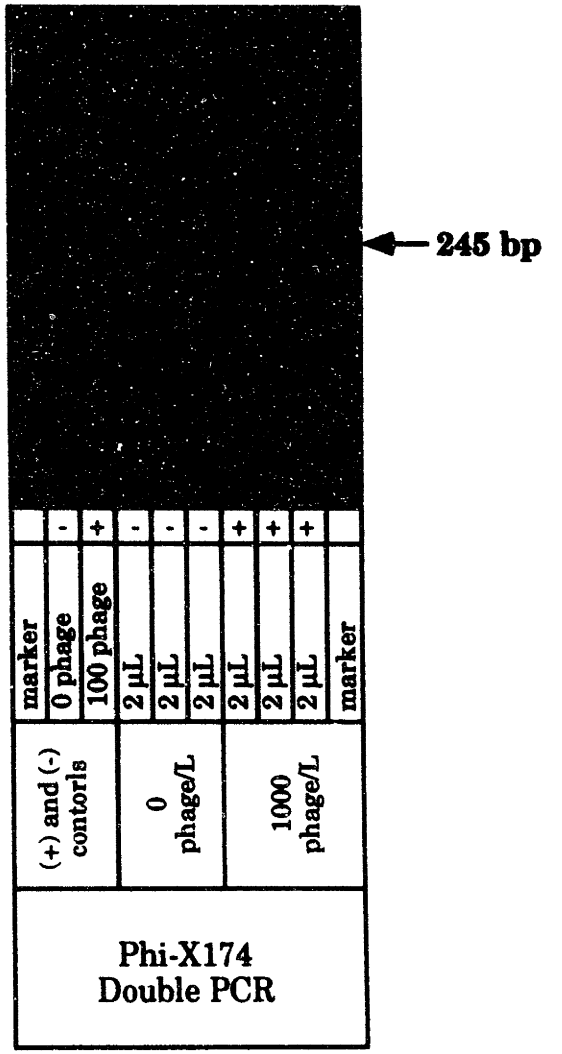


Table 25: Detection of Spiked Phi-X174 in Wachusett Reservoir Water

	Initial Spike into 10 L of WRW*	% of Sample used**	# of Phage in PCR Tube	Band ?	Recovery
	PFU		†		
DNA	10^4	16%	800	-	
		2%	100	-	
		0.2%	10	-	
	0	16%	0	-	
		2%	0	-	
		0.2%	0	-	
		pos. control	1	-	
		neg control	0	-	
DNA	10^4	2 µL	n/a	+	**10^3 PFU/ L**
		2 µL	n/a	+	**10^3 PFU/ L**
		2 µL	n/a	+	**10^3 PFU/ L**
	0	2 µL	n/a	-	
		2 µL	n/a	-	
		2 µL	n/a	-	
		pos. control	100	+	
		neg control	0	-	

* WRW = Wachusett Reservoir water
**40,000 times concentration 10 L to 250 µL
RMU to Centriprep to extractions to Centricon to Microcon to PCR (40 cycles)
† assumes 50% recovery efficiency
note: an aliquot of each tube from the previous experiment was subjected to 40 additional cycles of PCR using an internal nested primer

Figure 28: Detection of Spiked MS2 in Wachusett Reservoir Water

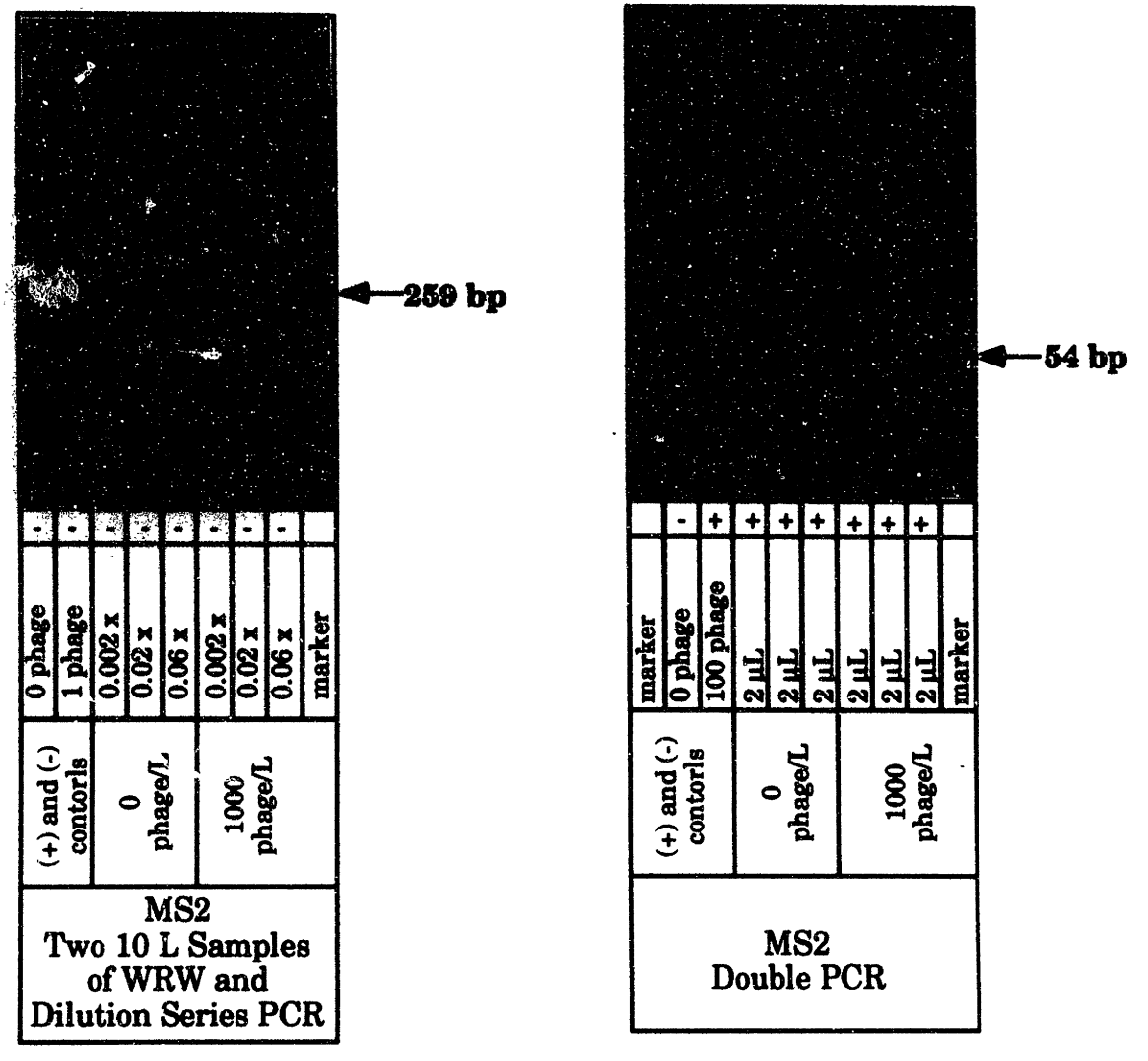


Table 26: Detection of Spiked MS2 in Wachusett Reservoir Water

	Initial Spike into 10 L of WRW*	% of Sample used**	# of Phage in PCR Tube	Band ?	Recovery
	PFU		†		
RNA	10^4	6%	300	-	
		2%	100	-	
		0.2%	10	-	
	0	6%	0	-	
		2%	0	-	
		0.2%	0	-	
		pos. control	1	-	
		neg control	0	-	
RNA	10^4	2 µL	n/a	+	
		2 µL	n/a	+	
		2 µL	n/a	+	
	0	2 µL	n/a	+	
		2 µL	n/a	+	
		2 µL	n/a	+	
		pos. control	100	+	
		neg control	0	-	

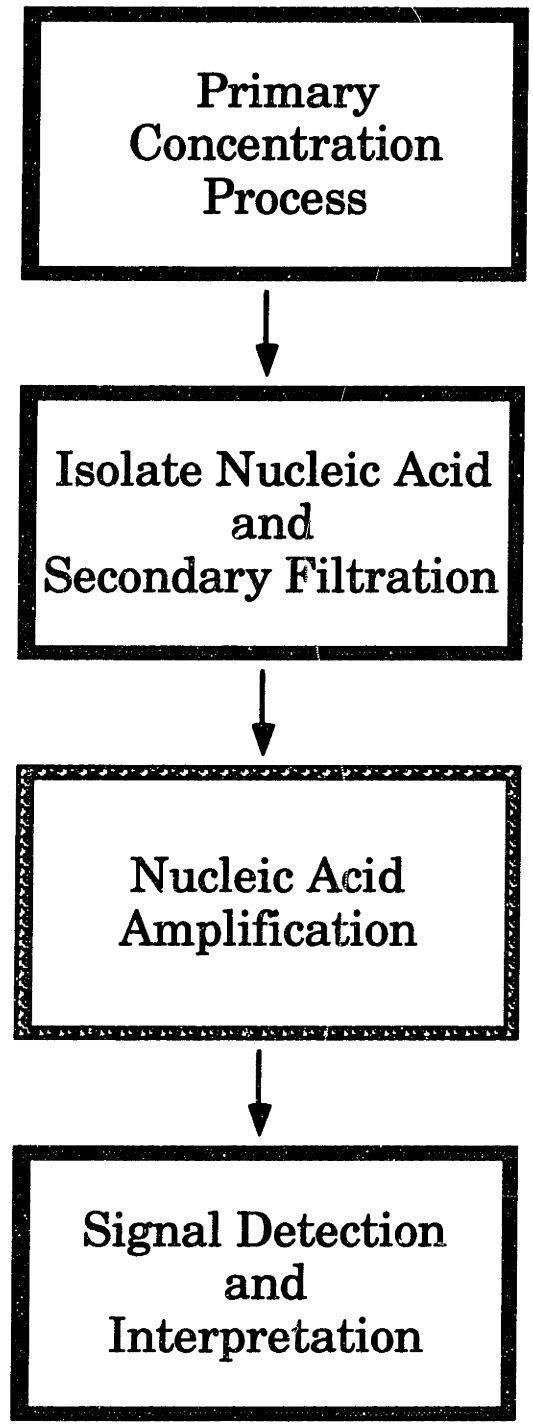
* WRW = Wachusett Reservoir water
**40,000 times concentration 10 L to 250 µL
RMU to Centriprep to extractions to Centricon to Microcon to PCR (40 cycles), nested PCR (40 cycles)
† assumes 50% recovery efficiency
note: an aliquot of each tube from the previous experiment was subjected to 40 additional cycles of PCR using an internal nested primer

6. Conclusions

The coupling of rotating membrane ultrafiltration (RMU) and the polymerase chain reaction (PCR) as a means to concentrate and detect microorganisms in environmental sources has been accomplished. Ultimately 10 DNA viruses per liter and 100 RNA viruses per liter could be detected in spiked 10 L samples of Cambridge drinking water. Furthermore, a concentration of 1000 DNA viruses per liter that had been spiked into 10 liters of Wachusett Reservoir water could be detected by PCR. In similar tests using an RNA virus, both spiked and unspiked samples gave positives. In both types of water samples, more than 10% of the sample was assayed. A description of the concentration and detection schemes is given in Chapters 3 and 4, respectively, while the results of these experiments are given in Chapter 5.

A protocol has been developed that allows for the efficient concentration of the smallest viruses from both drinking water and source water. This protocol provides a concentrated sample which is amenable to PCR detection. RMU has been used to quickly and efficiently concentrate 10 liter volumes of two environmental sources; but, 10 liters is not a limit, larger volumes could also be filtered. The protocol then calls for a number of successive filtrations and extractions, which are essential, using common laboratory apparatus and reagents. These secondary concentration steps were crucial to providing a sample that was not inhibitory to PCR and yet had a good recovery efficiency. Finally, PCR is used as a means of detection and PAGE is used for visualization of results. The protocol is shown schematically in Figure 29.

