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VIRUS EXCLUSION AND INACTIVATION DURING WATER TREATMENT BY REVERSE OSMOSIS

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This experimental work was conducted in the Water Resources Unit of the Department of Life Sciences, Trent Polytechnic, Nottingham.

SUMMARY

A river survey was undertaken to ascertain the virus load expected to be received by an RO unit treating river water, and it was shown that $Al(OH)_3$ flocculation, the concentration method originally employed, also concentrated heavy metals to levels toxic to assay cell lines.

Iron oxide adsorption was substituted, using a specially constructed electromagnet, and following a 1000 fold concentration of 20 litre water samples 87.46 % recovery of added viruses was achieved. With this method viruses were isolated at up to 2.4 pfu l^{-1}, with most isolations occuring during summer months. As a result a correlation between the number of virus isolations and improving water quality was established both between the sampling sites chosen, and during the course of the investigation.

Reverse osmosis, using artificially high loadings of test viruses was shown to effectively separate viruses from river water and tertiary treated sewage effluent, with 99.999+ % exclusion under normal operational conditions. Penetration of the membrane was shown not to occur, except at elevated pressures. A correlation was noted between decreasing recirculation water pH and increasing virus LOI.

Viral LOI was also thought to occur with heavy metal cations concentrated within the units and a series of laboratory studies were undertaken to examine this further. It was shown that LOI was temperature, metal ion concentration and

valency dependent for all cations tested. However, divalent mercury and lead, and trivalent cations were so effective as to totally inactivate the viruses at all ion concentrations examined. These studies also showed the presence of two virus sub-populations, one more sensitive to cationic action than the other.

Virus titre was partially recoverable by dialysis and electron microscopic examination revealed that viral aggregation was the main cause of LOI.

It was concluded that Enterovirus and MS 2 acted as hydrophilic colloids in water and that coat and A protein precipitation was the result of metal induced aggregation.

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CONTENTS

INTRODUCTION

Virus Dissemination in Water

By the beginning of the present century most human diseases of viral etiology had been characterised, but it was not until 1929 that the first theory of waterborne virus transmission was published (Kling, 1929). This 'theorie hydrique', for Poliomyeltis dissemination gained little general acceptance, however, until the 1940's, when Carlson, et al (1942) first demonstrated that Poliovirus was present in the faeces of people with the disease, and that the organism subsequently found its way into sewage and watercourses. Since then over one hundred identifiable viruses have been isolated from human faeces and more than seventy strains recovered from municipal wastewater and polluted surface water (Poduska & Hershey, 1972).

Any human virus excreted in faeces is theoretically water transmissible (Chang, 1948: Committee on Viruses in water, 1969) but consideration is usually restricted to those growing in the intestinal wall and discharged in large numbers in the faeces. These have been designated 'enteric' viruses and are listed, with their associated diseases, in Table 1.

Enteric viruses are usually classified into six groups, Polioviruses, Coxsackieviruses A and B, and Enteric, Cytopathogenic, Human Orphan (ECHO) viruses, which together form the Enteroviruses, plus Reovirus and the Hepatitis A virus.

Table 1. Diseases of humans caused by waterborne enteric viruses (Numbers refer to Serotypes)

Disease	Poliovirus	Coxsackie-virus A	Coxsackie-virus B	ECHOvirus	REOvirus	Infectious Hepatitis
Paralysis, Sustained	1,2,3	4,7,9,23	-	2,4,9,11, 13,16	-	-
Paralysis, transient	1,2,3	2,9	3,4,5	1,9,16	-	-
Encephalitis	1,2,3	-	3*	9,19	-	-
Aseptic mengitis	1,2,3	2,4,7,9, 23*	1,2,3,4,5* 6	1,2,3,4*, 6*,7,9*,11, 12,13,14, 15,16,18,19, 20,21,22,25	-	-
Enteritis	-	-	-	6*,8,11,14, 18,19,20,22, 23,24,28	-	-
Herpangina	-	2,3,4,5, 6,8,10	-	-	-	-
Epidemic pleurodynia	-	-	1,2*,3*, 4*,5*	-	-	-
Infectious hepatitis	-	-	-	-	-	A*
Respiratory illnesses	-	21*	1,3	6,8,11,20* 22,25,28*	1,2,3	-
Myocarditis	1,2,3	-	2,3,4,5	-	-	-
Pericarditis	-	-	2,3,4,5	-	-	-

Table 1 - continued

Disease	Poliovirus	Coxsackie-virus A	Coxsackie-virus B	ECHOvirus	REOvirus	Infectious Hepatitis
Rashes	-	2,4,9,16*, 23	1,3,5	2,4,6,9*, 14,16*	-	-
Hand, foot & mouth disease	-	5,16	-	-	-	-
Diarrhoea	-	-	-	-	1,2,3	-

Ref: Swain, 1970

* indicates serotypes known to have been associated with epidemics.

Also worthy of consideration are the Adenoviruses, causing infections of the eye and pharynx, for which some evidence of waterborne transmission exists (Becker, 1966). The characteristics of this organism, together with those of the enteric viruses, and the waterborne bacteriophage MS 2, are displayed in Table 2. The similarities between the enteric viruses are highlighted by reference to this table, as are those between the Enteroviruses and MS 2, frequently used as a model for predicting their behaviour.

It has been calculated (Clarke, et al, 1962), using human volunteers and Sabin's avirulent vaccine strain of Poliovirus that human faeces could contain up to 10^5 to 10^6 tissue culture infective doses (TCID 50) per gram, at times of peak excretion. Furthermore, 5 to 60 % of apparently healthy children, below 15 years of age, were shown to excrete enteric viruses, this being considered normal among young people (Berg, 1964). From this Clarke, et al (1962) reasoned that if 31.5 % of the population of the USA were within this age group, and that if 10 % of them were shedding viruses at any one time, then the mean 'per capita' enteric density would be two hundred virus units per gram of faeces. They also stated that with a mean coliform bacterial density of 1.95×10^9 per capita per day, and a mean faeces weight of 150 grams per capita per day, the ratio of coliforms to enteric viruses would be 65000:1. With an average coliform density of 4.6×10^7 per 100 ml in sewage, and 10^4 to 10^5 per 100 ml of polluted fresh water,

Table 2. Characteristics of waterborne human viruses and phage MS 2

Characteristics	Adenovirus	Reovirus	Infectious Hepatitis virus	Coxsackie-virus	ECHOvirus	Poliovirus	MS 2
Serotypes	30	3	1(?)	24A + 6B	30	3	1*
Diameter (nm)	70-80	76	27	28	27	28	25
Capsomeres, no.	252	92	-	32	32	60	180
Symmetry	cubic	cubic	cubic	cubic	cubic	cubic	cubic
Envelope	no	no	no(?)	no	no	no	no
Weight(Daltons)	175×10^6	100×10^6	-	7.0×10^6	7.0×10^6	8.4×10^6	3.8×10^6
Sved. Coeff.	-	630	-	153	157	158	150
Density (g ml^{-1})	1.34	-	1.39-1.41	1.34	1.34	1.34-1.44	
Type	DNA(2x)	RNA(2x)	RNA	RNA(1x)	RNA(1x)	RNA(1x)	RNA(1x)
Weight(Daltons)	20×10^6	2.4×10^6	-	1.7×10^6	1.8×10^6	2.4×10^6	1.3×10^6
%	11.5	15.0	-	25-30	20-30	25-31	31.5
A/G/U/C	-	-	-	28/28/ 24/20	-	30/23/ 24/23	23/27/24/ 26
Shape	-	-	-	linear	linear	linear	linear

Table 2 - continued

Characteristics	Adenovirus	Reovirus	Infectious Hepatitis virus	Coxsackie-virus	ECHOvirus	Poliovirus	MS 2
No. nucleotides	-	-	-	-	-	7700	3300
Phys/chem:							
Host			man	primate cells	primate cells	primate cells	'male' E. coli
Cell adsorption				cell surface	cell surface	cell surface	f pili sides
Cell entry				virion	virion	virion	RNA only
Release				vacuola-tion	vacuola-tion	surface vacuoles	cell lysis
pH range	Wide & stable	2.2-8.0	stable at 3.0	4.0-10.0	3.0-11.0	3.0-11.0	3.0-10.0
Ether sens.	resistant	resistant	resistant	resistant	resistant	resistant	resistant
Temp.stability	-	very stable	stable	cation stabilised	cation stabilised	cation stabilised	stable
LHT Family						napoviridae	napoviridae

Compiled principally from: Frankel-Conrat, 1974: Beladi, et al, 1972: Johnson, 1975.

*MS 2 is one of a group of serologically related male specific phage.

the expected enteric density was therefore predicted as 700 per 100 ml and 0.15 to 1.5 per 100 ml respectively. Actual enteric virus numbers were measured by Kelly and Sanderson (1962) as 100 per 100 ml of raw sewage, and Vajdic (1968) suggested values of 500 per 100 ml in sewage and 1.0 per 100 ml in polluted fresh water, both in reasonable agreement with the values calculated above.

Enteric viruses are readily detectable in many sewers and rivers and several detailed studies have been reported. Lamb, et al (1964) detected Coxsackieviruses and Polioviruses in the river Illinois, and the Chicago sewers feeding it, in one hundred and twenty-two out of one hundred and sixty-four samplings (74.4 %) and in a detailed examination of the river Isere at Grenoble (Seigneurin, et al, 1968) Adenoviruses were most commonly found, mainly in sewers from a large hospital. Coxsackieviruses and Polioviruses were also detected, together with a high percentage of unidentified viruses. In a recent report from Israel Fattal and Nishmi (1977) showed that of four hundred and eighty nine viral isolates from sewage, 74% were Poliovirus, 10% Coxsackievirus B, Coxsackievirus A9, or ECHOvirus 9, and 16 % other ECHOvirus or Enterovirus strains.

The Thames Water Authority virologists detected viruses on thirty three out of ninety four occasions (Annual Report, 1973) in samples of water from the Rivers Thames and Lee during 1972 and 1973, those most frequently isolated being Coxsackievirus B5 and Poliovirus 2. These were found in 20 % and 15 %

of all samples respectively, whilst Poliovirus 1, Poliovirus 3 and Coxsackievirus B1 were only detected on one occasion each. At the same time they found no viruses in three reservoirs and tap water samples in the same areas.

Finally Bagdasar'yan (1968) demonstrated that 34.1% of samples from the Rivers Moskva, Skhodnya, and Yauza, in Russia, contained cytopathogenic agents, although these were not identified further.

The sea is the ultimate recipient of sewage, from coastal settlements polluting it directly, or from contaminated rivers. Enteric viruses were shown to be very stable in sea water (Knott, et al, 1969: Slantez, 1966), the degree of salinity not being relevant to their survival, within usually accepted values.

Of much greater influence, however, in marine and fresh waters, was the temperature, as illustrated in Table 3, compiled from a number of sources. Several authors, notably Clarke, et al (1962) noted a direct correlation between survival time and water temperature, survival being less in all cases at elevated temperatures. Bagdasar'yan and Abieva (1971) also showed that the degree of pollution and individual variable resistance affected viral survival in water, but Clarke demonstrated that viruses survived equally well in clean and grossly polluted waters. However, Wellington, et al (1955) showed that bacterial survival was directly proportional to the degree of pollution, with longer survival at greater levels

Table 3. Survival time (days) of Various Viruses in Water of differing qualities

Virus	Sewage			Distilled water			River water			Sea water		
	4-8°	20°	28-37°	4-8°	20°	28-37°	4-8°	20°	28-37°	4-8°	20°	28-37°
Poliovirus 1	110(2)	23(2)	17(2)	100-140(2)	84(2)	-	118-180(1)	13-20 (3)	11-17(2)	140-154(3)	14-28 (3)	77(3)
Poliovirus 3	-	-	-	-	-	-	-	-	-	-	4(4)	-
ECHOvirus 6	-	-	20(2)	-	-	-	-	-	-	280(3)	42-56 (3)	77(3)
ECHOvirus 7	130(2)	41(2)	28(2)	-	-	-	15-26 (2)	7-16 (2)	5(2)	-	-	-
ECHOvirus 12	60(2)	32(2)	-	-	-	-	19-33 (2)	5-12 (2)	3-5(2)	-	-	-
Coxsackievirus A2	61(2)	41(2)	-	272+(2)	41-100 (2)	-	14-56 (1)	5-18 (1)	9(1)	-	-	-
Coxsackievirus A5	-	-	-	280(1)	210(1)	-	-	-	-	-	-	-
Coxsackievirus A9	12(3)	-	5(2)	-	-	-	8-20 (2)	8(2)	5-8(2)	-	-	-
Coxsackievirus B5	-	-	-	-	-	-	280(5)	210+ (5)	-	371+ (3)	14-56 (3)	77(3)

Table 3 - continued

Virus	Sewage			Distilled water			River water			Sea water		
	4-8°	20°	28-37°	4-8°	20°	28-37°	4-8°	20°	28-37°	4-8°	20°	28-37°
Adenovirus 5	-	-	-	-	68(6)	-	-	-	-	-	-	-
Infectious hepatitis virus	-	70+(1)	-	-	-	-	-	-	-	-	-	-
Bacteriophage T4	-	-	-	-	-	-	112(5)	28(5)	-	-	-	-

(1) Clarke & Change (1959) (2) Clarke, et al (1962) (3) Lo, et al (1976)

(4) Lycke, et al (1965) (5) Wellington, et al (1955) (6) Bagdasar'yan & Abieva (1971)

and they therefore suggested that the coliform index of water quality measurement was not after all a good indicator of viral contamination. Koff and Gloyna (1965) and Knott, et al (1974) suggested bacteriophage as alternative indicators of viral pollution, an idea supported by Fannin, et al (1977) who showed that coliphages behaved similarly to Enteroviruses but were present in greater numbers. They further suggested that RNA phages rather than T phages should be used for the modelling of Enterovirus removal because these shared a greater number of similar characteristics.

The other main factor influencing virus numbers in water has been shown to be the level of suspended solids. In an extensive review Bitton (1975) listed papers on virus adsorption to clays, silicates and sands, soils and a great many other substances not directly related to the aquatic environment. Carlson, et al (1968) showed that virus adsorption to clays and suspended solids was reversible and enhanced by the addition of metal cations, trivalent cations being more effective than divalent, which were in turn more effective than monovalent. Virus adsorption under laboratory conditions was shown to be rapid, i.e. 92 % for Encephalomyocarditis virus in one minute (Schaub and Sagik, 1975) and adsorbed viruses were found to be still infective (Moore, et al, 1975). As suspended solids were shown to adsorb metal cations (Toetz, 1970), pyridine (Baker and Luh, 1971), herbicides (Hague and Coshow, 1971), proteins (Murray 1973) and bacteria (Bitton and Mitchell, 1974:

Interaction with higher microorganisms ha
to influence virus numbers in water. Sobsey ar
detected a 99.999% reduction in Poliovirus 1 t
of bacteria and algae, in a stabilisation pond
days which they attributed to microbial antago
et al (1975), however, suggested that it was th
products of the bacterial-algal system, plus to
in the waste water, that was the predominant me
enteric virus inactivation.

Malherbe and Strickland-Cholmley (1967) s
waste water maturation ponds protozoa were part
responsible for the reduction in enteric virus
Electronic microscopic examination revealed tha
involved was ingestion of the virus particles.
supported by Chang (1970) who showed that proto
remove viruses by accidental ingestion.

Santoro and Stotzky, 1968; Stitzky and Bystricky, 1969: Lahan, 1962), it was clear that a major effect on virus survival in water would be adsorption to suspended solids, interactions with other species whilst adsorbed, and the degree of elution under favourable conditions.

INSERT.

In many viral studies (Berg, 1964; Chang, 1968; Lambert, et al, 1964; Melnick, et al, 1954; Seigneurin, et al, 1968; Wellington, et al, 1955; Bagdasar'yan, 1968; Grabow, 1968; Kelly, 1953; Clarke and Chang, 1959), a seasonal variation in virus numbers was demonstrated, with more isolations in Summer and Autumn than in Winter and Spring. These results were correlated circumstantially with increased incidences of non-specific gastroenteric and diahorreal diseases in warmer months which were not normally considered to be epidemic.

Whilst it was considered that personal contact was the most common means of enteric disease transmission (Berg, 1964), enteric viruses have been isolated from sewage, surface waters, swimming pools, potable waters and sea water (Moro, 1970). Further a considerable number of disease epidemics attributable to enteric viruses have been clearly demonstrated to be disseminated by water, although personal contact may have been the initial mode of infection. A list of authors reviewing such epidemics is given in Table 4. Infectious hepatitis, caused by the Hepatitis A virus, has been conclusively shown to be spread by water (Dennis, 1959), although Moseley (1967) deduced that the amount of infectious hepatitis in the USA

Table 4. List of reviewers of human diseases attributable to water contamination

Author	Period	Epidemic notes and comments
Moro, 1970	Up to 1970	All the reported cases in UK, USA, France and Italy.
Bancroft, et al, 1957	1952	Polio in Nebraska, USA. 150 cases per 100,000 population.
Becker, 1966	1955 - 1965	1 Polio epidemic in New York State, and 4 outbreaks of infectious hepatitis.
Clarke & Chang, 1959	1940-1958	Polio in Edmonton, Alberta, Canada; 17 epidemics of infectious hepatitis.
Moseley, 1967	1895-1964	8 confirmed epidemics of polio and 50 of infectious hepatitis.
Committee, 1969	1946-1960	142 gastroenteric and diarrheal outbreaks by waterborne viruses.
Weibel, et al, 1964	1946-1960	23 infectious hepatitis and 142 general gastroenteric outbreaks, not due to any known pathogen.
Dennis, 1959	1955-1956	Infectious hepatitis, New Delhi, India.
Lamb, et al, 1964	1962	Polio in sewers and rivers correlated with local disease cases.
Moseley, 1968	1916-1968	31 infectious hehatitis and several jaundice epidemics in Sweden.
Craun, et al, (1976)	1916-1974	13 proven infectious hepatitis and 66 outbreaks water implicated.

transmitted by water was less than one percent. However, the lack of direct evidence for other virus pathogens makes the establishment of conclusive links between disease outbreak and water transmission very difficult.

Finally it has been suggested that regions of poor sanitation probably produce sewage carrying enteric viruses all of the time (Chang, 1968) resulting in sporadic outbreaks of clinical disease which are merely the recorded tip of a pyramid of sub-clinical, unreported illnesses. Indeed Chang estimated that during the New Delhi outbreak of infectious hepatitis in 1955-1956 there were as many as ten times the number of confirmed cases not reported because they were not recognised as hepatitis.

Virus removal by treatment processes

Although a high proportion of domestic and industrial wastes receive some, if not total, treatment the viruses found in sewage are still detectable in effluent containing water. An examination of the literature on water reclamation processes showed that primary settling tanks had little effect on virus numbers during a three hour retention period (Clarke, 1966: Grabow, 1968). Clarke, et al (1962) recorded that the number of virus positive samples decreased from 32.6 % in influent water to 23.8 % in primary settled effluent and Nupen (1976) readily detected viruses in settled sewage effluent.

A comparison of activated sludge treatment and biological filtration by Clarke, et al (1962) demonstrated that the former was more effective, but neither totally removed viruses.

Grabow (1968) suggested that only about 40 % removal in filters was obtainable and Clarke (1966) showed that they reduced some, but not all Enterovirus numbers. In 1975 Clarke and Chang, using a bench scale rotary trickle filter obtained removal of 94, 95 and 83 % respectively for Coxsackievirus A9, Poliovirus 1 and ECHOvirus 12. They implied that loss was due to adsorption, although viruses could not be recovered from the filter slime layer. Adsorption, as well as bacterial antagonism, has been suggested as the main cause of loss during activated sludge treatment (Clarke, et al, 1962; Grabow, 1968). Removal efficiencies for Coxsackievirus A9 of 98 % and Poliovirus of 90 % have been demonstrated by Grabow, with Lund suggesting that a 1.5 to 2.0 log drop in titre would be common.

Anaerobic digestion was found to inactivate Poliovirus 1 (Ward and Ashley, 1976), particularly those particles that were solids bound. Loss of titre was found to be due to partial breakdown of protein components of the virus, and 'nicking' of its RNA core. Inactivation was, however, temperature dependent and a consequence of digestion as losses did not occur in undigested sludge. For these reasons it was suggested that the reduction of the viral infectivity might be due to bacterial proteolytic action rather than physiochemical degradation.

There is conflicting evidence on the efficiency of oxidation lagoons in virus removel. M^{c}Anulty (1964) and Kabler (1962) stated that these lagoons had no effect of Enteroviruses, but Christie (1966) showed that added Poliovirus was reduced from

10^5 to 10^3 in 72 hours. There was no evidence for antagonism and thus adsorption followed by settlement was thought to be the main method of removal.

Some effluents are chlorinated before discharge but it has been shown that not all such effluents are virus free (Lund, 1969: Clarke, et al, 1962). Poduska and Hershey (1972) showed that the effectiveness of chlorine depended on contact time, chlorine concentration, temperature and the extent of viral adsorption to particulate matter. Under ideal conditions Robeck, et al (1962) and Clarke and Chang (1959) achieved optimum removals of 99.99 %, but the presence of organic matter or lower temperature reduced the efficiency of HOCl (the viricidal component of chlorine in water) considerably. Other halogens (Clarke, 1968: Clarke and Chang, 1959) proposed for water sterilisation, have met with varied success, but none have replaced chlorination, despite its above mentioned disadvantages.

Alternatively, ozonisation, commonly used in Europe for more than fifty years, Poynter, et al (1973), has been shown to be more effective than chlorination in removing a number of water polluting substances (Chang, 1968: Clarke and Chang, 1959: Kessel, et al, 1947). Ozone was demonstrated by Majumdar, et al (1973) not to impart taste to the water, as did chlorine, and not to increase the inorganic salt content of the effluent. The pH had little influence on effectiveness, although the temperature and ozone concentration were found to be very important. Virus removals of up to 99.99 % were noted by Biedermann and Katzenelson (1976), the

only major disadvantage of ozone treatment being a lack of residual viricide.

One of the simplest means of reducing virus titre has been shown to be storage of the water (Clarke and Chang, 1959), removal being chiefly by adsorption to solids with subsequent settlement, and by bacterial antagonism. The Thames Water Authority noted a 99 % loss of viruses in their reservoirs and Chang (1968) suggested 99.0 % loss in a few weeks in warm seasons to a few months in cold seasons.

Flocculation, during which coagulants such as aluminium sulphate or ferric chloride were mixed with the water, was shown by Berg (1964) to remove 96 % of Coxsackievirus A2, whilst Clarke, et al (1962) recorded 93.8 to 99.9 % loss of Coxsackieviruses. Similar results were indicated by others (Chang, 1968: Grabow, 1968: Robeck, et al, 1962), viruses being concentrated but not inactivated in the floc blanket. Removal was shown to be by a nonspecific metal cation to virus complexation (Clarke and Chang, 1959), in the case of alum these being co-ordination complexes between aluminium ions and the -COOH groups of virus coat protein.

Flocculation was usually followed by sand rapid filtration, by itself shown to be ineffective in removing enteric viruses (Clarke and Chang, 1959). However the build up of an organic film or 'Schmutzdecke' over the sand (Chang, 1968), or impregnation of the filter with alum floc (Clarke and Chang, 1959), greatly improved the removal efficiency. Flow rate was clearly most important, slow sand filters removing viruses more

readily than rapid flow filters (Berg, 1964: Chang, 1968: Wellington, et al, 1955).

Water Sampling For Virus Detection

Virus samples from water are usually collected by a 'grab' sample, or by filtration through gauze and cotton pads. Comparative reviews of these techniques were made by Hill, et al, (1971), Melnick, et al (1954), Grabow (1968), Kelly (1953), Clarke, et al (1974) and Fattal and Katzenelson (1976) and were both shown to be effective in operation.

The grab, or dip, sample simply involved taking a known volume of water by bucket or bottle from the main water flow for further processing. This system collected all the viruses present in a given volume of water at a given time, although as a result any peak loads occuring between samplings would not be detected. The efficiency of grab sampling was therefore dependent on, and limited by, that of the subsequent concentration procedure.

Grab sampling has been shown to be less effective than gauze pads (Kelly, 1953: Fattal and Katzenelson, 1976), which were originally used by Moore (1948) for the isolation of salmonellae from sewers. Pads were found to have the advantage of sampling a larger volume of water and therefore concentrating any viruses present, as they were usually left submerged for at least twenty four hours. However, unlike grab sampling, this made quantitative viral assessment virtually impossible, although Bagdasar'yan (1968 a: b) attempted to circumvent this by collecting large grab samples and then suspending rotating

gauzes in them. Alternatively Liu, et al (1971) suggested a flow-through gauze pad water sampler, the flow rate of which was known. Fattal and Katzenelson (1976) reported that pads were capable of concentrating enteric viruses ninety times over the number in the surrounding medium, collection being by physical entrapment of solids-bound viruses.

Gause pads have proved very successful in enteric virus isolations from sewers (Duff, 1970: Kelly and Sanderson, 1962: Seigneurin, et al, 1968: Lambert, et al, 1964), which were enclosed systems but not so succesful in open water courses where there was often a danger of loss or damage, inadequate representative sampling or insecure fastening (Bagdasar'yan, 1968b).

Concentration of Water Samples

Following either grab or gauze pad collection methods it has usually been necessary to concentrate viruses for detection because of the low numbers present, and a great many techniques have been developed for this purpose. These are reviewed in Table 5, but detailed discussion is only made here of the two methods chosen for this investigation.

The first was aluminium hydroxide flocculation, originally described by Wallis and Melnick (1967) who, using Herpesvirus as a model, demonstrated up to 99.99 % virus adsorption to aluminium phosphate, aluminium hydroxide or calcium phosphate between pH values of 6.0 and 9.0, and at temperatures between $4^{o}C$ and $37^{o}C$. They subsequently showed that a very wide range of viruses were also adsorbed to aluminium hydroxide,

Table 5. A review of virus concentration methods

Method	Authors cited
Flocculation: $FeCl_3$	Manwaring, et al, (1971).
$Al_2(SO4)_3$	Manwaring, et al, (1971).
$Al_2(PO4)_3$	Wallis & Melnick (1967a: b).
$Al(OH)_3$	Wallis & Melnick (1967a: b) * ≠
$CaHPO_4$	Wallis & Melnick (1976)
Monkey kidney cells	Berg, et al (1966): Gravelle & Clun (1961).
Polyelectrolytes	Johnson, et al (1967): Grinstein, et al (1970): Wallis & Melnick (1970): Sattar & Westwood (1976)*
Ion exchange resins	Gravelle & Chin (1961): Sobsey, et al (1974).
Aqueous polymer phase	Lund & Hedstrom (1966, 1967): Lund, et al (1969): Philipson, et al (1960)* ≠ Shuval, et al (1967): Lund (1973).
Vegetable extracts	Konowalchuk, et al (1974): Konowalchuk & Speis (1973).
Membranes: epoxy/fibre glass	Liu, et al (1971): Jakubowski, et al (1974): Wallis, et al (1976): Jakubowski, et al (1975).
Millipore	* Wallis, et al (1972a): Tschider, et al (1971): Moore et al (1970): Cliver (1965): Vajdic (1966, 1967, 1970): Sobsey et al (1973)
Hollow fibre	Belfont, et al (1974).
Chromatography	≠ Cliver (1967).
Alginate	* ≠ Gardner (1967): Metropolitan Water Board (1971-73).
Ultrafiltration	Atoynatan & Hsiung (1964): Good, et al (1975).

