MODELLING THE TRANSPORT AND FATE OF VIRUSES IN THE AQUATIC ENVIRONMENT

BUTCHER, SA,* WILKINSON, J** JENKINS, A**
AND GOULD, EA*

* NERC Institute of Virology and Environmental Microbiology, Oxford.
** NERC Institute of Hydrology, Wallingford.

CEH CORE AREA 7
Final Report

Centre for Ecology & Hydrology

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Component Institutes
Institute of Freshwater Ecology
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Institute of Terrestrial Ecology
Institute of Virology & Environmental Microbiology

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PERSONNEL

Project Leader and Coordinator Project design, virological work and water sampling	Dr Ernest Gould Dr Sarah Butcher	IVEM IVEM
Project leader (IH) Project design and data analyses and modelling	Dr Alan Jenkins Dr Jeremy Wilkinson	IH IH

ABSTRACT

This project aims to study the distribution of viruses in the aquatic environment. The work entailed monitoring a sewage outfall and water from the River Thames at 5 strategic points both upstream and downstream, for the presence of viruses of human origin. Viruses were initially concentrated by positively charged membrane filtration followed by further concentration and purification. Rotaviruses and Enteroviruses were detected by reverse-transcription polymerse chain reaction (RT-PCR), and nested PCR was used for adenoviruses. These preliminary data were assessed in the context of the physical and chemical properties of the receiving water, and sedimentological characteristics to elucidate the key processes governing virus survival and transport. Daily and diurnal variation in viral load at sites were also assessed. In the limited dataset presented herein, viral titer was not found to undergo significant diurnal variation. Highly significant differences were found between viral loads at sampling sites at different distances from the outfall. Some correlations were found between pH and viral titer and these are being further investigated. Future data will be used together with existing models of bacterial transport to highlight differences in temporal and spatial behaviour between the indicator bacteria and viruses. Key processes determining viral dynamics will be incorporated into the models, which will then be calibrated using routine monitoring data.

PROJECT SIGNIFICANCE

Viruses of human enteric origin are widespread in the freshwater environment and may be viewed as a pollutant originating in sewage. Such viruses may themselves pose a significant health hazard to people coming in contact with river water; particularly those participating in water sports. The EC is currently revising the directives for microbiological (and viral) standards for bathing water. It is anticipated that viral monitoring will become a more important facet of water quality testing in view of the tightening of such regulations. This has been hampered in the past by the lack of suitable methods of detection. Hence the production of suitable methods for monitoring enteric viruses in environmental water samples, together with the increased understanding of the transport and persistence of such viruses will be of direct relevance both to the industries discharging effluents into the river system (water companies) and to the bodies becoming increasingly responsible for monitoring water quality in the environment.

Modelling transport and viral persistence would also be applicable to water quality problems in other countries; particularly in the third world. Currently gastrointestinal illness, predominantly caused by contaminated drinking water is the leading cause of morbidity and mortality in young children. Such methodologies, once validated would allow prediction of likely water quality problems without having to resort to large scale expensive monitoring regimes.

AIMS AND OBJECTIVES

The work has the following aims:

- To develop and apply improved molecular biological methods for detecting sewage-derived enteric viral pollution from both point and non-point sources of contamination.

- To describe the transport and persistence of human viruses from a point source in the aquatic environment.

- To compare and contrast the transport and persistence of viruses with existing models developed for the transport and persistence of bacterial indicators and to investigate the feasibility of preparing a similar model for viruses.

Objectives:

IVEM/IH:

- identify suitable field sites for sampling **COMPLETED**

- plan scale of field sampling **COMPLETED**

IVEM:

- develop suitable methods for recovery and detection of viruses from natural freshwaters **COMPLETED**

  identify suitable enteric viruses for study **COMPLETED**

- apply developed methods in the field to conduct surveying of viral load at multiple field sites, over varying time-scales **COMPLETED**

IH:

- to use modelling expertise to analyse field data for viral loads together with physicochemical data in order to describe viral persistence and transport **ONGOING**

- to assess feasibility of producing a model of viral persistence and transport, and to produce prototype model where appropriate **ONGOING**

INTRODUCTION

Human enteric viruses occur as contaminants in many aquatic environments and accurate assessment of the potential hazard they pose requires a detailed knowledge of their persistence and fate. The viruses potentially present in environmental waters include picornaviruses such as enteroviruses - including echoviruses, coxsakieviruses and hepatitis A, reoviruses - rotaviruses, adenoviruses and caliciviruses - small round structured viruses (SRSVs) other human calicviruses and hepatitis E. These virus groups differ in their physical properties, such as particle size, buoyant density, isoelectric point and also in their structure and nucleic acid type - ranging from single strand + sense RNA in the *Caliciviridae* and *Picornaviridae*, through double strand RNA in the rotaviruses and double strand DNA in the adenoviruses. They vary widely in their ability to cause frank illness in the human population and symptoms, where present, cover a wide spectrum from gastroenteritis, through cold-like symptoms, keratoconjunctivitis, to cardiomyopathies, neurologies such as paralyses and hepatitis. Since these viruses are only capable of replicating in a susceptible host, and are only shed into the sewage train by infected people, they are only present in untreated sewage when that virus is circulating in the population. This is in marked contrast to commonly used bacterial indicators for faecal pollution, such as faecal coliforms and faecal streptococci, which form an integral part of the gut flora and as such, are constantly present in sewage. During infection however, viruses are excreted in huge numbers - up to 10^{10} rotavirus virions per g faeces and 10^{6} pfu/g enteroviruses (Tyrrell & and Kapikian, 1982).

Models to predict the transport and survival of E. coli in streams and rivers have previously been developed and validated. Such models show the effects of various environmental factors on the bioavailability of viable bacteria in the water column with time. Although enteric viruses are potentially of great significance to human health, little is known about their interactions with natural waters. Little systematic monitoring of viruses has been carried out, in part due to the technical difficulties previously inherent in such work. Many of the current standards for microbiological water quality in legislation are expressed as permissible counts for indicator bacteria. One of the exceptions is the EC directive 76/160/EEC on bathing water quality which has a mandatory standard of 0 culturable enteroviruses per litre (Anon., 1976). Standard bacterial indicators of faecal pollution have previously been shown to be of limited or questionable use as accurate forecasters for enteric viruses in the environment. (Ashbolt *et al.*, 1993, Dufour, 1984).

Previously used methods for viral detection in the environment, based on plaque-assay in susceptible cell culture systems were time-consuming and relatively insensitive (Slade *et al.*, 1984, Morris & Waite, 1980). Moreover, some viruses cannot be detected at all, since the method of detection relies upon the ability of the virus to infect and replicate within cells in tissue culture. Some of the viruses most strongly linked to illness following exposure to water - such as Hepatitis A or human caliciviruses (SRSVs) are fastidious and have limited or no ability to grow in such

systems. As a consequence, little systematic surveying work has been done to track the movement of viruses through the aquatic environment.

More recently, the advent of other methods of viral detection has allowed the detection of a variety of viruses with greater speed and sensitivity and moreover has allowed the detection of nonculturable viruses. The method most widely applied has been the polymerase chain reaction, and its use in the detection of enteric viruses has been widely documented (Girones *et al.*, 1993, Jothikumar *et al.*, 1993, Jothikumar *et al.*, 1992 , Kopecka *et al.*, 1993, Marques *et al.*, 1993, Tsai *et al.*, 1993).

Viruses have markedly different physicochemical and biological properties from bacteria and these influence their interactions with the environment. Human enteric viruses are unable to multiply in the absence of susceptible host cells and hence cannot replicate in the aquatic environment. They are sufficiently resistant to environmental degradation processes to persist for appreciable periods of time. Enteric viruses have been shown to persist far longer than fecal coliform bacteria in freshwater and have also been shown to be more resistant to sewage treatment (Goyal, 1983). Once present in the water environment, they may be considered as very small particles which may interact passively with various constituents of that environment. They may remain suspended in the water column, slowly sedimenting out under the influence of gravity. Alternatively, they may adsorb to particles in the colloidal range, or larger matter including suspended sediments (Farrah & Preston, 1991), and may remain attached to such sediment indefinitely or may become unattached under certain conditions. Viral particles may become trapped in naturally occurring biofilms, or be ingested by bacteria or other organisms. However, in order to remain viable as pathogens, viruses have to retain the integrity of certain structures including their nucleic acid complement, and integral proteins and their ability to do this varies with virus type. Such complex molecules are damaged by environmental factors such as ultraviolet (solar) radiation and adverse temperature and pH ranges. They may also be damaged by proteolytic enzymes released by some environmental bacteria (Bitton *et al.*, 1976). Previous reports have indicated that enteric viruses have differing susceptibility to degradation by ultraviolet radiation (Battigelli *et al.*, 1993, Meng & Gerba, 1996) and also to temperature effects on degradation. Enteric viruses are more stable at lower temperatures and may persist for many months at temperatures near freezing (Yates *et al.*, 1985, Yeager & O'Brien, 1979). Studies have indicated that the adsorption of viruses to particulates in the water system may be an important factor both in viral survival and transport dynamics. (Metcalf & Melnick, 1983, Rao *et al.*, 1984) Such studies have suggested that suspended viruses readily adsorb to sediments and that adsorption may increase the infectious lifespan of the virus both in marine (Smith *et al.*, 1978) and freshwaters (Schaub & Sagik, 1975, Wellings *et al.*, 1976) - a phenomenon termed extended survival. Virus adsorption appears to proceed rapidly, and Vilker and colleagues (Vilker *et al.*, 1983a, Vilker *et al.*, 1983b) showed that poliovirus adsorbtion rates to clay particles approach equilibrium in under an hour. Virus adsorption to particulates, and solids in general, is highly dependent on charge interactions (Gerba, 1984) and hence pH, by influencing the surface charge on both particulate and virus, might be expected to influence the stability of such interactions. For instance, poliovirus, which has an isoelectric point of 4.5, carries a

net negative charge at pH 6, whereas at pH 3, it has a net positive charge (Bitton *et al.*, 1976). This would suggest that such a virus would adsorb more readily to a positively charged particulate at pH 6 than at pH 3.