Figure 29: Box Model of Detection Scheme



This research provides the basis on which can be built a detection scheme for a variety of important pathogens. The basic research is now complete and work on enhancing the system can begin.

Because RMU uses size separation and because the smallest viruses were tested in this research, other microorganisms can be expected to be recovered with equal or greater efficiency. Also, because the secondary concentration steps do not require the growth of target organisms or that any special physical or chemical properties be possessed by these organisms, no amendments need to be made to the protocol in order to include additional microorganisms of interest. Finally, PCR can be used to detect a variety of microorganisms requiring only that specific primer sets be available for each target organism.

The goal of this research was to develop a new method to detect low concentrations of microorganisms in the environment and this has been accomplished. Once in place, the system can be expanded to test for viral, bacterial, and protozoal pathogens, thus eliminating the need to culture indicator bacteria or pathogens. Importantly, the need for an indicator organism is eliminated; the pathogen itself is detected and the often undependable correlation between indicator and pathogen is avoided. Furthermore, indicator organisms also can be monitored, using the developed technology, alongside the pathogens of interest. The need for the inclusion of indicator organisms is two-fold. First, indicators are present at high concentrations and their presence does provide a warning signal. Secondly, they are familiar and widely used. Primer sequences for *E. coli* can be developed as well as primer sequences for fecal-associated bacteriophage.

Monitoring several important pathogens and indicator organisms simultaneously offers a significant improvement over the current system of fecal coliform.

The research described in this thesis provides a novel approach to detecting microorganisms in the environment and overcomes problems encountered in adsorption-elution filtration and gene probe or cell culture detection. The need for this detection method exists because an adequate method of monitoring environmental water sources for pathogens is not currently available. The culturing of pathogens cannot be used as a means of routinely monitoring water for pathogens, especially non-drinking water samples. Many pathogens cannot be grown; as well, they can enter viable, non-culturable states in the environment which make them undetectable by culturing. Most importantly, there are too many pathogens that would need to be monitored, each of which requires a different method for detection. This is clearly impossible. Gene probes offer a means to avoid the multiple tests required by culturing, but the high threshold of organisms needed for detection (even in the absence of an environmental concentrate) limits the use of the methodology. The only requirement for PCR detection is different primer sequences for the different pathogens. Once a library of primer sequences, which do not cross-react and are highly specific, are developed, a variety of pathogens or groups of pathogens can be monitored. As an example, all *Salmonella* could be detected by group-specific primers, while the species of *Salmonella* could be determined using nested amplifications of the original PCR amplification product.

Because RMU relies on sized-based separation and not chemical, biological, or other physical properties, water quality parameters and

particular characteristics of each virus do not affect recovery. Adsorption-elution cannot be optimized for consistent recoveries of multiple microorganisms from drinking water sources, much less from environmental samples. Further, protozoans, bacteria, and viruses can be concentrated simultaneously by RMU, which is not the case for adsorption-elution technology. These are important considerations with regard to the simultaneous detection of multiple pathogens.

This research has shown that RMU has proved effective at concentrating two model viruses in fresh water, drinking water, and various buffers. The ability to concentrate viruses is not dependent on water quality or on the type of virus or bacteria being concentrated. Relatively high recoveries were consistently achieved. In fact, little of even the smallest viruses (20 nm) is found in the filtrate after an RMU concentration.

RMU provides a rapid (15 L/hr untreated, 30 L/hr treated by flocculation/filtration) means of efficiently concentrating viruses from environmental samples. In future research, a larger pore filter (0.2 μm) should be used to concentrate and detect bacterial or protozoal organisms (without the viruses). The benefit being the faster flow rates as well as less material in the concentrate. Because the process is efficient and reproducible, the volume of the water sample need not be excessively large (100 liters). Negatively charged adsorption-elution filters require the pre-treatment of the sample, and for large volume samples this is onerous and costly. RMU requires no such amendments and, furthermore, it is not restricted in use by the pH, humic material content, and water quality parameters of the sample as is a positively-charged adsorption-elution filter. The only variation between different environmental samples was flow rate.

These characteristics make RMU an appealing means of processing water for pathogen detection.

Research concerning secondary filtration and extractions has revealed that: the use of Amicon filters along with (1) proteinase K digestion, (2) CTAB extraction, and (3) phenol:chloroform extractions provide a fast and efficient way of recovering both DNA and RNA viruses and a bacterium from potable water samples, and for DNA-based organisms in all environmental samples. The protocol that has been developed in this research provides a means of quickly and efficiently concentrating environmental samples with no need for amendments in order to detect additional pathogens. This is not true of the elution and organic flocculation steps in the adsorption-elution technology for which water quality and virus type are variables. No ultracentrifugation, growth of pathogens, or radioactivity is required in this procedure, allowing the technique to be useful to a broad range of possible users. Although CTAB, phenol, and chloroform are caustic chemicals, they are common in the laboratory and do not require highly skilled technicians for their use.

As mentioned above, PCR has been used in this research to show that, in treated drinking water, 100 RNA viruses per liter and 10 DNA viruses per liter have been recovered. In source waters to be used for drinking water, 1000 DNA virus per liter have been detected. Attempts to detect 1000 RNA viruses failed. It was hypothesized that the reverse transcriptase enzyme is much more sensitive to inhibition than *Taq* polymerase, because RT enzyme is very sensitive to buffer conditions, chelators, and metals. These experiments were conducted by spiking into a 10 L volume, concentrating the sample, and assaying all of the concentrate for drinking water and between 10% and 20% of the concentrate for environmental samples. This is

significant because RMU provides a concentrated sample that, after the developed secondary concentration process, allows successful amplification by PCR using a significant portion of the sample. This is true even when only a few target microorganisms are present. In the case of the adsorption-elution concentrate, the observed lower recoveries and failure of PCR (when significant portions of the sample are assayed) are likely due to sorbed humic material or other small charged molecules. Although the presence of humic material does not affect RMU recovery, the use of RMU and the secondary filtrations allow many of these small particles to pass through the filter, reducing the chance of inhibition in the detection scheme. Some problems with inhibition of PCR from non-drinking water environmental concentrates have been noted (and are discussed in Chapter 4.4.3), but nested "Double PCR" has proved effective in overcoming the interference in the detection of DNA viruses. DNA is detectable in large part because *Taq* polymerase is noted for sustaining activity under a broad range of buffer conditions. This first round of PCR allows a means to enrich for the target fragment, but not for amplification to a detectable level. The second round of PCR using nested primers provides an easily detectable product.

This research is unique in its ability to recover low numbers of viruses in large volumes of water due in part 1) to the filtration/extraction protocol that is used in conjunction with RMU and 2) to the development of unique primer sequences and proper reaction mixture conditions. No other research group has spiked in and recovered this level of virus using adsorption-elution and gene probe technology. Adsorption-elution combined with cell culture has (periodically, but not consistently) achieved similar results by filtering large quantities of contaminated drinking water. These results are inconclusive

because no recovery efficiencies were determined for the given water body and for the given virus. More importantly, cell culture is limited in its ability to detect many important pathogens and thus, does not provide a conservative means of monitoring water sources to which people may be exposed. In addition, the number of potential users of this technology is limited to those with well-equipped laboratories. The expense and difficulty of the procedure limit its use for anything more than well-funded surveys, not routine monitoring. Finally, results are not available in a timely fashion; an outbreak would be detected weeks after it had occurred. Because PCR does not require the growth of pathogens, but amplifies DNA and RNA (which is the same for all organisms except for sequence), there is no individuality between pathogens in regard to their ability to be detected. Furthermore, results can be obtained within one day.

This technique provides a means to detect low levels of virus in drinking water and source water samples. Although no method is available which can detect pathogens at concentrations calculated to result in 1 infection in a population of 10,000 per year (approximately 1 rotavirus per 10,000,000 L) given the dose-response model described in Chapter 2. Thus, either a new dose-response model will need to be developed that makes less conservative assumptions (e.g., obtain real data, which is likely not to be as conservative as the assumptions), or this level of detection will be unattainable. Thus, although routine monitoring cannot reduce low level exposure, it can be used to reduce exposure lower than current methods allow The EPA has not subscribed to this model to the author's knowledge, but the β-distributed dose-response model has been widely discussed in the literature. Accordingly, the EPA has not mandated a level of acceptable virus based on

this model. It should be noted that currently, coliform is the sole means of monitoring drinking water, which most certainly does not ensure a level of 1 in 10,000 annual risk of infection.

From Table 27, it can be seen that monitoring drinking water directly for pathogens would only be appropriate for a pathogen like *Vibrio cholera* for which this methodology can detect a level of pathogen that results in a 1 in 10,000 annual chance of infection. This would be important for several under-developed countries, like Peru, where recent out-breaks have occurred. The table was calculated under the assumption of twice weekly samplings of 10 liters. It should be noted that this table gives the chance of infection not the probability of disease. Importantly, disease from virus infection often occurs in 1% to 10% of those infected for many pathogens. Furthermore, in the table, enterovirus is measured in PFU and rotavirus in FFU which can contain 100 viral particles or more. Thus, because the detection scheme developed in this research is more sensitive than cell culture and immunological techniques (limited to 1 PFU as opposed to 1 viral particle), the numbers calculated in Table 27 should be reduced in order to account for units. Directly monitoring drinking water will only be useful for the detection of an outbreak of disease and will not be able to detect low levels of pathogens (1 in 10,000,000 liters); however, most large water-borne outbreaks occur due to a failure in the distribution system and pathogen levels are much higher (see Chapter 2).

Because source water is EPA-mandated to have a 10,000 fold reduction in pathogens through treatment before it can be distributed, the sensitivity of detection needed for source water is lower by four orders of magnitude than that for treated water. Furthermore, twice weekly testing of a 10 liter sample

Table 27: Expected Number of Yearly Infections (not Disease) in Various Communities

Organism	Village 100	Town 10,000	City 1,000,000
Campylobacter	20	2,000	200,000
Salmonella	8	800	80,000
Vibrio cholera	<1	2	200
Poliovirus 1	100	10,000	1,000,000
Rotavirus	100	10,000	1,000,000
Giardia	40	4,000	400,000

The table was calculated using a detection limit of 10 viruses per liter for DNA and 100 viruses per liter for RNA considering a twice weekly monitoring schedule for drinking water.

Organism	Village 100	Town 10,000	City 1,000,000
Campylobacter	<1	200	2,000
Salmonella	<1	8	800
Vibrio cholera	<1	<1	2
Poliovirus 1	*	*	*
Rotavirus	*	*	*
Giardia	<1	700	7,000

The table was calculated using a detection limit of 1000 viruses per liter for DNA considering a twice weekly monitoring schedule for source water which will undergo the mandated 10,000 fold reduction in pathogen concentration.