Table 5 - continued

Method	Authors cited
Iron Oxide	Lavin & Gallimore (1971): Rao, et al (1968): Warren, et al (1966): Warren et al (1969): Cunliffe (1967): Bitton & Mitchell (1974): Oberteiffer (1974): Bitton, et al (1976).
Coal	Oza & Chaudhuri (1975).
Talc-Celite	Sattar & Westwood (1976).
Glass powder	Sanette, et al (1977)
Centrifugation	Anderson, et al (1967): Melnick, et al (1954): Bagdasar'yan (1968a)
Ultracentifugation	* Duff (1970): Gravelle & Chin (1961): Kott (1966): Cliver & Yeatman (1965)
Freezing	Melnick, et al (1954): Rubenstein, et al (1971).
Polyethylene glycol	Cliver (1967)
Electrophoresis/ Electroosmosis	Bier, et al (1967): Ellender, et al (1972): Sweet & Ellender (1972) * ⁂
Hydroextraction	Shuval, et al (1967) ⁂
Passive haemagglutination	Smith & Couttney (1967).

* indicates Review by Hill, et al (1971)

⁂ indicates Review by Grabow (1968)

Reovirus being the only exception of those tested. For several reasons it was this technique that was chosen for the sampling programme, but it was soon apparent that the method was also concentrating heavy metal ions to levels toxic to the tissue cell

lines used for assay and therefore another technique was sought (see results and discussion sections).

Iron oxide adsorption was first suggested by Warren, et al (1966) for the adsorption of Myxoviruses in allantoic fluids, using ball-milled oxide at an average particle size of 0.5 to 1.0 micrometres (μm). Viruses were recovered by attraction of the oxide to a large horseshoe magnet, resulting in rapid settlement. The fluid was decanted and viruses eluted with a saturated aqueous solution of sodium carbonate or 10 % sodium phosphate for 15 to 20 minutes at room temperature. The kinetics of Myxovirus adsorption were outlined by Larin and Gallimore (1971), who showed that a pH range of 6.8 to 7.3 was most favourable for virus adsorption, the rate of adsorption being dependent on the rate of contact between adsorbate and adsorbent. They also showed that the magnetic property of the oxide did not influence the rates of adsorption and elution, and established the size range of organic compounds capable of being adsorbed as being between 3.0 x 15.0 to 80.0 x 120.0 mμ.

Rao, et al (1968) first suggested a practical technique for virus concentration from water, and using iron oxide, successfully recovered a wide range of human viruses, with a variety of eluents. Subsequently, several authors have reported on the use of iron oxide in the recovery of Hog Cholera Virus (Cunliffe, 1967), Respiratory Syncytial Virus and Mycoplasma pneumoniae vaccines (Kende, et al, 1968) and bacteriophage T7 (Bitton and Mitchell, 1974). Bitton, et al (1976) recently showed that Poliovirus adsorption was enhanced

by the presence of the cations sodium, calcium and aluminium and conformed to the Freundlich isotherm. They also demonstrated that adsorption did not vary significantly when the pH varied from 5.0 to 9.0 and therefore, after initial experiments using this technique, it was adopted as the regular method of virus concentration.

It has been suggested (Oberteuffer, 1974: Kohn, et al, 1975) that electromagnetic recovery of iron oxide adsorbed viruses, as well as other polluting species, could be achieved on a large scale, continuous basis, using high gradient magnetic separation, consisting of magnetised steel wool matrix filters. This was also investigated, although using a batch system, and was eventually adopted as a superior method of oxide/virus collection.

Reverse Osmosis

The increasing requirements for potable water have in recent years, prompted investigations into alternative water reclamation processes. These, as reviewed by Bailey, et al (1973) and Melbourne (1974), include coagulation, flotation, microstraining, activated carbon adsorption, ion exchange, ozonisation, distillation, freezing and several membrane techniques. Of these membrane separation methods Reverse Osmosis (RO) has been shown to be both economic and effective at demineralising sea and brackish waters (Carter, 1968), the process depending on the use of a membrane highly permeable to water but relatively impermeable to solutes.

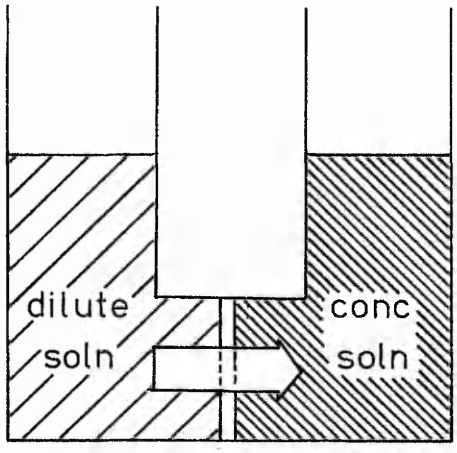
Osmosis is a natural phenomenon occuring when two solutions of differing concentration are separated by a semi-permeable membrane. Solvent flows from the dilute to the concentrated solution until an equilibrium is reached, the pressure just preventing flow being termed the osmotic pressure (Figure 1). A pressure in excess of the osmotic pressure, applied to the concentrated solution causes solvent to flow from the concentrated to the dilute side of the membrane, this being termed RO, also called exosmosis or hyperfiltration.

The first classical experiments, using pigs bladders, were conducted in 1748, and the first quantitative results were obtained by Pfeffer in 1877, using a membrane of copper ferrocyanide in porcelain, to study sucrose migration. There followed a period of intense study in the first quarter of the twentieth century by Hartley, Tinker, Van't Hoff and Berkley, but interest waned due to a lack of suitable membranes. There was a resurgence of interest in 1959, however, with the publication by Reid, Breton and Kuppers of an article on the high degree of semi-permeability of cellulose acetate membranes. They examined a wide range of synthetic and natural membranes and showed that cellulose acetate had a much greater capacity to reject sodium chloride solutions than any of the other (typically 96 % + compared with 0 - 35 %). Unfortunately their homogeneous 'dense' membrane had a very low flux rate, flux signifying the quantity of water passing through a unit area of membrane in a unit time, and was soon superceded by the 'asymmetric' membrane first described by Loeb and Sourirajan (1964).

FIGURE 1. Representation of the principles of R.O.

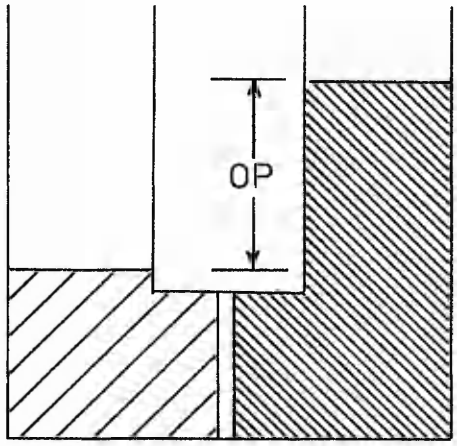
OSMOSIS

When solutions of different concentrations are separated by a semi-permeable membrane the dilute solution will flow through the membrane into the concentrated solution.



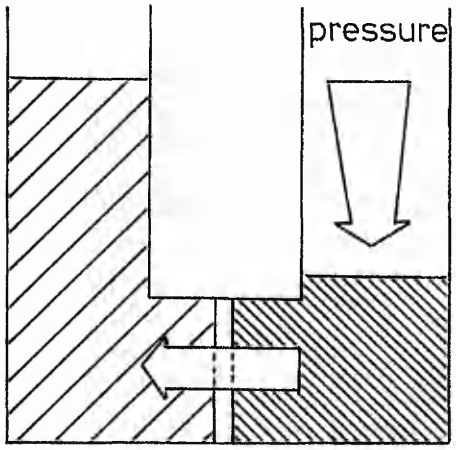
OSMOTIC PRESSURE

The level of dilute solution drops and the level of the concentrated solution rises to equilibrium. The pressure difference between these levels is the osmotic pressure (OP).



REVERSE OSMOSIS

A pressure in excess of the osmotic pressure applied to the concentrated solution reverses the flow, this phenomenon being reverse osmosis.



The asymmetric membrane was formed by adding magnesium perchlorate, or more recently formamide, to a solution of cellulose acetate in acetone, and casting the membranes on flat discs or around steel tubes at -10°C to ambient temperature to control acetone evaporation. They were then immersed in iced water to leach out soluble components and heat treated at 60 - 90°C to improve and vary the semipermeability, a higher temperature causing greater membrane shrinkage and therefore higher salt rejection, but lower flux (Cruver, 1973: Riley, et al, 1967: M^{c}Kinney, 1969). Electron microscopic examination of these membranes (Riley, et al, 1964: Riley, et al, 1966: Cruver, 1973) showed the structure to consist of a dense featureless skin, 0.2 μm thick supported on an integral spongy backing of up to 100 μm thickness, a porous support that gave the improved water transmission rates.

Membranes

The cellulose acetate membrane has been shown to consist of a network of long polymers, 1500 μm x 1-2 μm, with random orientation. When running together they form crystalline regions where the polymers are held together by hydrogen bonding and Van der Waal forces. There also exist disordered, amorphous regions with greater spacings between polymers, but as the membrane possessed a moderate degree of crystallinity these spacings were found to be extremely small (Reid and Breton, 1959). Increasing the acetyl content of the cellulose acetate increased this crystallinity (Riley, et al, 1964), thereby

increasing the semipermeability, but, unfortunately also decreasing the flux. Alternatively, the acetate could be substituted by a variety of groupings, such as carboxymethyl, acetyl-o-nitryl or cyanoethyl (El-Taraboulsi, et al, 1970). These were however found to be generally detrimental, with the exception of o-acetyl-o (cyanoethyl) which gave 21% more flux for the same salt rejection, or alternatively 352 % higher flux for slightly lower rejection. Cyanoethyl cellulose was also tougher and more elastic than standard cellulose acetate and more resistant to attack by bacteria and fungi.

Temperature, pressure and pH have been shown to be the three main parameters regulating membrane performance (Cruver, 1973). Permeation rate increased with increasing membrane curing temperature (15 - 32°C), but membrane hydrolysis occured at 38°C. Similarly membranes were found to operate over a range of pH values, with variable hydrolysis, this being minimal at pH 4 to 5. The optimum conditions for RO operation were therefore recommended as pH 5 and 25°C (McKinney, 1969). Flux proved proportional to pressure but at higher pressures, and after prolonged use at lower pressures, flux was found to decrease with time. Examination by Riley, et al (1967) showed this to be due to compaction of the porous substructure, and either membranes have to be renewed, or alternatives found for specialist separations (Rozelle, et al, 1973: Minturn, et al, 1974).

Theories of separation by RO

Reid and Breton (1959) considered that water molecules concentrated in the amorphous spacings of their homogeneous membranes and became associated by hydrogen bonding to carbonyl O-groupings of the acetate radicals, filling the voids with bound water. Ions not entering into H-bonding (e.g. NaCl, $MgCl_2$) would be capable of transport across membranes by hole type diffusion only and would therefore be rejected by the water-bound holes. Hydrogen bonding ions, e.g. water, NaF (86 % rejection) and NH_3^+ (30 % rejection), would fit into the water bound structure and migrate across the membrane. Large ions would be rejected on a size basis alone.

Loeb and Sourirajan (1964) and Lonsdale (1965), on the other hand, suggested a mechanism of preferential sorption-capillary flow, whereby the membrane surface had a preferential sorption for water and a preferential repulson for solute ions. The sorbed interfacial salt-free monolayer of water was removed by continuous skimming under pressure through membrane capillaries of a critical pore size (twice the depth of the sorbed water layer).

Several variations on a porous membrane mechanism have been proposed, Meares (1966), for example, suggesting that 99 % of transport of liquid water could be by viscous flow in tortuous channels 6.0 to 9.0 Å in diameter, occupying about 4 % of the total volume of the membrane. These channels were not thought of as pores at the molecular level, but as flowing molecules aggregated and freely moving, and therefore losing

little momentum with respect to the surrounding stationary medium. For organic rejection by RO, Duvel and Helfgott (1975) suggested that thermal motion on the polymer surface caused small, randomly changing gaps to open in the amorphous regions, into which molecules could enter by sorption and/or dissolution. Once in the membrane a molecule would move through the matrix by diffusion, differences in retention being caused primarily by differences in diffusion rates.

Finally, Glueckauf (1965) suggested that the electrostatic free energy of an ion in a pore filled with water surrounded by material of a low dielectric constant, i.e. a polymer matrix, would be much larger than in the bulk solution. Thus the equilibrium concentration of the ions in the mouths of the pores was much lower than in the adjacent solution. An ion entering a pore would experience a net force ensuring that it would be more likely to jump out of the pore than further in. This force would not occur if a second ion of opposite sign entered the pores, but this was thought highly unlikely if the pores were much less than the ionic separation which was 1.0 μm or less in up to 5.0 % NaCl solution.

Theoretical Considerations

There are two main required properties of RO membranes, flux and salt rejection factor.

Flux - each component dissolves in the membrane in accordance with equilibrium laws and diffuses through the membrane, with respect to concentration and pressure gradients. The flux of each component can therefore be given by:

$$F_1 = \frac{-D_1 C_1}{RT} \text{ grad } \mu_1 \qquad (1)$$

$$= \frac{-D_1 C_1}{RT} \left(\frac{\partial \mu_1}{\partial C_1} \text{ grad } C_1 - V_1 \text{ grad } P \right) \qquad (2)$$

where D_1 = diffusion coefficient of component 1.

μ_1 = chemical potential of component 1.

C_1 = concentration in membrane of component 1.

V_1 = partial molar volume.

P = applied pressure

There are therefore two contributors to the driving force of each component, a concentration gradient and a pressure gradient. Because very large pressures are required to change the chemical potential μ_1 by an amount achieved with only a small change in concentration, it is not normally practical to operate RO systems where large differences in concentration of solutes exist. RO is therefore usually restricted to solutions below 1.0 molar.

If water is the component C_1, integration of (2) yields:

$$F_w = \frac{-D_w C_w V_w}{RT} \cdot \frac{(\Delta p - \Delta \pi)}{\Delta X} \qquad (3)$$

where ΔP = Pressure drop across the membrane.

$\Delta \pi$ = osmotic pressure change.

ΔX = membrane thickness.

This expression can be reduced to give:

$$F_w = A (\Delta P - \Delta \pi) \quad (4)$$

where A = membrane constant.

For salt flux, in most cases, solute rejection by membranes is high and the first term of equation (1) is much higher than the second. Integration for solute flux therefore gives:

$$F_s = \frac{-D_s \cdot C_{sm}}{\Delta X} \quad (5)$$

$$= \frac{-D_s \cdot K \, C_{ss}}{\Delta X} \quad (6)$$

where C_{sm} = concentration of salt in the membrane.

C_{ss} = concentration of salt in the solution.

K = distribution coefficient for salt: ie. $K = \frac{C_{sm}}{C_{ss}}$

As $\frac{D_w C_w V_w}{RT \Delta X}$ in equation (3) can be considered a membrane constant (A), so $\frac{D_s K}{\Delta X}$ may be treated as a solute permeation constant (B). Therefore:

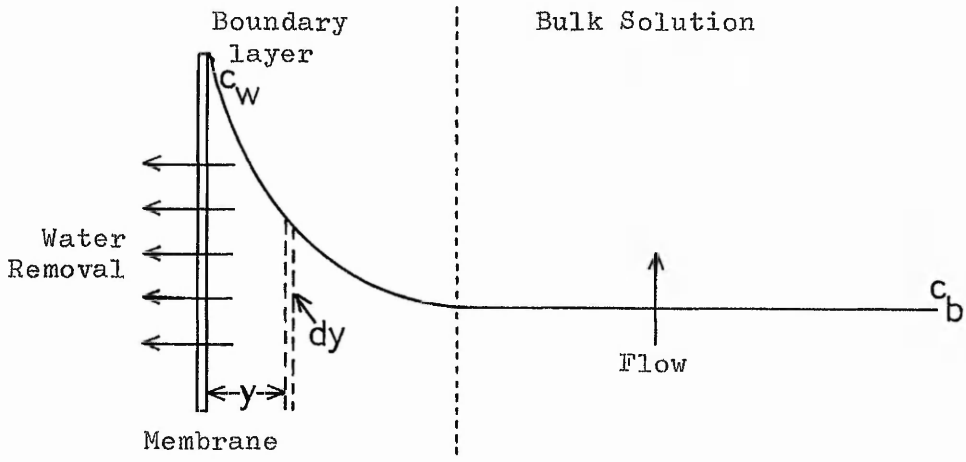
$$F_s = B \Delta C_{ss} \quad (7)$$

Equations (4) and (7) can therefore provide a description of membrane performance. It can be seen that while water flux is pressure dependent, salt flux is independent of pressure, and dependent only on concentration. Therefore the desalination factor, i.e. $\frac{\text{solution concentration in feed}}{\text{solution concentration in product}}$, is increased at higher pressures. As the pressure increases, the water flow increases, but the salt flow remains roughly constant: as

more water is extracted the salt concentration increases and the flux is reduced.

As well as increased pressure, flux can be affected by concentration polarisation at the membrane surface. Water is conducted to the phase boundary, ie, the membrane surface, by the bulk flow of the solution. Salts are carried by the water and, to maintain steady state conditions, salt must disperse back into the bulk solution. A salt gradient is therefore established on the concentrate side of the membrane (Figure 2).

Figure 2. Diagrammatic representation of concentration polarisation during RO separation



C_w and C_b = salt concentrations at the membrane and in the bulk solutions, respectively.

The salt balance at the boundary is therefore represented by:

$$F.\frac{C_w}{C_1} + D_s \cdot \frac{dc}{dy} = 0$$

where F = flux.

C_1 = water concentration at the membrane.

D_s = diffusion coefficient for salt in water.

Concentration polarisation reduces salt flux and rejection sensitivity and is fixed for a given flux rate. It can be decreased by turbulent flow, which is achieved by increasing concentrate velocity and optimisation of module design.

Rejection ratio - this is the ratio between the concentrations at both sides of the membrane, at a given location.

$$R = 100 \left(1 - \frac{C_p}{C_f} \right)$$

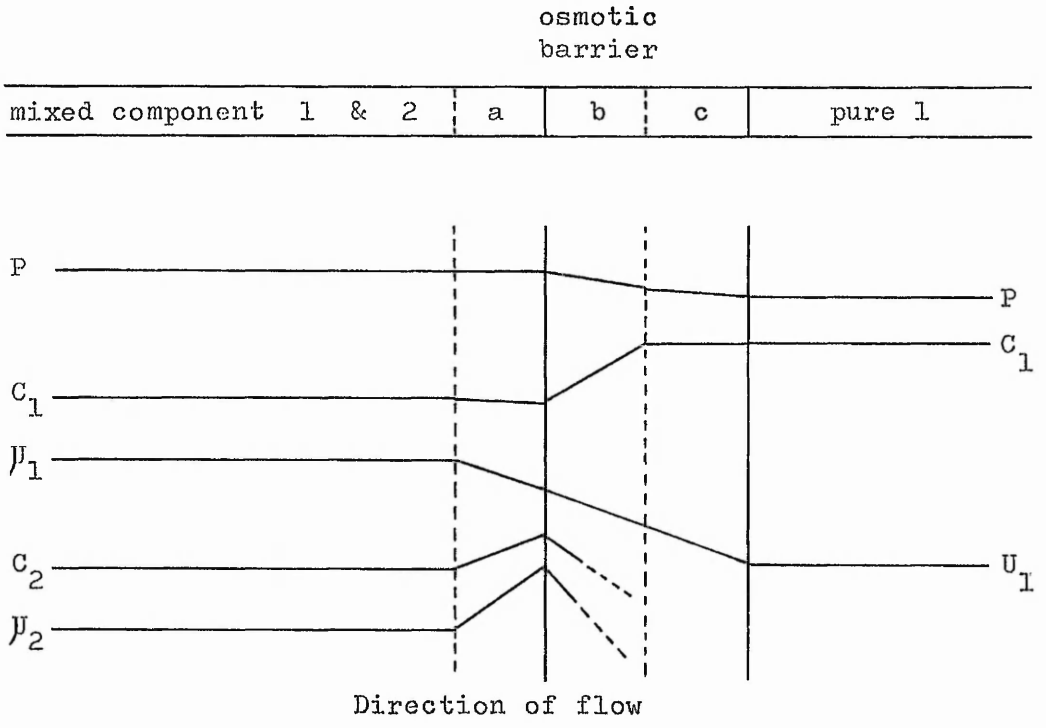
where R = percent rejection.

C_p = concentration in product water.

C_f = concentration in feed to module.

A perfect semi-permeable membrane should therefore be permeable to the solvent but completely impermeable to salt, as illustrated diagrammatically in Figure 3. In this case C1 and u_1 represent the concentration and chemical potentials of the solvent, and C2 and u_2 represent those of the salt component. Figure 3 also shows the pressure drop P across the membrane and the increase in concentration of the solvent as represented by C1.

Figure 3. RO cell showing qualitative variation of several parameters, during steady state, assuming an ideal semi-permeable membrane. (Merton, 1963)



where a = boundary layer.

b = diffusion region.

c = viscous flow region.

P = total pressure.

C_1 = concentration of solvent.

C_2 = concentration of component 2.

μ_1 = chemical potential of solvent.

μ_2 = chemical potential of component 2.

Membrane module design

Several RO systems are now commercially available and can be divided into five categories (Melbourne, 1974).

Tubular units - (Figure 4). The membrane is cast on the inside of a paper tube, eight to ten feet long and a half inch in diameter, which is inserted into a perforated support tube of stainless steel or glass fibre. One module is composed of several such tubes in a bundle, connected in series by 'U' tubes, and water is pumped under pressure through the membrane tubes. Permeated product water is forced through the membrane and support tube perforations and is collected in an outer plastic shroud.

Spaghetti rod units - (Figure 5). The membrane support in this system is an 1/8 inch diameter polypropylene solid rod, with four longitudinal grooves along its circumference, covered by a fabric braid on which the membrane is cast. The assembly is fitted inside a pressure tube in bundles of 37 - 240 rods, each rod sealed at one end and joined by a common outlet at the other. The whole system is surrounded by water under pressure and permeated water is forced through the membrane, along the grooves to be collected in the common end chamber. Spaghetti rod units have an increased membrane area/volume compared with the tubular units.

Spiral Wound units - (Figure 6). In this type a "swiss roll" consisting of two layers of membrane with porous backing between them, has one end attached to a perforated product pipe. The other edges are sealed together, the whole system

FIGURE 4. Tubular rod RO unit.

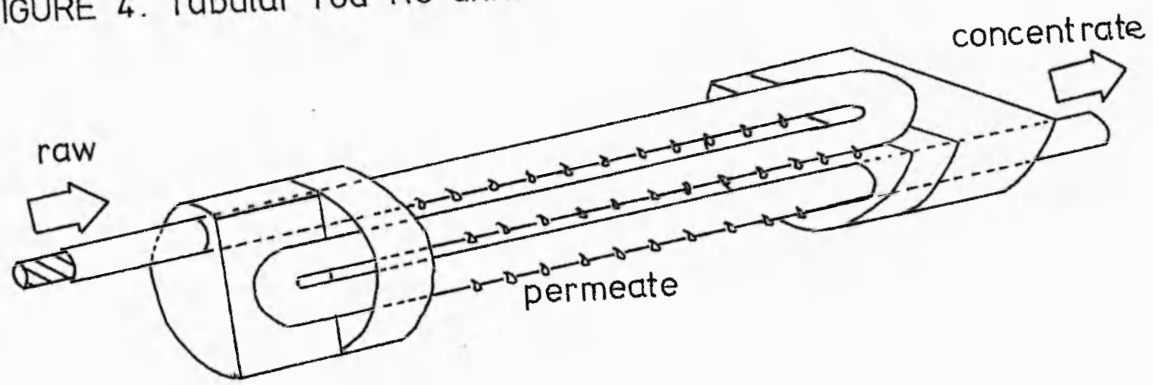


FIGURE 5. Spaghetti rod RO unit.

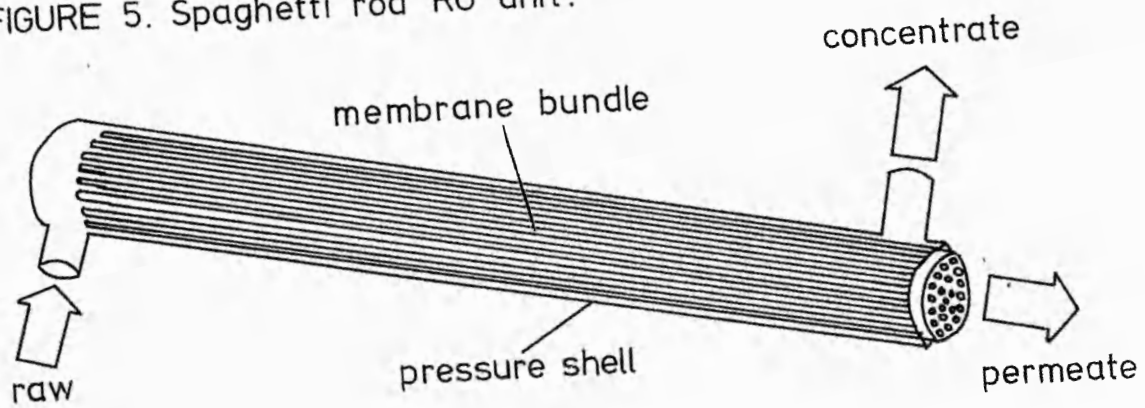
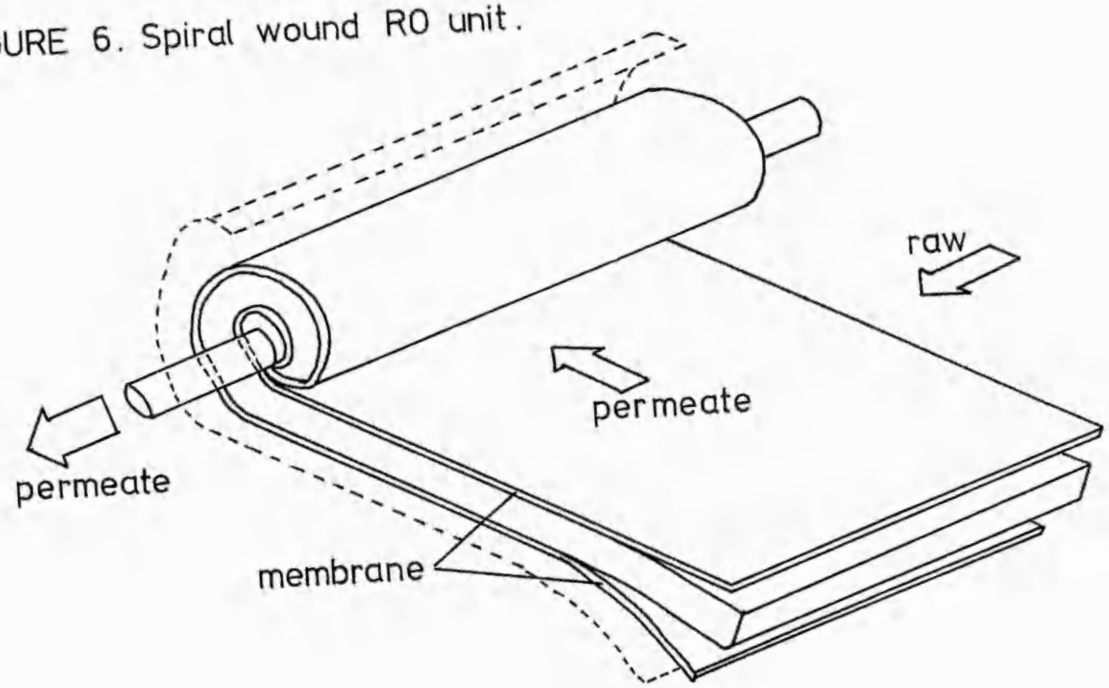


FIGURE 6. Spiral wound RO unit.



is rolled up around the pipe and placed within a pressure tube. Raw water flows axially through the system, product water spiralling radially towards the central tube. A very large surface area/unit volume is achieved.

Hollow fibre units - (Figure 7). Thousands of nylon fibres, with an outside diameter of 85 μm and inside diameter of 42 μm, are placed in bundles and sealed at one end, their small size ensuring that they don't collapse under pressure and therefore requiring no further support. This system gives very high membrane area/unit volume ratio's but is of little use for feed solutions with high suspended solids content.