METHOD
Project Design

Identification of suitable viruses for study

Standard indicator bacteria (including faecal coliforms) are part of the normal gut microflora and, as such, are always present in faecal material. Viruses are shed in faecal material only when a human is virally infected. The likelihood of finding a particular virus in a sewage sample will be influenced by the prevalence of that viral infection in the population from whence the sewage came. In order to produce a meaningful study of virus movement within the aquatic environment, the studied virus would need to be present in the contaminating source for a reasonable proportion of the study time.

A number of previous studies of viruses in water have utilised poliovirus. This is not suitable as a sole virus for study in this instance for a number of reasons. Poliovirus is known to be far more susceptible to inactivation by factors such as UV than numerous other human enteric viruses. Most poliovirus in UK sewage originates from transient human infections following administration of the live attenuated vaccine. The presence of this virus in the sewage train may well show seasonal variations in line with mass vaccination schedules. In the developed world, most poliovirus vaccine doses are administered to very young children. Most resultant virus tends to be collected in disposable nappies which are discarded to landfill sites, where the virus may persist for considerable lengths of time (Huber *et al.*, 1994). Thus a relatively small percentage of excreted virus enters the sewage train. Similarly, hepatitis A may not be suitable as a sole virus candidate. Studies have indicated that hepatitis A appears to be among the most resistant to inactivation of the human enteric viruses. Infection with hepatitis A is relatively uncommon in the UK and hence the recovery of this virus from any particular UK community is unlikely.

The nature of the study required that at least one of the viruses or virus groups monitored should be present at most of the sites a significant proportion of the time. This ensures that sufficient positive data are collected to support modelling. The exquisite sensitivity of the PCR method allows the differential detection of individual strains of a particular virus if required, but it can also be used to detect a broad range of viruses without distinction between them. In this instance it was decided that two sorts of viral detection would be carried out initially. The main part

of the project would collect data on the entire enterovirus group (termed Panenterovirus group - all echoviruses, coxsakieviruses polioviruses and enteroviruses), rotaviruses (multiple serotypes), and adenoviruses (multiple serotypes). An attempt would be made to distinguish relative levels of virus in these groups. Secondary to these data, attempts would be made to detect a number of other viruses - hepatitis A, poliovirus, and SRSVs. If any of these viruses were found to be present in a significant number of samples, further attempts would be made to characterise and enumerate them.

Choice of field sites

Local sewage outfalls were identified on the available Ordinance Survey maps (Pathfinder sheets 1116, 1136) and reconnoitred for suitability as field sites. Most of the sites marked on the maps were found to be defunct, however the outfall at the Thames Water sewage treatment works at Sandford-on Thames was found to be both accessible and functional. Sites both upstream and downstream of the outfall were then identified, and these are described in table 1 below

Table 1 - Field Sites

site	site name/location	description	distance from outfall
1	outfall	concrete culvert 20 m from discharge pipe	N/A
2	Littlemore Brook	shallow stream, sandy bottom	0.5 miles downstream
3	River Thames at Kings Arms Sandford - on Thames	next to Sandford lock, 0.25 miles downstream from confluence of Littlemore Brook	1 mile downstream from outfall
4	River Thames at Donnington Bridge, Oxford	sampling from launching ramp	2.2 miles upstream from outfall
5	River Thames at Radley	sampling from jetty 5m out from bank	2.6 miles downstream from outfall 1.6 miles downstream from site 3
6	River Thames at Abingdon Bridge	sampling from boathouse jetty	6.2 miles downstream from outfall 3.6 miles downstream from site 4

NB. Thames water Culham Intake water abstraction point is situated between sites 5 and 6.

Sample collection
Samples were collected from just below the water surface. In most cases, sampling was only possible from the bank, but Radley college site was sampled from a jetty out in midstream. The sampling vessel was introduced slowly, to minimise stirring of any bottom sediment. Ten litre water samples were collected at timepoints during January, March, April and June from some or all of the sites.

Samples were collected daily at the same time each day from all sites for 10 consecutive days in August in order to study short-term variation in a period of relatively low rainfall.

Samples were also collected from three sites (1, 4 and 6) every three hours over a single 24 hour period in order to study diurnal variation.

Sample Collection, Virus Concentration and Purification

Water temperature (°C) and pH were measured at the field site during each sampling session. Water samples were collected in 20L polypropylene commercial drinking water containers which were chemically disinfected with 1% Virkon after every use. A 500ml subsample was removed for separate suspended sediment analysis.

Suspended Sediment Analysis
Each 500ml subsample was filtered through a pre-weighed Whatman GF/C glass microfibre filter (70mm diam.) under negative pressure, baked to dryness and weighed to determine suspended sediment in mg/L.

Development of Methodology for Water Filtration
One of the major features of any methodology for the detection of viruses in environmental water samples is that of concentrating relatively small numbers of virus particles from relatively large volumes of water, with the concomitant removal of a number of naturally occurring substances such as humic and fulvic acids, which act as inhibitors in the methods of detection later used.

Literature research revealed a large number of papers describing complex methods for the concentration and purification of enteric viruses from such samples. Most involved the entrapment of viruses on a solid support - either a filter or glass wool, followed by elution of the virus from same, and subsequent rounds of purification by organic flocculation or polyethylene glycol precipitation, together with varying numbers of other purification steps.

More recent publications have made extensive use of positively charged filter membranes for viral entrapment. These have the advantage over negatively charged

membranes in that the water needs no pre-treatment. The water for filtration through negatively charged membranes has to be pH-adjusted by the addition of divalent cations to increase virus adsorption. This is difficult for large volumes, increases flocculation of humic acid and furthermore the pH may cause viral damage. The use of positively-charged filters - particularly those produced by Cuno Inc.(Meridan US) has been reported by several workers and adsorption rates of 99% of poliovirus have been quoted (Ma *et al.*, 1994) (Ma *et al.*, 1995, Schwab *et al.*, 1996). It was decided to base filtration methods around such filters. 10L volumes of water were studied since that is the agreed standard sampling volume for enteroviruses in the EC bathing water directive (Anon., 1976). Previously Schwab and colleagues have described methods of purification and concentration of viruses in conjunction with these filters; although their studies were concerned with far greater water volumes (Schwab *et al.*, 1995, Schwab *et al.*, 1996). The final methods used for this project are closely based around these methods, and are briefly described below. This complex methodology was chosen as its virus recovery rates were considered to be suitable for our purposes, and it had been validated for viral detection both by PCR and cell culture. Furthermore, it appeared to be capable of consistently minimising the carry-over of inhibitory contaminants; an important consideration if results were to be broadly comparable from different sites and timepoints.

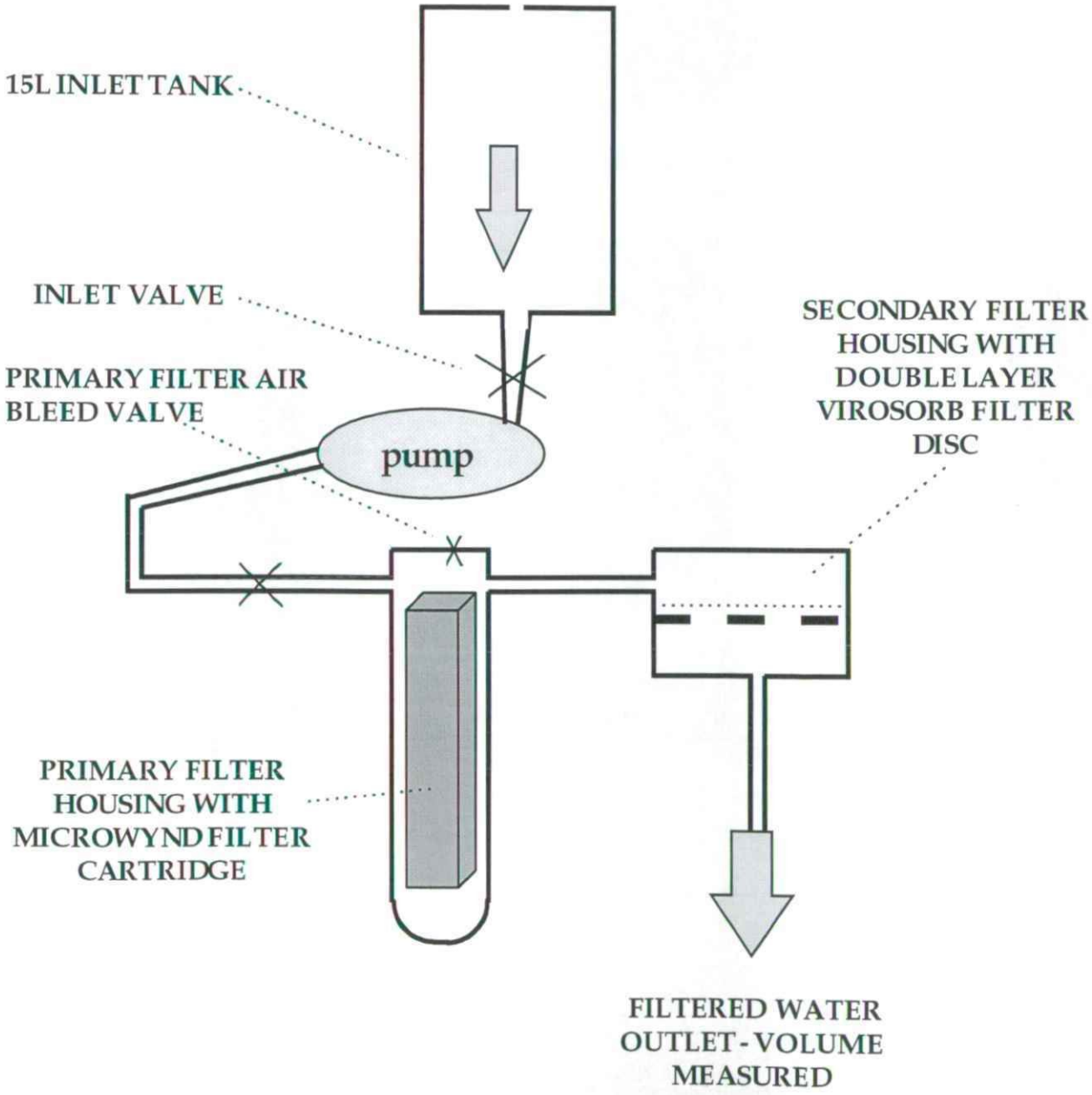
Water Filtration for Viral Concentration