Figure 30: Yearly Risk of Infection and Drinking Water Detection Limits

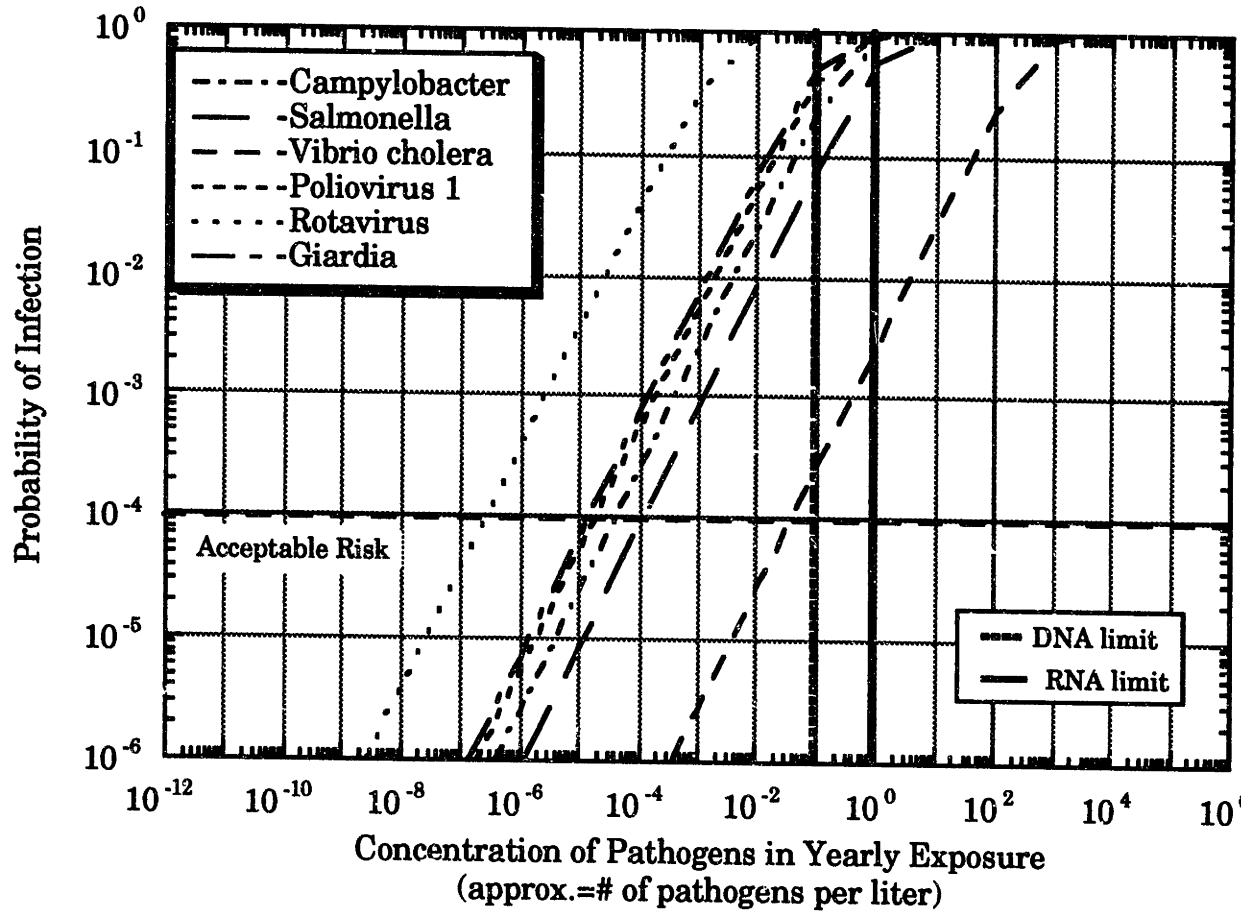
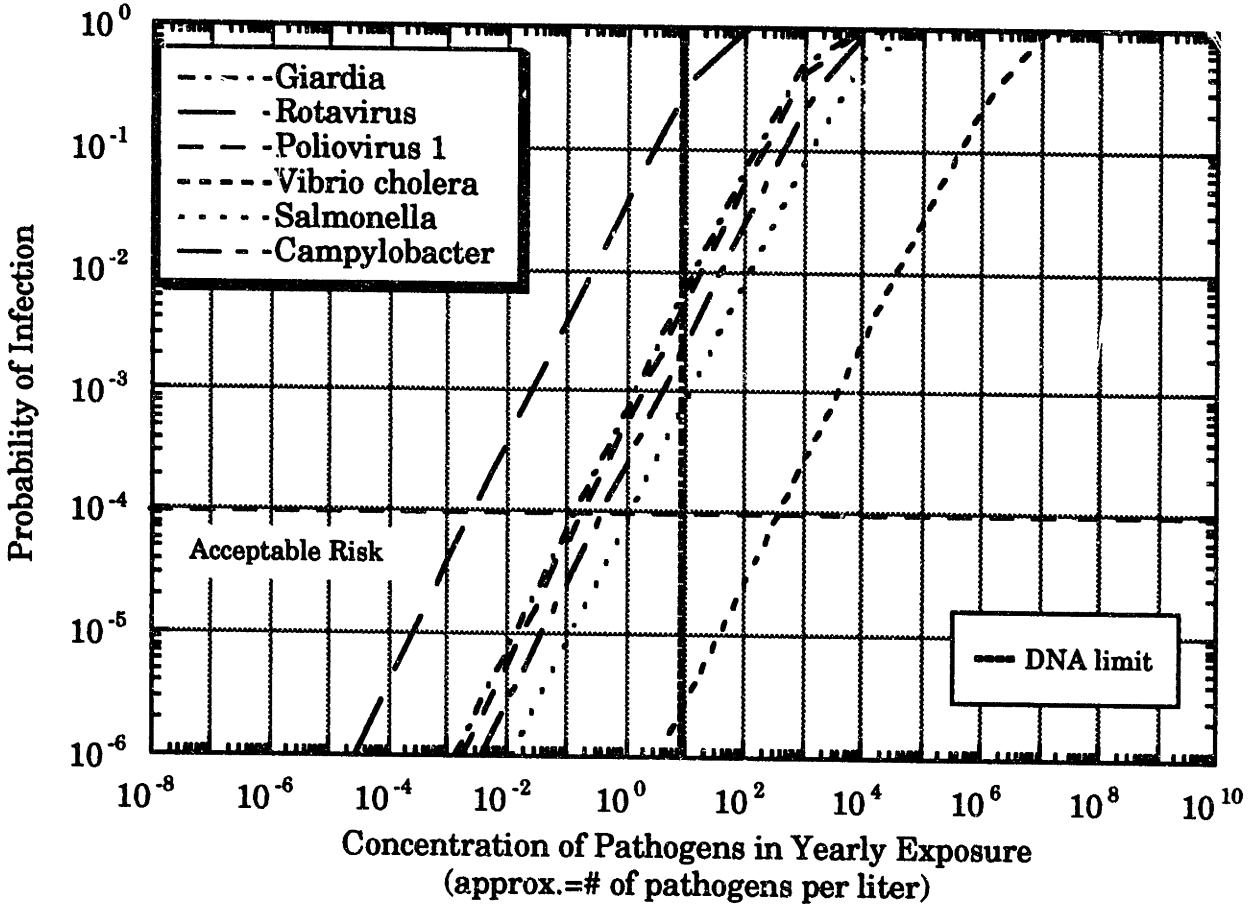


Figure 30: Yearly Risk of Infection and Source Water Detection Limits



provide sufficient safeguards against a number of pathogens (see below), and would not be difficult to do. By including indicator organisms, which would be present at much higher levels, this detection scheme would provide an additional level of safety over that which is currently available.

Different communities would need different levels of protection. A village of 100 people does not need to be protected from 1 virus in 1,000,000 liters as they will not consume that much water. Figure 30 and Figure 31 were used to calculate the data presented in Table 27 and was based on the assumption of the consumption of 2 L of water per day for a year.

Levels of virus in polluted environmental samples should be detectable by this methodology. In a recent study, levels of pathogens detected by cell culture at a sewage polluted site in Mamala Bay, Hawaii would have been detectable using this methodology (Gerba, 1994). *Giardia* and enterovirus were present at a concentration of about 2000 per liter. This technology would have provided a useful alternative to culturing viruses and pathogens, and perhaps additional no-culturable pathogens could have been detected as well. This technique would be useful for taking 10 L grab samples from surface water, groundwater, or ocean water sites thought to be polluted by sewage effluent. Because the concentrated sample is adjusted to 0.7 M NaCl in the secondary concentration steps, the salt present in ocean water would not be expected to provide any difficulty.

The developed methodology can be used to detect pathogens at low concentrations in the environment as a means of discovering the pathways of exposure for pathogens that are thought to be water-borne. Furthermore, after an outbreak of disease or in an area with a high incidence of infection,

water sources could be monitored. The ability to quickly process large volume samples for multiple pathogens would facilitate epidemiological studies. Many pathogens which are thought to be water-borne have not been epidemiologically linked to an outbreak. This method can be useful in a public health evaluation after an outbreak where exposure could be traced to several possible sources. Currently, only large outbreaks can be traced, and disease resulting from lower level contamination is untraceable because it goes largely unreported.

As amazing as it may seem, no one has reported levels of *Cryptosporidium* in Milwaukee drinking water that cause the March/April 1993 outbreak, which caused illness in 400,000 of 1.6 million people in a five county area [Gradus, 1994 #530]. Certainly, current indicators failed and no current technology has yet detected pathogens in the water. Because there are almost no published reports of recovery of pathogens from drinking water that has not been spiked, it is difficult to determine whether RMU/PCR technology could be useful in preventing outbreaks such as the one that occurred in Milwaukee. Perhaps tracing the source of pollution either up or down gradient in order to determine the source or fate, respectively, of contamination.

There are several potential users of this technology, most obviously water treatment plant operators. The ability to monitor drinking water and the incoming source water for pathogens would be important. Despite the fact that the level of virus associated with an acceptable risk of 1 infection in 10,000 per year cannot be detected, the developed methodology outperforms the current indicator system. If the Pacesetter RMU is used to achieve higher flow rates in drinking water, this procedure would be more useful for

the direct monitoring of drinking water. In addition, disinfection efficiency of the treatment process for a variety of pathogens could be determined using this methodology.

The ability to monitor pathways for exposure would be useful to epidemiologists. The ability to determine the presence of pathogens in a water body would establish a link to any observed disease. As mentioned above, many pathogens have not been proven to be water-borne because they have not been recovered from environmental samples.

Microbial ecologists would find a use for this technology. The ability to monitor for the presence of genetically-engineered microorganisms or for the presence of a particular gene in a population of microorganisms from different water bodies would be particularly useful. (i.e., studying the water column in addition to the sediments where bacterial densities are higher). Microbial ecological examinations outside of soils and sediments would be facilitated.

The concentration of 10 liters of water should take between 20 and 40 minutes depending on the source of the sample, while the lab work would take around one day with much less hands-on time than the traditional methods. Large volumes can be filtered on the Benchmark unit, although filtration times become longer (e.g., the final liter of a 20 liter volume of CRW had a flow rate approaching 9 liter per hour). However, the most time-consuming steps in the procedure are filtration and amplification, which require minimal supervision. Also, it should be noted that several samples can be processed at one time and multiple organisms can be assayed from one sample. The protocol is inexpensive, and savings will be recognized once a

streamlined method is developed so that all the reagents are prepackaged. One can foresee disposable tubes containing the digestion and extraction chemicals to which the sample would be added, mixed, incubated, and recovered. Furthermore, pre-made reaction mixtures could be produced which contain the appropriate primers and to which the sample can be added and subjected to PCR. Finally, no radioactive and extremely caustic materials are used, and the methodology is straightforward so that specialized lab technicians are not required.

For regular monitoring, a small-volume blank distilled water sample must be run in order to ensure no cross-contamination occurs. As a positive control, phi-X174 could be spiked into any sample to be tested. This would ensure proper recovery and would be a control on PCR inhibition, and yet would not add significantly to the work required.

Several tasks must be accomplished before this technology can be universally accepted. Primer sequences that can be used to multiplex, or amplify several sequences at once in the same reaction mixture tube, must be developed. Primer sequences capable of amplifying several important groups of pathogens and indicators would be ideal. Because pathogens are likely to be present in low concentrations, only a single type of pathogen would be expected in a given sample unless massive contamination was present. Thus, the need to multiplex is less significant than the need for primers that do not cross-react and that are highly specific. Importantly, RNA detection in source water should be pursued. Perhaps an initial DNA degrading step would allow for recovery of RNA, if the RT enzyme was swamped by the amount of nucleic acid. Then again, perhaps the concentrate salt levels must

be analysed as RT enzymes are very sensitive to variations in reaction mixture conditions.