Plate and frame units - (Figure 8). These consist of a pile of flat membranes on top of flat circular support plates within a pressure container, feed and product water compartments being separated by "O" ring seals. The system is expensive to install and has low surface area/unit volume ratios.

Wastewater Treatment and Virus rejection

RO is becoming a widely applied technique for the separation, concentration or fractionation of inorganic or organic substances in aqueous and non-aqueous solutions, permeated product usually being removed at ambient temperature and atmospheric pressure. No heating or phase change is required and the process can therefore be used for processing sensitive biological substances, as well as refining sugar, concentrating fruit juices and whey solids, recycling metal effluents and recovering and purifying water.

FIGURE 7. Hollow fibre RO unit.

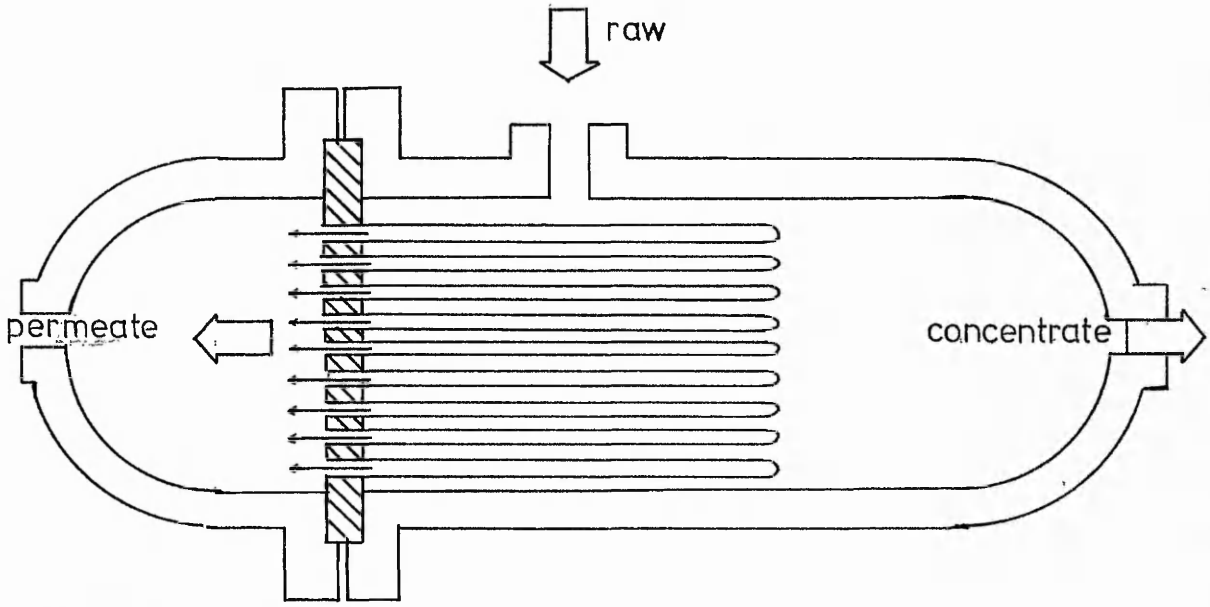
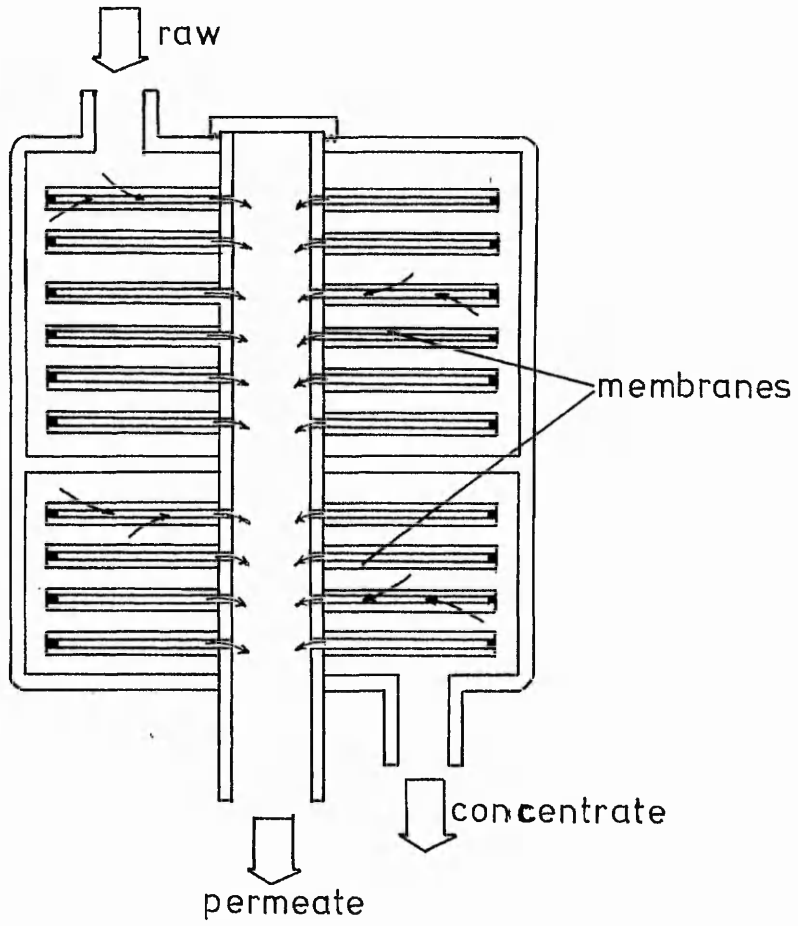


FIGURE 8. Plate and frame RO unit.



For water purification to potable quality, it is necessary to establish the fate of the multitude of contaminating species whether suspended, colloidal or dissolved, organic or inorganic, present in the influent stream. Duvel and Helfgott (1975) used a tubular unit to study the removal of organic species from water at flux rates of 0.65 to 0.77 m^3 day^{-1} m^{-2}, a pressure of 42 Kg m^{-2} and concentrations of 0.01 M and showed that large molecules were easily retained, whereas small molecules had variable fates, depending on size, shape and chemistry. Besik (1972) concluded that RO excluded most wastewater constituents and suggested that the tubular unit could be used in sewage treatment. This suggestion was expanded upon by Wechsler (1977) who showed that a tubular RO system was most effective during sewage treatment if placed after activated sludge treatment, settlement, alum flocculation and filtration.

Recovery of reusuable water from laundry wastes was achieved by Minturn, et al (1974) and Bhattacharyya, et al (1974) despite the elevated temperatures and the presence of anionic and nonionic surfactants associated with such wastes. Typical removal efficiences of many contaminant species are shown in Table 6.

Table 6. Efficiency of removal of various water constituents by RO (compiled from several sources)

Constituent	% removal	Constituent	% removal
Sodium	83.0-97.0	Organic carbon	94.0-98.0
Potassium	83.0-97.0	Various viruses	50.0-99.9
Ammonium	77.0-95.0	Poliovirus 1	32.0-84.0
Calcium	96.3-99.7	MS 2	97.0
Magnesium	93.0-99.9	Poliovirus	99.2
Boron	38.0-60.0	Optical density	99.0+
Manganese	100.0	COD	96.0-99.0
Silicon	80.0-95.0	BOD	87.9-97.0
Iron	99.9+	PV5	85.0
Aluminium	97.3-99.9	TDS	89.0-99.0
Chromium	92.6-98.6	Suspended solids	100.0
Bicarbonate	80.0-98.0	Colour	100.0
Chloride	86.97	Conductivity	91.8
Nitrate	58.0-86.0	Hardness	95.0
Nitrite	33.3	Anionic detergents	100.0
Fluoride	88.98	Lignin sulphonates	96.0-99.0
Sulphate	97.0	Sucrose	99.9
Phosphate	99.0+	Glucose	99.5+
Polyphosphate	99.0+	Proteins	98.0-100.0
		Phenol	negative rejection

Potentially among the most important wastewater constituents interacting with RO membranes are animal viruses already shown to be present in the types of water suitable for this treatment. Ultrafiltration on a bench scale was used to concentrate Poliovirus, Adenovirus, Australian Antigen, Reovirus, Herpesvirus, Rubellavirus, Turnip Mosaicvirus, Murine Leukemiavirus, Cytomegalovirus and various bacteriophages (Ellender and Sweet, 1972) with recoveries of 50.0 to 99.9 %, from distilled or tap water using flat disc membranes. Further, Belford, et al (1974) achieved an overall 84.0 % recovery of seeded Poliovirus input, with backwashing to regenerate membrane bound virus particules. This adsorption was stated to be due to a lack of an inner, dense skin to their cellulose acetate hollow fibre membrane, which therefore represented a homogenous 'sponge'. The unrecovered 16.0 % of viruses were assumed to be inactivated.

Sorber, et al (1972) used flat disc Loeb-type membranes to recover 99.5 to 100 % of Poliovirus and coliphage T2 inoculum, under a variety of flux rates. Higher pressures and fluxes were shown to rapidly inactivate T2 by structural damage to the tail fibres, but Poliovirus was unaffected. Viruses were detected in the permeated water at 0.0 to 16.0 pfu per run, more being detected at higher flux rates. They concluded that random penetration of the membrane must be due to mechanical defects as there was no pore structure in the dense layer of their membranes observable down to 10.0 nm, making penetration impossible, without fissure, for viruses of 25 nm in diameter.

The above conclusion was also reached by Bennett (1973) working from this laboratory and using coliphage MS 2 to investigate a pilot scale tubular RO plant operating on River Trent water. She detected 2.0 to 134.0 pfu ml^{-1} in permeate water, although 97.0 % of input viruses were rejected. It was suggested that this high level of permeated viruses was due to mechanical failure of the membranes, a fact subsequently proven by measurement of other parameters.

It can therefore be seen that human viruses are only partially removed by sewage treatment systems, and, having entered water courses, enjoy considerable longevity. They could therefore prove to be a problem should such surface waters be required for supplementing potable supplies.

Further, despite theoretical considerations, viruses seem capable of passing through 'impermeable' membranes during RO, a system already used for the desalination of sea and brackish waters, and likely to be used for preparing potable water from fresh supplies.

It is therefore necessary to understand the conditions that would influence virus movement and survival during such treatment and for these reasons the following investigations were undertaken.

METHODS

In all experimental work two virus types were used, the attenuated strain of Poliovirus type 1 (Glaxo oral vaccine) and the male specific coliphage MS 2. All Poliovirus manipulations were performed in a LEEC vertical flow laminar cabinet with ultraviolet sterilisation facilities, and all contaminated glassware was sterilised at 121°C for 15 minutes in a Griffin bench top autoclave. Tissue culture techniques were practised in a separate, enclosed room in a LEEC horizontal flow laminar cabinet.

All glassware was boiled for one hour in stainless steel buckets containing detergent ('Serve'; Dubois Chemicals) and glass distilled water from a Jencons Autostill 8 Plus still, and then rinsed in three changes of tap water and five changes of glass distilled water. After drying for 18 hours all bottles were sterilised by autoclaving at 121°C for 15 minutes, and all pipettes, tubes and other glassware sterilised for two hours at 180°C in a LTE hot air oven.

Host cell culture

For all media see Appendix 1.

(a) Tissue culture for Enterovirus assay. Vero, HeLa and HEp2 cell lines were obtained from Flow laboratories Ltd., or Gibco Ltd., as 50 cm^2 monolayers. These were delivered in bottles full of the appropriate maintenance medium, and all but 10 ml. of this was discarded upon receipt. Bottles were incubated at 37°C overnight to test for contamination and then

the medium aseptically replaced with 5.0 ml of 0.25 % trypsin in Hank's basal salt solution. This was incubated for three minutes at room temperature and then the trypsin was discarded and the cells incubated at 37°C until the monolayer detached itself from the glass surface of the bottle. For HeLa and HEp2 this time was usually three to five minutes, but for Vero the cells often took fifteen to twenty minutes to become detached. To a bottle of trypsinised cells was added 10 ml of growth medium and the bottle agitated to deaggregate cell clumps. A further 50 to 100 ml of growth medium was then added, with mixing and the cell suspension transferred to sterile 75 x 13 mm tubes, sterile 125 ml. flat prescription bottles or one sterile 1.0 litre Roux flask, depending on the requirement. Tubes were inoculated with 0.5 ml of suspension, shaken to distribute cells and incubated at 37°C in racks tilted at 7° to the horizontal. The tops of these racked tubes were covered with sheets of 'Parafilm' and a clip-on lid, to prevent contamination. When cell layers were confluent the growth medium was removed by aseptically tipping the contents into a Pyrex glass dish, and 1.0 ml of maintenance medium added to each tube. These were then reincubated until required, for up to seven days.

Prescription bottles received 10 ml of cell suspension and were incubated horizontally at 37°C until monolayers were confluent. Growth medium was then replaced by maintenance medium, at 10 ml per bottle, and the bottles stored at 37°C until cell layers were required, for a maximum of seven days.

Roux bottles were used to quickly propagate cells, one bottle producing, upon trypsinisation, enough cells to seed twenty 125 ml bottles.

(b) Bacterial culture for coliphage MS 2. Escherichia coli K12 Hfr H (NCIB 10235, EMG 23) was originally obtained as a freeze-dried culture from the National Collection of Industrial Bacteria, Aberdeen. The glass phial was aseptically opened and a few drops of nutrient broth from a pasteur pipette were added to distribute the contents. Using the same pipette the suspension was removed to 10 ml of nutrient broth in a Universal bottle, and incubated for 24 hours at 37°C. Samples of the resulting bacterial suspension were then inoculated by streaking with a wire loop onto the surface of Blood agar base slopes and again incubated at 37°C for 24 hours. The suspension was also streaked onto a blood agar base Petri plate and incubated as above in order to check for contaminants.

After growth, slopes were stored at 4°C until required. For use the surface of a slope was washed by adding a few ml of nutrient broth from a pipette and agitating the bottles by hand to create a bacterial suspension. This was then transferred to 100 ml of nutrient broth in a 250 ml conical flask and an alcohol sterilised 'bean' of plastic coated iron added. The whole was then stirred on a Chilton magnetic stirrer at moderate speed, for two to three hours at 37°C to obtain a cell density of 10^8 to 10^9 cells ml^{-1}.

Virus culture

(a) Poliovirus. Attenuated Poliovirus was preserved as 1.0 ml aliquots of cell-free supernatant extract in 10 ml Bijoux bottles at -20°C. The virus was cultured by inoculation of the contents of one bottle onto a confluent monolayer of Vero or HEp2 cells, in a 125 ml bottle, and incubated at 37°C for one hour. To this was then added 10 ml of maintenance medium and the bottle reincubated for 72 hours at 37°C. After microscopic examination (Gillet and Sibert inverted microscope, 37.8 x magnification) for characteristic CPE, the bottle contents were shaken to dislodge any remaining cells and transferred to a 25 ml Universal bottle. Cell debris was deposited by centrifugation for 15 minutes at 3000 rev min^{-1} (805 g) in a Janetski T32B centrifuge with a 6 x 25 ml fixed head rotor, and the supernatant divided into 1.0 ml volumes in Bijoux bottles. These were frozen to -20°C and then one of them thawed and assayed by the plague assay method of Dulbecco in order to determine the titre.

(b) MS 2. This bacteriophage was stored as a suspension at 4°C in 10 to 100 ml volumes. Two methods of preparation were used, agar plate culture and liquid culture.

(i) Agar plate culture - To each of 2.5 ml volumes of molten soft agar at 48°C in ten 75 x 13 mm tubes was added five drops (approximately 0.15 ml) of host bacteria from a pasteur pipette, and 0.1 ml of MS 2 suspension. These were immediately mixed and poured into ten Petri plates containing

15 ml each blood agar base. When they were set plates were inverted and incubated for 18 hours at 37°C. After this time the surface of the first plate was flooded with 10 ml of Ringer's solution and carefully agitated with a glass spreader to macerate the soft agar layer, without disturbing the base layer. The macerated contents were then poured onto the second plate, and the process repeated until the tenth plate was macerated. The final macerate was divided between two Universal bottles and centrifuged at 3000 rev min^{-1} (805 g) for 30 minutes to deposit the agar. The supernatant was decanted into a 100 ml bottle and 1.0 ml chloroform added to kill the remaining bacteria. To each of the two Universal bottles was added a further 5.0 ml of Ringers solution and the deposited agar resuspended by mixing for five seconds on a Fisons 'Whirlimixer' vortex mixer. These bottles were then centrifuged a second time as above and the two pooled supernatents added to the 100 ml bottle. After further shaking the bottle was allowed to stand for 1 hour at 4°C and then the virus containing supernatant was decanted from the chloroform into a fresh, sterile 100 ml bottle. The supernatant was assayed using the standard phage plaque assay (Eisenstark, 1967) and the suspension stored at 4°C until required.

(ii) Liquid Culture - Into a 250 ml conical flask containing 100 ml of growth medium A was inoculated 1.0 ml of E. coli k12, HfrH suspension prepared from a stock slope. An alcohol sterilised plastic coated 'bean' was added and the flask stirred at 37°C on a Chilton magnetic stirrer at moderate

speed for 2 hours. This was then inoculated with 5.0 ml of coliphage MS 2 at 10^9 viruses ml^{-1} and reincubated at 37°C with stirring for a further 4 hours.

After this time 1.0 ml chloroform and 10.0 ml lysing solution (Appendix 1) were added by pipette, and the flask shaken vigerously. The contents of the flask were divided equally amongst four 25 ml Universal bottles and these were centrifuged at 3000 rev min^{-1} (805g) for 30 minutes to deposit bacterial debris. The four suspernatents were pooled, a few drops of chloroform added and assayed for virus titre.

Virus Enumeration

(a) Enterovirus Assay. Two assay methods were employed for Enteroviruses, tube CPE assay, and monolayer plaque assay. In the former the medium from the required number of tubes was decanted, and 0.5 ml of sample or its dilution added per tube, one sample using 5 or 10 replicates. Tubes were returned to their racks and incubated at 37°C for one hour to allow virus adsorption. The fluids were then discarded into a dish of sodium hypochlorite ('Chloros', ICI Ltd.) and the cells washed with 1.0 ml of PBSA per tube. Into each tube was then carefully pipetted 1.0 ml of maintenance medium and a separately sterilised silicon rubber bung added to seal the top. Tubes were incubated at 37°C for a maximum of seven days, care having been taken at all stages to ensure that the cell layer, and subsequently the virus suspension and media, remained on the correct, i.e. lower side of the tube. All tubes were

examined daily, using the inverted microscope, for viral CPE, and the number of tubes showing a positive result was recorded. From these the virus titre was calculated according to the method of Reed and Muench (1938). All samples, including negative results, were passaged for a total of five times, by shaking each tube and then pipetting 0.1 ml of its contents into a fresh tube of the same cell line and adding 1.0 ml of maintenance medium.

For the plaque assay method, viral samples were diluted, if required, in sample dilution medium and then 1.0 ml of each added to replicate bottles of cell monolayers from which the medium had previously been removed. Inoculated bottles were incubated at 37°C for 1 hour, ensuring that the entire cell sheet had been covered by the inoculum. After one hour equal portions of double strength molten Bacto-agar and warmed overlay medium were mixed at 48°C, and 10 ml added to each bottle. These were laid horizontally, when the agar had cooled by a few degrees, and then allowed to solidify. They were then incubated at 37°C for up to seven days, and examined daily. At three days 5.0 ml of 0.01% neutral red solution in Hanks basal salts were added to each bottle, the bottles returned to 37°C and covered by cloths to exclude any light. After five hours the bottles were examined against a dark background for plaques in the red stained cell monolayer. Bottles were reincubated and re-examined if required and plaques from water samples were further processed by isolation

as follows. A pasteur pipette containing 1.0 ml of growth medium was pushed through the agar layer above any plaque, the media expelled and recollected several times and then transferred into two 75 x 13 mm tubes of the same cell line. These were passaged twice to confirm the presence of viruses.

(b) Bacteriophage Enumeration. Tenfold dilutions of MS 2 sample or lysate were made in sterile, glass-distilled water, and assayed by inoculation of 0.1 ml of dilution into 2.5 ml molten soft agar at 48°C in 75 x 13 mm glass tubes. Dilutions were mixed on a Fisons 'Whirlimixer' in Universal bottles, and inoculations were performed with Sigma MP-100 or MAP-1000 fixed volume automatic pipettes of 100 and 1000 µl capacities respectively. Three replicates per dilution were usually employed, the tube contents being mixed by gentle agitation, and poured onto pre-dried blood agar base Petri plates. When the overlays were set, plates were inverted and incubated at 37°C for eighteen hours. They were then examined against a dark background, and viral plaques were counted.

Water Sampling

At the six river sites 20 litres of water were collected using a 'grab' sample technique. A 10 litre plastic bucket, previously rinsed in river water and attached to a length of 'Nylon' rope, was cast into the middle of the water flow and, when full, hauled back avoiding the banks of the river. Each sample was poured through a plastic funnel into a 20 litre plastic screw-capped container for storage and transport

to the laboratory. All six samples were collected on the same morning, at fortnightly intervals and processed during the afternoon. At each site the water temperature was measured and a further 100 ml sample taken in a 125 ml screw capped glass bottle for pH determination.

From December 1973 to May 1974, because of problems with the virus concentration technique, gauze pads only were used at Colwick and Eggington. These were 10 cm square bags of surgical gauze filled with approximately 15.0 g of absorptive cotton wool. Two 20 cm lengths of string were sewn onto each pad with cotton and the gauze bag closed with cotton to retain the cotton wool.

Two pads were deployed at each site, tied by their strings to the downstream side of a screen. These screens were constructed of 50 x 50 mm softwood, made into a painted framework measuring 1.0 m by 0.6 m, across which was positioned a length of 25 mm mesh plastic netting (Netlon). At Eggington a screen was suspended in a reservoir intake stream adjacent to the river and at Colwick to a floating boom housing the pilot plant intake pump. In both cases screens were immersed so that the pads were at a depth of about 0.5 m, and left for seven days before collection.

Reverse Osmosis-Water Collection

(a) Natural Virus Load. Monthly samples were collected from the 'B' type tubular unit at Colwick, from September 1973 to June 1974. At each collection 10 litres of inlet water, 10

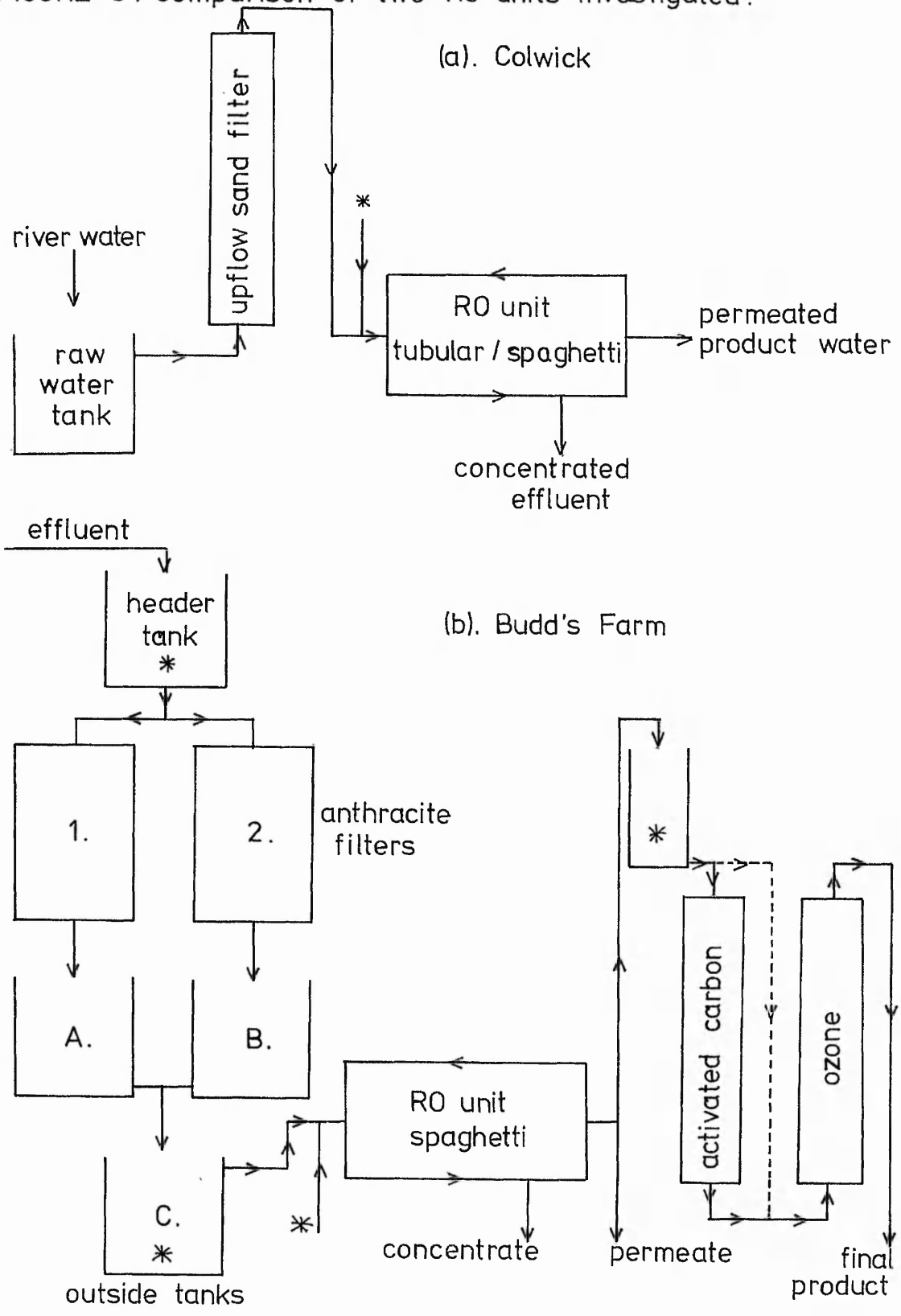
litres of concentrate and 100 litres of permeate were collected in 10 or 20 litre plastic screw capped containers during the middle of normal machine operation. These were returned to the laboratory for virus concentration. After June 1974 the 'B' type unit was closed down due to membrane failure and no further samples were taken for viral examination. Details of this machine, and the other RO units examined were listed in Table 7.

(b) Artificial Loading. Inoculation of viruses into the influent water of an RO plant was undertaken from June 1974 until March 1975, using a modified 'R3' type spagetti unit, installed at Colwick in the summer of 1974. The viruses used, attenuated Poliovirus and coliphage MS 2, were transported to the unit in Bijoux bottles in a 'Thermos' vacuum flask of five litre capacity, containing crushed ice. The RO unit was modified to include an inoculation port in the influent flow, prior to the main pressurising pump, (for diagram of RO unit see Figure 9). This consisted of a 12 mm Plastic T-piece insert in the water pipe over which was fitted a rubber 'Suba Seal 45' cap. Viruses, as a high titre (10^7 - 10^9 ml^{-1}), low volume (1-2 ml) 'slug', were injected rapidly through this port using 2 ml plastic disposable syringes. Ten ml aliquots of concentrated effluent water were collected from the reject line at thirty second intervals for ten to thirty minutes in sterile Universal bottles and packed in crushed ice in the vacuum flask. In most cases permeate water was collected by hose, for the duration of the experiment, into a 20 litre plastic container.