In conjunction with consultation from Cuno Inc., a suitable filtration circuit was designed, and fabricated at IVEM. The apparatus is shown in figure 1.

Water samples were filtered within 12 hours of collection - usually within 3 hours. The filter system consisted of a Microwynd^{TM} (Cuno Inc.) prefilter to remove gross debris (nominal pore size 1µm) followed by a positively charged double thickness Virosorb^{TM} filter membrane (Cuno Inc.) to adsorb viruses (nominal pore size 0.2. µm ,). Filters, once used, were stored sealed in plastic at 4 °C in the dark for up to 14 days before further processing. Cuno data suggest that recovery of infectious enteroviruses from such membranes does not significantly change for periods of 10 weeks, when stored under these conditions. Following each apparatus use, the entire circuit was drained, flushed with 40L clean tap water and drained again. Samples of 10L volumes of tap water were filtered after this cleaning procedure and attempts were made to detect viruses from these, in order to verify the lack of sample carry-over. (see results). Chemical flushing of the system was not thought to be feasible since the prefilter was reused on multiple samples and it would be almost impossible to rinse the apparatus sufficiently. Similarly, it was not feasible to use a new prefilter for each sample as the smallest filter available had a capacity of 10,000L, and a cost of £19 for each unit. Furthermore, optimal performance was only achieved after conditioning the filter (see results).

Viruses were eluted from the Virosorb filters into 300ml 1% beef extract/0.05 M glycine pH 9.5 at 4 °C for 1 hour with gentle stirring. The resulting eluate was pH adjusted to 7.4 and virus precipitated by the addition of 13% polyethylene glycol (PEG) 8000/0.2 M NaCl pH 7.4 at 4 °C for 2-15 hours with stirring, followed by sedimentation at 7000g. Schwab and colleagues (Schwab *et al.*, 1995) have previously

Figure 1 - Plan of Water Filtration Apparatus



shown that virus recovery does not change significantly within this time period. The resultant precipitate was resuspended in 3ml TE buffer [10mM Tris, 1mM EDTA pH 8], extracted with an equal volume of chloroform and the aqueous phase reprecipitated as above but with the addition of 8% PEG. The precipitate was resuspended in 0.4 ml TE buffer and underwent gel chromatography through a Sephadex G200 spin column. The column was packed and used following the standard procedure outlined by Sambrook and colleagues (Sambrook *et al.*, 1989). This final eluate was concentrated by ultrafiltration using a Centricon 100™ (Amicon Ltd) concentrator following the manufacturers instructions and standardised to a final volume of 150 ml. Samples were stored at 4 °C in the dark.

METHOD
Viral Detection

Viruses were detected in a 1 µl subsample by polymerase chain reaction (PCR). Each sample was individually tested in a number of PCR reactions of differing specificities, designed to detect a number of different viruses.

Target specificity in PCR is chiefly determined by the design of the oligonucleotides used to prime the reaction, together with the annealing conditions used. A number of PCR primer sets have previously been described for the viruses of interest herein. Previously published primers were used for the detection of poliovirus, panenterovirus group, adenoviruses and hepatitis A. No suitable rotavirus-specific primers were identified, and so a set was designed specifically for this project. Primer sets used are shown in figure 2.

PCR itself, is not a quantitative method. It produces massive amplification of very small copy numbers of DNA and is exquisitely sensitive. It can be adapted however, to give a true reflection of the initial number of copies of a target piece of DNA. In order to do this, every single reaction has to contain an identical number of copies of another template, in addition to the DNA of interest. These control templates are competitively amplified under the same reactions using the same, or a separate set of oligonucleotide primers. The control is designed so that the resultant product is clearly identifiable; being a slightly different size from the test product. The test product is then quantified relative to the control standard reaction. This allows for in-tube variation due to minute differences in the reaction constituents and heating block temperature profiles, and inhibitors present in the individual samples. For accurate results, the DNA is amplified using fluorescent dye-tagged primers, and quantified by spectrofluorimetry. Although IVEM is currently developing controls for use in such a system, their use was outside the scope of this pilot study; particularly since the initial equipment outlay necessary would be considerable.

For this pilot study, a far more simple, but less accurate method of quantification was chosen, to give a relative measure of viral load in samples. Briefly, a log dilution

series was performed on the initial concentrated water sample. PCR was performed on a 1 µl volume of neat or diluted samples corresponding to 1:10, 1:100, 1:1,000 or 1:10,000 dilutions of the original. The end result was an expression of the most diluted sample still capable of being PCR-positive. Thus viral loads were expressed on an arbitary log scale as follows: -

titer = 1 sample was PCR positive at neat.
titer = 2 sample was PCR positive at 1:10 dilution
titer = 3 sample was PCR positive at 1:100 dilution
titer = 4 sample was PCR positive at 1:1,000 dilution
titer = 5 sample was PCR positive at 1:1,000 dilution

This method is frequently used to express relative quantities in other biological assays, such as serum antibody titer determination. In-tube variation due to pipetting inaccuracy was minimised wherever possible by making up bulk reaction mixes for each PCR and running the reactions simultaneously on large batches of samples. However, any variation in amplification efficiency due to inhibitory substances carried into the PCR by individual water samples could not be taken into account by this method.

Different PCR methods were followed for different viruses. Adenoviruses are DNA viruses and a nested PCR was used for their detection. All other viruses examined contain an RNA genome, and hence required an initial reverse transcription step.

RT-PCR viral samples were lysed by heating to 99°C for 5 minutes in 15µl PCR buffer [75 mM Tris, 20 mM $(NH_4)_2SO_4$, 0.01% Tween, 1.5 mM $MgCl_2$, 40 U ribonuclease inhibitor (RNasin - Promega) pH 9] with 50 pmols reverse PCR primer. Samples were chilled on ice for 5 minutes followed by reverse transcription at 42°C for one hour with the addition of 20U AMV reverse transcriptase (NBL). Samples were heated to 95°C for 5 minutes, then chilled on ice for 15 minutes. 35µl PCR buffer containing 50 pmols corresponding forward PCR primer and 1.5 U Red Hot DNA polymerase (Advanced Biotech. Ltd) was added to each tube and 30 cycles PCR performed using identical Omnigene thermal cyclers (Hybaid Ltd). Annealing temperatures are shown in figure 2.

Samples for adenovirus detection were lysed as above and used directly in the outer nested PCR. Reactions were carried out in 50µl volumes as above, with 8 pmols each of the outer primers. 1µl of the outer reaction was re-amplified using 16 pmols each of the inner primers.

PCR products (10µl) were visualised on 0.7% (w/v) Synergel™ (Diversified Biotech), 0.5% agarose gels stained with 0.3 mg/ml ethidium bromide and band sizes compared to a standard 50-1000 bp DNA ladder (Amersham) in order to identify the correct product. The viral load in the initial sample was expressed as previously described.