In conclusion, the ultrafiltration unit has been demonstrated with two viruses to allow high recoveries. Because phi-X174 and MS-2 are among the smallest viruses and because RMU uses size-based separation, larger viruses can be expected to have equal or greater removal efficiency. A Membrex ultrafiltration unit is commercially available which can achieve flow rates of 100 liters per hour by increasing filter surface area and operating at higher pressures. Thus, from all of the results to date, RMU can provide a powerful tool in filtering environmental samples. It would be interesting to test adsorption/elution techniques against RMU under various sampling scenarios.

The developed methodology allows one or a few pathogens to be detected (viable or not) from a host of pathogens that may be present, but impossible to detect . In other words, a single pathogen would be effectively hidden amongst the plethora of pathogens that could be present, much like a needle in a haystack. The knowledge of which pathogen is present allows more expensive and time-consuming tests to be performed for additional information, but only for one pathogen.

BIBLIOGRAPHY

Abbaszadegan, M., Huber, M. S., Gerba, C. P., & Pepper, I. L. (1993). Detection of enteroviruses in groundwater with the polmerase chain reaction. *Applied and Environmental Microbiology*, **59**(5), 1318-1324.

Akopyanz, N., Bukanov, N. O., Westblom, T. U., & Berg, D. E. (1992). PCR-based RFLP analysis of DNA sequence diversity in the gastric pathogen *Helicobacter pylori*. *Nucleic Acid Research*, **20**(23), 6221-6225.

Allison, R. C., Durand, R., & Schwebach, G. (1988). Risk: a key water policy issue. *Journal WPCF*, **60**(7), 1211-1213.

APHA (Ed.). (1989). *Standard Methods for the Examination of Water and Wastewater*. Baltimore, MD: Port City Press.

Armon, R., Arella, M., & Payment, P. (1988). A highly efficient second-step concentration technique for bacteriophages and enteric viruses using ammonium sulfate and Tween 80. *Canadian Journal of Microbiology*, **34**(5), 651-655.

Barkay, T., & Sayler, G. S. (1988). *Gene probes as a tool for the detection of specific genomes in the environment*. Philadelphia: ASTM.

Bej, A. K., DiCesare, J. L., Haff, L., & Atlas, R. M. (1991a). Detection of Escherichia coli and Shigella spp. in water by using the polymerase chain reaction and gene probes for uid. *Applied and Environmental Microbiology*, **57**(4), 1013-1017.

Bej, A. K., Mahbubani, M. H., & Atlas, R. M. (1991b). Detection of viable Legionella pneumophila in water by polymerase chain reaction and gene probe methods. *Applied and Environmental Microbiology*, **57**(2), 597-600.

Benbow, R. M., Hutchison, C. A., Fabricant, J. D., & Sinsheimer, R. L. (1971). Genetic map of bacteriophage phi-x174. *Journal of Virology*, **7**(5), 549-558.

Berg, G. (Ed.). (1978). *Indicators of Viruses in Water and Food*. Ann Arbor, MI: Ann Arbor Science.

Berg, G. (Ed.). (1987). *Methods for Recovering Viruses from the Environment*. Boca Raton, FL: CRC Press.

Bisson, J. W., & Cabelli, V. J. (1980). Clostridium perfringens as a water pollution indicator. *Journal WPCF*, **52**(2), 241-248.

Biziagos, E., Passagot, J., Crance, J. M., & Deloince, R. (1989). Hepatitis A virus concentration from experimentally contaminated distilled, tap, waste and seawater. *Water Science and Technology*, **21**(3), 255-258.

Bloch, A. B., Stramer, S. L., Smith, J. D., Margolis, H. S., Fields, H. A., McKinley, T. W., Gerba, C. P., Maynard, J. E., & Sikes, R. K. (1990). Recovery of hepatitis A virus from a water supply responsible for a common source outbreak of hepatitis A. *American Journal of Public Health*, **80**(4), 428-430.

Boardman, G. D., Shannon, R. D., & McBrayer, T. R. (1988). Detection and occurrence of waterborne bacterial and viral pathogens. *Journal WPCF*, **60**(6), 1121-1132.

Borchardt, J. A., Cleland, J. K., Redman, W. J., & Olivier, G. (Eds.). (1977). *Viruses and Trace Contaminants in Water and Wastewater*. Ann Arbor, MI: Ann Arbor Science.

Borrego, J. J., Arrabal, F., de Vicente, A., Gomez, F., & Romero, P. (1983). Study of microbial inactivation in the marine environment. *Journal WPCF*, **55**(3), 297-301.

Borrego, J. J., Moriñigo, M. A., de Vicente, A., Córnax, R., & Romero, P. (1987). Coliphages as an indicator of faecal pollution in water. Its relationship with indicator and pathogenic microorganisms. *Water Research*, **21**(12), 1473-1480.

Borrego, J. J., & Romero, P. (1985). Coliphage survival in seawater. *Water Research*, **19**(5), 557-562.

Bosch, A., Gajardo, R., Abad, F. X., Diez, J. M., & Jofre, J. (1991). Concentration of hepatitis A virus in environmental samples. *Water Science and Technology*, **24**(2), 229-234.

Bosch, A., Pinto, R. M., Blanch, A. R., & Jofre, J. (1988). Detection of human rotavirus in sewage through two concentration procedures. *Water Research*, **22**(3), 343-348.

Bridger, J. C. (1987). *Novel rotaviruses in animals and man*. New York: John Wiley & Sons.

Brock, T. D., & Madigan, M. T. (1988). *Biology of Microorganisms*. Englewood Cliffs, NJ: Prentice-Hall.

Buck, J. D. (1977). *Candida albicans*. Philadelphia: ASTM.

Buck, J. D., & Bubucis, P. M. (1978). Membrane filter procedure for enumeration of Candida albicans in natural waters. *Applied and Environmental Microbiology*, **35**(2), 237-242.

Buras, N., Duek, L., Niv, S., Hepher, B., & Sandbank, E. (1987). Microbiological aspects of fish grown in treated wastewater. *Water Research*, **21**(1), 1-10.

Byrd, J. J., Xu, H. S., & Colwell, R. R. (1991). Viable but nonculturable bacteria in drinking water. *Applied and Environmental Microbiology*, **57**(3), 875-878.

Cabelli, V. J. (1977). *Clostridium perfringens as a water quality indicator*. Philadelphia: ASTM.

Cabelli, V. J. (1978a). *New standards for enteric bacteria*. New York: John Wiley & Sons.

Cabelli, V. J. (1978b). *Obligate anaerobic bacterial indicators*. Ann Arbor, MI: Ann Arbor Science.

Cabelli, V. J., Dufour, A. P., McCabe, L. J., & Levin, M. A. (1983). A marine recreational water quality criterion consistent with indicator concepts and risk analysis. *Journal WPCF*, **55**(10), 1306-1313.

CDC (1990a). Viral agents of gastroenteritis: public health importance and outbreak management. *MMWR*, **39**(RR-5), 1-24.

CDC (1990b). Waterborne disease outbreaks, 1986-1988. *MMWR*, **39**(SS-1), 1-13.

Chang, S. L. (1978). *Zoomicrobial indicators*. Ann Arbor, MI: Ann Arbor Science.

Christensen, M. L. (1989). Human viral gastroenteritis. *Clinical Microbiology Reviews*, **2**(1), 51-89.

CIBA (Ed.). (1987). *Novel Diarrhea Viruses*. New York: John Wiley & Sons.

Clausen, E. M., Green, B. L., & Litsky, W. (1977). *Fecal streptococci: indicators of pollution*. Philadelphia: ASTM.

Craun, G. F. (1991). Causes of waterborne outbreaks in the United States. *Water Science and Technology*, **24**(2), 17-20.

De Leon, R., & Gerba, C. P. (1991). Detection of rotavirus in water by gene probe. *Water Science and Technology*, **24**(2), 281-284.

den Blanken, J. G. (1985). Comparative disinfection of treated sewage with chlorine and ozone. *Water Research*, **19**(9), 1129-1140.

DePasquale, D. A., Law, C. B., & Bissonnette, G. K. (1987). Comparative survival and injury of Candida albicans and bacterial indicator organisms in streams receiving acid mine drainage. *Water Research*, **21**(12), 1525-1530.

Divizia, M., de Filipps, P., di Napoli, A., Gabrieli, R., Santi, A. L., & Panà, A. (1989). HAV recovery from tap water: evaluation of different types of membranes. *Annali di Igiene, Medicina Preventiva e di Comunità*, **1**(1/2), 57-64.

Donnelly, R. J. (1991). Taylor-Couette flow: The early days. *Physics Today*, *, 32-39.

Dubrou, S., Kopecka, H., Lopez Pila, J. M., Maréchal, J., & Prévot, J. (1991). Detection of hepatitis A virus and other enteroviruses in wastewater and surface samples by gene probe assay. *Water Science and Technology*, **24**(2), 267-272.

Dufour, A. P. (1977). *Escherichia coli: the fecal coliform*. Philadelphia: ASTM.

Duncanson, R. A., & Saad, D. L. (1989). *Alternative indicators for the sanitary quality of shellfish resources*. Woods Hole, MA: Woods Hole Oceanographic Institution.

Düreth, S., Herrmann, R., & Pecher, K. (1986). Tracing faecal pollution by coprostanol and intestinal bacteria in an ice-covered Finnish lake loaded with both industrial and domestic sewage. *Water, Air, and Soil Pollution*, **28**(1/2), 131-149.

Dutka, B. J. (1979). *Microbiological indicators, problems and potential of new microbial indicators of water quality*. Chichester, UK: John Wiley & Sons.

Dutka, B. J., & El-Shaarawi, A. (1975). Relationships between various bacterial populations and coprostanol and cholesterol. *Canadian Journal of Microbiology*, **21**(9), 1386-1398.

Dutka, B. J., El-Shaarawi, A., Martins, M. T., & Sanchez, P. S. (1987). North and South American studies on the potential of coliphage as a water quality indicator. *Water Research*, **21**(9), 1127-1134.

Dutka, B. J., Jones, K., Kwan, K. K., Bailey, H., & McInnis, R. (1988). Use of microbial and toxicant screening tests for priority site selection of degraded areas in water bodies. *Water Research*, **22**(4), 503-510.

Emde, K. M. E., Mao, H., & Finch, G. R. (1992). Detection and occurrence of waterborne bacterial and viral pathogens. *Water Environment Research*, **64**(4), 641-647.

Engelbrecht, R. S., & Greening, E. O. (1978). *Chlorine-resistant indicators*. Ann Arbor, MI: Ann Arbor Science.

Enriquez, C. E., Abbaszadegan, M., Pepper, I. L., Richardson, K. J., & Gerba, C. P. (1993). Poliovirus detection in water by cell culture and nucleic acid hybridization. *Water Research*, **27**(7), 1113-1118.

EPA (1993). In *Virus Monitoring Workshop*, . Cincinnati, Ohio: EPA.

Evison, L. M. (1979). Microbial parameters of raw water quality. In A. James & L. Evison (Eds.), *Biological Indicators of Water Quality* (pp. 16:1-19). Chichester, UK: John Wiley & Sons.

Farmer, J. J., & Brenner, D. J. (1977). *Concept of a bacterial species: importance to writers of microbiological standards for water.* Philadelphia: ASTM.

Farrah, S. R., Preston, D. R., Toranzos, G. A., Girard, M., Erdos, G. W., & Vasuhdivan, V. (1991). Use of modified diatomaceous earth for the removal and recovery of virus in water. *Applied and Environmental Microbiology*, **57**(9), 2502-2506.

Feachem, R. G., Bradley, D. J., Garelick, H., & Mara, D. D. (1983). *Sanitation and Disease: Health Aspects of Excreta and Wastewater Management*. New York: John Wiley & Sons.

Figueroa, M. E., & Rasheed, S. (1991). Molecular pathology and diagnosis of infectious disease. *American Journal of Clinical Pathology*, **95**(4, Suppl.1), 442-444.

Frankel-Conrat, H. (1985). *The Viruses: Catalogue, Characterization, and Classification*. New York: Plenum Press.