Table 7. Details of RO experimental units

B Type Unit	R Type Unit
Normal pressure range 25 to 30 bar: maximum 80 bar.	Normal pressure range 20 to 30 bar: maximum 65 bar.
Water pH range 6.0 to 7.0.	Water pH range 3.0 to 7.0.
Membranes cast on inside of stainless steel tubes: Plastic collection shroud.	Membranes cast on outside of grooved polypropylene bars: Steel collection tubes.
Dual strainer prefilter of stainless steel (pore size 50 μm).	Three cartridge prefilters (pore cartridge size 125 μm).
Archimedean positive displacement screw main pump.	Centrifugal main pump.
Recirculating water joins feed before dual strainer.	Recirculating water joins feed before cartridge filters.
Membrane area 18.9 ft^2 per module.	Membrane area 10.4 ft^2 per module.
Cleaning by flushing and/or foam plugs, or chemicals.	Chemical cleaning.
Chlorine or formalin sterilisation.	Chlorine or formalin sterilisation.
Membranes - Colwick.	Membranes - (a) Colwick
9 T2/15	4 PCI 75: flow rate 0.826 l min^{-1}
	3 H-75-O: flow rate 0.672 l min^{-1}
	3 H-75-N: flow rate 0.704 l min^{-1}
	3 H-70-O: flow rate 0.695 l min^{-1}
	3 J-70-N: flow rate 0.658 l min^{-1}
	(b) Budd's Farm.
	16 W2 : flow rate 3.14 l min^{-1}

FIGURE 9. Comparison of two RO units investigated.



After February 1975 reject water samples were diluted 1.0 ml into 9.0 ml nutrient broth on collection, this having been shown to protect the viruses from the toxic effects of the water during transport from the unit to the laboratory.

Duplicate loadings were performed in September 1976 on a second 'R' type spagetti unit operated by Portsmouth Polytechnic, and situated at Budd's Farm sewage treatment works, Havant, Hampshire. This unit received secondary settled effluent from activated sludge treatment tanks via a 4500 litre header storage tank and two 0.6 m diameter tentiary filters, each containing anthracite of 1.8 to 2.5 mm particle size, to a depth of 0.3 to 0.68 m. Effluent percolated through either or both of these filters at a surface loading rate range of 145 to 600 M^3 M^{-2} day^{-1}, into three 4500 l capacity polythene tanks, and hence to the RO unit, as shown in Figure 9. With this machine viruses were loaded continuously from the third tank (labelled C), or as inoculated 'slugs' through an installed injection port as previously described.

In addition to the Budd's Farm RO unit, the activated carbon and ozone sterilisation units, present in the event of RO membrane failure, were also examined for virus removal efficiency.

The activated carbon was housed in a 204 mm diameter 'Perspex' column with a bed depth of 867 mm. Into the side of the column were let sampling ports with rubber bungs and clamped outlet tubes at bed depths of 140, 394, 660 and 865 mm.

In use 10 ml of MS 2 at 10^9 ml^{-1} were mixed for one minute in 250 litres of RO permeate water and then three bed volumes, 190 litres, run through the column before samples were taken. Samples of 10 ml were collected from the inlet and four sampling ports at 0, 15 and 30 minutes and assayed for phage.

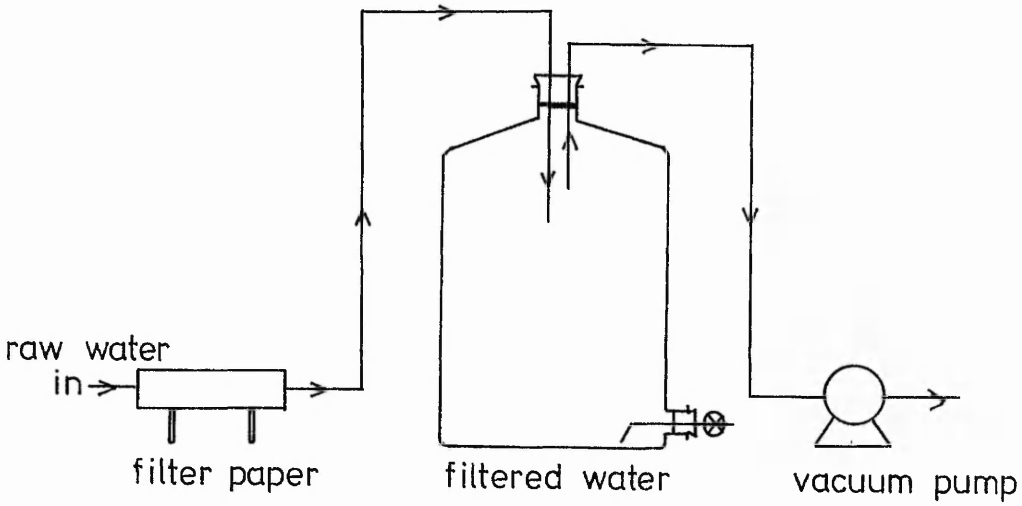
The ozonising tube was 2.4 m tall and 200 mm in diameter, with an ozone bubble path length of 2.13 m, and approximately 70 litres capacity. To 250 litres of permeate water were added 10 ml of MS 2 at 10^{11} ml^{-1}, mixed thoroughly and the tube flushed with 50 litres of this. The tube was then filled with virus containing water and ozone produced by passing dry air at 100 litres hr^{-1} over a discharge tube and applying a voltage of 150 volts. This produced 0.274 g of ozone hr^{-1} and the water leaving the ozonator was sampled over fifteen minutes.

Virus Concentration Techniques

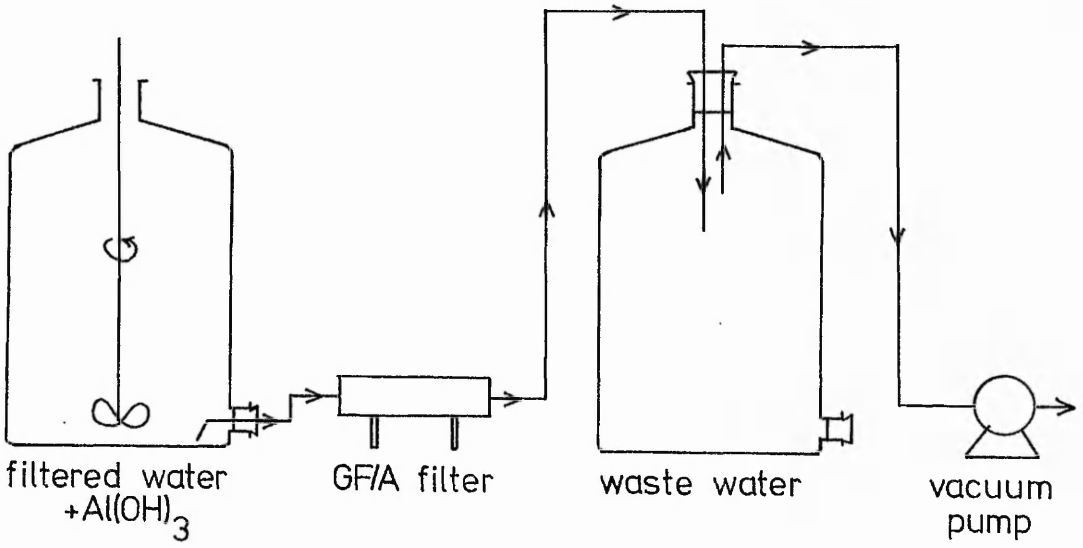
(a) Aluminium Hydroxide Flocculation. The concentration of earlier river water samples and water from the 'B' type RO unit was undertaken using the aluminium hydroxide technique of Wallis and Melnick. After prefiltration through a 150 mm diameter Whatman cellulose filter paper in a Sartorius membrane holder type SM 16505, to remove suspended solids, sterile aluminium hydroxide was added to the water in a 20 litre glass aspirator, at 100 ml per 20 litres. (Figure 10a: for hydroxide preparation see Appendix 1). The water was then stirred rapidly with a Gallenkamp overhead stirrer at room temperature for one hour to allow virus adsorption to the

Figure 10. $Al(OH)_3$ concentration apparatus and technique.

(a). Water pretreatment



(b). Virus concentration



hydroxide and then filtered through a 150 mm Whatman GF/A glass fibre filter paper under negative pressure from an Edwards EB3A vacuum pump/compresor, to collect the virus floc complex (Figure 10b). This complex was removed by scraping with an alcohol sterilised spatula into 10 ml of tissue culture maintenance medium in a Thunberg tube and 1.0 ml of ether added to kill contaminating bacteria. These latter operations were performed within the glass fronted hood of a LEEC negative pressure cabinet with exhaust filter and ultra-violet sterilisation facilities, to prevent the escape of any viruses present. After one hour the ether was removed over thirty minutes with a water vacuum pump attached to the side arm of the Thunberg tube, and the sample inoculated onto replicate tissue culture cells for assay. Water samples concentrated by this method were inoculated into five replicate 75 x 13 mm tubes of Vero and HEP2 as described and all tubes passaged five times.

(b) Iron Oxide Adsorption. From November 1973 iron oxide adsorption was used as the preferred concentration technique for water-borne viruses. Two methods of oxide collection were employed, filtration and later, electromagnetic removal, and these are described in turn.

(i) Oxide Filtration - To twenty litres of prefiltered water in an aspirator was added 2.0 ml of wet ball milled iron oxide suspension (200 mg l^{-1}), prepared in a Pascall ball milling machine, and 20 ml of 20 mM aluminium chloride (see Appendix 1 for oxide preparation). This was stirred for

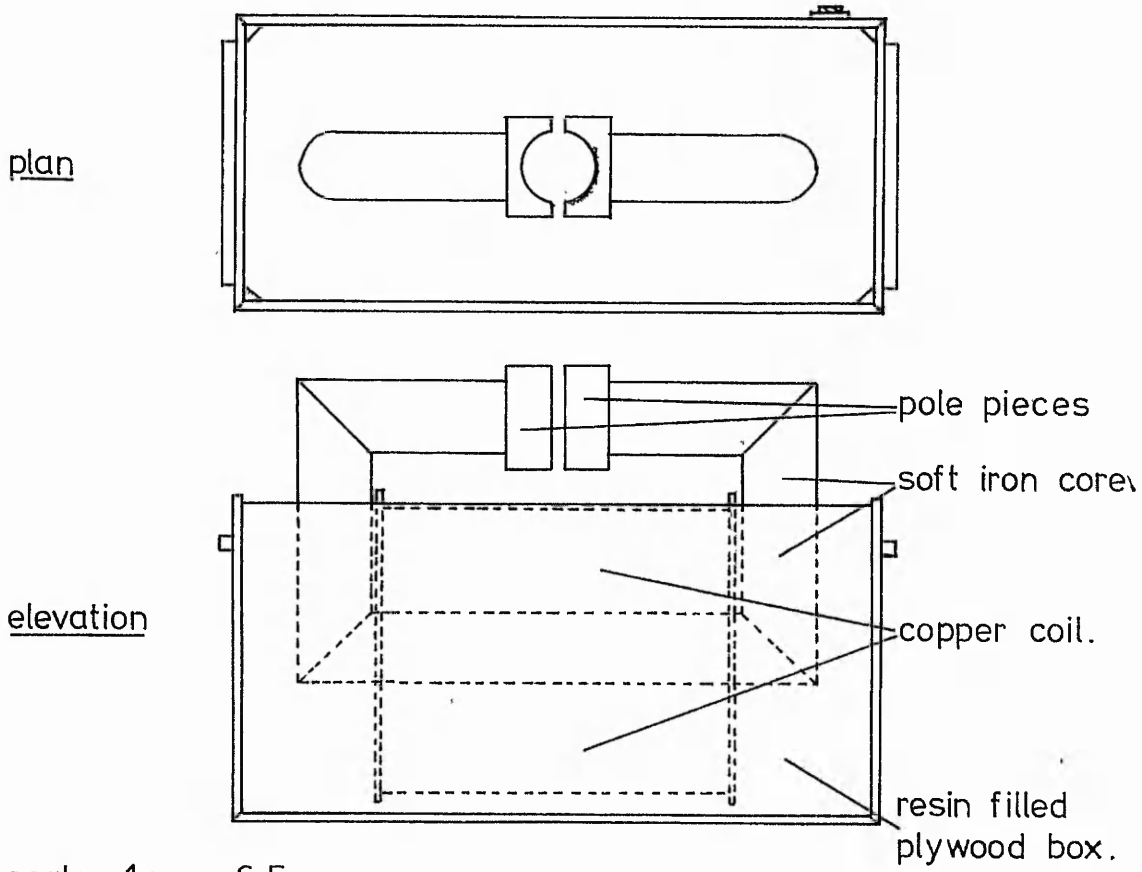
thirty minutes and then filtered through a 150 mm Whatman GF/A filter paper precoated with 1.5 g of Keiselguhr as filter aid. This apparatus was identical with that in Figure 10b, substituting iron oxide for aluminium hydroxide. Oxide plus filter were transferred to a 250 ml glass centrifuge tube and macerated with sterile spatula and forceps in 50 ml 1.0% lab-lemco pH 9.0. The tube was then agitated for thirty minutes on a laboratory constructed rotary mixer at approximately 10 rev min^{-1} to elute any viruses. These latter operations were performed in a LEEC vertical laminar flow cabinet to prevent virus aerosol contamination of the laboratory. After thirty minutes the 50 ml of concentrate was centrifuged at 1000 rev min^{-1} (450 g) in a MSE 4/64 Mk 2 magnum centrifuge with a 6 x 250 ml swing out rotor for fifteen minutes to deposit oxide and filter paper, and the lab-lemco was decanted, ether treated and assayed on replicate tissue cultures.

The gauze pad samples of November 1973 to May 1974 were treated by placing them one at a time, into wide bore 100 ml plastic syringes and expressing as much liquid as possible. They were then rinsed in 50 ml sterile distilled water and compressed again, pooling the total of four volumes of liquid obtained from two pads. To the resulting volume of approximately 200 ml was added iron oxide at 200 mg l^{-1} with thirty minutes agitation, followed by centrifugation and elution as described.

(ii) Electromagnetic Separation - An electromagnet was ordered from and constructed by the Mechanical Engineering Department of Trent Polytechnic in 1974. This consisted of

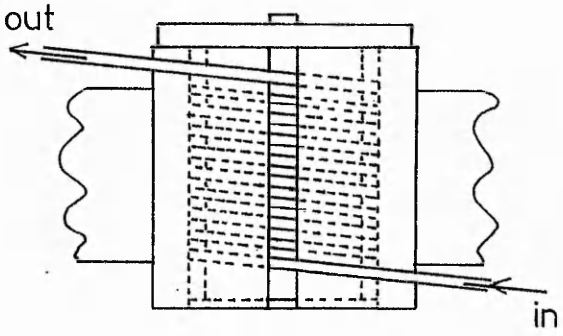
2.0 m of 60 mm diameter Swedish soft iron, machined into a rectangle, as shown in Figure 11(a), with a cylindrical space 87 mm wide in the middle of the top side, which constituted the magnet pole pieces. The lower side of the rectangle was enclosed by a large copper coil and the whole was 'potted' in 25 litres of epoxy resin in a 9-ply varnished wooden box, measuring 580 x 300 x 287 mm, the whole assembly weighing 100 Kilograms. Electrical connections were taken from five positions on the coil via a water resistant seven pin multi-socket at 240 volts DC. A variable voltage rheostat permitted control over the strength of the magnetic flux and a function switch facilitated oxide demagnetisation, by switching from DC to AC current. Using a Bell 600A Gaussmeter with T-601 transverse probe, the maximum magnetic flux density was measured as 150 mT (or 1500 Gauss) with an accuracy of 14% FS. The oxide collecting head in the original design consisted of a central iron cylindrical core 77 mm long by 55 mm diameter, supported by a brass plate resting on the top of the pole pieces. Around this cylinder was tightly spiraled 2.0 m of 40 mm diameter clear plastic tubing and the whole placed into the magnet as indicated in Figure 11 (b). To twenty litres of unfiltered water was added 2.0 ml of oxide as before with 20 ml of 20 mM aluminium chloride, and the whole stirred for thirty minutes at room temperature. The liquid was then passed at up to 50 ml min^{-1} into the bottom of the spiral within the magnetisied electromagnet, by negative pressure from the Edwards vacuum pump/compressor. Oxide plus viruses were retained within

Figure 11. Electromagnet specifications and collecting heads.
(a). magnet

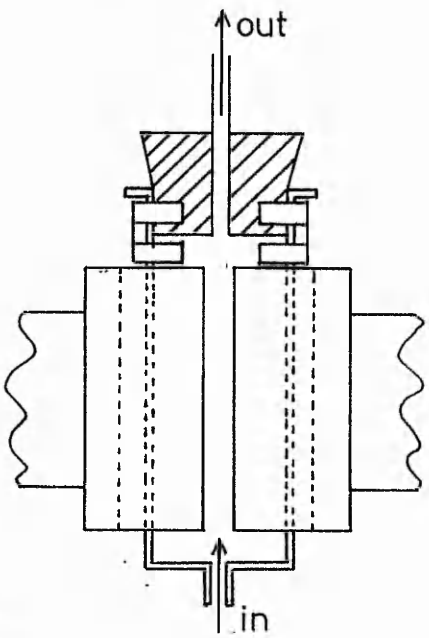


scale: 1cm = 6·5cm.

(b). plastic coil



(c). magnetic filter



scale for (b) and (c): 1cm = 2·5cm.

the spiral by the magnetic flux density and effluent liquid removed at the upper end to waste.

After demagnetisation the plastic coil was carefully detached and the oxide forced out with 20 ml of 1% lab lemco, pH 9.0. This was achieved by inserting a 25 ml pipette containing the eluent in one end of the tubing and applying gentle positive pressure by mouth. The eluted oxide was removed into a 25 ml Universal bottle and mixed for thirty minutes at room temperature on the laboratory made rotary mixer. The oxide was then deposited by centrifugation at 1000 rev min^{-1} (80 g) for fifteen minutes in the Janetzki bench top centrifuge and the elutent decanted. This was ether treated and assayed as required.

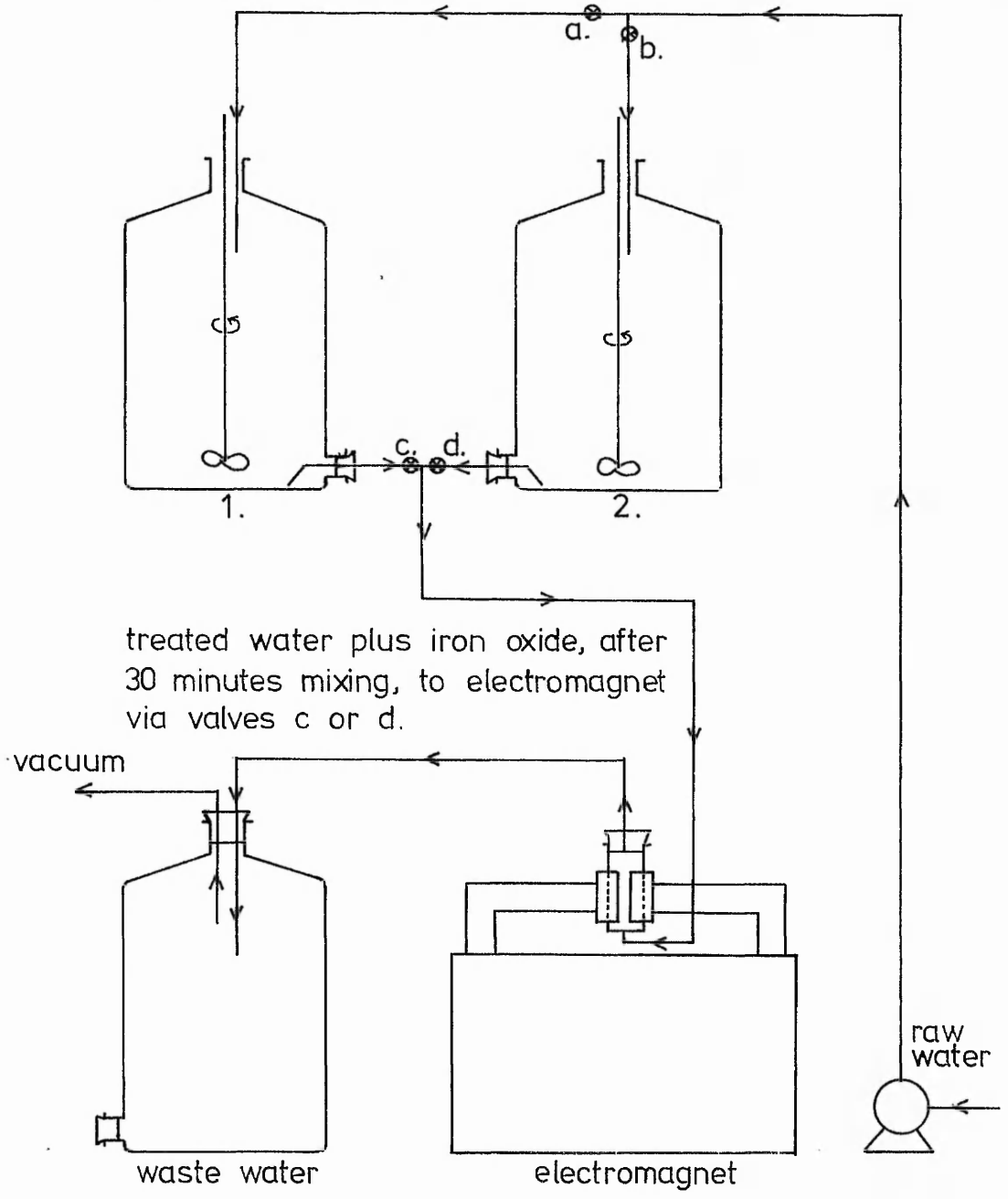
The plastic spiral was found to be inefficient at flow rates greater than 50 ml min^{-1} and so a different oxide collecting head was designed. This consisted of a 100 ml plastic syringe containing 3.0 g of loosely packed steel wool capped with a rubber bung with outlet. The complete assembly was suspended between the pole pieces of the magnet by a clamp, as in Figure 11(c). In operation a water sample and iron oxide were mixed as before and after thirty minutes passed vertically through the steel wool at up to 1.5 l min^{-1}, under negative pressure. After demagnetisation the lower end of the syringe was sealed with a small length of rubber tubing and a Hoffman clip, the bung at the wide end removed and 20 ml of 1% lab lemco, pH 9.0 added by pipette. The syringe plunger was inserted so that it sealed the tube and reduced the internal volume to 50 ml, thus compressing the steel wool and increasing

the contact between eluent and iron oxide. Air was expelled into a negative pressure cabinet by controlling the Hoffman clip, which was subsequently resealed. The complete assembly was then mounted on a second laboratory made rotary mixer and mixed for 60 minutes at approximately 5.0 rev min^{-1}. After 60 minutes elution the syringe was removed from the mixer, the Hoffman clip released and the eluent expressed into a 25 ml Universal bottle by depression of the plunger. This bottle was then centrifuged on the Janetzki centrifuge at 1000 rev min^{-1} (80 G) for fifteen minutes to deposit oxide, the supernatant decanted and treated as above.

For more efficient processing of water samples the apparatus was developed as shown in Figure 12. This arrangement enabled two samples to be processed in quick succession, the second being magnetically filtered as soon as possible after the first.

(c) Two Phase Separation/Concentration. This method was employed to concentrate coliphage MS 2 prior to electron microscope experiments, where high titres were necessary to ensure adequate particle numbers per field. Using the liquid culture method for MS 2 production, 100 ml of lysate were produced and used to seed two litres of medium A. This was treated in essentially the same way, the medium being divided between twenty 50 ml conical flasks and E. coli K12 grown for two hours at 37°C at 250 rev min^{-1} on a Griffin orbital shaking incubator. MS 2 was then added at a multiplicity of 10

Figure 12. Iron oxide concentration apparatus and technique.



(or 1.0 ml at 10^9 ml^{-1} per flask), and the flasks reincubated at 37°C and 150 rev min^{-1}.

After four hours the contents of the flasks were pooled into a non-sterile five litres separating funnel and 20 ml of chloroform and 200 ml of lysing solution added. After vigorous shaking 43.75 g sodium chloride (0.3 M) 172.5 g polyethylene glycol 6000 (BDH, 6.5%) and 5.0 g sodium dextran sulphate (Sigma 500-s, 0.2%) were added, shaken to dissolve and the funnel placed vertically at 4°C for twenty four hours.

After twenty four hours standing, a heavily turbid bottom phase of approximately 30 ml had formed under a light top phase of about two litres. This bottom phase was slowly collected into a 50 ml glass centrifuge tube and centrifuged for 15 minutes at 2000 rev min^{-1} (1100 g) in a Griffin P669 centrifuge with 6 x 50 ml swing out rotor. Two phases were clearly seen after this treatment, phage having collected at the interface between them. The top and bottom phases were removed using a pasteur pipette and water vacuum pump and the remaining 'cake' resuspended in 20 ml of 1.0% sodium dextran sulphate. Then 5.25 ml of 3.0 M potassium chloride was added to the suspension, mixed, and allowed to stand at 4°C for two hours to precipitate the sodium dextran sulphate. The tube was again centrifuged at 2000 rev min^{-1} for ten minutes and the phage containing supernatent removed by pipette to a sterile 100 ml bottle. A few drops of chloroform were added and the suspension stored at 4°C until required for assay.

Electron microscopy

Virus support films for electron microscope use were prepared by dissolving 5.0 ml of 1.0 % formvar solution in 15.0 ml of chloroform and dipping a clean glass microscope slide, to half its depth, into this solution. The slide was withdrawn vertically in a slow, even manner and allowed to dry for 30 minutes. The edges of the formvar film formed on the slide were gently scraped with a sharp blade to free them and then the slide dipped slowly, at an acute angle, into a Petri dish of distilled water. The film was floated free of the slide onto the water surface, and G-200 3.05 mm copper grids were positioned closely together upon it, matt side down. A strip of 'Parafilm' tape was applied over the grids and the whole assembly removed from the Petri dish and inverted. After 15 to 20 minutes of air drying the grids were placed film side uppermost onto a piece of filter paper and mounted inside the dome of a Nanotech 300 shadowing unit. The grids were carbon coated from a sharpened graphite rod under a 10^{-5} TORR vacuum and then removed individually with fine pointed forceps to a second piece of filter paper, carbon/formvar surface uppermost. This second piece of filter paper was positioned in the bottom of a glass Petri dish and chloroform added until it just floated. The grids were left for five minutes until the formvar was dissolved, and then the filter paper removed to a second glass Petri dish. This was left

with lid ajar until the chloroform had evaporated, and the resulting carbon coated, formvar eroded grids were ready for use.

Two methods of specimen preparation were used, the first simply involving the dipping of a prepared grid, held with carbon side downwards in fine forceps, into a suspension of viruses, or host bacteria plus viruses, for ten seconds. After air drying the film, the grid was dipped into a 2.0% solution of phosphotungstinic acid stain, pH 6.8, in a watch-glass for two seconds, removed and air dried.

Alternatively 0.2 ml phage was pipetted into a Fullam nebuliser-sprayer (No. 5275) which was held vertically in a fume cupboard and aimed at a number of grids fixed to filter paper by very small strips of adhesive tape. The rubber bulb of the sprayer was squeezed forty times to produce a very fine aerosol which settled on the grid, positioned about 10 cm from the nozzle of the sprayer. After air drying, the preparations were stained as above.

All grids were examined in an AEI 108 Electron Microscope at a voltage of eighty Kv and a magnification of one hundred or one hundred and sixty thousand. Photographs were taken with the inbuilt plate camera at six seconds exposure and further enlarged for detailed examinations of individual fields to be made.

RESULTS

River Sampling

After consultations with the then Trent River Authority (now the Severn-Trent Water Authority, STWA) a six point viral survey of the River Trent and some of its major tributaries was initiated in May 1973. Water sampling was undertaken from sites already chosen for chemical analyses by STWA and representative of differing water qualities within the Trent basin.

The sites, numbered 1 to 5 on Figure 13, included the water intakes to two reservoirs, at Egginton on the River Dove and Church Wilne on the River Derwent. The River Soar, known to be polluted by domestic and industrial effluents, was sampled at Kegworth, as was the Trent itself at Shardlow after confluence with the polluting River Tame and the clean River Dove. The River Trent was also assessed for virus population at the intake to the WRC/STWA pilot treatment plant at Colwick, this being an important consideration in the calculation of removal by RO of naturally occuring viruses.

The final effluent from the main Nottingham sewage treatment works at Stoke Bardolph was also ananlysed during 1975 (number 6 on Figure 13).