Fig 2 Design of PCR Primers

Rotavirus: (Butcher, S. - unpublished) correspond to a 256 bp portion of the VP6 region. Primers were designed using a multiple sequence alignment of 29 rotavirus sequences created using the Wisconsin Package (GCG v.8) pileup program.
Anneal = 47°C

TCC ACC AGG TAT GAA TT - forward
GGT CAC ATC CTC TCA CTA - reverse

Panenterovirus: (Rotbart, 1990) correspond to a highly conserved 155 bp region in the 5′ untranslated region and have been shown to amplify multiple environmental and laboratory strains of Polio, Echoviruses and Coxsakieviruses (Lees *et al.*, 1995). Anneal = 37°C

CCT CCG GCC CCT GAA TGC GGC TAA T - forward
ATT GTC ACC ATA AGA AGC CA - reverse

Adenovirus: (Girones *et al.*, 1993, Puig *et al.*, 1994) correspond to a 300 bp (outer) and 142 bp (inner) region of the hexon gene. They have been shown to detect 47 different strains (Girones *et al.*, 1993)
Anneal = 55°C (outer and inner)

GCC GCA GTG GTC TTA CAT GCA CAT C - forward outer
CAG CAC GCC GCG GAT GTC AAA GT - reverse outer
GCC ACC GAG ACG TAC TTC AGC CTG - forward inner
TTG TAC GAG TAC GCG GTA TCC TCG CGC TC - reverse inner

Hepatitis A: (Lees *et al.*, 1995) correspond to a 438 bp region of the VP3 gene.
Anneal = 53°C.

AAT GTA TAA CTG CTT TGG CTT CTA - forward
TCC ATA GCA TGA TAA AGA GGA ACA AAA CA - reverse

Poliovirus: (Tsai *et al.*, 1994) correspond to a 394 region of the 5′ untranslated region and amplify all 3 serotypes of polio, but no other enteroviruses.
Anneal = 55°C.

AGC ACT TCT GTT TCC C - forward
ACG GAC ACC CAA AGT A - reverse

series was performed on the initial concentrated water sample. PCR was performed on a 1 µl volume of neat or diluted samples corresponding to 1:10, 1:100, 1:1,000 or 1:10,000 dilutions of the original. The end result was an expression of the most diluted sample still capable of being PCR-positive. Thus viral loads were expressed on an arbitary log scale as follows: -

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PCR products (10µl) were visualised on 0.7% (w/v) Synergel™ (Diversified Biotech), 0.5% agarose gels stained with 0.3 mg/ml ethidium bromide and band sizes compared to a standard 50-1000 bp DNA ladder (Amersham) in order to identify the correct product. The viral load in the initial sample was expressed as previously described.

An example of typical PCR products using rotavirus and adenovirus-specific primers is shown in figure 3.

Due to the very nature of PCR, this method is prone to cross-contamination problems - both between individual samples being amplified at the same time, and by the accidental introduction of the relatively abundant specific DNA product from previous positive reactions. In order to detect any false-positive results due to cross-contamination, every PCR batch included a number of negative control reaction tubes which contained all reagents necessary for amplification, in the absence of template. These were interspersed throughout the batch - at least 6 per run, and were subjected to exactly the same manipulations as the actual samples. Any product in these tubes would have indicated the presence of some degree of cross contamination, leading to the complete discard of that batch. Similarly, known positive samples were also included in every PCR batch to show that the method was working correctly. All normal procedures were followed to minimise any problems of cross-contamination, such as the physical separation of areas used for sample manipulation pre- and post- amplification, the use of use-once batch-aliqotted reagents, and aerosol-reducing filter containing pipette tips.

RESULTS

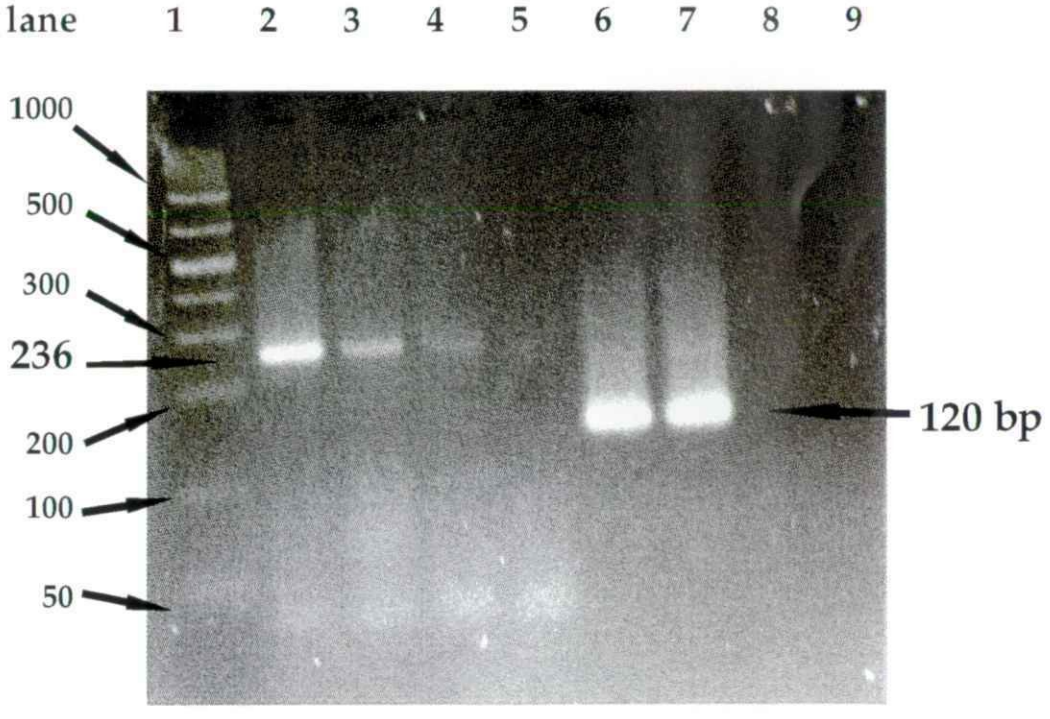
Raw data are shown tabulated in appendix 1.

Flow data for the Thames Water outfall, and River Thames sites downstream of the outfall, have been solicited from Thames Water PLC. We are currently awaiting their arrival, and so, as yet, no attempt has been made to link viral loads with perhaps the most important physical data of all - flow dynamics. Thames Water PLC have also agreed to supply water quality data for both the outfall and Culham Intake water abstraction point, situated on the River Thames between the Radley and Abingdon Bridge sites.

Initial Sampling

Briefly, water samples were collected from site 1 and site 3 in January, during a period of relatively high flow. These samples contained high levels of suspended sediment (45.5-56.75 mg/L) and it proved impossible to filter a full 10L sample due to repeated clogging of the secondary virus-trapping filter. Viruses were detected in samples corresponding to the maximum volumes actually filtered. These were 3.2 and 4.4L respectively and so these results cannot be directly compared with later samples where the full 10L were filtered. Conditioning the primary filter by running a large volume of sediment-laden water through a closed circuit in the absence of a secondary filter for several hours appeared to alleviate the problem and it was not encountered later in the project when sediment levels dropped. Initially, it was

Figure 3 - Agarose Gel Electrophoresis of Typical PCR Products from Environmental Water Samples



10ul PCR product run on a 2.5% Synergel 0.7% agarose gel and bands visualised by ethidium bromide staining

lane 1 dsDNA markers corresponding to the sizes shown (bp)

lanes 2-5 show a log dilution series of a sample taken from site 1 (outfall) and amplified with rotavirus-specific primers. The specific PCR product is 236 bp. Lanes correspond to neat, 1:10, 1:100 and 1:1,000 sample dilution. This sample was assigned a titer of 4

lanes 6-9 show a log dilution series of a sample taken from site 1 (outfall) and amplified with adenovirus-specific primers. The specific PCR product is 120. Lanes correspond to neat, 1:10, 1:100 and 1:1,000 sample dilution. This sample was assigned a score of 2

unknown how far downstream from the outfall viruses would remain detectable and so sites 1-3 only were sampled. This was done while purification and viral detection methods were being validated, in order to maximise the likelihood of water samples actually containing viruses. Once it became obvious that a number of viruses were persisting at the furthermost site (3), three further sites were repeatedly sampled - one upstream of the outfall (4), and two further downstream (5 and 6) - see table 1. Samples collected from sites during January, March and April were tested for the presence or absence of hepatitis A and polioviruses - a total of 16 samples. Of these, all samples were negative for the presence of hepatitis A virus, and only two samples were positive for poliovirus - taken from site 1 (the outfall) on January 26 and 31 respectively. Since the majority of samples were negative, despite the correct amplification of known positive samples in each test batch, further testing of samples specifically for hepatitis A and polioviruses was not performed. Initially all these samples were tested for the presence of caliciviruses (results not shown) but results are not presented due to problems with the primer specificity which could not be fully addressed within the scope of this study.

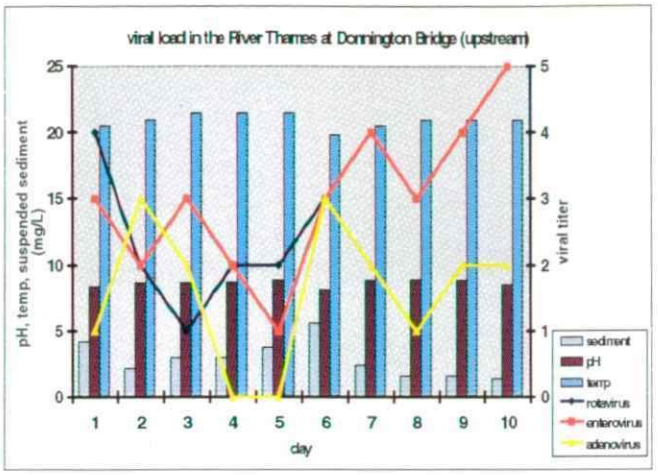
These early samples collected between January and April show water temperatures uniformly lower than those for samples collected during the later 10 day and diurnal series; ranging from 8-10 °C in January (see appendix 1A) to 11-16 °C in April. These sampling points show a distinct temperature difference between sampling sites, with the outfall water several degrees above those of the river Thames sites. Later samples, taken on 21st June, show higher water temperatures at all sites 20.5-22°C and the temperature gradient along the sites is no longer apparent. A pH gradient can also be identified in all these sampling sets (January-June), with the pH increasing from the outfall downstream.