Freeman, B. A. (1985). *Burrows Textbook of Microbiology*. Philadelphia: W.B. Saunders.

Fujioka, R. S., Hashimoto, H. H., Siwak, E. B., & Young, R. H. F. (1981). Effect of sunlight on the survival of indicator bacteria in seawater. *Applied and Environmental Microbiology*, **41**(3), 690-696.

Fujioka, R. S., & Shizumura, L. K. (1985). Clostridium perfringens, a reliable indicator of stream water quality. *Journal WPCF*, **57**(10), 986-992.

Funderburg, S. W., & Sorber, C. A. (1985). Coliphages as indicators of enteric viruses in activated sludge. *Water Research*, **19**(5), 547-555.

Geldenhuys, J. C., & Pretorius, P. D. (1989). The occurrence of enteric viruses in polluted water, correlation to indicator organisms and factors influencing their numbers. *Water Science and Technology*, **21**(3), 105-109.

Geldreich, E. E. (1976). Fecal coliform and fecal streptococcus density relationships in waste discharges and receiving waters. *Critical Reviews in Environmental Control*, **6**(4), 349-369.

Geldreich, E. E. (1978). *Bacterial populations and indicator concepts in feces, sewage, stormwater, and solid waste*. Ann Arbor, MI: Ann Arbor Science.

GenBank, & EMBL. (Eds.). (1985). *Nucleotide Sequences 1985*. Washington, D.C.: IRL Press.

Gerba, C. P. (1994). *Incidence of pathogens in Mamala Bay* (Project Report No. #2). Mamala Bay Project MB-7.

Gerba, C. P., & Goyal, S. M. (Eds.). (1982). *Methods in Environmental Virology*. New York: Marcel Dekker.

Gerba, C. P., Margolin, A. B., & Hewlett, M. J. (1989). Application of gene probes to virus detection in water. *Water Science and Technology*, **21**(3), 147-154.

Gerba, C. P., Margolin, A. B., Trumper, B., Rose, J. B., & Zhang, C. Y. (1990). Low cost rapid methods for enterovirus detection in water. *unpublished*.

Gerba, C. P., & McLoed, J. S. (1976). Effect of sediments on the survival of Escherichia coli in marine waters. *Applied and Environmental Microbiology*, **32**(7), 114-120.

Gerba, C. P., Rose, J. B., & Singh, S. N. (1985). Waterborne gastroenteritis and viral hepatitis. *Critical Reviews in Environmental Control*, **15**(3), 213-236.

Goyal, S. M., Zerda, K. S., & Gerba, C. P. (1980). Concentration of coliphages from large volumes of water and wastewater. *Applied and Environmental Microbiology*, **39**(1), 85-91.

Guttman-Bass, N., Hostovsky, T., Lugten, M., & Armon, R. (1985). A comparison of current methods of poliovirus concentration from tap water. *Water Research*, **19**(1), 85-88.

Haas, C. N. (1983). Estimation of risk due to low doses of microorganisms: A comparison of alternative methodologies. *American Journal of Epidemiology*, **118**(4), 573-582.

Haas, C. N., Severin, B. F., Roy, D., Engelbrecht, R. S., Lalchandani, A., & Farooq, S. (1985). Field observations on the occurrence of new indicators of disinfection efficiency. *Water Research*, **19**(3), 323-329.

Hahn, T., Karst, M., Tougianidou, D., & Botzenhart, K. (1989). Comparison of methods for concentrating coliphages and enteroviruses in raw and drinking water. *Water Science and Technology*, **21**(3), 259-262.

Hallström, B., & Lopez-Leiva, M. (1978). Description of a rotating ultrafiltration module. *Desalination*, **24**(1/2/3), 273-279.

Hendricks, C. W. (1978). *Exceptions to the coliform and the fecal coliform tests*. Ann Arbor, MI: Ann Arbor Science.

Heufelder, G. (1989). *Pollution sources in Buttermilk Bay: keeping it all in perspective*. Woods Hole, MA: Woods Hole Oceanographic Institution.

Hickey, J. M. (1989). *Massachusetts shellfish closures, monitoring programs, regulations, and the concern over the bacterial standard*. Woods Hole, MA: Woods Hole Oceanographic Institution.

Ho, S. A., Hoyle, J. A., Lewis, F. A., Secker, A. D., Cross, D., Mapstone, N. P., Dixon, M. F., Wyatt, J. I., Tompkins, D. S., Taylor, G. R., & Quirke, P. (1991). Direct polymerase chain reaction test for detection of Helicobacter pylori in humans and animals. *Journal of Clinical Microbiology*, **29**(11), 2543-2549.

Holdeman, L. V., Good, I. J., & Moore, W. E. C. (1976). Human fecal flora: variation in bacterial composition within individuals and a possible effect of emotional stress. *Applied and Environmental Microbiology*, **31**(3), 359-375.

Holm, S. E. (1987). *Sewage indicators in sediments*. Rockville, MD: Office of Oceanography and Marine Assessment.

Hull, R., Brown, F., & Payne, C. (1989). *Virology: Directory and Dictionary of Animal, Bacterial and Plant Viruses*. New York: Stockton Press.

Hurst, C. J., Benton, W. H., & Stetler, R. E. (1989). Detecting viruses in water. *Journal AWWA*, **81**(9), 71-80.

Ignazzitto, G., Volterra, L., Aulicino, F. A., & D'Angelo, A. M. (1980). Coliphages as indicators in a treatment plant. *Water, Air, and Soil Pollution*, **13**(4), 391-398.

Jofre, J., Blasi, M., Bosch, A., & Lucena, F. (1989). Occurrence of bacteriophages infecting Bacteroides fragilis and other viruses in polluted marine sediments. *Water Science and Technology*, **21**(3), 15-19.

Jofre, J., Bosch, A., Lucena, F., Gironés, R., & Tartera, C. (1986). Evaluation of Bacteroides fragilis bacteriophages as indicators of the virological quality of water. *Water Science and Technology*, **18**(10), 167-173.

Johnson, P. C., Hoy, J., Mathewson, J. J., Ericsson, C. D., & DuPont, H. L. (1990). Occurrence of Norwalk virus infections among adults in Mexico. *Journal of Infectious Diseases*, **162**(2), 389-393.

Joret, J. C., Hassen, A., Hartemann, P., & Foliguet, J. M. (1986). Some limits in current adsorption-elution methods for the detection of viruses in large volumes of tapwater. *Water Science and Technology*, **18**(10), 133-140.

Josephson, K. L., Gerba, C. P., & Pepper, I. L. (1993). Polymerase chain reaction detection of nonviable bacterial pathogens. *Applied and Environmental Microbiology*, **59**(10), 3513-3515.

Kalter, S. S. (1986). The role of animals in the waterborne transmission of viruses. *Water Science and Technology*, **18**(10), 241-263.

Kapikian, A. Z. (1993). Viral Gastroenteritis. *Journal of the American Medical Association*, **269**(5), 627-630.

Knight, I. T., DiRuggiero, J., & Colwell, R. R. (1991). Direct detection of enteropathogenic bacteria in estuarine water using nucleic acid probes. *Water Science and Technology*, **24**(2), 261-266.

Kopecka, H., Dubrou, S., Prévot, J., Maréchal, J., & Lopez Pila, J. M. (1993). Detection of naturally occurring enteroviruses in waters by reverse transcription, polymerase chain reaction, and hybridization. *Applied and Environmental Microbiology*, **59**(4), 1213-1219.

Kott, Y. (1977). *Current concepts of indicator bacteria*. Philadelphia: ASTM.

Kraus, M. P. (1977). *Bacterial indicators and the potential health hazard of aquatic viruses*. Philadelphia: ASTM.

Kroner, K. H., Nissinen, V., & Ziegler, H. (1987). Improved dynamic filtration of microbial suspensions. *Bio/Technology*, **5**(9), 921-926.

Lambden, P. R., Caul, E. O., Ashley, C. R., & Clarke, I. N. (1993). Sequence and organization of a human small round-structured (Norwalk-like) virus. *Science*, **259**, 516-519.

Leclerc, H., Mossel, D. A., Trinel, P. A., & Gavini, F. (1977). *Microbiological monitoring-- a new test for fecal contamination*. Philadelphia: ASTM.

Levin, M. A. (1977). *Bifidobacteria as water quality indicators*. Philadelphia: ASTM.

Lewis, R. G. (1990). *Detection of the Alu Sequence Using the Polymerase Chain Reaction*. Massachusetts Institute of Technology.

Mack, W. N. (1977). *Total coliform bacteria*. Philadelphia: ASTM.

Mahbubani, M. H., Bej, A. K., Miller, R., Haff, L., DiCesare, J. L., & Atlas, R. M. (1990). Detection of Legionella with polymerase chain reaction and gene probes methods. *Molecular and Cellular Probes*, **4**(3), 175-187.

Mara, D. D., & Oragui, J. I. (1981). Occurrence of Rhodococcus coprophilus and associated actinomycetes in feces, sewage, and freshwater. *Applied and Environmental Microbiology*, **42**(6), 1037-1042.

Mara, D. D., & Oragui, J. I. (1983). Sorbitol-fermenting bifidobacteria as specific indicators of human faecal pollution. *Journal of Applied Bacteriology*, **55**(2), 349-357.

Margolin, A. B., Hewlett, M. J., & Gerba, C. P. (1991). The application of a poliovirus cDNA probe for the detection of enterovirus in water. *Water Science and Technology*, **24**(2), 277-280.

Margolin, A. B., Richardson, K. J., De Leon, R., & Gerba, C. P. (1989). *Application of gene probes to the detection of enteroviruses in groundwater*. Chelsea, MI: Lewis.

Martinez, J. L., & Baquero, F. (1990). Epidemiology of antibiotic-inactivating enzymes and DNA probes: the problem of quantity. *Journal of Antimicrobial Chemotherapy*, **26**(3), 301-305.

McFeters, G. A., Bissonnette, G. K., Jezeski, J. J., Thomson, C. A., & Stuart, D. G. (1974). Comparative survival of indicator bacteria and enteric pathogens in well water. *Applied Microbiology*, **27**(5), 823-829.

Metcalf and Eddy, I. (1991). *Wastewater Engineering: Treatment, Disposal, and Reuse*. New York: McGraw-Hill.

Metcalf, T. G. (1978). *Indicators of viruses in shellfish*. Ann Arbor, MI: Ann Arbor Science.

Milne, D. P., Curran, J. C., & Wilson, L. (1986). Effects of sedimentation on removal of faecal coliform bacteria from effluents in estuarine water. *Water Research*, **20**(12), 1493-1496.

Mitchell, R., & Chamberlin, C. (1978). *Survival of indicator organisms*. Ann Arbor, MI: Ann Arbor Science.

Muñoa, F. J., & Pares, R. (1988). Selective medium for isolation and enumeration of Bifidobacterium spp. *Applied and Environmental Microbiology*, **54**(7), 1715-1718.

O'Keefe, B., & Green, J. (1989). Coliphages as indicators of faecal pollution at three recreational beaches on the Firth of Forth. *Water Science and Technology*, **23**(8), 1027-1030.

Ochert, A. S., Boulter, A. W., Birnbaum, W., Johnson, N. W., & Teo, C. G. (1994). Inhibitory effects of salivary fluids on PCR: Potency and removal. *PCR Methods and Application*, **3**, 365-368.

Ofsthun, N. J. (1989). *Cross-flow Membrane Filtration of Cell Suspensions.* Massachusetts Institute of Technology.

Ohgaki, S., Ketratanakul, A., Suddevgrai, S., Prasertsom, U., & Suthienkul, O. (1986). Adsorption of coliphages to particulates. *Water Science and Technology*, **18**(7/8), 267-275.

Olson, B. H., & Nagy, L. A. (1984). *Microbiology of potable water.* Orlando, FL: Academic Press.

Oragui, J. I. (1982). *Bacteriological Methods for the distinction between human and animal faecal pollution.* University of Leeds.