Originally, all samples from the five river sites were concentrated by the aluminium hydroxide flocculation technique and concentrates were assayed by the tube CPE method in replicates of five or ten, but in no case was CPE detected by the fourth and fifth passages. However, it was noted that

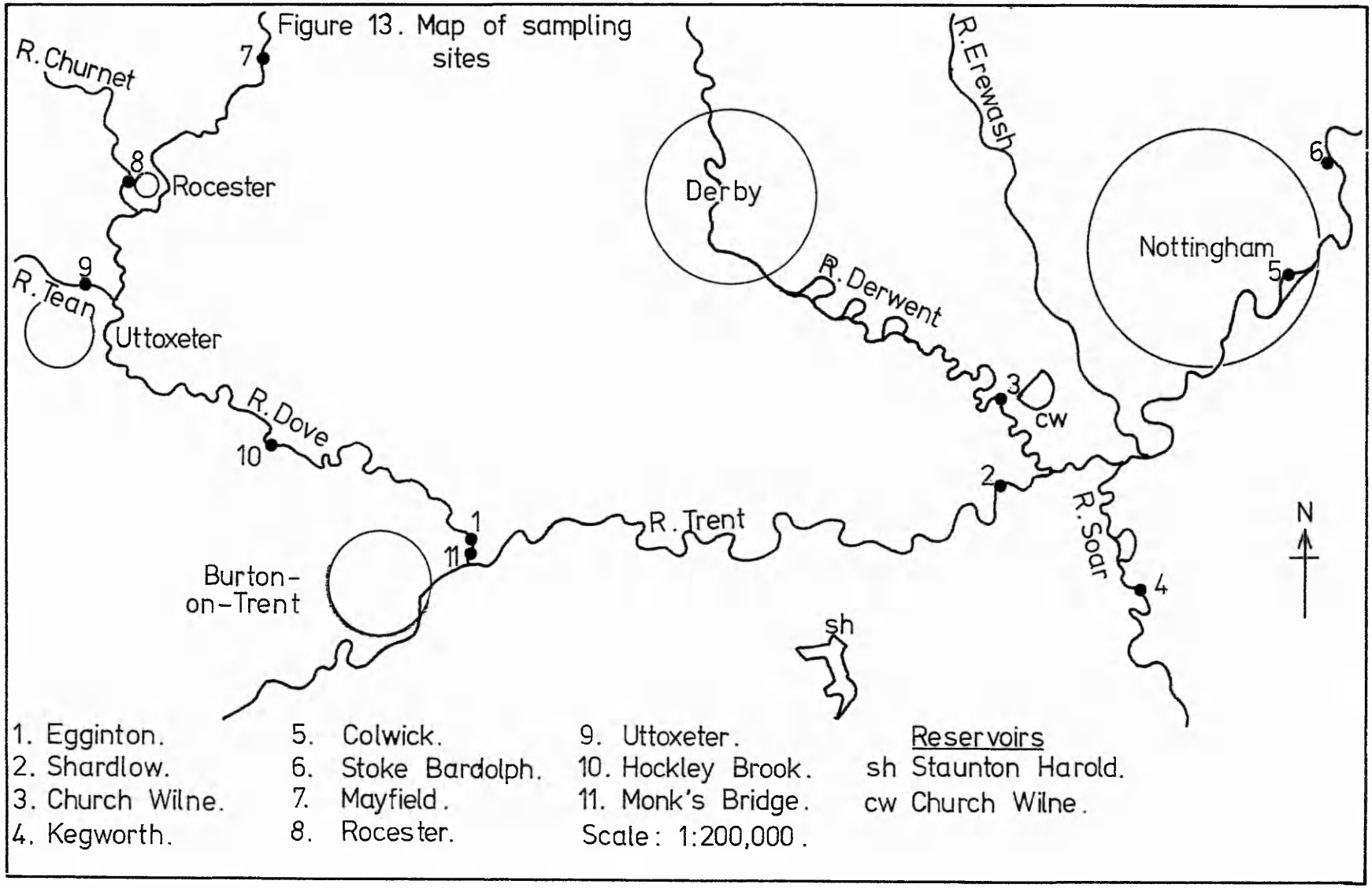


Figure 13. Map of sampling sites

1. Egginton.
2. Shardlow.
3. Church Wilne.
4. Kegworth.
5. Colwick.
6. Stoke Bardolph.
7. Mayfield.
8. Rocester.
9. Uttoxeter.
10. Hockley Brook.
11. Monk's Bridge.

Scale: 1:200,000.

Reservoirs

sh Staunton Harold.
cw Church Wilne.

Bloom, et al (1959) suggested that some cytopathogenic agents lose their ability to produce a visible CPE after isolation. They therefore suggested that for positive identification there should be detectable CPE for at least two passages and reference to the results in Table 8 showed this to occur in some samples (marked *). These results were also shown in Figure 14, together with river pH and temperature readings obtained at the same time. Assuming the conclusions of Bloom, et al to apply, they could be interpreted as showing that viruses were demonstr-able during Summer and Autumn only, with the majority of isolations at Kegworth on the River Soar. This river received large amounts of domestic sewage effluent from Leicestershire and so it was not perhaps surprising that more isolations were made from this site. The two sites on the River Trent each yielded only half the number of isolations of those from the Soar, the River Dove showed only two isolates due to its lack of contamination, and none were detected in the River Derwent because of the suspected presence of chemical pollutants. This was confirmed by the high initial cyto-toxicity of all River Derwent samples, as shown in Table 8, this being quickly removed by dilution on passage.

Within a few river assays it became noticeable that some component of the concentrated samples, other than possible viruses, was exerting a dramatic cytotoxic effect on tissues, and masking virus isolations. Reference to Table 8 showed this to occur in many cases, particularly the Derwent samples,

Table 8. River survey results (1973)

(* indicates possible virus isolation)

Date	HEp 2				Vero			
	Isolation	1	2	3	Isolation	1	2	3
Colwick								
18/05/73	6/10	2/10	6/10	6/10*	0/10	0/10	0/10	0/10
13/06/73	5/10	1/10	5/10	2/10*	2/10	2/10	0/10	0/10
25/06/73	1/10	1/10	1/10	0/10*	1/10	1/10	0/10	0/10
12/07/73	10/10	6/10	6/10	0/10*	0/10	0/10	0/10	0/10
24/07/73	4/10	4/10	0/10	0/10	10/10	10/10	3/10	0/10*
14/08/73	6/10	6/10	0/10	0/10	-	-	-	-
05/09/73	10/10	0/10	0/10	0/10	-	-	-	-
18/09/73	10/10	0/10	0/10	0/10	-	-	-	-
02/10/73	-	-	-	-	0/5	0/5	0/5	0/5
18/10/73	10/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
08/11/73	-	-	-	-	0/5	0/5	0/5	0/5
22/11/73	4/10	0/10	0/10	0/10	-	-	-	-
Church Wilne								
18/05/73	10/10	0/10	0/10	0/10	10/10	5/10	0/10	0/10
13/06/73	10/10	0/10	0/10	0/10	10/10	0/10	0/10	0/10
25/06/73	10/10	0/10	0/10	0/10	10/10	0/10	0/10	0/10
12/07/73	10/10	8/10	0/10	0/10	10/10	0/10	0/10	0/10
07/08/73	-	-	-	-	0/5	0/5	0/5	0/5
30/08/73	10/10	0/10	0/10	0/10	3/10	0/10	0/10	0/10
10/09/73	10/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
24/09/73	10/10	0/10	0/10	0/10	-	-	-	-
08/10/73	10/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
05/11/73	9/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10

Table 8 - continued

Date	HEp 2				Vero			
	Isolation	1	2	3	Isolation	1	2	3
Kegworth								
18/05/73	10/10	2/10	0/10	0/10	9/10	2/10	1/10	0/10*
13/06/73	10/10	3/10	0/10	0/10	9/10	1/10	0/10	0/10
25/06/73	10/10	3/10	0/10	0/10	9/10	1/10	1/10	1/10*
12/07/73	10/10	6/10	0/10	0/10	10/10	4/10	4/10	4/10*
24/07/73	2/10	2/10	0/10	0/10	10/10	10/10	4/10	0/10*
07/08/73	2/10	0/10	0/10	0/10	8/10	8/10	1/10	1/10*
05/09/73	7/10	1/10	1/10	2/10*	1/10	0/10	0/10	0/10
18/09/73	9/10	2/10	2/10	2/10*	4/5	1/5	0/5	0/5
02/10/73	9/10	3/10	2/10	0/10*	1/5	1/5	1/5	0/5*
16/10/73	10/10	0/10	0/10	0/10	5/5	2/5	0/5	0/5
08/11/73	-	-	-	-	2/5	1/5	0/5	0/5
Shardlow								
04/05/73	9/10	9/10	9/10	8/10*	0/10	0/10	0/10	0/10
18/05/73	10/10	8/10	8/10	6/10*	0/10	0/10	0/10	0/10
13/06/73	10/10	4/10	9/10	4/10*	2/10	1/10	0/10	0/10
25/06/73	8/10	3/10	7/10	4/10*	0/10	0/10	0/10	0/10
12/07/73	0/10	0/10	0/10	0/10	10/10	10/10	0/10	0/10
24/07/73	0/10	0/10	0/10	0/10	10/10	8/10	0/10	0/10
07/08/73	0/10	0/10	0/10	0/10	10/10	9/10	2/10	0/10*
05/09/73	-	-	-	-	3/5	0/5	0/5	0/5
18/09/73	-	-	-	-	2/5	0/5	0/5	0/5
02/10/73	-	-	-	-	1/5	0/5	0/5	0/5
16/10/73	-	-	-	-	0/5	0/5	0/5	0/5
08/11/73	-	-	-	-	1/5	0/5	0/5	0/5

Table 8 - continued

Egginton								
Date	HEp 2				Vero			
	Isolation	1	2	3	Isolation	1	2	3
04/05/73	7/10	1/10	3/10	1/10*	0/10	0/10	0/10	0/10
20/05/73	0/10	0/10	0/10	0/10	8/10	2/10	0/10	0/10
12/06/73	10/10	3/10	0/10	0/10*	0/10	0/10	0/10	0/10
25/06/73	6/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
26/07/73	-	-	-	-	0/5	0/5	0/5	0/5
07/08/73	-	-	-	-	0/5	0/5	0/5	0/5
23/08/73	-	-	-	-	0/5	0/5	0/5	0/5
06/09/73	-	-	-	-	0/5	0/5	0/5	0/5
17/09/73	-	-	-	-	0/5	0/5	0/5	0/5
15/10/73	-	-	-	-	0/5	0/5	0/5	0/5
29/10/73	-	-	-	-	0/5	0/5	0/5	0/5

as noted above. Such samples showed an initial high degree of cell death which decreased to little or no effect upon the first passage, whereas samples assumed to be virus-containing demonstrated a gradual reduction in the number of positive CPE tubes with passage. It was therefore considered that 'virus positive' samples contained sufficient of these agents to overcome any background cytotoxic effect of the non-viral toxic agents. Viruses, however, could have been present at other times, but at levels too low to be detected above the background level of chemically induced cell death.

Figure 14. River survey results.

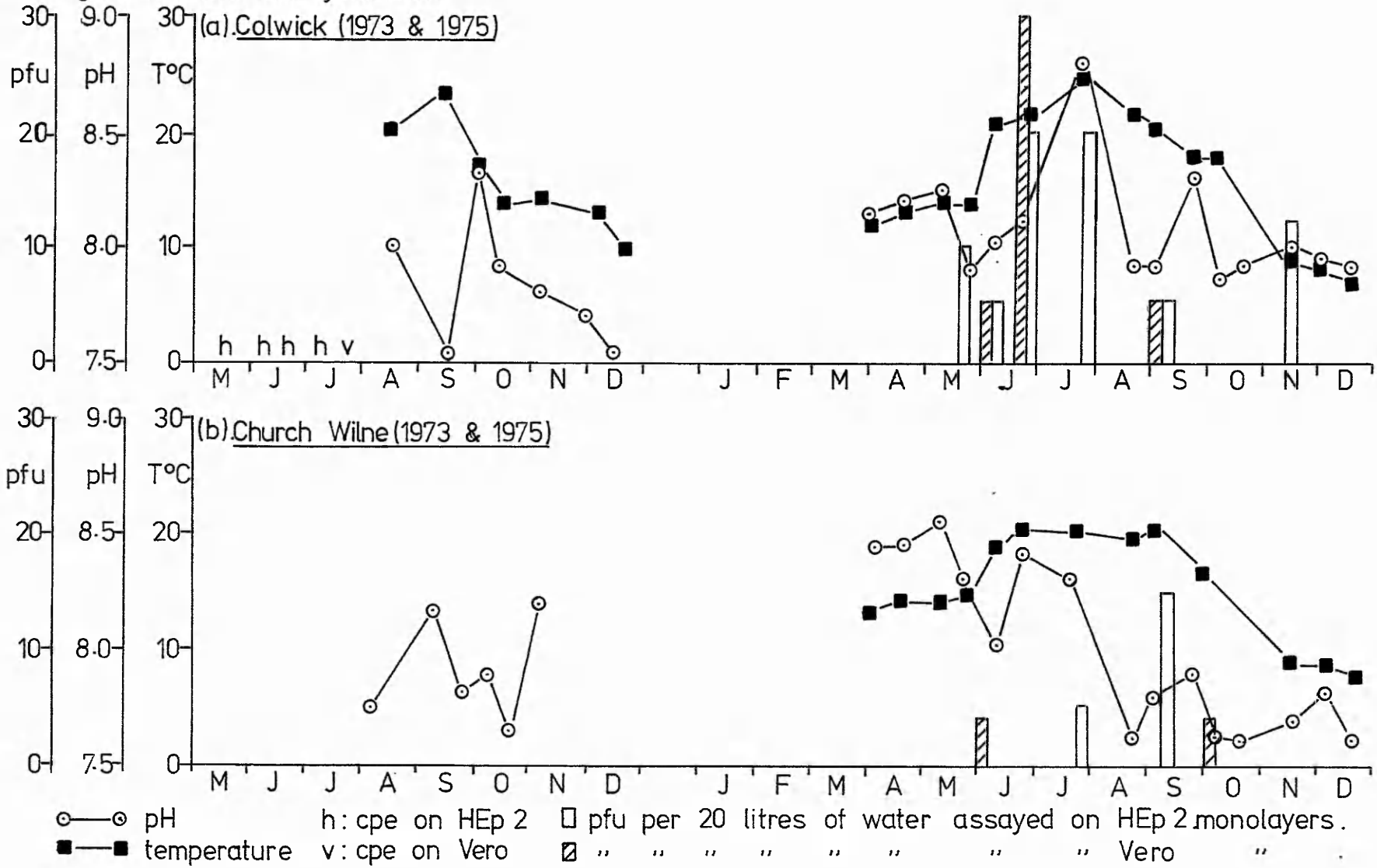


Figure 14. cont.

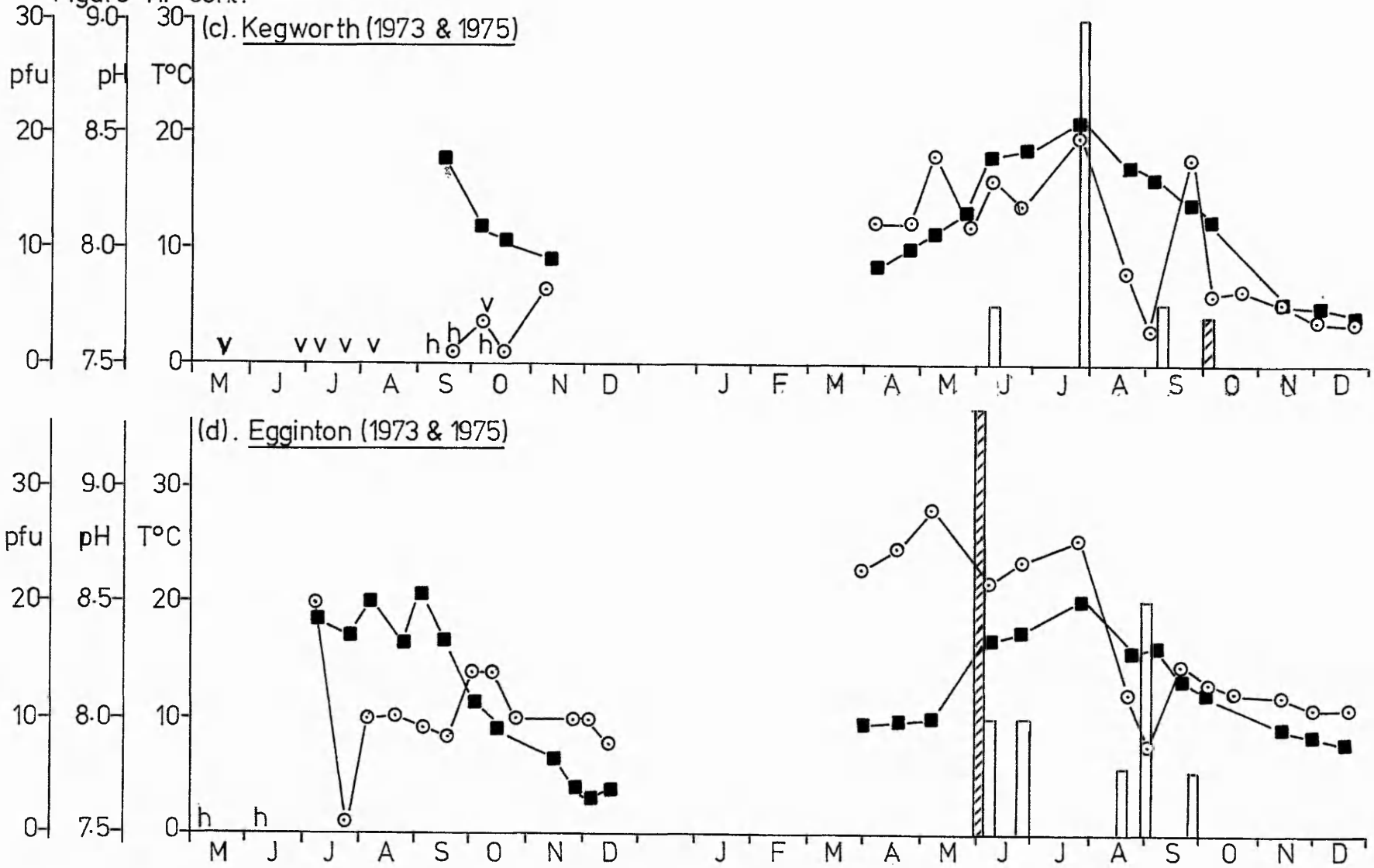
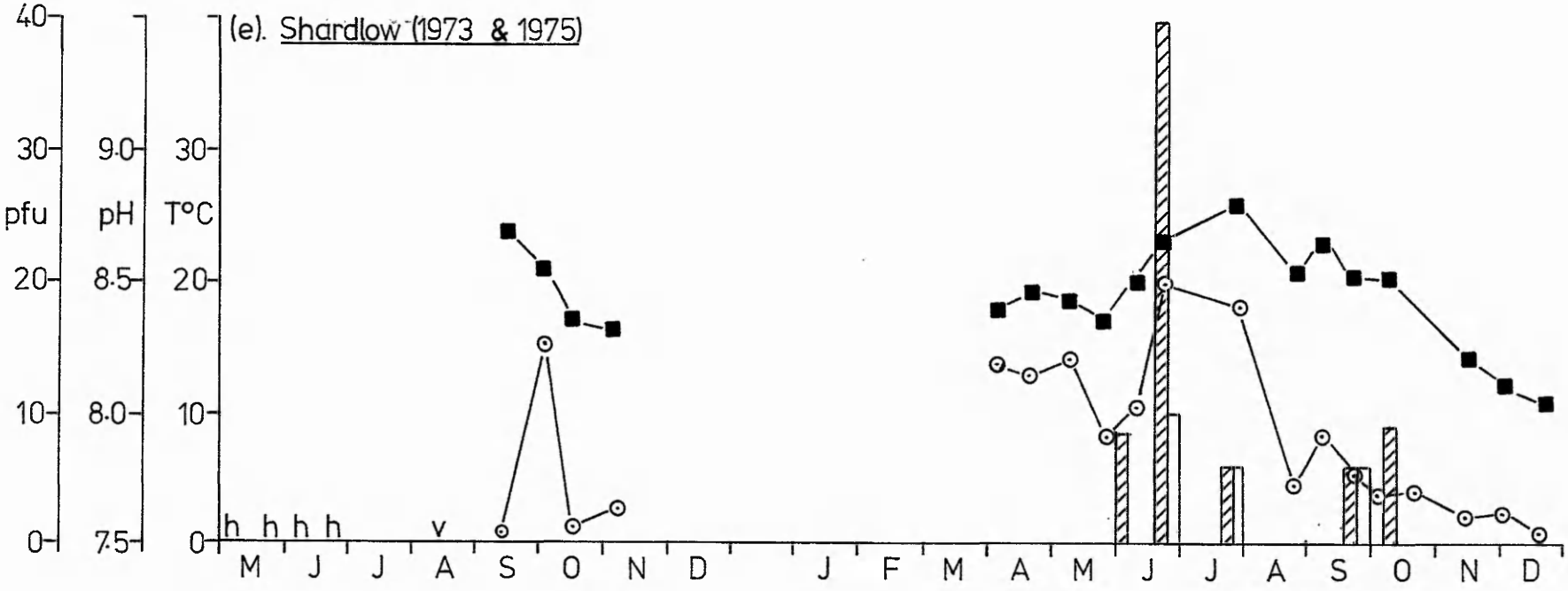


Figure 14. cont.



In order to resolve this problem a small, intensive survey, subordinate to the main one, was initiated using selected points of the River Dove system (see Figure 13), where waters of differing quality could be collected. The waters of the upper reaches of the Dove were known to be of very good quality and so samples were taken at Mayfield Bridge. The River Churnett, however, received large amounts of industrial effluents and domestic sewage and, as a result, was grossly polluted over much of its length, and so was sampled at Rocester. On the other hand the River Tean received only one major discharge of sewage effluent which caused a decrease in quality downstream of the water reclamation works, and this river was sampled at Uttoxeter. Therefore, in one small area several different qualities of water could be collected, which was done in collaboration with the STWA. From each site ten litres of water were taken, concentrated to 5.0 ml by aluminium hydroxide flocculation and inoculated onto five replicates of Vero and HEp 2 cells. The results of this assay were shown in Table 9 and illustrated the same interpretative problems as the major survey results.

Table 9. Results of virus survey of the River Dove system

Location	Cells	Isolation	1	2	3	4	5
Uttoxeter	Vero	0/5	0/5	0/5	-	-	-
(R. Tean)	HEp 2	5/5	3/5	2/5	1/5	0/5	0/5*
Hockley Brook	Vero	5/5	2/5	0/5	0/5	-	-
(R. Dove)	HEp 2	5/5	1/5	1/5	0/5	0/5	-*
Rocester	Vero	2/5	0/5	0/5	-	-	-
(R. Churnett)	HEp 2	5/5	0/5	0/5	-	-	-
Monk's Bridge	Vero	1/5	1/5	1/5	0/5	0/5	-*
(R. Dove)	HEp 2	5/5	2/5	2/5	0/5	0/5	-*
Mayfield Bridge	Vero	2/5	2/5	1/5	0/5	0/5	-*
(R. Dove)	HEp 2	5/5	0/5	0/5	-	-	-

* indicates probable virus isolation.

Using the same criteria as before, viruses could be detected at Uttoxeter (R. Tean), Monk's Bridge (R. Dove), Hockley Brook (R. Dove), and Mayfield (R. Dove), but not at Rocester (R. Churnett). It was evident from Table 9, however, that non-viral agents were causing cytotoxicity, and that the high cell loss on initial inoculation was seen to be greater in HEp 2 cells.

Water samples taken at the same time as those for viral assay were analysed chemically by the STWA (Table 10), and whilst it was difficult to clearly demonstrate which component of the water might be causing cell death, it was noted that the River Churnett, in which no viruses were detected (Table 9), did

Table 10. Chemical analyses of the River Dove and its principal tributaries

Parameter	Tean	Mayfield	Hockley	Monk's Br	Churnett
suspended solids	9.0	4.0	7.0	10.0	7.0
BOD	2.4	1.0	0.9	1.8	3.9
TOC	6.3	2.3	3.9	4.5	7.5
Anionic detergents	0.14	0.0	0.07	0.07	0.14
pH	7.8	8.0	8.0	8.0	7.8
Conductivity (µmhos)	630	440	490	570	420
Ammonia N	1.6	0.2	0.5	0.4	1.1
Iron	0.28	0.13	0.24	0.31	0.42
Manganese	0.09	0.01	0.09	0.08	0.1
Aluminium	0.09	0.04	0.07	0.1	0.09
Sodium	23.3	9.0	16.0	20.0	18.2
Potassium	7.1	2.1	3.7	4.1	4.7
Cadmium	0.004	0.001	0.001	0.001	0.001
Chromium	0.008	0.004	0.006	0.008	0.004
Copper	0.015	0.011	0.037	0.039	0.069
Lead	0.016	0.014	0.016	0.016	0.016
Nickel	0.022	0.006	0.012	0.01	0.02
Zinc	0.048	0.012	0.018	0.019	0.025

All measurements in mg l^{-1} unless stated otherwise.

have the highest level of many of the parameters measured. As the River Churnett received the greatest levels of both

domestic and industrial effluents there appeared to be an inverse correlation between the water quality in this river system and virus removal, and a direct correlation with cellular toxicity.

The evidence from both the main river survey and the detailed River Dove survey suggested that the aluminium hydroxide concentration technique might be responsible for the cellular toxicity observed by the collection and concentration of toxicity. If this were true then any non-viral components of the water, when concentrated into the hydroxide floc, would be detectable by chemical analysis, and so sterilised samples from the various stages of the process were sent to STWA for analysis, the results being shown in Table 11.

These results clearly indicated considerable concentration of metal cations by the aluminium hydroxide floc, in the cases of zinc and chromium at least, to levels shown by laboratory experiments to be toxic to the same cell lines used for virus assay (Jones, P., 1977: M. Phil. thesis, Trent Polytechnic and Norman, 1974: HND dissertation, Trent Polytechnic). Therefore metal toxicity to cells during viral assay, as a result of $Al(OH)_3$ concentration was seen to occur, as well as the possibility of metal inactivation of viruses in the raw water and during and after concentration.

Table 11. Chemical analyses of two virus concentration methods

Parameter	$Al(OH)_3$ flocculation				Iron oxide adsorption		
	R.Trent Colwick	Coarse filtn.	GF/A filtn.	Conc. in 5.0 ml	R.Trent Colwick	Waste water	Conc. in 5.0 ml
TOC	5.3	5.5	5.6	-	-	-	-
Anionic Detergents	0.15	0.16	0.14	0.0	0.26	0.36	0.0
Iron	0.31	0.05	0.05	290	0.24	0.02	1.5
Manganese	0.08	0.07	0.02	8.0	0.03	0.02	1.5
Cadmium	0.003	0.004	0.002	0.0	0.002	0.002	0.0
Chromium	0.008	0.01	0.006	8.0	0.012	0.004	4.0
Copper	0.023	0.029	0.025	12.0	0.02	0.019	2.5
Lead	0.03	0.038	0.022	16.0	0.024	0.024	3.0
Nickel	0.066	0.062	0.062	0.0	0.08	0.06	0.0
Zinc	0.07	0.072	0.054	58.0	0.061	0.052	2.5

All measurements in mg l^{-1}

This last possibility was explored further by the establishment of a series of tests in which 1.0 ml aliquots of Poliovirus 1 at 8.0×10^{6} ml^{-1} were inoculated into 10.0 ml of each of the five main river waters. These were incubated at room temperature for 24 hours on a laboratory constructed rotary mixer at 10 rev min^{-1}, a high initial titre being employed to avoid the necessity of a concentration procedure. Samples were diluted 1.0 ml into 9.0 ml of tissue culture maintenance medium after incubation, in order to remove any residual chemicals that might produce cytotoxicity. They were then assayed by plaque assay, because this involved a further dilution into the agar layer, following the virus adsorption period. It was also felt that plaque assays gave more easily assessable, quantitative results, which allowed percentage losses to be calculated, as in Table 12. The test showed a trend of increasing virus loss with increasing BOD and decreasing Biotic Index (BI), BI, as used by STWA, was a scale of biological activity in water ranging from 0, indicating grossly polluted water with no biological activity, to 10, indicating very clean water containing a wide variety of biological life. Therefore virus survival seemed to be closely linked with water quality, as shown during the River Dove samplings, although loss of titre was quite rapid in all cases. Therefore it would seem that in the polluted waters of the River Trent system, with the possible exception of the River Dove, any influent viruses would be quickly diluted and

Table 12. Survival of Poliovirus 1 in waters from five sampling sites on the River Trent system

	% loss	mean BOD	mean ss	mean NH_3/N	BI
Colwick	97.2	7.3	23.0	0.9	5
Shardlow	69.4	9.5	31.0	0.8	4
Kegworth	58.3	7.2	17.0	4.0	6
Church Wilne	44.4	4.3	20.0	0.6	7
Egginton	33.3	2.7	17.0	0.2	9

BOD = Biological oxygen demand.

ss = Suspended solids.