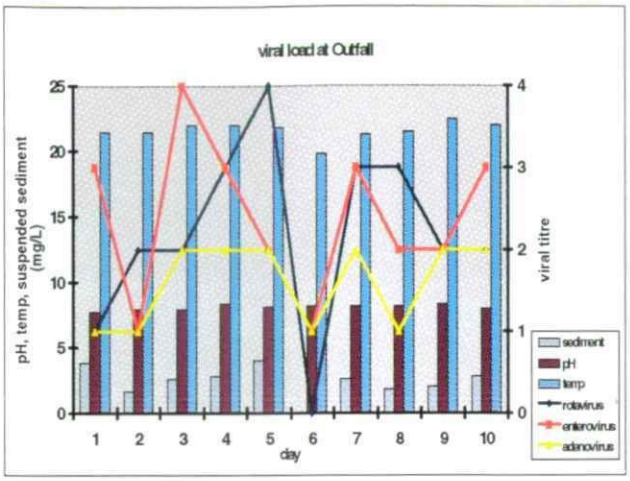
Daily Variation Series

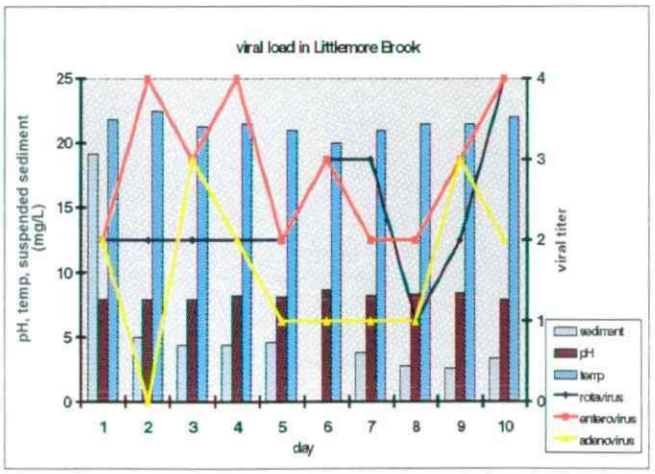
All six sites were sampled at the same time every day for 10 consecutive days during the period 21-30 July. This was done in order to investigate the daily variation in viral loads and physical data at each site, as well as collecting data to investigate how these varied with distance from the outfall.

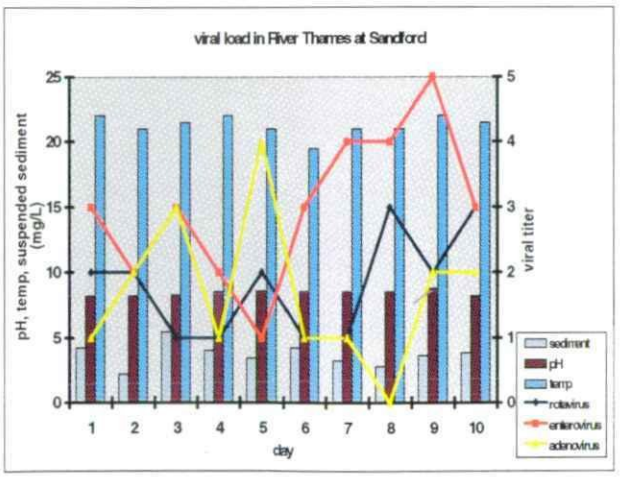
Water temperature, pH and suspended sediment values are shown plotted together with a measure of viral titer for ten consecutive sampling days at six sites (fig 4). As can be seen from this figure, physical parameters varied little throughout the sampling period. Water temperature ranged between 19.5-22.5 °C - a difference of only 3°C. Suspended sediment varied between 1.2-6.2 mg/L, although a single measurement recorded 19.2 mg/L at site 2, when sand was stirred up from the stream bed into the sample due to poor sampling technique. pH remained relatively constant within the 7.7-8.9 range. Generally, pH was lowest at the outfall, and highest at Abingdon Bridge. No strong trends in water temperature or suspended sediment were identified between different sites. Before the ten day series, no rain fell in the area for at least 2 weeks. Sparse rain fell immediately after the day 4 sample was collected. It then rained heavily for approximately 10 hours before the next (day 5) sample was collected. All three viral loads measured (adeno-, entero- and rota-) showed a drop in titer at the outfall on day 6. Rotavirus titer decreased

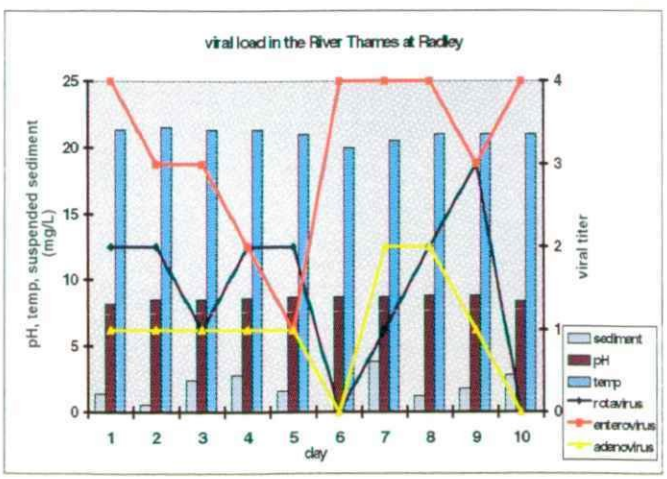
Fig 4 - DailyVariation in Detectable Viruses With Water Temperature, pH, and Suspended Sediment at Different Sites Above and Below Outfall Over a Ten Day Period - July 1997

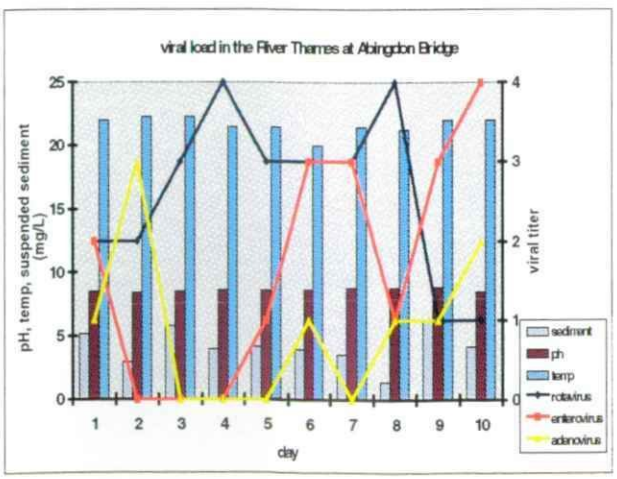












from 4-0, and both enterovirus and adenovirus titers decreased from 2-1. This pattern was not seen at any of the other sampling sites. This would perhaps reflect increased flow in Littlemore Brook upstream of the outfall, which would then dilute the outfall effluent being sampled at the outfall site. Conversely, this drop in apparent viral load may reflect rainwater dilution of the effluent itself. This may become more clear when flow data become available for the outfall, and other sites.

Diurnal Variation Series

Three sites comprising the outfall, one site upstream (Donnington Bridge) and the furthermost downstream site (Abingdon Bridge) were selected for intensive sampling over a single 24 hour period in order to investigate diurnal variation in viral load and physical features. No rain fell during this period, or for the proceeding 7 days.