Oragui, J. I., & Mara, D. D. (1983). Investigation of the survival characteristics of Rhodococcus coprophilus and certain fecal indicator bacteria. *Applied and Environmental Microbiology*, **46**(2), 356-360.

Palmer, E. L., & Martin, M. L. (1982). *An Atlas of Mammalian Viruses.* Boca Raton, FL: CRC Press.

Paul, J. H., Jeffrey, W. H., David, A. W., DeFlaun, M. F., & Cazares, L. H. (1989). Turnover of extracellular DNA in eutrophic and oligotrophic freshwater environments of Southwest Florida. *Applied and Environmental Microbiology*, **55**(7), 1823-1828.

Paul, J. H., Jeffrey, W. H., & DeFlaun, M. F. (1987). Dynamics of extracellular DNA in the marine environment. *Applied and Environmental Microbiology*, **53**(1), 170-179.

Paul, J. H., Jiang, S. C., & Rose, J. B. (1991). Concentration of viruses and dissolved DNA from aquatic environments by vertex flow filtration. *Applied and Environmental Microbiology*, **57**(8), 2197-2204.

Payment, P., Richardson, L., Siemiatycki, J., Dewar, R., Edwardes, M., & Franco, E. (1991). A randomized trial to evaluate the risk of gastrointestinal disease due to consumption of drinking water meeting current microbiological standards. *American Journal of Public Health*, **81**(6), 703-708.

Pinto, R. M., Jofre, J., Abad, F. X., González-Dankaart, J. F., & Bosch, A. (1993). Concentration of fish enveloped viruses from large volumes of water. *Journal of Virological Methods*, **43**, 31-40.

Rao, V. C., & Melnick, J. L. (1986). *Environmental Virology.* Berkshire, UK: Van Nostrand Reinhold.

Resnick, I. G., & Levin, M. A. (1981). Assessment of bifidobacteria as indicators of human fecal pollution. *Applied and Environmental Microbiology*, **42**(3), 433-438.

Rhodes, M. W., & Kator, H. (1988). Survival of Escherichia coli and Salmonella spp. in estuarine environments. *Applied and Environmental Microbiology*, **54**(12), 2902-2907.

Rivera, S. C., Hazen, T. C., & Toranzos, G. A. (1988). Isolation of fecal coliforms from pristine sites in tropical rain forest. *Applied and Environmental Microbiology*, **54**(2), 513-517.

Rosas, I., Mazari, M., Saavedra, J., & Báez, P. (1985). Benthic organisms as indicators of water quality in Lake Patzcuaro, Mexico. *Water, Air, and Soil Pollution*, **25**(4), 401-414.

Rose, J. B., Gerba, C. P., & Margolin, A. B. (1987). *Viruses in drinking water: an overview of gene probes and their usage for detection of viruses in water*. Denver, CO: AWWA.

Ruano, G. (1989). *Prestidigitation and the Polymerase Chain Reaction* (unpublished report No. Yale University.

Rutkowski, A. A., & Sjogren, R. E. (1987). Streptococcal pollution profiles as indicators of water quality. *Water, Air, and Soil Pollution*, **34**(3), 273-284.

Sartory, D. P. (1986). Membrane filtration enumeration of faecal clostridia and Clostridium perfringens in water. *Water Research*, **20**(10), 1255-1260.

Scarpino, P. V. (1978). *Bacteriophage indicators*. Ann Arbor, MI: Ann Arbor Science.

Seeley, N. D., & Primrose, S. B. (1982). A review: the isolation of bacteriophages from the environment. *Journal of Applied Bacteriology*, **53**(1), 1-17.

Shuval, H. I. (1986). *Thalassogenic diseases. UNEP Regional Seas Report and Studies No. 79*. Nairobi, Kenya: UNEP.

Sinton, L. W. (1986). Microbial contamination of alluvial gravel aquifers by septic tank effluent. *Water, Air, and Soil Pollution*, **28**(3/4), 407-425.

Sinton, L. W., & Ching, S. B. (1987). An evaluation of two bacteriophages as sewage tracers. *Water, Air, and Soil Pollution*, **35**(3/4), 347-356.

Sobsey, M. D., Cromeans, T., Hickey, A. R., & Glass, J. S. (1984). Effects of water quality on microporous filter methods for enteric virus concentration. *Water Science and Technology*, **17**(4/5 Pt.2), 665-679.

Sobsey, M. D., & Glass, J. S. (1984). Influence of water quality on enteric virus concentration by microporous filter methods. *Applied and Environmental Microbiology*, **47**(5), 956-960.

Stanier, R. Y., Ingraham, J. L., Wheelis, M. L., & Painter, P. R. (1986). *The Microbial World*. Englewood Cliffs, NJ: Prentice-Hall.

Steffan, R. J., & Atlas, R. M. (1991). Polymerase chain reaction: Applications in environmental microbiology. *Annual Reviews in Microbiology*, **43**, 137-161.

Straub, T. M., Pepper, I. L., Abbaszadegan, M., & Gerba, C. P. (1994). A method to detect enteroviruses in sewage sludge-amended soil using PCR. *Applied and Environmental Microbiology*, **60**(3), 1014-1017.

Struck, P. H. (1988). The relationship between sediment and fecal coliform levels in a Puget Sound estuary. *Journal of Environmental Health*, **50**(7), 103-107.

Tartera, C., & Jofre, J. (1987). Bacteriophages active against Bacteroides fragilis in sewage-polluted waters. *Applied and Environmental Microbiology*, **53**(7), 1632-1637.

Tartera, C., Lucena, F., & Jofre, J. (1989). Human origin of Bacteroides fragilis bacteriophages present in the environment. *Applied and Environmental Microbiology*, **55**(10), 2696-2701.

Tougianidou, D., & Botzenhart, K. (1991). Detection of poliovirus in water by direct isolation of the RNA and hybridization with gene probes. *Water Science and Technology*, **24**(2), 273-276.

Tsai, Y. L., & Olson, B. H. (1992). Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. *Applied and Environmental Microbiology*, **58**(7), 2292-2295.

Tsai, Y. L., Palmer, C. J., Yahya, M. T., McGee, C. D., & Sangermano, L. R. (1993). Detection of enteroviruses and Hepatitis A virus in sewage and ocean water by reverse transcriptase-polymerase chain reaction. In *66th Annual Conference and Exposition*, (pp. 115-122). Anaheim, CA: Water Environment Federation.

Van Zwet, A. A., Thijs, J. C., Kooistra-Smid, A. M. D., Schirm, J., & Snijder, J. A. M. (1994). Use of PCR with feces for detection of *Helicobacter pylori* Infections in patients. *Journal of Clinical Microbiology*, **32**(5), 1346-1348.

Vlassoff, L. T. (1977). *Klebsiella*. Philadelphia: ASTM.

Volterra, L., Bonadonna, L., & Aulicino, F. A. (1985). Comparison of methods to detect fecal streptococci in marine waters. *Water, Air, and Soil Pollution*, **26**(2), 201-210.

Volterra, L., Piccininno, G., Palliola, E., Aulicino, F. A., & Gianfranceschi, M. (1984). Environmental fecal pollution and concentration power of the clam Chamelea gallina. *Water, Air, and Soil Pollution*, **21**(1/2/3/4), 415-424.

Ward, R. L., & Akin, E. W. (1984). Minimum infective dose of animal viruses. *Critical Reviews in Environmental Control*, **14**(4), 297-310.

Wentsel, R. S., O'Neill, P. E., & Kitchens, J. F. (1982). Evaluation of coliphage detection as a rapid indicator of water quality. *Applied and Environmental Microbiology*, **43**(2), 430-434.

Wheater, D. W. F., Mara, D. D., & Oragui, J. I. (1979). *Indicator systems to distinguish sewage from stormwater run-off and human from animal faecal material*. Chichester: John Wiley & Sons.

Williams, F. P., & Akin, E. W. (1986). Waterborne viral gastroenteritis. *Journal AWWA*, **78**(1), 34-39.

Witte, A., Bläsi, U., Halfmann, G., Szostak, M., Wanner, G., & Lubitz, W. (1990). Phi-x174 protein E-mediated lysis of Escherichia coli. *Biochemie*, **72**(2/3), 191-200.

Young, C. C., Burghoff, R. L., Keim, L. G., Minak-Bernero, V., Lute, J. R., & Hinton, S. M. (1993). Polyvinylpyrrolidone-agarose gel electrophoresis purification of polymerase chain reaction-amplifiable DNA from soils. *Applied and Environmental Microbiology*, **59**(6), 1972-1974.

Young, K. D., Anderson, R. J., & Hafner, R. J. (1990). Lysis of Escherichia coli by the bacteriophage phi-x174 E protein: inhibition of lysis by heat shock proteins. *Journal of Bacteriology*, **171**(8), 4334-4341.

APPENDIX I: Alternative Indicator Systems

i Streptococci and Enterococci

The genus *Streptococcus* are Gram-positive, non-sporing, facultative anaerobic cocci that are consistently isolated from the intestines of warm-blooded animals (Geldreich, 1976). The fecal streptococci are commonly used to indicate water quality. As mentioned above, the concentration of these organisms is currently used in standards in many European countries (Shuval, 1986). In addition, the U.S. EPA published a report stating that enterococci or *E. coli* should be used as indicators instead of fecal coliform (Allison, *et al.*, 1988). Further support for the use of the enterococci comes from Cabelli. He found that the enterococci best correlated to 'highly credible' GI illness with *E. coli* a poor second (Cabelli, *et al.*, 1983).

The fecal streptococci are composed of Lancefield's serologic group D streptococci and of the viradans group streptococci. Lancefield's serologic group D streptococci include *Streptococcus faecalis*, *S. faecum*, *S. bovis*, and *S. equinus* as well as the group Q streptococci [*S. avium*]; these organisms have the D antigen (Rutkowski & Sjogren, 1987; Volterra, *et al.*, 1985). *S. bovis*, *S. avium*, and *S. equinus*, while they have the D antigen, are also part of the viradans group along with the oral streptococci, *S. mitis*, *S. sanguis*, and *S. salivarius*. The oral streptococci are considered to be part of the fecal streptococci, because they too can be enteric bacteria. The enterococci are *S. faecalis* and *S. faecum* (Volterra, *et al.*, 1985); *S. durans* is sometimes included (Wheater, *et al.*, 1979). Another streptococcal group is the dairy streptococci, which include *S. lactis*, *S. cremoris*, and *S. thermophilus* (Rutkowski & Sjogren, 1987). As with the fecal coliform, the fecal

streptococcus are operationally defined by the biochemical tests used to detect them.

Rutkowski and Sjogren found that in human waste the percentage of enterococci [out of the total streptococci] was much higher than in animal waste. The opposite was true regarding enterococci in animal wastes, where it was found the percentage of group D, non-enterococci (*S. bovis*, *S. avium*, and *S. equinus*) was higher. In other words, the group D non-enterococci dominated the enterococci in animal waste, while the enterococci dominated in human waste. Apparently, the oral streptococci were entirely absent in non-human waste and the dairy streptococci were absent when dairy wastes were absent (Rutkowski & Sjogren, 1987).

S. bovis is considered animal specific as it could not be isolated from humans or domestic sewage. It was recovered from cattle, sheep, horses, pigs, dogs, cats, hens, ducks, and seagulls. Ruminants had the highest percentage, while horses had higher percentages of *S. equinus*. Wheater, *et al.* reports that others obtained similar results (Wheater, *et al.*, 1979). Thus, *S. bovis* could be used to differentiate human and animal wastes. The problem is that *S. bovis* dies-off very quickly in the environment. This organism could be used to indicate the proximity or age of a pollution source.