NH_3/N = Ammonia nitrogen.

BI = Biotic index

All figures, except BI, are quoted as mg l^{-1}.

inactivated, making their detection uncertain. Also, if the concentration technique concentrated toxicants as well as viruses, the chances of virus detection and the distinguishing of viral from chemical CPE would be extremely difficult.

Finally it was noted that aluminium hydroxide was used in the coprecipitation of metals from sea water by entrapment of the cations in the floc, with a 95 - 100 % yield (Rodier, 1975 and Table 13).

Both surveys, and the experiments above, showed that although tube CPE has been useful in the detection of chemical pollutants in polluted surface waters, it was inadequate for

Table 13. Element entrainment by aluminium hydroxide

(Rodier, 1975)

H															
Li	(Be)											B	C	N	O
Na	Mg											Al	Si	P	S
K	Ca	Se	(Ti)	(V)	(Cr)	Mn	(Fe)	(Co)	(Ni)	Cu	(Zn)	(Ga)	(Ge)	As	Se
Rb	Sr	Y	(Zr)	(Nb)	(Mo)	Tc	(Ru)	(Rh)	Pd	Ag	Cd	In	(Sn)	Sb	Te
Cs	Ba	(La)	(Hf)	Ta	(W)	Re	Os	(Ir)	(Pt)	Au	Hg	Tl	Pb	(Bi)	Po
Fr	Ra	Ac													
		Ce	Pr	Nd	Pm	Sm	(Eu)	Gd	Tb	Dy	Ho	Er	Tm	Yb	Lu
		Tn	Pa	(U)	Np	Pu	Am	Cm	Bk	Cf	Es	Fm	Mv	No	

Encircled elements are those which are entrained by $Al(OH)_3$.

the demonstration of viruses under these conditions. Further, the aluminium hydroxide concentration technique, because of its ability to coadsorb metal cations capable of affecting both viruses and cell lines, was undesirable for polluted waters. Thus it was decided that the technique should be replaced, and iron oxide adsorption was examined as described in the next section. Chemical analyses on the stages of this procedure were performed as before and the results, displayed in Table 11, showed considerably less metal cation concentration than the $Al(OH)_3$ method.

During 1974, whilst these problems were being examined, gauze pads were deployed at Colwick and Egginton, virus recovery being by ferric oxide adsorption and inoculation onto

replicates of cell monolayers. As pads were able to collect and concentrate viruses but not excessive amounts of heavy metals it was hoped that their use would remove the problem of cytotoxicity. Assay was by plaque assay, but no plaque forming units (pfu) were detected in any sample.

In June, July and August 1974, following successful laboratory trials with the electromagnet and spiral collector, 10.0 litre water samples were collected from the five sampling sites, and concentrated by this method. Only one PFU was detected, at Shardlow on 3/6/74, and identified as Poliovirus 3.

However, following laboratory studies under controlled conditions (see iron oxide section) it was decided that further work was required on the magnetic collector and so the sampling programme was abandoned for that year.

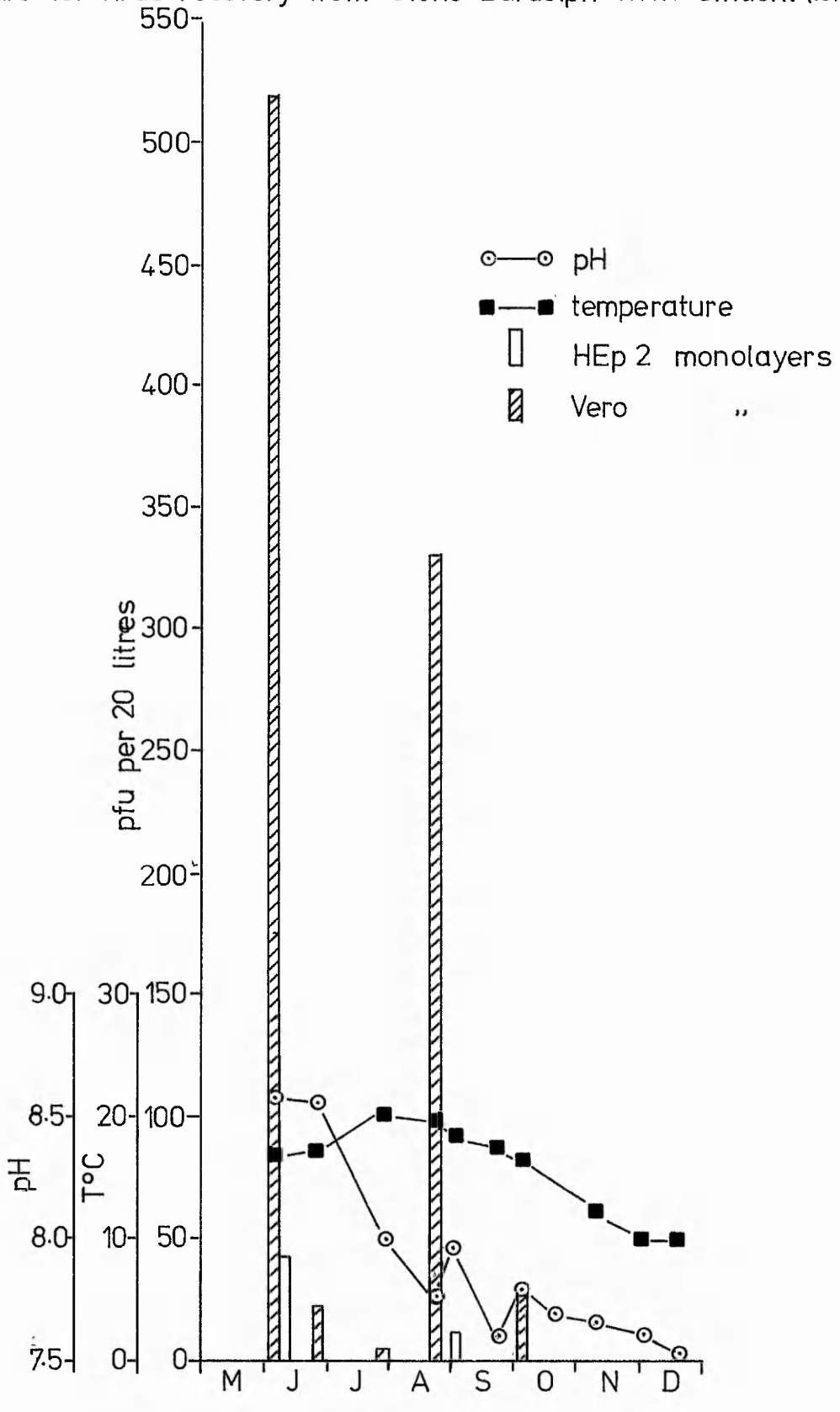
Following design improvements, magnetic filtration, in conjunction with 20.0 litre samples, was used to sample the five river sites, plus the effluent stream from the Stoke Bardolph water reclamation works. This survey gave the results displayed in the second half of Figure 14 and in Table 13. The results for Stoke Bardolph were illustrated separately in Figure 15 and showed the much higher numbers expected from such an effluent. Again it was noted that viruses were recovered in Summer and Autumn with highest numbers of 1.5 to 2.0 pfu l^{-1} in Summer, although it should also be stated that no samples were taken in January, February and March of 1975.

Table 14. Results of River Survey 1975 (Figures as Pfu 20 l^{-1} water sample)

Date	Colwick		Church		Wilne		Kegworth		Shardlow		Egginton		Stoke Bardolph	
	VERO	HEp2	VERO	HEp2	VERO	HEp2	VERO	HEp2	VERO	HEp2	VERO	HEp2	VERO	HEp2
12/05/75	-	-	-	-	-	-	-	-	-	-	NT	NT		
25/05/75	-	10	-	-	-	-	-	-	-	-	NT	NT		
09/06/75	5	5	4	-	-	5	8	-	36	10	520	40		
23/06/75	40	20	-	-	-	-	48	10	-	10	20	-		
30/07/75	-	20	-	5	-	30	5	5	-	-	5	-		
25/08/75	-	-	-	-	-	-	-	-	-	5	320	-		
08/09/75	5	5	-	15	-	5	-	-	-	20	-	10		
23/09/75	-	-	-	-	4	-	5	5	-	5	-	-		
07/10/75	-	-	4	-	-	-	8	-	-	-	32	-		
21/10/75	-	-	-	-	-	-	-	-	-	-	-	-		
18/11/75	-	12	-	-	-	-	-	-	-	-	-	-		
02/12/75	-	-	-	-	-	-	-	-	-	-	-	-		
15/12/75	-	-	-	-	-	-	-	-	-	-	-	-		

- indicates below limit of assay (4 Pfu 20 l^{-1}) NT indicates sample not taken

Figure 15. Virus recovery from Stoke Bardolph WRW effluent (1975).



From the number of isolates obtained several observations concerning virus distribution and survival were made. Firstly, for the same Summer/Autumn period more isolations were made using iron oxide adsorption than aluminium hydroxide flocculation, which was a reflection of both the improved concentration and assay procedures. However, it was noted that available data (STWA Water Quality, 1975/1976) showed a general decrease in mean BOD, suspended solids and ammonia nitrogen between 1973 and 1975, as well as a decreased water flow, reflecting the dry weather of that period. As virus survival had been shown to be linked to BOD and water quality in Table 12, it would therefore seem possible that improved virus survival conditions were prevalent. This was an indication that water quality improvement, although highly desirable, could present unseen problems, such as increased enteric virus survival.

An RO plant operating on polluted waters, such as those of the River Trent, would therefore receive a virus load proportional to the degree of pollution of that river. At Colwick, during 1975 the unit was being dosed with viruses at levels of up to 1.5 PFU l^{-1}, which would be an extremely low number to detect during and after RO processing of the water. Consequently, it would be necessary, to artificially load RO units with higher titres of viruses, in order to assess virus loss and this was the procedure adopted for the studies undertaken at Colwick and Budd's Farm.

Iron Oxide Adsorption Studies

The adoption of the iron oxide virus concentration technique necessitated a considerable amount of preliminary investigation to establish the optimum parameters for a large scale use. The two methods of virus collection by iron oxide suggested in the literature were examined, these being static columns and oxide suspensions. The static columns as described by Rao, et al (1968), constructed from various configurations of 76 mm and 150 mm QVF glass tubing with sintered glass discs, filter papers or nylon mesh iron oxide retainers presented several practical problems, not least of which was rapid clogging or 'ponding' of the oxide when using water volumes greater than 1.0 litre. This problem was also discussed by Rao who suggested mixing oxide with keisulguhr to circumvent 'ponding'. Emphasis was, however, altered to viral adsorption by iron oxide in suspension as it was felt that this presented a more practical alternative. Most initial experiments were performed with 10 ml aliquots of tap or river water, virus and oxide being mixed together in suspension in Universal bottles on a laboratory constructed rotary mixer. They were then centrifuged at 1000 rev min^{-1} (80 g) for 15 minutes using a Janetski T32B centrifuge with 6 x 25 ml fixed head rotor to deposit the oxide.

The kinetics of adsorption were established by reacting dilutions of coliphage MS 2 and iron oxide at 500 mg l^{-1} in Universal bottles as described above for 2, 5, 10, 20 and 30

minutes. Assay of the decanted supernatants lead to the percentage adsorption in each case being determined, as in Figure 16. The experiment, performed at room temperature (20°C) was repeated at 37°C and at a refrigerated temperature (6°C) with more rapid, and greater adsorption being noted at the lower temperature, although a change in incubation temperature had only a slight effect on adsorption rate. The rate of virus adsorption was seen to obey first order kinetics, the initial fast rate being diffusion limited only and due to nearly every collision between virus and oxide resulting in adsorption. As the viruses were adsorbed the rate slowed to zero, maximum adsorption however occuring within the thirty minutes of the experiment. This time was therefore chosen for all subsequent studies as it allowed maximal virus adsorption with apparently no desorption.

The effect of pH on adsorption was also monitored, using distilled water of pH's 2.9, 4.1, 6.05, and 7.6. After 30 minutes rotary mixing the virus numbers were assayed and the calculated percent adsorptions plotted against pH as in Figure 17. A marked decrease in adsorption below pH 6 was noted, but as most natural water pH's have been found to be above this value, it was not thought to be a potential problem during virus concentration.

As adsorption was a balance between the forces bringing the adsorbate (viruses) to the adsorbent surface (iron oxide) and thermal agitation which tended to distribute it uniformly,

Figure 16. Effect of temperature on MS 2 adsorption to iron oxide.

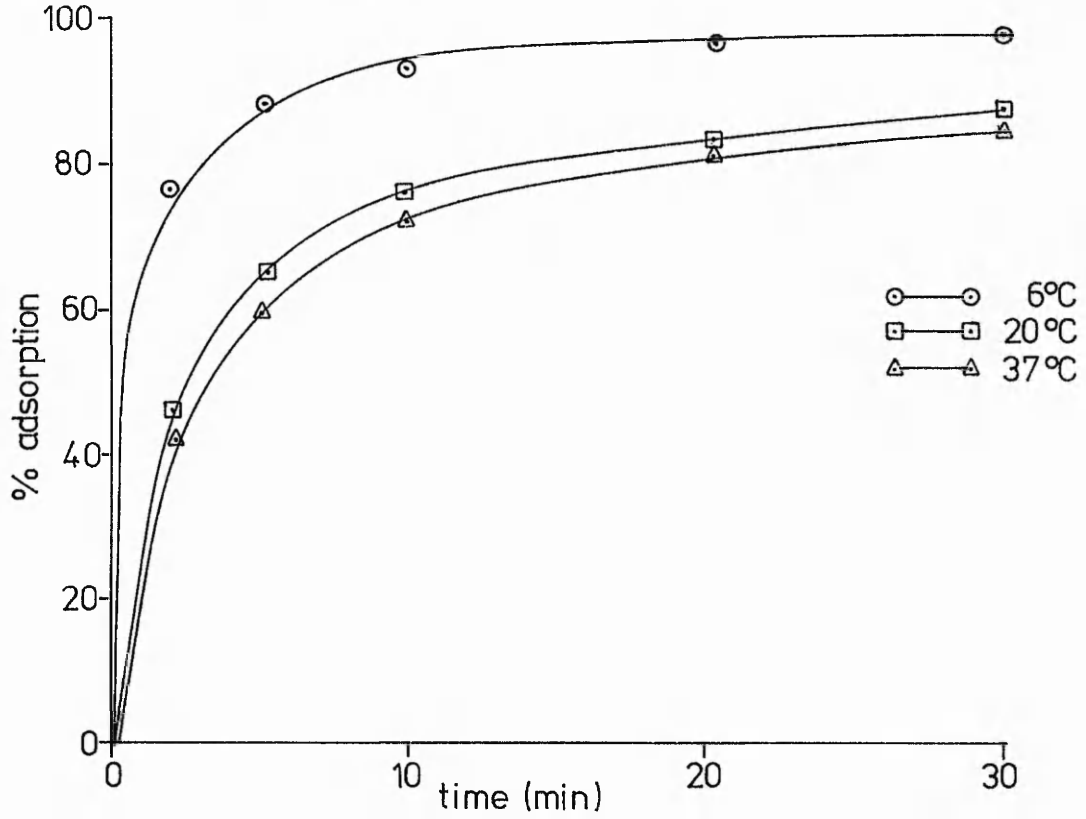
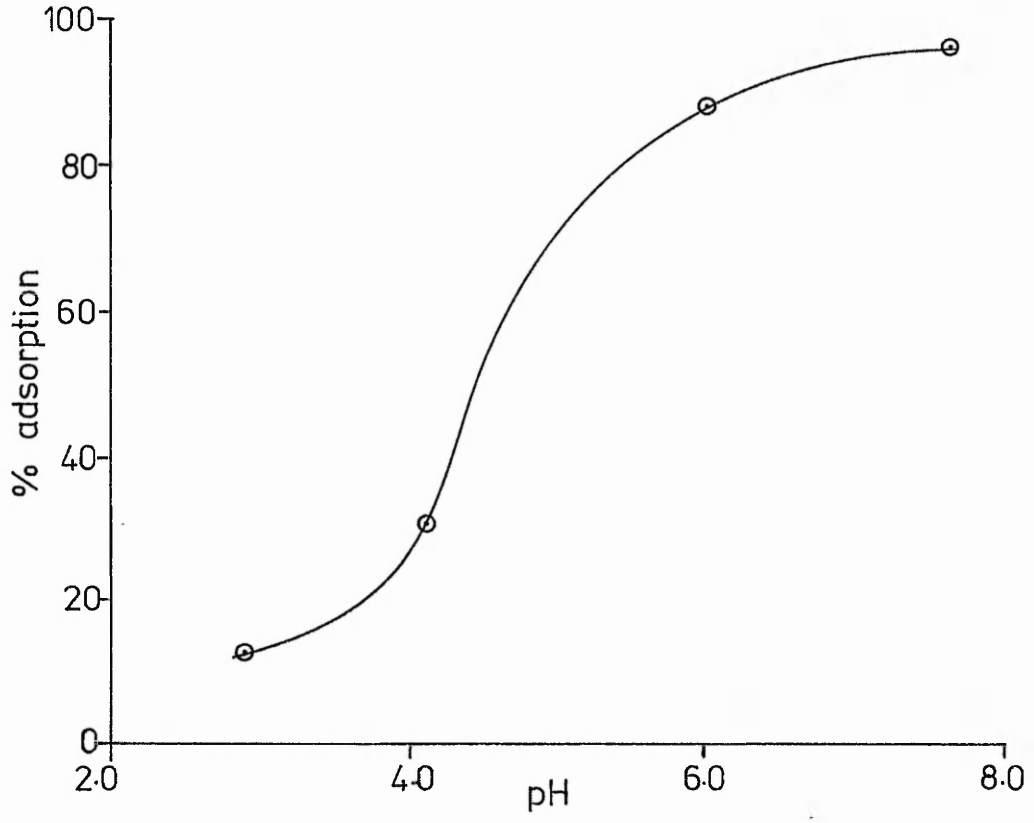


Figure 17. Effect of water pH on MS 2 adsorption to iron oxide.



a study of the effect of altering the oxide concentration was performed at one temperature ($20^{o}C$). This was done in order to determine the optimum oxide concentration for maximum virus recovery, and the concentration was varied from 10 to 6250 mg l^{-1}. The experiment was repeated in the presence of 2.0 mM magnesium chloride or 0.002 mM aluminium chloride which, as it will be explained, enhanced virus adsorption to, and removal from, iron oxide. MS 2 was again added and samples assayed after 30 minutes. As the results were taken from several different experiments, they were presented together in the form of Freudlich adsorption isotherms, as these reduced the different sets of data to common figures (Figure 18). They took the form:

$$x/w = kc^{1/n}$$

where: x = amount of coliphage added.

w = weight of iron oxide.

c = concentration of residual viruses.

k and n are constants, k deriving from the intercept of the isotherm and n from its slope.

Plotting the logarithm of the virus concentration adsorbed per mg of adsorbent against the logarithm of viruses remaining after 30 minutes, defined a straight line, showing the Freundlich isotherm to apply. Adsorption was therefore shown to be physical and monomolecular as multimolecular types would not conform to simple isotherms such as that of Freundlich.

Figure 18. Freundlich isotherms for MS 2 adsorption to iron oxide.

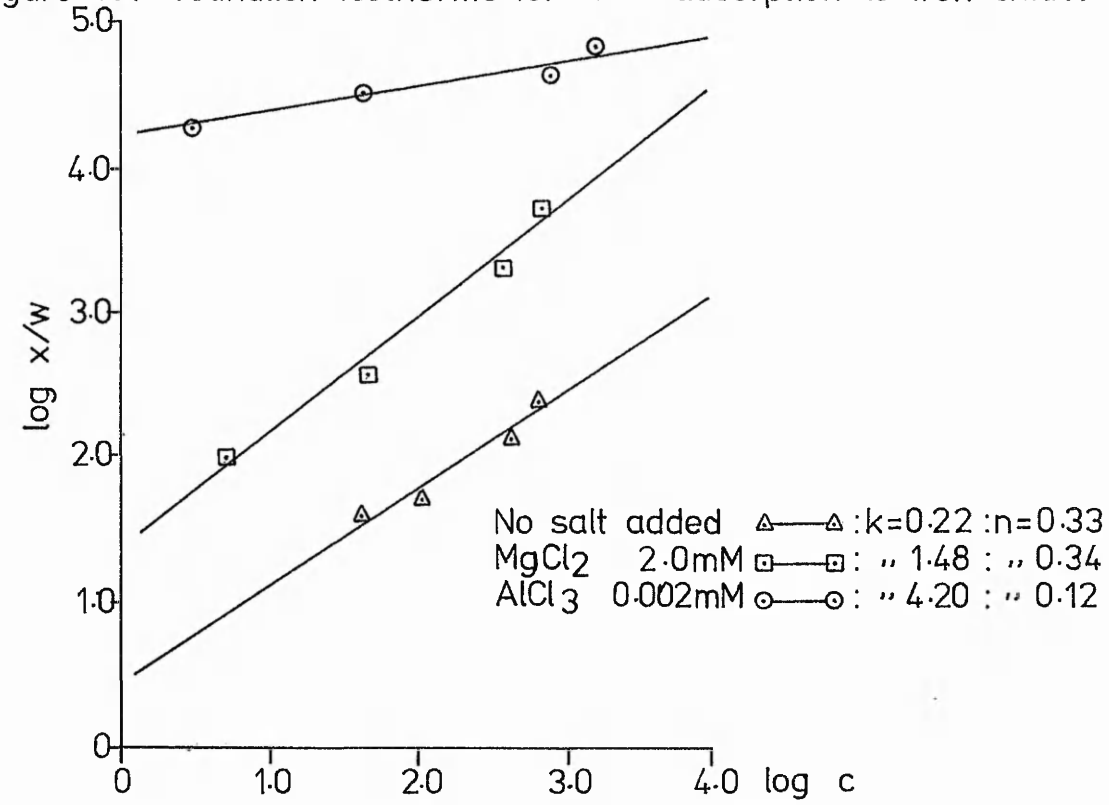
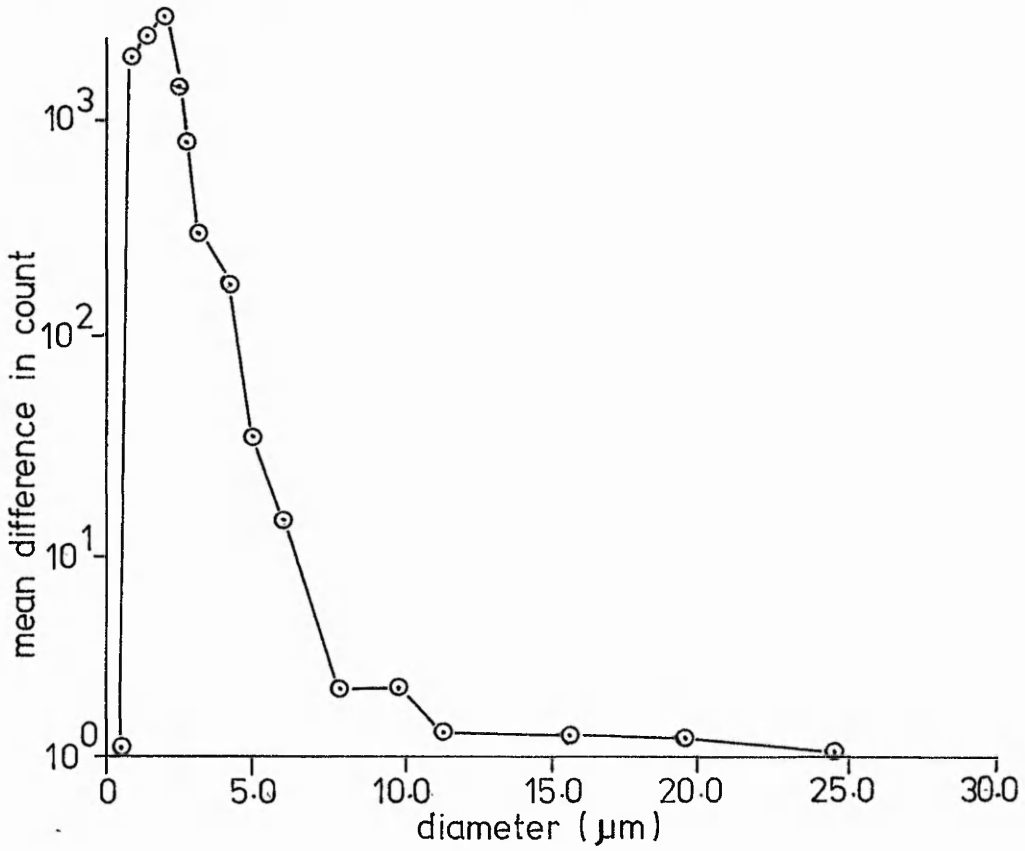


Figure 19. Particle size distribution of iron oxide.



The graph also indicated the amount of iron oxide required, for a given initial concentration of viruses, to achieve complete virus removal or to leave a known residual concentration. As will be explained later, the enhancing effect of metal cations was also demonstrated, greater removal for the same weight of oxide being achieved with $MgCl_2$ than without any salt addition. This effect was exaggerated even further with $AlCl_3$, very efficient removal of viruses being obtained for the same oxide concentration.

It had been determined by Warren, et al (1966) that Ballmilling of iron oxide for 24 hours would produce a particle of 0.5 to 1.0 µm diameter, and this was therefore examined using an Industrial Model D Coulter Counter, with a 50 µm diameter aperture. (Coulter Electronics Ltd.) Oxide was suspended in 'Isoton' at about 100 mg l^{-1}, counts taken and, from mean figures the distribution determined. Plotting the difference between each group of particles, as determined by Coulter counting, against particle diameter, produced the distribution indicated in Figure 19. From this it could be seen that the majority of the iron oxide particles had diameters between 1.0 and 8.0 µm, with a mean size of 2.25 µm.

Examination of iron oxide particles on electron micrographs, prepared using the dip method described, but substituting oxide for viruses or bacteria, produced a calculated mean particle size, for fifty particles, of 1.92 µm, in excellent agreement with the above recorded 2.25 µm. Both measurements however were in disagreement with the advised diameter of 0.5 to 1.0 µm,

but, as virus adsorption was unhindered by the difference in size, this was not thought to be a potential problem.