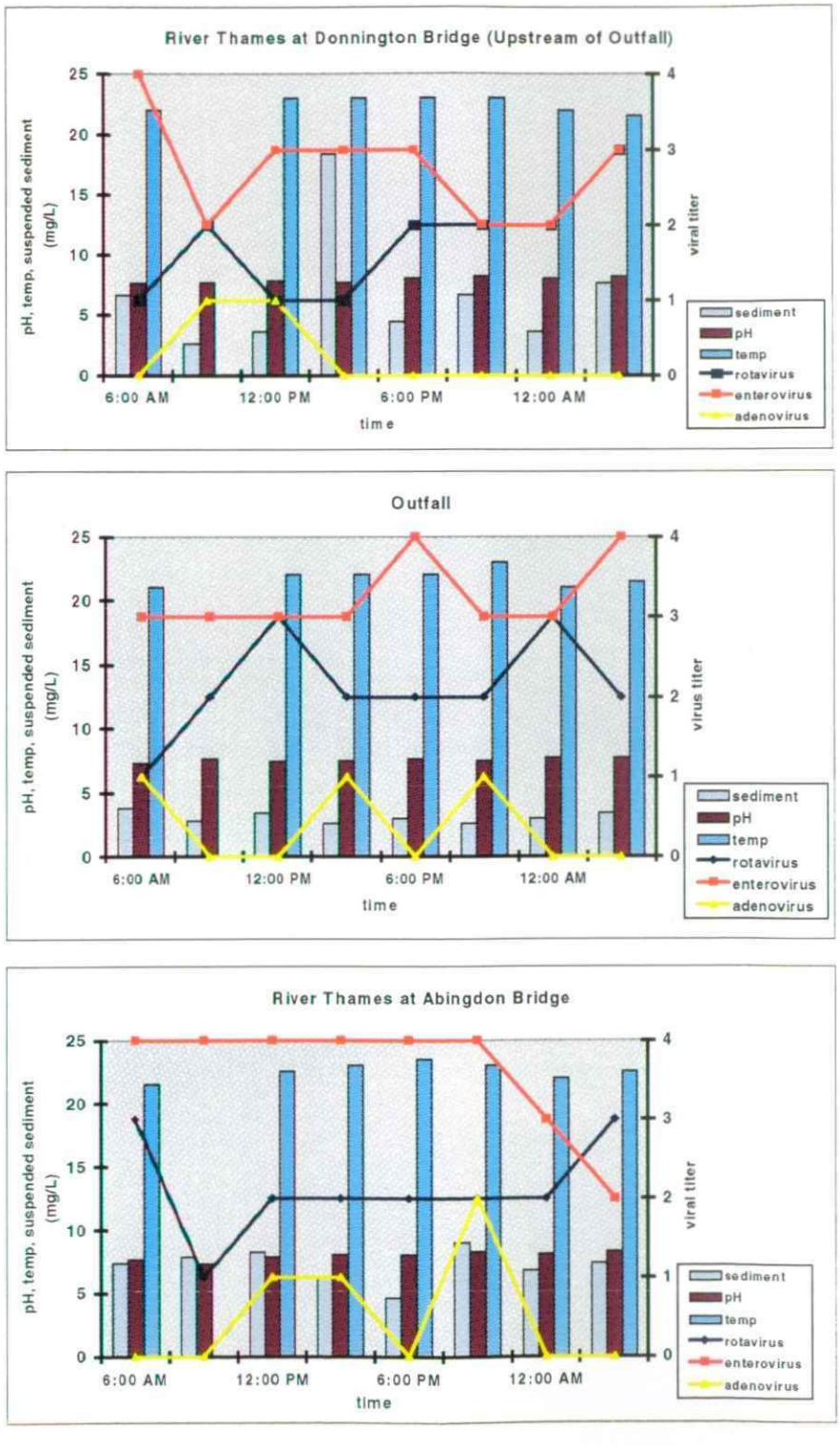
Water temperature, pH and suspended sediment values are shown plotted together with a measure of viral titers for the three sites with sampling every three hours over a 24 hr period (fig 5). As can be seen from figure 5, again there was little variation in either water temperature (21-23 °C) or pH (7.3 - 8.3); both between different sites or different sampling times. Also, suspended sediment was generally low, varying between 2.6-9 mg/L, with a single high measurement of 18.4 (see appendix 1 C). This was attributed to children riding bicycles off the jetty into the water at the sampling site while the sample was being collected. This can be contrasted with values of 45-58 mg/L recorded in January.

Accuracy of Determination of Viral Titer

The sensitivity of PCR with the previously-published primer sets has been determined elsewhere (Girones *et al.*, 1993, Lees *et al.*, 1995) (Tsai *et al.*, 1994).These studies have shown that, under ideal conditions, such PCR methods are capable of detecting as little as 1 (adenovirus) or 10 (hepatitis A, poliovirus, panenterovirus) virus genomes. Field samples, however carefully prepared, are likely to still contain inhibitory molecules and these decrease the apparent method sensitivity. As mentioned previously, the current methodology makes no provision for the measurement of degrees of inhibition, if any, in individual samples. Any PCR inhibitors inherent in the samples would be identical for all PCR tests on the same sample. While these cannot be calculated, the extensive and well-characterised purification protocol was designed to minimise inhibitory effects.

The percentage recovery of viruses during sample preparation for PCR is also currently unknown. Reports in the region of 90% recovery for poliovirus during similar filtration/purification have been made previously (Schwab *et al.*, 1995). Once decided upon, the methodology for sample filtration and purification was rigorously adhered to and so recoveries should be comparable between samples. A further source of inaccuracy stems from differences in the efficiency of both reverse transcription and amplification steps between individual tubes. These were minimised as far as possible by keeping rigorously to an exact protocol, and running samples in batches using bulk-produced reagent mixes to minimise pipetting

Fig 5 -Diurnal Variation in Detectable Viruses With Water Temperature pH, and Suspended Sediment at Different Sites Over 24 Hour Period - August 1997



inaccuracies. This should enable direct comparison of samples from the same trial e.g. all the ten-day samples were run simultaneously in the same batch.

Assuming a PCR detection limit of 10 viral particles, and a virus recovery of 100% during filtration/purification, viral titer values of 1, 2, 3, 4 and 5 are equivalent to 150, 1.5×10^3, 1.5×10^4, 1.5×10^5 and 1.5×10^6 genomes per L respectively. Since all viruses have only a single genome per virus particle, this could also be expressed in particle numbers. In practice, both detection limits and recovery of virus would be significantly lower, and so actual viral load would be correspondingly higher.

This method of detection does not require that virus particles infect cells, and so, technically, it is not possible to distinguish between viable infectious and damaged noninfectious viral particles. This would have important implications were viral titer to be directly used as a measure of hazard to human health posed by exposure to water. PCR can detect naked viral genomes, providing that the nucleic acid is intact within the region spanned by the PCR primers. There is little agreement in the literature as to the probability of virus detected in environmental water samples being noninfectious (Enriquez *et al.*, 1993). Some studies suggest that degradation of nucleic acid is rapid once the virion is sufficiently damaged to lose infectivity. Other reports suggest that this is not always the case. Many of the published investigations are not directly relevant since they address the problem in seawater rather than freshwater (Tsai *et al.*, 1995). This may well depend upon the type of viral genome as different types of nucleic acids are more resistant to degradation with nucleases. Single strand RNA, including the enterovirus genome, is highly sensitive to degradation - so much so that it is commonly protected by the addition of inhibitors of degradation, while undergoing manipulations in the laboratory. The double strand RNA genome of Rotaviruses is perhaps more resistant to degradation in the environment, and the adenovirus DNA genome even more so. These viruses become noninfectious mainly due to damage to the integrity of the outermost protein layer(s) comprising the viral capsid. This damage also opens the viral genome to attack by environmental factors. Thus infectivity and integrity of genome are functionally linked.

As previously discussed in this report, the estimation of viral load by PCR on a log dilution series cannot be expected to give truly consistent results without internal tube comparative controls.

Nevertheless, even within the scope of this trial, a number of significant results can be shown.

Significant Results

- A method for viral concentration, purification and detection from environmental freshwater samples has been developed.

- Viral loads were significantly different at different sampling sites, at differing distances up- and downstream.

- Neither viral loads nor physical parameters measured differed significantly over the 24 hour sampling trial, at any of the three sites investigated. This suggests that, at least during a 24 period with no rain, diurnal variation may be ignored in future calculations.

- All three viruses (adeno-, rota- and entero-) were frequently detected at the Donnington Bridge site upstream of the test outfall. This suggests that there is significant persistence of viruses in the river, and these are being detected from outfalls upstream of the test site.

- Neither poliovirus nor hepatitis A are suitable model viruses for monitoring fecal contamination of river water in view of their very rare presence.

- Rotaviruses, enteroviruses and adenoviruses were all commonly detected in riverwater at all sampling points and, as such, fulfil the necessary modelling criterion of being present at detectable levels in a high proportion of samples.

- There is no simple correlation between viral titer at a particular site and suspended sediment, water temperature or pH within these data.

- Viruses are more stable in the freshwater environment than fecal indicator bacteria such s *E. coli* and persist at high titers over 7 miles downstream from a sewage outfall.

Samples from the ten day sampling series, and the 24 hour sampling series were considered as separate datasets, and analysis of covariance was performed on all measured parameters. Enterovirus, adenovirus and rotavirus titers were found to differ significantly between sites ($p > 0.05$). Site 4, upstream of the outfall, was found to have significantly different viral titers from all other sites. Sites 5 and 6 (downstream) had no statistically significant differences between them, but were significantly lower than titers at sites 1, 2 and 3. Likewise, sites 1, 2 and 3 did not differ significantly between themselves, but were significantly different from sites 4, 5 and 6. No significant correlations between viral titer and water temperature, pH or suspended sediment were found. Furthermore, when the 24hr. dataset was considered, there was found to be no significant change in any of the three viral loads measured at any of the sites over the time-period.

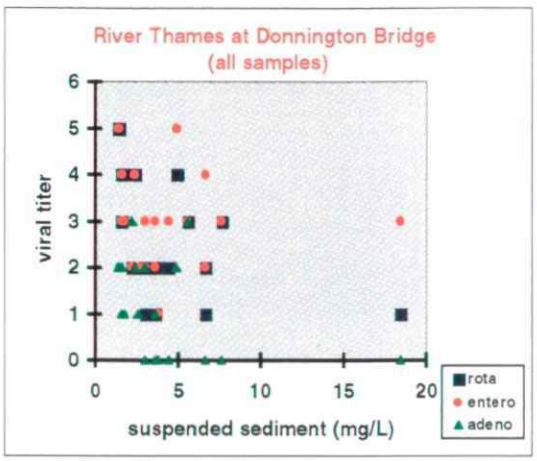
These datasets were pooled for each site, together with relevant sample points from earlier months, and the pairwise degree of linear correlation between different parameters was measured (Pearson's). The relationships between suspended sediment, water pH and viral titer at three sites are shown as scatter plots in fig. 6. There was a weak positive correlation between adenovirus titer and water pH at all three sites shown in fig 6., but this was only significant at the outfall ($p < 0.001$). There was a significant inverse correlation between pH and enterovirus titer at Abingdon Bridge ($p < 0.01$). There were no significant correlations found between any viral titer and either suspended sediment or water temperature. There was a significant correlation between rotavirus titer and enterovirus titer at all three sites. Since both viruses come from the same sewage source, this is perhaps not surprising. However, this was a positive correlation at Donnington Bridge ($p<0.01$) and the outfall ($p< 0.1$) but an inverse correlation at Abingdon Bridge ($p<0.001$) and this cannot be explained at present. There was also a significant correlation between rotavirus titer and pH but this was only significant at Donnington Bridge.