One of the most notable uses of the fecal streptococci is in the fecal coliform to fecal streptococcus ratio (FC/FS ratio). It has been claimed that the FC/FS ratio is greater than four for humans and less than about one for animals. This has been shown not to be the case by numerous researchers. The problem is that the two sets of organisms have differential die-off rates in the environment, thus as time passes, the ratio changes. To complicate the

problem, the viradans streptococci are less resistant than the fecal coliform, while the enterococci are more resistant than the fecal coliform. So, one would expect that initially the FC/FS ratio to go up in animal wastes and down in human wastes. To further confuse matters, environmental factors, such as sunlight and salinity, more dramatically reduce the level of fecal coliform than the levels of fecal streptococcus (Fujioka, *et al.*, 1981), so after a significant exposure to the environment the FC/FS ratio will continually decrease over time. Four days from the time of release is given as the limit for the usefulness of the ratio, after which differential die-off will have obscured the results (Rutkowski & Sjogren, 1987); however, other researchers used 24 hours as the limit (McFeters, *et al.*, 1974) Furthermore, the author has seen unpublished data that show that diet can greatly affect the ratio. The diet of Canada geese was found to cause the FC/FS ratio to reach four in that animal's intestines. The FC/FS ratio must be used with caution.

Another ratio of interest is the ratio of *S. faecalis* to *S. faecum*. *S. faecalis* concentrations are higher in animals than *S. faecum* concentrations, the opposite is true for human wastes (Wheater, *et al.*, 1979). A final ratio of interest is the ratio of total streptococci to sorbitol-fermenting streptococci. The ratio is about four for humans and it is between 15 and 175 for animals (Wheater, *et al.*, 1979). These ratios are generalizations and are dependent on diet and other factors; therefore there could be a great deal of variation in the ratios between regions and animals (Evison, 1979). Very little information has been published on these ratios, and only the FC/FS ratio is extensively used.

Many problems exist with the use of fecal streptococci. These organisms are less resistant to chlorination and environmental stresses than spore and

cyst formers as well as many viruses. Thus, there absence does not necessarily indicate the absence of pathogens. The enterococci are, however, more resistant than the fecal coliform (Clausen, *et al.*, 1977). McFeters gives the following list in order of resistance: *Aeromonas*> the *Shigellae* > fecal streptococci ≈ enterococci > *Salmonella* spp. ≈ fecal coliform (McFeters, *et al.*, 1974). Although the enterococci are more resistant than the fecal coliform, they are still less resistant than many pathogens. A further difficulty with the use of the fecal streptococci is that certain biotypes of *S. faecalis* are associated with insects and plants (Kott, 1977), although no particular species can be consistently recovered from these non-mammalian sources (Clausen, *et al.*, 1977). Geldreich found that *S. faecalis* var. *liquificans* can be consistently recovered from 'unpolluted' environments and because of this fecal streptococci counts below 100 should not be used (Geldreich, 1976).

ii Clostridia

Members of the genus *Clostridium* are Gram-positive, obligately anaerobic endospore-forming rods, which, in general, are "ubiquitous" in the environment (Holt 1984, 1141). Several groups or species within this genus have been suggested (and are currently used in Europe) as water quality indicators as they are consistently found in sewage and feces; they are the fecal clostridia, sulfite-reducing clostridia, and *Clostridium perfringens* [especially Type A]. Of these groups, *C. perfringens* is the most commonly used as an indicator because it is considered to be exclusively of fecal origin, although not exclusively of human origin (Sartory, 1986). There is a

controversy as to how broad the clostridia indicator group should be (Cabelli, 1977)

Because the fecal clostridia are spore-formers, they can survive in the environment for extended periods; thus providing a fairly conservative tracer of fecal pollution. In fact, spore-formers tend to correlate better with other spore and cyst forming pathogens as well as some of the more resistant viruses than do more traditional indicators of pollution. *C. perfringens*, as is the case for the other fecal clostridia, are fairly resistant to an array of adverse environmental conditions and are not dramatically affected by chlorination (Fujioka & Shizumura, 1985). Their ability to grow in the environment is considered both good and bad depending on why the indicator is being used. Because clostridial spores are so resistant there is the possibility that they will outlast all the pathogens. And, because clostridial spores are often concentrated in the sediments, sediment resuspension due to turbulent water conditions could cause high counts when no danger is actually present, so that in effect the sediments become a reservoir for clostridia (Bisson & Cabelli, 1980). Some researchers feel that their long survival time causes the clostridia to be concentrated in the sediments to the point where they could be considered ubiquitous in nature. In fact, Cabelli found that *C. perfringens* concentrations did not vary between slightly polluted and polluted sites as did the more traditional indicators (Cabelli, 1977). On the other hand, if the water being examined is to be used as drinking water, then this conservative approach would be valued. In fact the absence of *C. perfringens* is a "reliable indication of the absence of pathogenic bacteria and enteroviruses" and is also a good measure of water treatment efficiency (Sartory, 1986). Another use of *C. perfringens* is the ratio of

vegetative cells to spores, or the ratio of other indicators, like *E. coli,* to *C. perfringens*. Because the physical states of the organism and the different indicators have different survival times in the environment, these ratios could be used to determine the age and proximity of a pollution source (Bisson & Cabelli, 1980).

The fecal clostridia were given by Sartory as *C. perfringens, C. perenne, C. celatum, C. ghoni, C. clostidiiforme, C. sphenoides*, and *C. novyi*. He stated that these species were "predominately of fecal or urino-genital origin". The use of these organisms to determine water treatment and purification efficiency, pollution in extreme environments, and pollution in the presence of toxics have been noted (Sartory, 1986). Another good quality of the fecal clostridia is the fact that they do not grow well in the environment, because they are anaerobic bacteria with special growth requirements and temperature optima. Nevertheless it seems more research needs to be done regarding the growth and non-fecal occurrence of fecal clostridia in the environment.

Some problems with the use of the clostridia involve their low concentration in the intestines [two orders of magnitude less than *E. coli*], but this is balanced by their long survival time. In other words, even though, initially, the clostridia occur at lower concentrations than other enteric bacteria, over time there relative number increases (Bisson & Cabelli, 1980). Another problem is this group's inability to distinguish between animal and human wastes because the clostridia are common to the intestines of most warm blooded animals. This is no more limiting, though, than it is for the other popular indicators such as *E. coli*, fecal coliform, and fecal streptococcus. Although sulfite-reducing clostridia, or the broader group of

sulfite-reducing, spore-forming anaerobes (SSA), are more easily recovered by selective media, they are not necessarily of fecal origin and are not necessarily good indicators of pollution (Cabelli, 1977). The SSA are used in Europe as indicators, because they are easily detected by stormy fermentation of milk (Cabelli, 1978b). Because *C. perfringens* is anaerobic and requires more specific tests for its detection, it is more difficult and more expensive to work with in the lab than the SSA or more traditional indicators. One benefit over other anaerobes is that the fecal clostridia can stand microaerophillic conditions (Geldreich, 1978).

iii Bacteriophages

Bacteriophages are viruses that infect bacteria (Brock & Madigan, 1988). The presence of these viruses has been suggested as an indicator of the possible presence of viral pathogens. There are a number of bacteriophages [coliphages, cyanophages, pseudomonaphages] that have been suggested as pathogen indicators or sewage tracers (Kraus, 1977). The reasoning is that a bacteria cannot model the transport and survival characteristics of viruses because the two differ in size and, more basically, in the form of life. But because bacteriophages are viruses, they would be expected to better mimic pathogenic viruses. On the other hand, bacteriophages would not necessarily be expected to correlate well with bacterial pathogens, although Dutka *et al.* found a high degree of correlation between coliphages, fecal coliform, and *E. coli* (Dutka, *et al.*, 1987). An advantage of using phage as indicators is that water samples can be frozen and saved, unlike bacterial samples (Sinton & Ching, 1987). Although there was an initial sharp decline in the

concentration of phage in the environment, phage concentrations were found to level off, and afterwards were able to survive for extended periods [1 year in stored samples] (Ignazzitto, *et al.*, 1980). Another study showed inactivation or dilution below detection could occur in as little as 15 minutes after discharge to a saline water body, but no measurement of viral pathogen inactivation was made (Borrego & Romero, 1985).

Several different bacteriophages have been researched; they include bacteriophages of *Bacteroides fragilis*, *Bacteroides* spp., *E. coli* , and others. Bacteriophages of *E. coli* are referred to as coliphages. Phage of other organisms have been suggested, but there is a paucity of data concerning alternative bacteriophages (Boardman, *et al.*, 1988). The author would like to suggest the use of bacteriophages of *Bifidobacterium* spp. as they may also give some insight into the source of pollution [see below]. Human specificity in virus detection would not be too limiting in that viruses are fairly host specific.

It is important in choosing an indicator phage to select one that infects the enteric bacteria of a large percentage of population, and it should be consistently associated with human feces. The genus *Bacteroides* is the most concentrated bacteria in the human intestines of 'healthy people' (Holdeman, *et al.*, 1976), so looking for a *Bacteroides* bacteriophage makes sense. Because *Bacteroides* are obligate anaerobes, these organisms are unlikely to grow in the environment and their bacteriophages are not believed to reproduce outside the body. The problem is that working with anaerobes can be difficult (Seeley & Primrose, 1982). If not for this difficulty, *Bacteroides* spp. would themselves be considered as indicator organisms. The author notes very little information was found concerning *Bacteroides* spp. as indicators.

From twelve *Bacteroides* spp. tested, *Bacteroides fragilis* HPS 40 was the chosen as the host bacteria because bacteriophages that attacked this host had the highest detection rate from rivers in a densely populated area in North Barcelona. In this study, phage infecting this host were recovered (1) from all samples of sewage polluted waters, (2) from 10% of human fecal samples, (3) never from animal samples, and (4) never from unpolluted waters (Tartera & Jofre, 1987; Tartera, *et al.*, 1989). In another study, *Bacteroides fragilis* 40 was used as host and the bacteriophage assayed was B40-8. Again, similar results to those presented above were obtained. Furthermore, the phage concentration was shown not to increase in aerobic and anaerobic extra-intestinal environments. The decay rate of the phage studied modeled the decay rate of coliphage f2, poliovirus, and a rotavirus in both fresh and saltwater (Jofre, *et al.*, 1986). These bacteriophages seem to be good indicators of human viral pathogens; however, the difficulty of working with anaerobes and bacteriophages in the laboratory remains a problem.

Coliphages have also been examined for their use as indicators. It seems, though, that there is a problem associated with the use of many coliphages. The habitat for many coliphage is the environment, meaning they not only survive but can thrive in the natural milieu. This situation can be avoided by using male-specific [F-phage including f2 and MS-2] (Cabelli, 1978a). The attachment site for these coliphages is the F pilus, which is not formed below 30°C (Seeley & Primrose, 1982). Also, broad spectrum coliphage hosts can be used if the background coliphage level is known so that the indicator coliphage level can be discounted for the background.

Different researchers have used a variety of *E. coli* strains which are infected by numerous of coliphages. The following is a list of hosts and/or phage used recently by researchers: *E. coli* C603 host which has morphologically distinct phage (O'Keefe & Green, 1989) P2-like phage (fMWD 1) of *E. coli* (H2S+) (Sinton, 1986) *E. coli* 9484B was chosen as host from nine other strains examined because it had the best recovery rate from polluted samples (Ignazzitto, *et al.*, 1980), and a combination of four strains of *E. coli* were used to detect coliphages by replicate sample testing (Borrego, *et al.*, 1987; Borrego & Romero, 1985).