Efficient elution of bound viruses from iron oxide was considered essential and the literature suggested that this could be achieved with either a salt or protein solution. Consequently a selection of these were examined, using attenuated Poliovirus 1 and MS 2, with the results displayed in Table 15. Total percentage recovery was derived from:

$$\frac{\text{Number of plaque forming units ml}^{-1}\text{ observed}}{\text{Total number of plaque forming units ml}^{-1}\text{ expected}} \times 100$$

Similarly, percentage recovery from the number of viruses actually adsorbed was calculated from:

$$\frac{\text{Number of plaque forming units ml}^{-1}\text{ observed}}{\text{Total number of pfu ml}^{-1}\text{ - number of pfu ml}^{-1}\text{ not adsorbed}} \times 100$$

The extent of adsorption of both viruses was high, but in all cases recovery was very disappointing. Although sera were amongst the better of the eluents, they were considered to be too expensive for routine use, and lab-lemco was substituted for all further investigations.

In order to overcome the problem of poor elution the addition of metal cations to the virus adsorption system was attempted, the enhancing effect of metals having been shown to be true for all examined virus adsorptive systems as reviewed by Bitton (1975). A series of experiments were therefore conducted with $MgCl_2$, $CaCl_2$ and $AlCl_3$ at varying concentrations, using 500 mg l^{-1} iron oxide, 3.0 % lab-lemco, pH 8.0 and

Table 15. Range of iron oxide eluents tested and the recoveries achieved with each, in tap water.

Eluent	Virus	Titre (pfu/ml)	Adsorption %	% recovery A	B
1 % peptone, pH 8	MS 2	$7.2x10^3$	90.42	1.71	1.84
1 % tryptone, pH 8	MS 2	$7.2x10^3$	90.42	3.99	4.41
10% K_2HPO_4, pH 8	MS 2	$7.2x10^3$	90.42	0.74	0.81
10% Na_2HPO_4, pH 8	MS 2	$7.2x10^3$	90.42	0.32	0.35
10% Na_2HPO_4, pH 8	Poliovirus	$8.0x10^3$	100.00	0.0	0.0
Nutrient broth, pH 8	MS 2	$7.2x10^3$	90.42	1.94	2.15
7.5% $NaHCO_3$, pH 8	MS 2	$7.2x10^3$	90.42	0.56	0.61
3% Lab lemco, pH 8	MS 2	$7.2x10^3$	90.42	4.96	5.48
3% Lab lemco, pH 8	Poliovirus	$8.0x10^3$	100.00	5.87	5.87
Bovine serum	MS 2	$7.2x10^3$	90.42	0.86	0.97
Bovine serum	Poliovirus	$8.0x10^3$	100.00	8.50	8.50
Calf serum	MS 2	$7.2x10^3$	90.42	0.14	0.15

Table 15 - continued

Eluent	Virus	Titre (pfu/ml)	Adsorption %	% recovery A	% recovery B
Foetal calf serum	MS 2	7.2×10^3	90.42	2.44	2.70
0.05M. glycine, pH 11.5	MS 2	6.2×10^3	95.65	0.0	0.0
0.1M. EDTA	MS 2	6.2×10^3	95.65	10.16	10.61
Satd. Na_2PO_4	MS 2	2.5×10^3	40.91	4.45	10.87
Satd. $MgPO_4$	MS 2	2.5×10^3	78.46	0.20	0.25

A = % recovery from the initial number added.

B = % recovery from the number adsorbed.

coliphage MS 2. The experiments were duplicated in tap water and River Trent water collected from sampling site 5, at Colwick, to assess the effect of water quality on the adsorption process. Results, expressed in Table 16, indicated a ten to one hundred fold increase in virus adsorption for all three cations in tap water and greatly increased recoveries of MS 2 at higher concentrations of the divalent cations.

For trivalent Aluminium the reverse was found, with higher recoveries at the lower molarities, this echoing reports that trivalent cations were more efficient adsorbers, at the same concentration, than divalent cations. The other observation noted was a decreased efficiency of the system in river water for all three cations, this difference however being minimal with the more effective trivalent aluminium. Finally, these results supported those shown in Figure 18 which also clearly demonstrated the superior enhancing effect of $AlCl_3$ on virus adsorption to iron oxide.

Recovery of MS 2 was attempted using 3.0 % lab-lemco over a pH range of 4.0 to 10.0, and iron oxide at 500 mg l^{-1} without cationic enhancement, to determine whether pH had any effect on recovery. No significant differences between the observed results were noted, however, with recoveries ranging from 4.35 to 6.85 %. It would therefore seem that, although pH influenced adsorption, it did not have a great effect on elution. Rao, et al (1968), however, suggested that a pH of 8.0 to 9.0 was the best for virus elution and so lab-lemco was

Table 16. Enhancement of iron oxide virus recovery by metal ions

Salt concn. (mM)	Mg^{2+}				Ca^{2+}				Al^{3+}			
	Tap		River		Tap		River		Tap		River	
	A	B	A	B	A	B	A	B	A	B	A	B
20.0	99.6	24.1	100	38.3	98.6	28.8	98.2	15.1	100	4.8	100.0	4.0
2.0	96.8	25.5	97.7	22.3	99.6	23.8	97.8	15.5	100	5.7	100	6.24
0.2	96.2	22.7	94.4	4.4	98.8	13.6	92.5	3.0	100	5.0	99.5	5.9
0.02	99.1	17.2	93.6	4.0	97.6	6.4	90.2	0.7	98.1	20.2	97.8	15.4
0.002	74.2	5.3	68.0	1.3	78.2	2.4	67.5	0.3	67.4	21.8	68.6	26.8

A = % adsorption

B = % recovery

used at pH 9.0 for all adsorption/elution work.

The eluent concentration was also investigated to determine whether it had an influence on virus recovery, MS 2 being adsorbed from tap or river water in the absence of salts or with (a) $MgCl_2$ at 20 mM, (b) $CaCl_2$ at 20 mM, or (c) $AlCl_3$ at 0.02 mM. Elution was then attempted using lab-lemco pH 9.0, at 1.0, 2.0, 3.0, 5.0 and 10.0% (Table 17). The results obtained lead to the conclusion that a lab-lemco concentration of 1.0% was the most effective, within the range tested, for recovering the added viruses. Secondly, aluminium was again found to be the superior cation of those assessed, and was therefore used for most of the subsequent scaling up experiments.

Table 17. Effect of eluent concentration on virus recovery from iron oxide

Lab-lemco concn (%)	No cations		20 mM mg^{2+}		20 mM Ca^{2+}		0.02 mM Al^{3+}	
	% recovery		% recovery		% recovery		% recovery	
	Tap	River	Tap	River	Tap	River	Tap	River
1.0	23.7	2.9	20.9	19.8	36.6	15.1	84.3	73.4
2.0	1.2	1.4	39.9	14.3	39.8	15.0	48.1	49.5
3.0	6.3	1.3	31.5	10.6	32.3	14.5	34.5	40.0
5.0	3.3	1.5	16.3	12.0	18.4	11.8	27.5	29.4
10.0	14.5	2.0	12.3	7.9	14.9	8.0	30.7	21.7

The first of these larger scale experiments utilised 1.0 litre of tap water to which was added 200 mg l^{-1} iron oxide as a slurry, $AlCl_3$ to 0.02 mM and coliphage MS 2. It was found,

when increasing the water volume, that 500 mg l^{-1} iron oxide was greatly in excess, this amount having been calculated from small scale experiments where only 10 ml amounts of reaction mixture were involved. In these small scale experiments 500 mg l^{-1}, suspected of being in excess, was the amount delivered in one drop from a pasteur pipette. When multiplied one hundred times, for one litre experiments, the experimental error involved was likewise increased resulting in an excessive inoculumn of iron oxide slurry. Further, these conclusions were supported by the oxide concentration experiments, illustrated as isotherms (Figure 18), which showed that 200 mg l^{-1} was sufficient for maximum virus adsorption, especially with the addition of divalent and trivalent cations.

Following the 30 minutes adsorption period, oxide was deposited by centrifugation in four 250 ml MSE centrifuge bottles in a MSE 4/64 Mark II Magnum refrigerated centrifuge at 3000 rev min^{-1} (4000 g) for 15 minutes at ambient temperature. Then 5.0 ml of 1.0 % lab-lemco, pH 9.0 was added to each bottle, the contents resuspended for 30 minutes by intermittent hand shaking, centrifuged as before and the supernatants pooled. By this method, for three experiments, a mean of only 8.3 % of the input viruses were recovered and so oxide filtration was attempted as an alternative collection method.

To 10 litre aliquots of tap water was added MS 2 at 10^3 ml^{-1}, 200 mg l^{-1} iron oxide and final metal chloride concentrations of 20 mM $MgCl_2$, 20 mM $CaCl_2$ or 0.02 mM $AlCl_3$.

These were mixed and filtered at approximately 2.0 litres per minute, using an Edwards vacuum pump, through a Whatman GF/A glass fibre filter paper, each precoated with 1.5 g keisulguhr as filter aid, in a 15 cm Sartorius membrane holder. Filter papers were then treated by one of two methods. Initially the oxide and keisulguhr were scraped by an alcohol sterilised spatula into 10 ml of 1.0 % lab-lemco, rotary mixed for 30 minutes and then centrifuged at 3000 rev min^{-1} (800 g) for 15 minutes. This proved to be inefficient because of the amount of oxide remaining in the filter paper and so, for the second series of filtration experiments, the whole filter paper plus oxide and filter aid were placed in 100 ml plastic syringes and macerated with 50 ml of 1.0 % lab-lemco, pH 9.0, using alcohol sterilised spatulas. After 30 minutes the pulped contents of the syringes were expressed into Universal bottles, and centrifuged at 3000 rev min^{-1} (800 g) for 15 minutes. In both types of operation pooled supernatants were mixed with a few drops of chloroform to kill contaminating bacteria, and stood for one hour at 5.0°C, after which time the chloroform was removed by sterile pasteur pipette. The results of these experiments, summarised in Table 18, showed little difference between the two methods and, for Ca^{2+} and Mg^{2+}, good agreement for recoveries, with previous small scale experiments.

Table 18. Comparison of GF/A scraping and maceration for MS 2 Recovery from 10 litres of tap water plus iron oxide.

Cation	% recovery after scraping	% recovery after maceration
20 mM Mg^{2+}	25.15	16.80
20 mM Ca^{2+}	37.25	36.16
0.02 mM Al^{3+}	8.30	11.85

Aluminium chloride, however, gave very low recoveries, when compared with, for example, those in Table 17, where 1.0 % lab-lemco recovered 84.3 % of those viruses seeded into 10 ml tap water. Using the oxide scraping method 49.2 % of input Poliovirus 1 was recovered, an improvement over the results obtained for MS 2.

Therefore, at this stage it was obvious that the scaling up of virus concentration by iron oxide was presenting problems of virus/iron oxide collection and virus recovery, and so electromagnetic removal of oxide was attempted, as an alternative to those mentioned above.

As described under methods (see Figure 11b) the magnet was supplied with facilities for removal of oxide by the use of a plastic spiral wrapped around the inside of the pole pieces. Experiments were undertaken using 1.0 litre volumes of tap water, each containing 0.02 mM $AlCl_3$, 200 mg iron oxide and Poliovirus 1 or MS 2. Each volume, after 30 minutes mixing, was passed under negative pressure through the

collecting coil at 50 ml min^{-1} using an Edwards vacuum pump and the collected oxide eluted with 20 ml of 1.0 % lab-lemco, pH 9.0 as described. Results, in Table 19, showed good adsorption and recovery percentages using attenuated Poliovirus 1, although recoveries of the phage were considered rather low.

Table 19. Virus recoveries using an Electromagnet spiral collector

Virus	% adsorption	% recovery of imput	% recovery from number adsorbed
Poliovirus	90.76	61.16	63.95
MS 2	87.37	17.29	17.33

Unfortunately when a ten fold increase in water volume to be processed was attempted it became necessary to increase both the oxide concentration and the flow rate to permit a reasonable passage time per sample. At 100 ml min^{-1} Poliovirus recovery dropped to 22.7 %, and that for MS 2 to 3.6 %, with large oxide carryover into the effluent stream in both cases. The effluent from one coliphage experiment was collected and filtered through a GF/A filter paper, with maceration, as previously described. This produced 96.3 % of the estimated MS 2 input, indicating that the lost viruses were being washed from the spiral by the increase in flow rate which affected the oxide concentration within the collector.

As an alternative to reducing the flow rate or increasing the pipe bore, which was not considered readily feasible, the use of a magnetic depth filter was investigated.

This initially consisted of a 100 g plastic chemical bottle, measuring 90 x 61 mm which was filled to capacity with standard quality steel wool. A rubber bung with inlet tube running to the bottom of the bottle and a shorter outlet tube, was forced into the top of the container and the whole suspended with a clamp in the pole pieces of the magnet. This arrangement with modifications, such as moving the inlet pipe to the bottom of the bottle, was used for preliminary investigations, in magnetic filtration. With 10 litre water volumes, flow rates of 0.6 to 1.03 l min^{-1} were easily achieved and oxide was usually collected within the bottom 50 % of the filter depth. No virus studies were performed with these early models because, despite their efficiency, they had a tendency to leak, and this was not considered conducive to safe virus handling.

A watertight filter container was constructed from a 76 mm diameter 'Perspex' cylinder, 90 mm long, sealed at the bottom by a disc of 'Perspex' into which was let an inlet tube. The top, containing the outlet tube was bolted to a 'Perspex' flange glued to the cylinder, and sealed with a QVF 50 mm rubber seal and petroleum jelly. This construction permitted flow rates of 1.1 to 1.3 l min^{-1} and mean virus recoveries of 56.4 % were obtained with MS 2.

There was, however, a problem in virus elution using this construction, it being impossible either to remove all the steel wool plus oxide to a suitable elution container, or to elute within the pot with a sufficiently small volume of lab-lemco. Therefore the final filter container was developed, the 100 ml plastic syringe described under methods. At the same time the amount of added steel wool was reduced from approximately 8.0 g to 3.5 g, this mass being teased out to fill the whole syringe volume. This was done primarily because it was found that the wool retained the last 20 to 30 ml of water sample, altering the eluent concentration when this was added. No attempt was made to remove either the wool or the oxide after collection, but eluent was added and the whole syringe rotary mixed as described.

Water samples were no longer prefiltered and experiments were performed to detect any removal of naturally occuring suspended solids by magnetic filtration (Table 20).

Table 20. Removal of river water constituents by magnetic filtration

Water type	filter rate $l\ min^{-1}$	susp. solids $mg\ l^{-1}$	Turbidity FTU
Tap water.	-	0.0	0.0
River water (R.Trent: Colwick)	-	20.0	16.0
River water after magnetic filtration	1.44	11.1	11.0
River water plus 200 mg l^{-1} and 0.002 mM $AlCl_3$ after magnetic filtration	1.44	10.4	5.5

Turbidity measurements were made on a Hach 2100A turbidometer in Formazin Turbidity Units.

These experiments clearly showed a reduction in both turbidity and suspended solids content as a result of magnetic filtration. The addition of $AlCl_3$ and iron oxide, whilst reducing suspended solids by a minimal amount, caused a further 50 % loss of turbidity, presumably as a result of small particle adsorption.

The removal of seeded MS 2 from tap water was attempted by this final experimental configuration, with recoveries ranging from 13.02 to 50 %. The method of elution was suspected as the cause of these low, variable results, and so a series was devised in which the syringe plunger was set at either the 100 ml or the 50 ml mark for elution, and the elution time was varied from 30, through 45 to 60 minutes in order to see whether this increase in time improved recoveries. For the 100 ml set syringe volume, the eluent volume was also varied at 20, 40 and 60 ml, the 40 and 60 ml additions not being possible with the smaller, 50 ml set syringes. The mean results for MS 2, shown in Table 21, indicated that 60 minutes was preferable to the other elution times for this technique, contrary to the quite adequate 30 minutes of small volume experiments. It was also shown that the reduction in syringe volume during elution increased the intimacy of oxide and virus to eluent, leading to a higher recovery.

Table 21. Recovery of MS 2 from tap water by magnetic filtration, effect of eluent volume and elution time

Syringe setting	eluent vol. added (ml)	eluent vol. actual (ml)	% total recovery and elution time (min)		
			30 min	45 min	60 min
50 ml	20	27.5	4.33	44.82	87.46
100 ml	20	31.0	5.38	-	66.0
	40	51.3	17.16	31.35	56.4
	60	69.0	37.23	-	57.65

Finally the results also showed that a fairly constant 10 ml of water was still retained by the magnetic filter, despite the reduction in steel wool mass, and the improved filter unit design.

The 'short barrel', 50 ml syringe setting experiment was repeated for attenuated Poliovirus 1 with mean recoveries of 84.86 % from tap water and 72.84 % from River Trent water, from the Colwick site, after 60 minutes elution time.

Thus it could be seen that, with the addition of metal cations, and in particular aluminium chloride at a final concentration of 0.002 mM, iron oxide at 200 mg l^{-1} proved a very effective adsorber of the viruses MS 2 and Poliovirus 1, especially at neutral pH's. Elution was found to be most effective using 1.0 % lab-lemco at pH 9.0 with 30 minutes mixing when using small volumes, but 60 minutes when using the magnetic filter. Magnetic filtration was shown to be superior to glass fibres paper filtration, which proved to be unsafe in practice, with a risk of operator contamination because of the manipulations involved, and to consistently recover less of the imput virus titre.

The final mean recovery figures for Poliovirus 1 of 84.86 % from tap water and 72.84 % from river water compared very favourably with those of alternative concentration techniques cited in the literature.

Reverse Osmosis

While RO has been shown capable of separating most waste water constituents, whether colloidal or dissolved, organic, inorganic or biological, one aspect of this removal requiring further investigation was the extent of its exclusion of water-borne viruses. The investigation was initiated with the study of the virus population of the River Trent at Colwick, the water of which was used as feed to a 'B type' tubular rod RO unit. During the eleven months from September 1973 to July 1974 nine monthly samples were collected, concentration being by aluminium hydroxide flocculation initially, and subsequently iron oxide adsorption. Samples taken in 1973 were assayed by $TCID_{50}$, producing the results in Table 22, which intimated the presence of viruses in raw and concentrate water, but their absence in permeate water. However, due to the difficulty of interpreting results and the cytotoxicity evident during assay of concentrates, those samples taken during 1974 were assayed by the plaque technique. No viruses were, however, demonstrated in any of these samples, taken over a Winter and Spring period. Further to this, artificial loadings of River Trent water from Colwick with Poliovirus 1 had shown a 97.2 % loss of infectivity within 24 hours, suggesting that viral numbers would be low or unassayable some distance below a sewage outfall. Equally, Winter and Spring waters were known to contain few viruses, and so, in either case, the low titres realised illustrated an inability to analyse naturally occurring viruses in order to determine RO plant operational efficiency.

Table 22. Detection of river-borne viruses during B type RO operation

Date	Sample	Isolation	Passage number 1	2	3	4	5
25/09/73	Raw	5/5	5/5	2/5	0/5	0/5	0/5
	Concentrate	5/5	3/5	1/5	0/5	0/5	0/5
	Permeate	4/5	1/5	0/5	0/5	0/5	0/5
30/10/73	Raw	4/5	1/5	0/5	0/5	0/5	0/5
	Concentrate	2/5	2/5	0/5	0/5	0/5	0/5
	Permeate	2/5	0/5	0/5	0/5	0/5	0/5
20/11/73		Not taken due to pump failure					
13/13/73	Raw	4/5	2/5	0/5	0/5	0/5	0/5
	Concentrate	5/5	3/5	1/5	0/5	0/5	0/5
	Permeate	2/5	0/5	0/5	0/5	0/5	0/5
31/01/74	Raw	0/5 on Vero plaque assay					
	Concentrate	0/5 "	"	"	"		
	Permeate	0/5 "	"	"	"		
28/02/74	Raw	0/5 "	"	"	"		
	Concentrate	0/5 "	"	"	"		
	Permeate	0/5 "	"	"	"		
12/03/74	Raw	0/5 "	"	"	"		
	Concentrate	0/5 "	"	"	"		
	Permeate	0/5 "	"	"	"		
24/04/74	Raw	0/5 "	"	"	"		
	Concentrate	0/5 "	"	"	"		
	Permeate	0/5 "	"	"	"		
16/05/74	Raw	0/5 "	"	"	"		
	Concentrate	0/5 "	"	"	"		
	Permeate	0/5 "	"	"	"		
26/06/74	Raw	0/5 "	"	"	"		
	Concentrate	0/5 "	"	"	"		
	Permeate	0/5 "	"	"	"		
30/07/74		Not taken due to membrane failture: after this time the unit was permanently shut down					

This therefore necessitated the loading of the units with an artificially high titre of viruses and on two occasions these consisted of 1.0 ml 'slugs' of attenuated Poliovirus at 8.0×10^{6} ml^{-1}, inoculated as described in methods. Twenty ml samples of concentrate were then taken from the reject water line at 1.0 minute intervals from 4 to 25 minutes after inoculation. They were assayed by TCID 50 and the results (Table 23) suggested the same cyclic pattern of recovery as demonstrated for coliphage MS 2 by Bennett, 1973 using the same equipment. The previous studies had shown that the passage time through the B type unit could be calculated as 4.5 minutes ignoring permeated water loss, or approximately 5 to 5.5 minutes including permeate flow. Therefore a 'peak' of virus recovery in the concentrate stream could be expected every 5 to 5.5 minutes, diminishing with each cycle if no more viruses were added. Three MS 2 peaks had been noted at 5.5, 11.0 and 16.5 minutes, and reference to Table 23 indicated similar results at 5.0 to 6.0, 14.0 to 15.0 and 18.0 to 20.0 minute intervals showing that the plant was operating in a similar manner. It proved difficult, however, to calculate further information from these results or to draw graphs, owing to the previously mentioned inadequacies of the assay technique.

From June 1974 to March 1975 artificial seedings of attenuated Poliovirus 1 and MS 2 were applied to the replacement R3 spagetti rod RO unit at Colwick. From these, graphs of viruses detected in concentrated water were plotted.

Table 23. Loading of B type RO unit with Poliovirus 1

Run 1: 11/12/73				Run 2: 12/03/74			
Time (min)	Passage number			Time (min)	Passage number		
	1	2	3		1	2	3
0	4/5	2/5	2/5	0	4/5	0/5	0/5
4	4/5	0/5	0/5	4	4/5	0/5	0/5
5	5/5	5/5	0/5	5	5/5	5/5	0/5
6	4/5	5/5	1/5	6	4/5	5/5	1/5
7	5/5	5/5	0/5	7	5/5	5/5	0/5
8	5/5	5/5	0/5	8	5/5	5/5	0/5
9	2/5	0/5	0/5	9	2/5	0/5	0/5
10	2/5	0/5	0/5	10	2/5	0/5	0/5
11	2/5	0/5	0/5	11	2/5	0/5	0/5
12	1/5	0/5	0/5	12	1/5	0/5	0/5
13	2/5	0/5	0/5	13	2/5	0/5	0/5
14	0/5	0/5	0/5	14	0/5	1/5	0/5
15	4/5	5/5	1/5	15	4/5	5/5	1/5
16	4/5	5/5	1/5	16	4/5	5/5	0/5
17	2/5	5/5	0/5	17	0/5	5/5	0/5
18	1/5	5/5	1/5	18	1/5	5/5	0/5
19	2/5	5/5	1/5	19	2/5	5/5	1/5
20	3/5	3/5	1/5	20	3/5	3/5	1/5
21	4/5	0/5	0/5	21	4/5	0/5	0/5
22	3/5	0/5	0/5	22	3/5	0/5	0/5
23	5/5	0/5	0/5	23	5/5	0/5	0/5
24	3/5	0/5	0/5	24	3/5	0/5	0/5
25	4/5	0/5	0/5	25	4/5	0/5	0/5
Permeate	5/5	0/5	0/5	Permeate	5/5	0/5	0/5
Permeate flow rate: 2.57 $l\ min^{-1}$ from one module				Permeate flow rate: 2.36 $l\ min^{-1}$ from one module			

As with the 'B' type unit assays permeate water was collected over the duration of the experiment and, after iron oxide concentration, assayed for virus content. In no cases were viruses detected in concentrated permeate samples. Experimental data was summarised in Table 24, and results of concentrate water assays plotted in graphic form (Figure 20). These typically showed the diminishing cyclic peaks of virus numbers as demonstrated with the B type unit, but at much shorter intervals of 1.5 to 2.0 minutes, reflecting the faster recirculating rate of the R type unit.

Computer enhancement of the results by Legrange Interpolation using an ICL 1905 computer was followed by calculation of the percentage losses at each cycle (Table 25: see Appendix 2 for Legrange programme and Appendix 3 for calculations).

Table 24. Summary of data from R type RO unit (Colwick)

No.	Virus	Titre (pfu ml^{-1})	pH				Flow (l min^{-1})			
			River	Inlet	Perm. ($\bar{x}$)	Conc.	Inlet	Perm. ($\bar{x}$)	Conc.	Recycle
1	MS 2	1.1×10^7	7.43	7.85	6.18	7.85	6.60	0.33	2.04	30.0
2	MS 2	1.1×10^7	7.70	8.02	6.40	8.00	4.44	0.21	1.59	30.0
3	MS 2	1.1×10^7	7.45	6.27	5.36	6.27	6.30	0.27	1.72	12.0
4	MS 2	1.0×10^8	7.17	2.80	3.59	2.80	5.58	0.25	1.71	30.0
5	MS 2	1.0×10^8	7.25	3.20	4.31	3.20	4.92	0.28	1.84	30.0
6	MS 2	1.0×10^8	7.57	5.65	5.42	5.65	4.98	0.22	1.79	30.0
7	MS 2	3.0×10^7	7.70	5.15	5.26	5.30	4.20	0.23	0.59	30.0
8	Polio	1.6×10^7	7.80	5.41	5.20	5.00	4.44	0.24	1.03	30.0
9	Polio	1.6×10^7	7.90	5.10	4.96	4.85	4.44	0.20	1.44	30.0
10	Polio	1.6×10^7	7.90	5.10	4.96	4.20	4.08	0.20	1.44	28.5
11	MS 2	3.0×10^7	7.80	5.58	5.45	5.35	3.60	0.18	0.93	27.6
12	MS 2	1.1×10^9	7.85	5.55	5.40	5.10	3.60	0.18	0.88	30.0

Table 24 - continued

No.	Virus	Titre (pfu ml^{-1})	pH				Flow (l min^{-1})			
			River	Inlet	Perm. ($\bar{x}$)	Conc.	Inlet	Perm. ($\bar{x}$)	Conc.	Recycle
13	MS 2	1.1×10^9	7.80	4.75	5.15	5.20	4.50	0.18	1.60	28.2
14	MS 2	1.1×10^9	8.00	4.75	5.15	7.40	4.32	0.18	1.60	27.0
15	MS 2	1.1×10^9	7.97	5.55	5.30	7.31	4.02	0.18	1.16	27.0
16	MS 2	1.1×10^9	7.97	5.55	5.30	5.30	4.20	0.20	1.80	27.6
17	MS 2	1.1×10^9	8.00	5.55	5.40	5.20	4.08	0.19	1.02	27.6
18	MS 2	3.1×10^9	8.00	5.55	5.40	7.40	4.44	0.19	1.01	28.5
19	Polio	1.6×10^7	8.00	5.55	5.40	7.45	4.44	0.19	1.01	28.5
20	Polio	1.6×10^7	7.95	5.30	5.30	5.35	3.69	0.18	0.95	27.0

Perm. = permeated water

Conc. = concentrate water

Table 24 - continued

No.	Conductivity (umhos $^{-1}$ cm^{-1})				Temperature (^{o}C)				Turbidity (JTU)				A	B
	River	Inlet	Perm. ($\bar{x}$)	Conc.	River	Inlet	Perm. ($\bar{x}$)	Conc.	River	Inlet	Perm. ($\bar{x}$)	Conc.		
1	880	2230	70.5	2460	-	28.4	28.2	28.2	2.6	-	0.10	4.5	+	+
2	1030	2350	87.0	2470	-	28.5	27.6	27.2	2.3	-	0.08	3.5	+	+
3	970	2630	80.7	2890	-	26.1	25.5	25.8	2.4	-	0.09	8.3	+	-
4	775	2450	232	2676	-	29.0	29.0	29.0	2.8	-	0.18	18.0	+	-
5	680	1650	170	1760	-	23.6	22.8	23.4	5.5	-	0.24	25.0	+	-
6	820	1690	234	1800	-	24.0	23.6	24.0	2.5	-	0.24	5.2	+	+
7	625	2650	102	2970	6.5	20.0	19.5	18.3	43.0	45.0	0.09	53.0	+	-
8	785	2170	99.0	2450	8.3	19.5	19.1	19.3	8.5	13.0	0.19	32.0	+	-
9	810	2050	119	2170	10.0	22.8	21.7	22.3	8.2	9.8	0.15	11.0	+	+
10	810	2050	119	2170	10.0	22.8	21.7	22.3	8.2	9.8	0.15	11.0	-	-
11	635	1790	478	1920	7.2	22.4	21.5	22.0	33.0	43.0	0.26	44.0	+	-
12	670	2130	190	2340	8.2	22.7	21.5	21.9	12.0	15.0	0.33	17.0	+	+

Table 24 - continued

No.	Conductivity (umhos $^{-1}$ cm^{-1})				Temperature (^{o}C)				Turbidity (JTU)				A	B
	River	Inlet	Perm.	Conc.	River	Inlet	Perm.	Conc.	River	Inlet	Perm.	Conc.		
13	715	1780	133	1850	8.5	20.0	19.0	19.5	13.0	11.0	0.35	12.0	+	+
14	715	1780	133	1850	8.5	-	19.0	18.5	13.0	11.0	0.35	12.0	+	+
15	740	2100	163	2230	8.5	22.3	21.5	22.0	9.8	14.0	0.20	14.0	+	+
16	740	2100	163	2230	8.5	21.0	20.1	20.8	9.8	14.0	0.20	14.0	-	-
17	680	1360	122	1470	9.1	23.0	22.2	22.5	9.2	9.8	0.18	10.5	-	+
18	680	1360	122	1470	-	-	-	-	9.2	9.8	0.18	10.5	+	+
19	680	1360	122	1470	-	-	-	-	9.2	9.8	0.18	10.5	+	+
20	665	2000	175	2120	10.4	25.1	24.2	24.5	13.0	16.0	0.23	17.0	+	-

A = presence or absence of three cartridge prefilters.