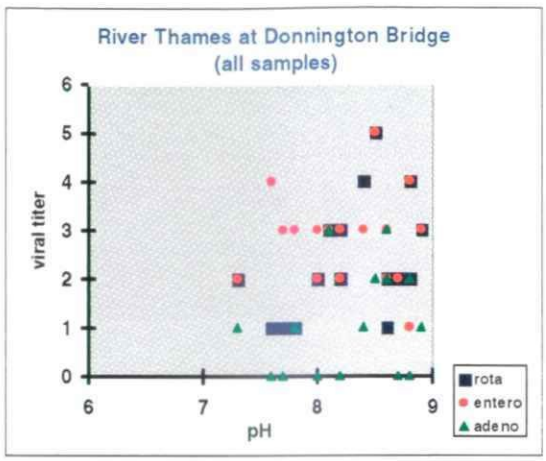
DISCUSSION

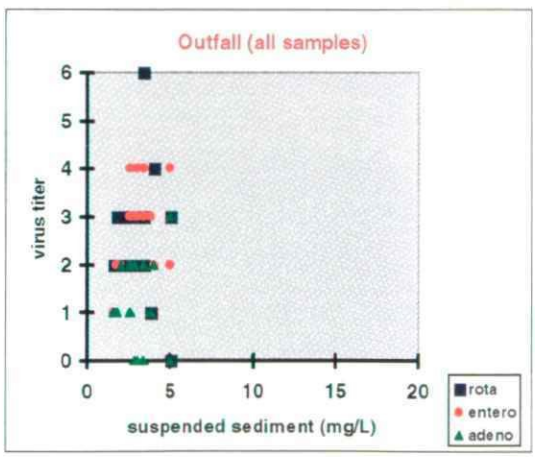
The presence of significant levels of virus at Site 4 (Donnington Bridge) suggests that viruses are able to persist in the river and are being carried downstream appreciable distances, from outfalls upstream of the sample area. No outfalls have been identified in the immediate area upstream. This is hampered currently by the fact that available maps are very out of date and numerous changes to the sewage system are not marked. Thames Water PLC have again been approached to give details of upstream discharges into both the Cherwell and Thames, together with any water quality data, and these will help to show how far these viruses are being transported downstream. This will make the further study of viral transport and persistence more complicated than first envisaged, since this alternatively sourced virus will have to be accounted for. The inability to distinguish between viral loads at sites 1, 2 and 3 is perhaps not surprising since they are relatively close together spatially, compared with sites 5 and 6, which also could not be distinguished from each other with statistical significance. This again reflects the finding that viruses appear to be very persistent in the system. Viral loads were lower at the more distant sites, as may be expected due to viral dispersal and inactivation in the river. All these data suggest that enteric viruses appear to be more persistent in the river than faecal indicator bacteria.

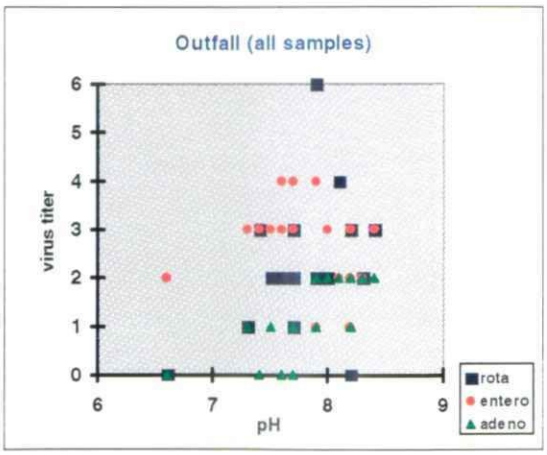
The failure to find any significant correlation between physical data - suspended sediment, temperature and pH - and viral load in these data may partially be explained by the relatively small datasets, and the relatively small level of variation of these parameters. It may also be that the combined inaccuracies inherent in the PCR-based viral detection/quantification method currently used are sufficient to mask relationships - particularly during periods with little variation in physical parameters. Furthermore, it is also possible that the primary filter may influence the

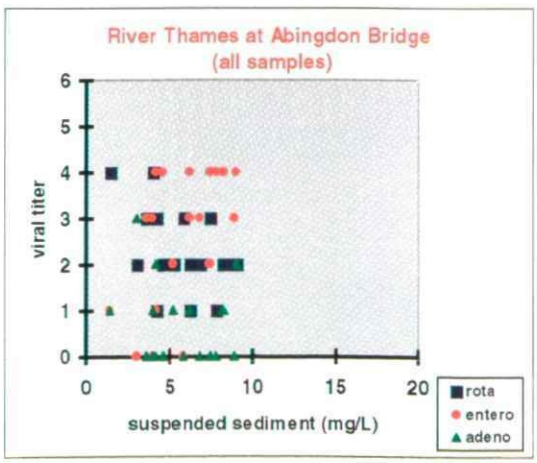
Fig 6 - Scatter Plots Showing the Relationship Between Suspended Sediment, pH and Detectable Virus At Different Sites Over 8 Month Period

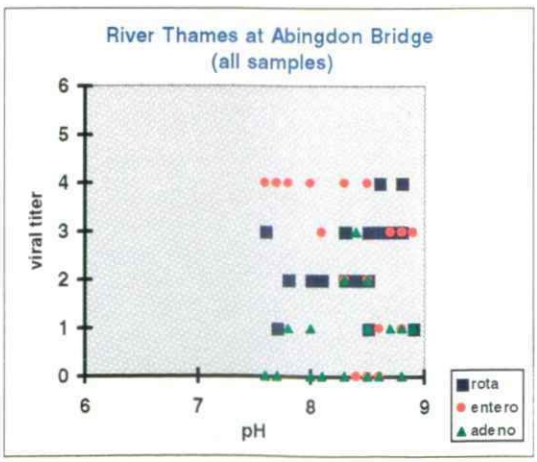












relation between suspended sediment and viral titer, by trapping a proportion of the suspended sediment, together with any virus adsorbed to it.

Certainly, all the physical parameters measured would be expected to influence viral persistence, either by speeding viral degradation, or by influencing the interactions between viral particles and sediment as previously discussed in the introduction.

The lack of evidence for significant diurnal variation in viral loads at different sites has implications for further experimental design since this suggests that sample timing may not be crucial in order to make samples broadly comparable. This sampling was undertaken during a period of dry weather, with no rainfall for the preceding 10 day period. This result may not hold for periods where conditions in the river are changing more rapidly; particularly where flow changes rapidly due to a significant rainfall, with concomitant changes in suspended sediment.

ONGOING WORK

The work discussed herein has already been presented, as both a poster and an invited short oral presentation at the 2nd UK Conference on Health-Related Water Microbiology (Warwick UK).

As mentioned earlier in the results section, crucial flow and water quality data are still being awaited from Thames Water PLC. Once these are forthcoming a considerable amount of further data analyses will be undertaken, to further elucidate the relationships between physical factors and viral loads. It is hoped that these analyses, along with the work already completed will be presented for publication.

FUTURE WORK

As a pilot scheme, this project has shown that it is feasible to undertake environmental water sampling work of this kind. The data it has provided have enabled a number of results to be presented. However, by the very nature of being a limited trial, a number of important points have been raised which need to be addressed. Further work will need to encompass two major areas:

1. Validation of methodology by further experimentation and substitution of more accurate, but more expensive methodologies for viral quantification where necessary

2. Greatly increased sampling to produce datasets of sufficient size to be used for full statistical analyses of the sampling sites under diverse conditions. This would need to include actual sampling and quantification of *E. coli* levels at all sampling points if viral loads are to be directly compared with fecal bacteria

3. The provision of (2) will enable the construction of a mathematical model which would describe the factors influencing the transport and persistence of viruses in a similar vein to the model for *E. coli* previously developed by IH.

Validation of Methodology

Laboratory experiments will be used to determine levels of virus recovery during filtration/purification by using known quantities of poliovirus as a model virus to spike water samples undergoing treatment. Accurate titers of virus recovered will then be measured by conventional tissue culture plaque assay. This will also determine loss of infectivity during sample processing since this method will only detect infectious virus. Plaque assay cannot be used for most of the other viruses of interest due to their inability to either grow or produce plaques in tissue culture. This will be repeated using PCR methodology.

The sensitivity of the Rotavirus-specific RT-PCR will be measured by using a strain of rotavirus capable of growth in tissue culture as a titrated test template. Use of in-tube standardisation controls will also be investigated for the PCR. This will require the development of suitable RNA control templates where these are not currently available. This method will also require significant input in terms of initial equipment outlay since accurate quantification of PCR products needs specialised spectrofluorometric equipment.