Problems exist with the use of coliphages. They include the lack of a accepted host/coliphage to use in a standardized test, although *Standard Methods* gives a proposed method that used *E. coli* C, 13706, as the host (APHA, 1989). One of the problems that must be overcome is that of the difficult and time-consuming nature of working with viruses and phage as compared to bacteria. A rapid [6 hour] procedure for determining the presence of coliphages was tested at water quality laboratories across the U.S. and was found to be accurate and easily performed (Wentsel, *et al.*, 1982). Other laboratory difficulties have not been so easily solved. It was discovered that different bacteriophage concentration techniques yield widely different results (Sinton & Ching, 1987) thus affecting sensitivity and repeatability of those experiments. Coliphage survival is affected by sunlight, sediment association [organic matter], pH, temperature, salinity, and grazing by predators (Borrego & Romero, 1985), but one would expect pathogenic viruses to react in much the same way. Funderburg and Sorber, however, found no correlation between viral pathogens and coliphages in primary and secondary waste effluents (Funderburg & Sorber, 1985). "The

absence [of coliphages] is not a guarantee of absolutely clean water", but during this survey coliform bacteria were never found in the absence of coliphages (Borrego, *et al.*, 1987). On the other hand, this implies that traditional methods would be just as inaccurate. Borrego, *et al.* found coliphages type B and K-12 had longer survival times than traditional indicator bacteria (Borrego, *et al.*, 1983). Some researchers found that coliphage T2 did not survive as well as enteroviruses, while others found coliphages T1 through T7 survived equally as well as enteroviruses (Scarpino, 1978). The point being that different bacteriophages have different survival characteristics in different environments, and more work must be done in the standardization of procedures and understanding the ecological effects of varying environments on different bacteriophages.

Bacteriophage 80, which is active against *Staphylococcus aureus* (Heatley Oxford strain; ATCC 9144), was determined to have "considerable potential as a tracer". Other bacteriophages that have been examined include *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Serrate*, and phages of *Bacillus* sp. (Sinton & Ching, 1987).

iv Bifidobacteria

The genus, *Bifidobacterium*, is composed of Gram-positive, obligate anaerobic, non-sporing rods that are common inhabitants of human intestines (Holt 1984, 1418). These organisms occur in the intestines at a concentration that is about 100 times higher than *E. coli* and other facultative anaerobes (Holdeman, *et al.*, 1976). It has been noted that

bifidobacteria, especially particular species, are specific to humans and a limited number of other higher animals (Mara & Oragui, 1983).

Bifidobacteria have been suggested as indicator organisms for many years. In fact, researchers in the late 1950's and early 1960's began work on the use of bifidobacteria as a fecal specific indicator (Resnick & Levin, 1981) It was also known that some species showed specificity to humans and a limited number of higher animals; however, the difficulty of culturing these organisms outweighed their usefulness as indicators. This was the case because bifidobacteria must be grown anaerobically, they have difficult growth requirements, and at that time there was no selective medium. Selective media has since been developed (Muñoa & Pares, 1988). Thus, these organisms have recently been reexamined as possible indicators.

Oragui states that sorbitol-fermenting bifidobacteria are human-specific (Oragui, 1982). Although the number of animals tested seemed limited, Oragui and Mara could not find bifidobacteria [grown on a specific medium, YN-17], in horses, rabbits, rats, mice, chickens, cats, and dogs. He found bifidobacteria in cattle, sheep, pigs, and humans, but only humans and pigs had sorbitol-fermenting bifidobacteria. The sorbitol-fermenting bacteria that they were able to isolate exclusively from humans were *B. breve* and *B. adolescentis* (Mara & Oragui, 1983). Others have found these two bifidobacteria species in pigs (Resnick & Levin, 1981), while others have isolated them from cattle and dogs (Holt 1984, 1426). Bifidobacteria is also commonly isolated from sewage (Resnick & Levin, 1981),

It is possible that organisms could be specific to humans due to special growth requirements provided only in the human intestines or due to

something special in the diet of humans that is not common to the diet of animals. Even if bifidobacteria are not exclusively associated with the human intestines, they still show a certain specificity to humans in that they are consistently isolated from human feces and usually occur in large numbers in human intestines whereas this is not the case with animals. In these terms they could be said to be 'more human specific' than any other enteric bacteria currently suggested as indicators Another favorable aspect to the use of these bacteria is that, because they have complicated growth requirements, there is little chance for regrowth in the environment

There are problems with the use of the bifidobacteria as indicator bacteria. Because they are anaerobic, they are much more difficult and expensive to grow in the laboratory. Also, they are not very resistant to chlorination and they die-off relatively quickly in the environment (Muñoa & Pares, 1988). Because bifidobacteria have a quick die-off time, it has been suggested that the ratio of bifidobacteria to other longer-lived indicator organisms could be used to indicate the age of or the distance from the contamination source. As an example, near a fecal input *E. coli* concentrations were on the order of 5,200 / 100 mL, while bifidobacteria concentrations were around 22,000 / 100 mL. Further downstream, the bifidobacteria levels numbers dropped off dramatically as compared to E. coli. (Resnick & Levin, 1981). However, several researchers found that the rate of die-off of bifidobacteria was about the same as that of *E. coli* (Dutka, 1979; Levin, 1977).

v *Rhodococcus coprophilus*

Rhodococcus is a genus of aerobic, nocardioform actinomycete (Stanier, *et al.*, 1986). This organism has been offered as an animal specific bacteria, especially for the indication of farm runoff. Mara and Oragui state that they were able to recover *R. coprophilus* from cattle, chickens, sheep, pigs, horses, turkeys, ducks, geese, and other fowl, but not from humans (Mara & Oragui, 1981). They also found that *R. coprophilus* could survive for extended periods in the environment, much longer, in fact, than fecal coliform and fecal streptococcus (Oragui & Mara, 1983).

Little information is currently available on these and other actinomycetes as indicators of pollution. Their usefulness will be limited if they are shown to grow in the environment or if they are indigenous to the natural environment. Another problem with this organism's use as an indicator is the difficulty in culturing it and the long incubation period necessary for its detection, which is about 20 days (Oragui & Mara, 1983).

vi Coprostanol and Fecal Sterols

The fecal sterols are composed of chemicals like cholesterol and coprostanol. Research has been completed that concerns the use of these chemicals as indicators. Coprostanol, 5b-Cholestan-3b-ol, is a degradation product from a reduction of cholesterol performed by the enteric bacteria of higher mammals (Düreth, *et al.*, 1986) It is reported to be detectable even after being in the environment for extended periods (Holm, 1987). The only source of coprostanol is the feces of higher mammals. This chemical's concentration may be reduced or completely remove during biological treatment depending on treatment efficiency. Cholesterol and coprostanol

are consistently recovered from sewage and feces, but cholesterol has been found from non-fecal sources (Dutka & El-Shaarawi, 1975).

Dutka and El-Shaarawi found that no consistent relationship exists between the fecal sterols and total coliform, fecal coliform, fecal streptococci, or the heterotrophic population. This evidence may not preclude the use of fecal sterols as many researcher have found that under varying conditions these traditional indicator organisms [discussed in respective sections] do not correlate with pathogens. Dutka and El-Shaarawi also suggest standards for the fecal standards of 0.5 ppb coprostanol and 0.75 ppb cholesterol for recreational waters (Dutka & El-Shaarawi, 1975).

The fecal sterols are suggested as possible indicators of viruses as they are not affected by chlorination. Moreover, because they tend to sorb to particles, they would travel more like a virus than would a bacterium. The lack of correlation with bacteria may, in fact, involve this differential partitioning onto particles (Düreth, *et al.*, 1986). Results from papers published by both Dutka and Düreth *et al.*, mentioned above, refer to coprostanol as an absolute indicator of higher mammalian pollution, which models the virus's ability to escape chemical treatment and be transported by sorption to particulates. One major problem, though, is that the fecal sterols are difficult to work with in the laboratory.

vii *Candida albicans*, Sewage Yeasts, and Acid-fast Bacteria

The group designated sewage yeasts and the acid-fast bacteria have been offered as a possible indicator of wastewater treatment efficiency and/or

water quality in sewage affected waters. Sewage yeast are fungi often associated with, but not necessarily exclusively from, sewage. *Candida albicans* is a opportunistically pathogenic, facultative anaerobic yeast that has received attention in recent years as a possible indicator of pollution in stressed environments. Because it can survive adverse conditions, such as variations in pH, temperature and chlorination, better than most indicator bacteria, it has been used to indicate pollution in streams also contaminated with acid mine drainage (DePasquale, *et al.*, 1987). *C. albicans* is also proposed as an indicator in fresh and salt water systems. Although *C. albicans* is only isolated from about 18% of human fecal samples, it is consistently found in domestic sewage (Cabelli, 1978a) Problems occur because *C. albicans* has been found in 'unpolluted' areas and therefore may not be exclusively of fecal origin (Buck & Bubucis, 1978) On the other hand, Dutka found the yeast was "a rarity in non-polluted areas" (Dutka, 1979) and that no truly autochthonous populations exists. Although *C. albicans* is not especially difficult to culture because the yeast has a morphologically distinctive formation called a germ tube that can be easily identified, it can be confused with *C. stellatoidea*. A problem with *C. albicans* is that it is a natural inhabitant of the skin so that bathers would be an extra-fecal source (Buck, 1977).

The acid-fast bacteria are those with large amounts of lipids and long chain mycolic acids in their cell walls. Because this is true, the acid-fast bacteria are difficult to stain unless an elevated temperature is used during the staining process, and once they are stained they do not easily lose the stain. Several acid-fast bacteria have been suggested as indicators because they are fairly chlorine resistant and are associated with sewage. Examples

include *Mycobacterium* sp. and *Nocardia* sp. (Engelbrecht & Greening, 1978). The nocardioform actinomycetes, like *Rhodococcus*, are often acid-fast (Stanier, *et al.*, 1986). The acid-fast bacteria are much like the sewage yeasts in that they, as a group, are much more resistant to chlorination and environmental effects than the traditional indicators, namely the coliform bacteria (Haas, *et al.*, 1985). On the other hand, they are also not exclusively of fecal origin. Much more work needs to be completed with these organisms, especially in defining what exactly the sewage yeasts are and looking for more specific groups or types of these organisms.

ix Benthic and Natural Water-Body Organisms

For continuous or sporadic high level, long term pollution, an ecologically based indicator system can be developed. In this type of indicator system, effects on the natural flora and fauna would be examined for changes in species diversity and number or physical characteristics like dissolved oxygen (D.O.) and biochemical oxygen demand (B.O.D.). Low level pollution or intermittent pollution would probably not be detected by this system.

A study has been done using benthic microorganisms and macroinvertebrates as indicators. The researchers looked at variations in the autotrophic and heterotrophic communities [i.e. diversity, relative numbers, *etc.*]. The *Oligochaeta* were dominant in polluted regions, and accounted for more than 90% of the living population in very polluted waters (Rosas, *et al.*, 1985). Thus, the death of intolerant species, low D.O., and competition from B.O.D.-fed heterotrophs sent a signal that could be used as an indication of pollution. This information, along with the knowledge that a large city

treatment plant was nearby, makes sewage pollution the likely candidate. This method is not very exacting and makes it virtually impossible to develop standards. It is, on the other hand, a good method to judge water quality without regard to the presence of pathogens.

Zoomicrobial indicators have also been suggested. Information about non-pigmented protozoans and nematodes concentrations and speciation are obtained. With this information a zoomicrobial pollution index (ZPI) is derived which gives insight into the history of the pollution (Chang, 1978). Although this method would not give information about sporadic, low level pollution, it does give information about how the pollution affected the environment.

APPENDIX II: Stock Chemicals

Human Placental RNase inhibitor — Sigma

Humic acid standard — Aldrich

Marker — New England Biolabs

pBR322 DNA-Msp.1 Digest — 1 μg / μL stock solution

Fragment	# of base pairs	Fragment	# of base pairs
1	622	14	147
2	527	15	123
3	404	16	110
4	309	17	90
5	242	18	76
6	238	19	67
7	217	20	34
8	201	21	34
9	190	22	26
10	180	23	26
11	160	24	15
12	160	25	9
13	147	26	9

Oligonucleotide primers — Research Genetics

5 μM stock solution

Taq DNA polymerase — Perkin-Elmer/Cetus

5 Units / μL solution

TetZ RT/DNA polymerase — Amersham

5 Units / μL solution