There would also be significant scope for the investigation of loss of viral infectivity of samples *in vitro* under varying conditions.

Future Sampling

This project has highlighted the need to increase the scope for sampling. Many of the sites showed little variation in physical data during the sampling periods and this may be a function of the timescale. Future sampling should be targeted within other seasons, to increase the likelihood of sampling at more widespread flow rates. It is possible that the weak correlations noted between viral titer and pH or other parameters may become significant when more data becomes available. It may also prove important to sample from other sites, perhaps even further from the outfall.

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SITE	DATE	TIME	water temp	pH	sediment (	volume (L)	rotavirus	entero	adeno	polio	hep A
1	1/26/97	11.45	10	7.5	45.5	3.2	+	+	ND	+	0
3	1/26/97	12.22	8	7.8	56.75	4.4	+	+	ND	0	0
1	3/1/97	15	12.5	7	-	18	2	2	1	+	0
1	3/27/97	10.2	16	7.9	3.4	10	6	4	2	0	0
2	3/27/97	12.05	15	8	11.4	10	2	4	1	0	0
3	3/27/97	10.41	12	8.6	9.8	10	2	3	2	0	0
Weir	3/27/97	11.25	12	8.8	9.8	10	0	2	3	0	0
1	4/4/97	17.25	16	6.6	5	10	0	2	0	0	0
2	4/4/97	16.45	16	6.8	8.6	10	0	3	0	0	0
3	4/4/97	17.45	12	7.8	4.8	10	0	4	3	0	0
1	4/21/97	16.45	16	ND	5	10	3	4	3	0	0
2	4/21/97	17.2	15	ND	4.2	10	2	4	1	0	0
3	4/21/97	17	12	ND	8.3	10	1	3	0	0	0
donnington	4/21/97	14.35	11	ND	4.9	10	4	5	2	0	0
radley	4/21/97	15.29	12	ND	7.7	10	3	4	1	0	0
abingdon	4/21/97	16.1	11	ND	8.9	10	2	3	0	0	0
1	21/7/97	12.32	21.5	7.7	3.8	10	1	3	1	ND	ND
2	21/7/97	13	21.8	7.9	19.2	10	2	2	2	ND	ND
3	21/7/97	14.47	22	8.2	4.2	10	2	3	1	ND	ND
donnington	21/7/97	11.58	20.5	8.4	4.2	10	4	3	1	ND	ND
radley	21/7/97	14.22	21.3	8.2	1.4	10	2	4	1	ND	ND
abingdon	21/7/97	13.3	22	8.5	5.2	10	2	2	1	ND	ND

APPENDIX 1A - Initial Sampling

APPENDIX 1B - 10 Day Trial

SITE	DATE	TIME	water temp	pH	sediment (	volume (L)	rotavirus	enterovirus	adenovirus
1	21/7/97	12.32	21.5	7.7	3.8	10	1	3	1
2	21/7/97	13	21.8	7.9	19.2	10	2	2	2
3	21/7/97	14.47	22	8.2	4.2	10	2	3	1
donnington	21/7/97	11.58	20.5	8.4	4.2	10	4	3	1
radley	21/7/97	14.22	21.3	8.2	1.4	10	2	4	1
abingdon	21/7/97	13.3	22	8.5	5.2	10	2	2	1
1	22/7/97	12.35	21.5	7.9	1.6	10	2	1	1
2	22/7/97	13.03	22.5	7.9	5	10	2	4	0
3	22/7/97	13.12	21	8.2	2.2	10	2	2	2
donnington	22/7/97	11.55	21	8.6	2.2	10	2	2	3
radley	22/7/97	13.54	21.5	8.5	0.6	10	2	3	1
abingdon	22/07/97	14.25	22.3	8.4	3	10	2	0	3
1	23/7/97	12.36	22	7.9	2.6	10	2	4	2
2	23/7/97	12.1	21.3	7.9	4.4	10	2	3	3
3	23/7/97	12.25	21.5	8.3	5.4	10	1	3	3
donnington	23/7/97	11.5	21.5	8.6	3	10	1	3	2
radley	23/7/97	1.15	21.3	8.5	2.4	10	1	3	1
abingdon	23/7/97	13.4	22.5	8.5	5.8	10	3	0	0
1	24/7/97	13.3	22	8.4	2.8	10	3	3	2
2	24/7/97	13	21.5	8.2	4.4	10	2	4	2
3	24/7/97	13.14	22	8.5	4	10	1	2	1
donnington	24/7/97	12.35	21.5	8.7	3	10	2	2	0
radley	24/7/97	14.3	21.3	8.6	2.8	10	2	2	1
abingdon	24/7/97	14.5	21.5	8.6	4	10	4	0	0
1	25/7/97	13.03	21.8	8.1	4	10	4	2	2
2	25/7/97	12.4	21	8.1	4.6	10	2	2	1
3	25/7/97	12.49	21	8.6	3.4	10	2	1	4
donnington	25/7/97	11.43	21.5	8.8	3.8	10	2	1	0
radley	25/7/97	13.4	21	8.7	1.6	10	2	1	1
abingdon	25/7/97	14.05	21.5	8.6	4.2	10	3	1	0
1	26/7/97	13.58	19.8	8.2		10	0	1	1
2	26/7/97	13.1	20	8.7		10	3	3	1
3	26/7/97	13.45	19.5	8.5	4.2	10	1	3	1
donnington	26/7/97	13.3	19.8	8.1	5.6	10	3	3	3
radley	26/7/97	14.3	20	8.7		10	0	4	0
abingdon	26/7/97	15	20	8.7		10	3	3	1
1	27/7/97	13.05	21.3	8.2	2.6	10	3	3	2
2	27/7/97	12.4	21	8.2	3.8	10	3	2	1
3	27/7/97	12.5	21	8.5	3.2	10	1	4	1
donnigton	27/7/97	12.2	20.5	8.8	2.4	10	4	4	2
radley	27/7/97	13.3	20.5	8.7	3.8	10	1	4	2
abingdon	27/7/97	13.5	21.5	8.8	3.6	10	3	3	0
1	28/7/97	2.4	21.5	8.2	1.8	10	3	2	1
2	28/7/97	2.15	21.5	8.3	2.8	10	1	2	1
3	28/7/97	2.25	21	8.5	2.8	10	3	4	0
donnington	28/7/97	1.45	21	8.9	1.6	10	3	3	1
radley	28/7/97	3.05	21	8.8	1.2	10	2	4	2
abingdon	28/7/97	3.25	21.3	8.8	1.4	10	4	1	1

APPENDIX 1B - 10 Day Trial

1	29/7/97	12.55	22.5	8.3	2	10	2	2	2
2	29/7/97	12.33	21.5	8.4	2.6	10	2	3	3
3	29/7/97	12.44	22	8.8	3.6	10	2	5	2
donnington	29/7/97	12.15	21	8.8	1.6	10	4	4	2
radley	29/7/97	13.2	21	8.8	1.8	10	3	3	1
abingdon	29/7/97	13.45	22	8.9	6.2	10	1	3	1
1	30/7/97	13.35	22	8	2.8	10	2	3	2
2	30/7/97	13.12	22	7.9	3.4	10	4	4	2
3	30/7/97	13.2	21.5	8.2	3.8	10	3	3	2
donnington	30/7/97	12.55	21	8.5	1.4	10	5	5	2
radley	30/7/97	14.05	21	8.4	2.8	10	0	4	0
abingdon	30/7/97	14.45	22	8.5	4.2	10	1	4	2

APPENDIX 1C - Diurnal Sampling over 24 hour period

SITE	DATE	TIME	water temp	pH	sediment (	volume (L)	rotavirus	enterovirus	adenovirus
1	12/8/97	6.3	21	7.3	3.8	10	1	3	1
donnington		6.05	22	7.6	6.6	10	1	4	0
abingdon		6.55	21.5	7.6	7.4	10	3	4	0
1		9.25		7.6	2.8	10	2	3	0
donnington		9		7.3	2.6	10	2	2	1
abingdon		9.55		7.7	7.8	10	1	4	0
1		12.03	22	7.4	3.4	10	3	3	0
donnington		12.22	23	7.8	3.6	10	1	3	1
abingdon		12.48	22.5	7.8	8.2	10	2	4	1
1		15.25	22	7.5	2.6	10	2	3	1
donnington		15.1	23	7.7	18.4	10	1	3	0
abingdon		15.55	23	8	6.2	10	2	4	1
1		18.15	22	7.6	3	10	2	4	0
donnington		18	23	8	4.4	10	2	3	0
abingdon		18.47	23.5	8	4.6	10	2	4	0
1		21.25	23	7.5	2.6	10	2	3	1
donnington		21	23	8.2	6.6	10	2	2	0
abingdon		21.5	23	8.3	9	10	2	4	2
1	13/08/97	0.25	21	7.7	3	10	3	3	0
donnington		0.01	22	8	3.6	10	2	2	0
abingdon		1	22	8.1	6.8	10	2	3	0
1	13/08/97	3.2	21.5	7.7	3.4	10	2	4	0
donnington		3	21.5	8.2	7.6	10	3	3	0
abingdon		3.5	22.5	8.3	7.4	10	3	2	0