B = Positive or negative virus assay result.

Data collected on site, or supplied by Dr. J.D. Melbourne, and staff Water Research Centre, Medmenham.

Figure 20. Typical graph of virus rejection in RO concentrate water.

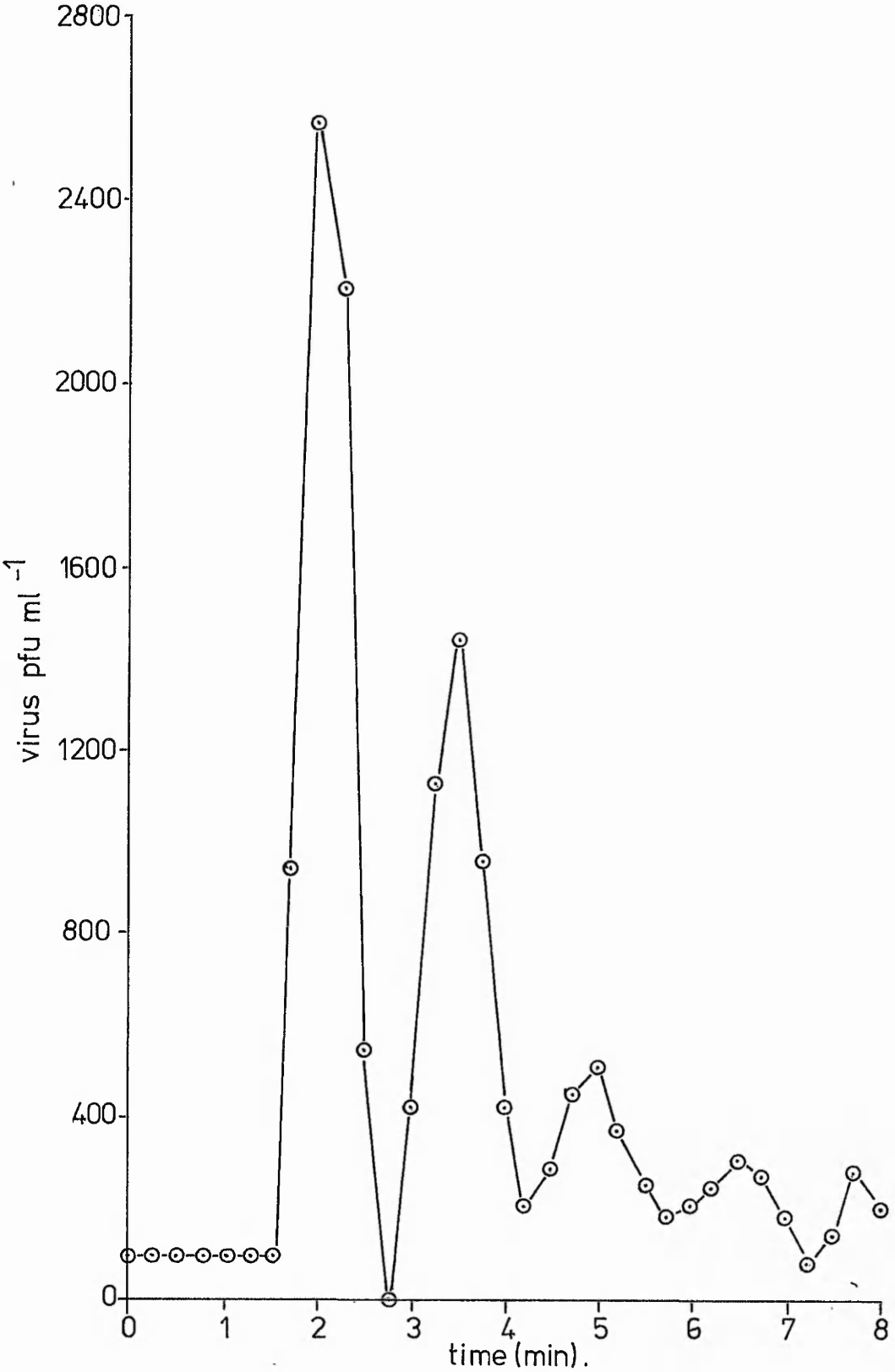


Table 25. Percentage losses of viruses from R type RO unit (Colwick)

Run No.	Virus	Flux ($l\ day^{-1}\ cm^{-2}$)	Initial titre ($pfu\ ml^{-1}$)	Cycle 1		Cycle 2	
				% loss	load for 2 ($pfu\ ml^{-1}$)	% loss	load for 3 ($pfu\ ml^{-1}$)
1	MS 2	7.08	1.1×10^7	57.48	3.59×10^6	6.2	2.93×10^6
2	MS 2	4.51	1.1×10^7	12.7	7.94×10^6	32.76	4.42×10^6
6	MS 2	4.72	1.0×10^8	81.61	1.58×10^7	49.5	6.46×10^6
9	Polio	4.02	1.6×10^7	4.9	1.26×10^7	99.87	-
12	MS 2	3.86	1.08×10^9	99.88	1.1×10^6	85.96	-
13	MS 2	3.86	1.08×10^9	99.99	5.35×10^5	72.15	-
14	MS 2	3.86	1.08×10^9	95.87	3.63×10^7	16.65	2.46×10^7
15	MS 2	3.86	1.08×10^9	99.5	4.46×10^6	88.01	4.46×10^5
17	MS 2	4.07	1.08×10^9	99.9	2.4×10^5	40.4	-
18	MS 2	4.07	3.1×10^9	99.33	1.75×10^7	62.1	5.55×10^6
19	Polio	4.07	1.6×10^7	95.03	6.66×10^5	77.85	-

- indicates below limit of assay

Table 25 – continued

Run No.	Cycle 3		Cycle 4		Rejection ratio %	Concentrate pH
	% loss	load for 4 (pfu ml^{-1})	% loss	load for 5 (pfu ml^{-1})		
1	24.66	2.8×10^6	22.44	2.6×10^6	100	7.85
2	49.97	1.83×10^6	96.5	–	100	8.00
6	19.3	4.22×10^6	98.5	–	100	5.65
9	–	–	–	–	100	4.85
12	–	–	–	–	100	5.10
13	–	–	–	–	100	5.20
14	42.34	1.15×10^7	26.89	6.84×10^6	100	7.40
15	17.92	3.05×10^5	53.84	–	100	7.30
17	–	–	–	–	100	5.20
18	46.83	2.46×10^6	50.82	1.01×10^6	100	7.40
19	–	–	–	–	100	7.10

– indicates below limit of assay

Table 26. Cumulative theoretical virus losses and observed losses within each cycle of an R type RO unit.

Run no.	Cycle 1		Cycle 2		Cycle 3		Cycle 4	
	Calculated	Observed	Calculated	Observed	Calculated	Observed	Calculated	Observed
1	78.57	57.48	95.42	73.36	99.02	74.54	99.79	76.36
2	75.67	12.70	94.09	59.82	98.56	83.36	99.65	-
6	77.13	81.61	94.76	93.54	98.81	95.78	99.73	-
9	77.25	4.90	94.82	-	98.82	-	99.73	-
12	75.70	99.88	94.09	-	98.56	-	99.65	-
13	64.48	99.99	87.41	-	95.51	-	98.41	-
14	62.94	95.87	86.29	97.72	94.92	98.93	98.93	99.37
15	72.50	99.50	92.43	99.96	97.92	99.97	99.43	-
17	75.67	99.90	94.08	-	98.56	-	99.65	-
18	77.26	99.33	94.84	99.82	98.82	99.92	99.73	99.97
19	77.25	95.03	94.82	-	98.82	-	99.73	-

Cumulative losses, based on dilution and exclusion, were calculated (Table 26 and Appendix 3) and, on the basis of the figures in Table 24 it was observed that the results could be divided into two groups.

The first of these groups consisted of those results whose observed percentage removal was lower than, or approximated to, the calculated values based on dilution alone (numbers 1, 2, 6 and 9). The second group being those with a much larger initial loss (12, 13, 14, 15, 17, 18 and 19). The only immediately obvious difference between these two groups was the initial inoculation titre, being 10^7 to 10^8 ml^{-1} in all the former cases and 10^9 ml^{-1} in the latter. This suggested that, at the higher titre, viral aggregation had occured prior to inoculation, this phenomenon being frequently encountered during laboratory experiments. Under laboratory conditions this could be overcome by dilution of the virus suspension 24 hours prior to use, an operation which deaggregated virus clumps. However, for effective virus assay after seeding of an RO unit, a high inoculum titre was required, which of necessity excluded the possibility of using a dilution deaggregation technique.

Thus the actual number of viruses could have been larger than the observed number, which would result in a higher apparent loss of titre during the first cycle than had actually occurred. The dilution by influent water, and the turbulent flow within the machine, necessary to overcome concentration gradients at the membrane, would subsequently deaggregated the remaining viruses producing recorded losses closer to the calculated

values on analysis of subsequent cycles.

However, for those runs in the second group for which results beyond the first cycle were obtained, it could be seen that the recorded cumulative loss of viruses was about ten times greater than the calculated losses. Thus some influence other than inoculum dilution was considered to be causing the apparent loss.

Additionally, detailed examination of the physical data in Table 24 showed that for runs 1, 2 and 6, the river turbidity readings and consequently the readings for the RO concentrates and permeates were relatively low. These ranged from 2.3 to 2.6 JTU's for the river, 0.08 to 0.24 for permeate and 3.5 to 5.2 for concentrate. Comparative ranges for runs 12, 13, 14, 15, 17, 18, and 19 were 9.2 to 13.0 for the river, 0.18 to 0.35 for permeate and 10.5 to 17.0 for the concentrate streams. All the latter runs, together with run 9, which showed intermediate turbidity figures, were performed during February and March 1975, whilst the former were taken in June and October 1974. This suggested that as well as aggregation, adsorption of viruses to the increased levels of suspended solids associated with faster Winter river flows had occured. However, as viruses adsorbed to suspended solids normally retain their infectivity, (Moore, et al, 1975), and in this case no infectivity was demonstrable it was reasoned that other mechanisms were involved in the loss of virus titre.

A second noted division within Table 25 was found between those runs producing one concentrate graph peak only, and those producing multiple peaks. Further comparison of viral and physical data showed that one peak runs occured during correct acid dosing, when a pH of 5.0 to 5.5 was achieved. Multiple peak runs, with one exception, occured when the unit was not correctly dosed with acid and thus operating close to the raw water pH of 7.5 to 8.0. The unit was acid dosed with sulphuric acid using a simple metering pump and frequent pH fluctuations were recorded during operation. Indeed, a number of loading experiments conducted towards the end of 1974 yielded no results at all, this coinciding with recorded pH levels of as low as 2.8. It therefore seemed that pH played an important role in virus removal during RO operation, being effective at operational pH's of 5.0 to 5.5.

A series of laboratory experiments was therefore undertaken to examine the pH stability of MS 2 using phosphate-citrate buffer over a pH range of 2.2 to 8.5. It was clear from Figure 21 that MS 2 did not survive well at low pH's, but remained stable in the range of pH 5.0 to 8.5. There was also increased inactivation with time, this not being signficant at neutrality but very marked at acid pH's.

A similar experiment was performed using RO water from Colwick, taken at pH 7.4 and acidified with concentrated sulphuric acid to pH's 2.5, 5.6, 6.3 and 7.4 (Figure 22). Increased loss of titre was noted at all pH's, although this was exaggerated at those values closer to neutrality.

Figure 21. pH stability range of MS 2 in phosphate-citrate buffer.

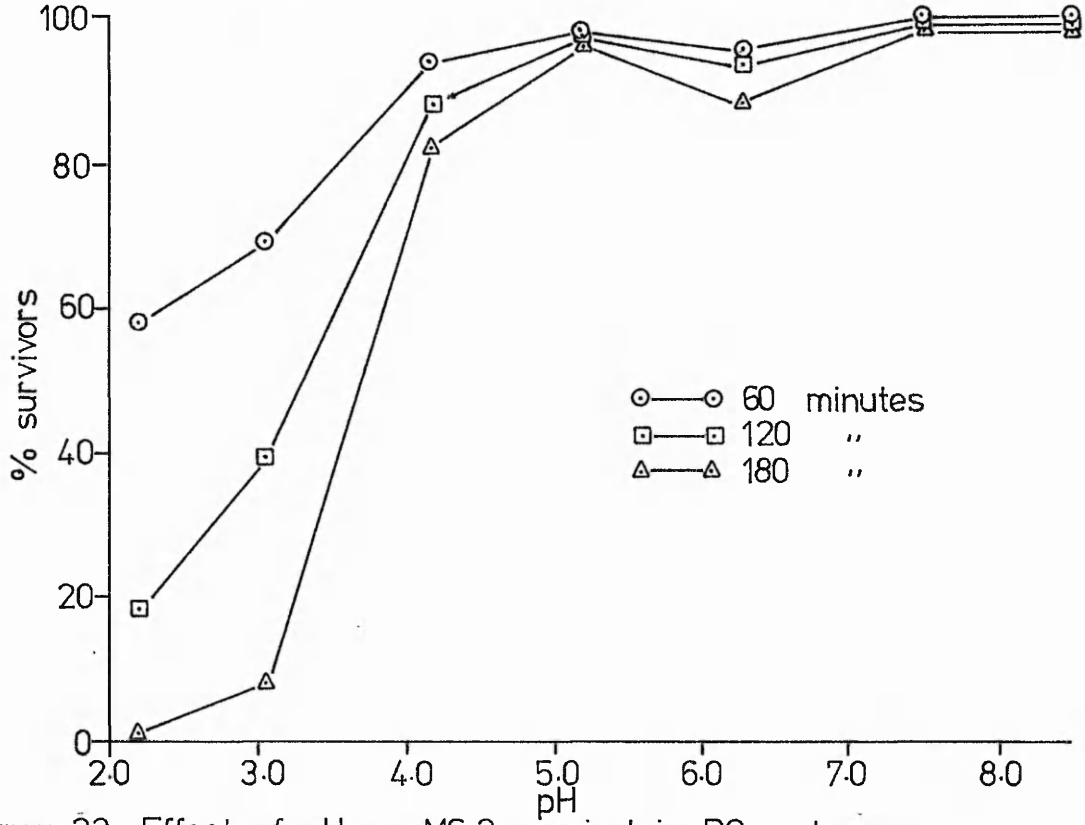
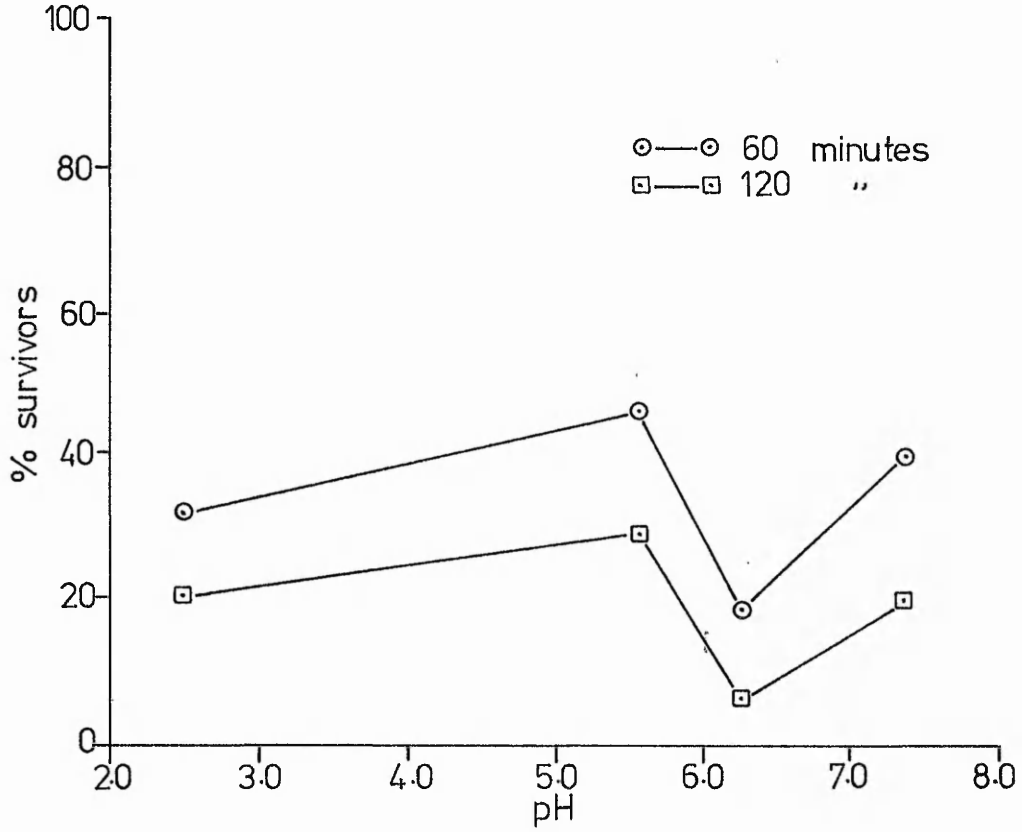


Figure 22. Effect of pH on MS 2 survival in RO water.



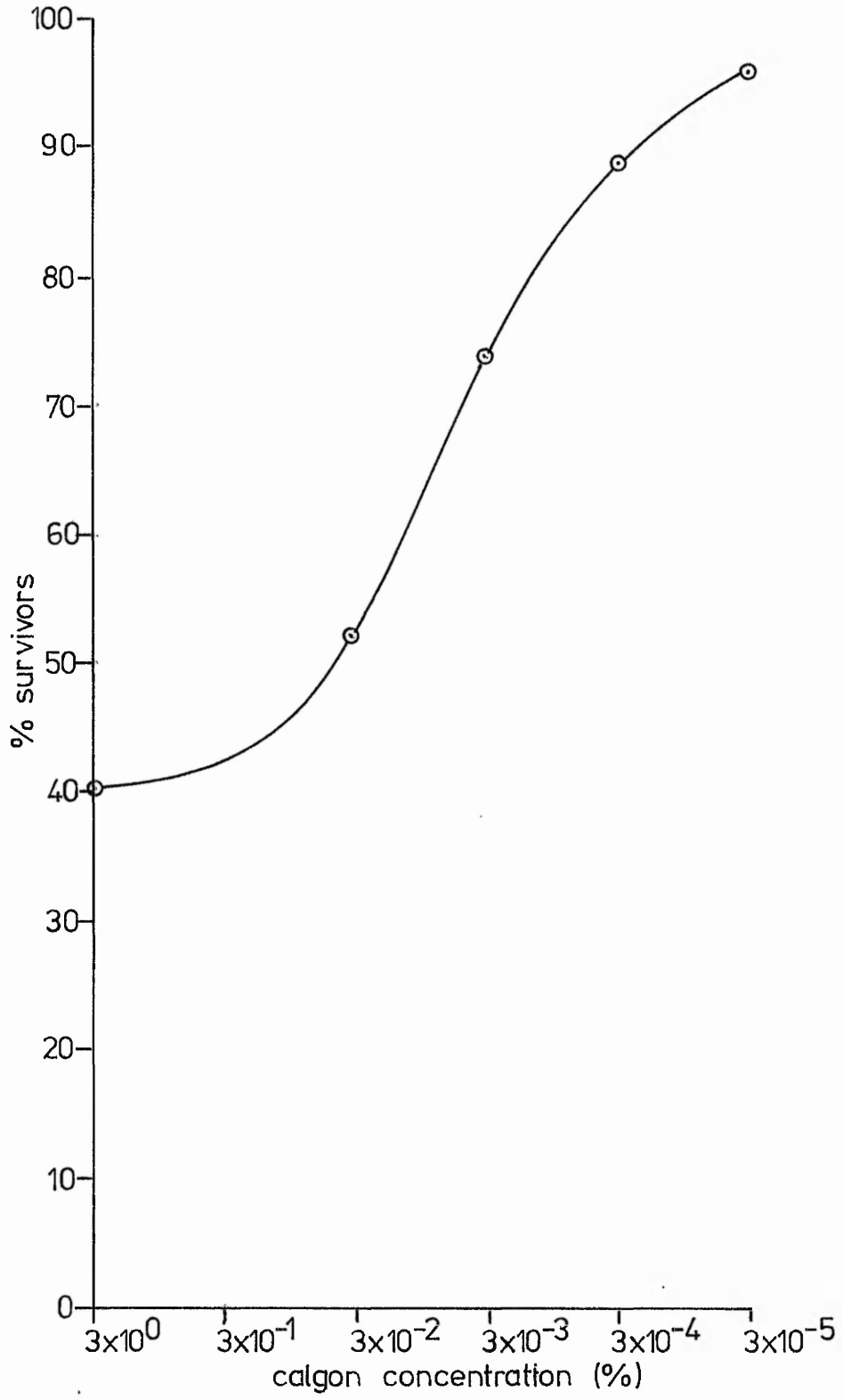
Secondly, at or around pH 6.0, in both Figures 21 and 22, a decrease in the number of survivors was noted, this loss however being greater in RO water. It was known from other experiments (see later) that MS 2, along with other viruses, could exist in two forms, one generally more sensitive to external physical and chemical influences than the other. Consequently, the two populations migrated at different rates under an electrical field, and it was shown (Irwin, 1977) that MS 2 possessed two isoelectric points, at pH 4.5 and 7.0. Therefore, the double peaked curves evidenced in Figures 21 and 22, at pH's of 5.0 and 7.0 could represent the combined inactivation curves of the 'sensitive' and 'resistant' strains of the virus.

Therefore the loss of viruses at low pH's was, in both bufffer and RO water, due to the inactivation of both types of particle, and probably a result of protein denaturation. At pH's closer to neutrality, however, an overall increased loss in RO water was noticed, as was a deepening in the 'trough' of the curves. This suggested that inactivation of both types of virus particle had occured in RO water. There was also increased inactivation with time, this being constant in the RO water, as would be predicted by a mechanism of chemical inactivation, but significant at acid pH's only in the buffer. It would therefore seem that with increased acidity the pH was the overriding influence on virus numbers. However, at pH 5.0 to 6.0 pH alone could not account for the loss of titre.

Because of the unexplained loss during RO operation, it was postulated that the RO cleaning agent, sodium hexametaphosphate ('Calgon'), might be exerting a toxic effect on the viruses within the machine. This chemical was used as a 3.0 % solution to wash the membrane bundles in situ, and as can be seen from Figure 23 there was a significant loss at this concentration. However 'Calgon' was known to be highly water soluble, and it was considered that it would not remain in the vicinity of the RO membrane long enough after its application to affect added viruses. At lower concentrations 'Calgon' showed little or no toxicity to MS 2 after 60 minutes, supporting the hypothesis that it would have no influence during normal machine operation.

A further consideration in the loss of viruses was the observed tissue damage experienced during many assays for Poliovirus in concentrated samples, these cytotoxic agents possibly also causing virus inactivation. It was postulated that either the chloroform used to kill bacteria in the water prior to assay, or constituents of the water itself, were causing this extensive cytotoxicity, and this was investigated by inoculating 1.0 ml aliquots of waters in various combinations onto Vero monolayers. These were incubated for one hour at 37°C and then overpoured with 10 ml of overlay medium as used in standard plaque assays. All bottles were incubated at 37°C for three days and then stained with 5.0 ml each of neutral red solution. Results, in Table 27, showed that sterile,

Figure 23. Effect of 'Calgon' on MS 2 survival.



distilled water had no effect on Vero cells but RO water, which had to be membrane filtered in order to sterilise it, caused an 85 to 90 % loss within the three days of incubation under overlay medium. The addition of serum to RO water

Table 27. Effect of RO water and chloroform on Vero monolayers

Treatment	Cell layers	
	1	2
(a) 1.0 ml maintenance medium	+++	+++
(b) 1.0 ml sterile distilled water	+++	+++
(c) 1.0 ml (b) + 0.1 ml chloroform	++	+++
(d) 1.0 ml RO water	+	+
(e) 1.0 ml (d) + 0.05 ml calf serum	+	+
(f) 1.0 ml (d) + 0.1 ml calf serum	++	++
(g) 1.0 ml (d) + 0.1 ml calf serum + 0.1 ml chloroform	++	+++

Key: + less than 15 % of cell layer remaining
++ 15.0 to 75.0 % " " " "
+++ more than 75 % " " " "

reduced the cell loss, and chloroform seemed to have little or no effect at all. As only 0.005 ml of chloroform would be expected to dissolve in 1.0 ml of water at 25°C, and proportionally less at 37°C, it was clear that this solvent, when used to remove bacteria from samples, was not responsible for cell damage. Therefore the loss was due to the RO water itself, clearly showing the toxic nature of the concentrate. This suggested that the

concentrated water constituents were also causing the virus loss during RO operation, and it was these aspects which were examined further.

The success of calf serum in protecting tissues from the toxic qualities of RO water prompted an examination of protection which might be afforded to viruses within the same water. Because of the expense of sera nutrient broth was used, being of consistent quality, low cost and ready availability. MS 2 was inoculated into 100 ml of Colwick RO water in a stirred container and immediately 10 ml of this water was placed in a 25 ml Universal bottle on ice. A further 1.0 ml was inoculated into 9.0 ml of cooled nutrient broth, also placed on ice. The remaining 89 ml of water in a 250 ml conical flask was magnetically stirred at room temperature and assayed with time (Figure 24). It was seen that the virus titre in cooled nutrient broth exhibited very little loss, whereas those cooled in RO water were inactivated at a faster rate. In the 89 ml of water remaining at room temperature a 10 fold reduction in titre occured within 35 minutes. The protection of MS 2 by nutrient broth, plus cooling, was therefore apparent, with a subsequent and similar experiment indicating that the decrease in titre could be halted and partially reversed at any time by removal of the viruses to the broth (Figure 25). The protection of viruses by the addition of a protein solution was therefore seen as a strong indication of the viricidal nature of RO water, and all concentrate samples were therefore treated by dilution into

Figure 24. Effect of nutrient broth and low temperature on MS 2 survival in RO water.

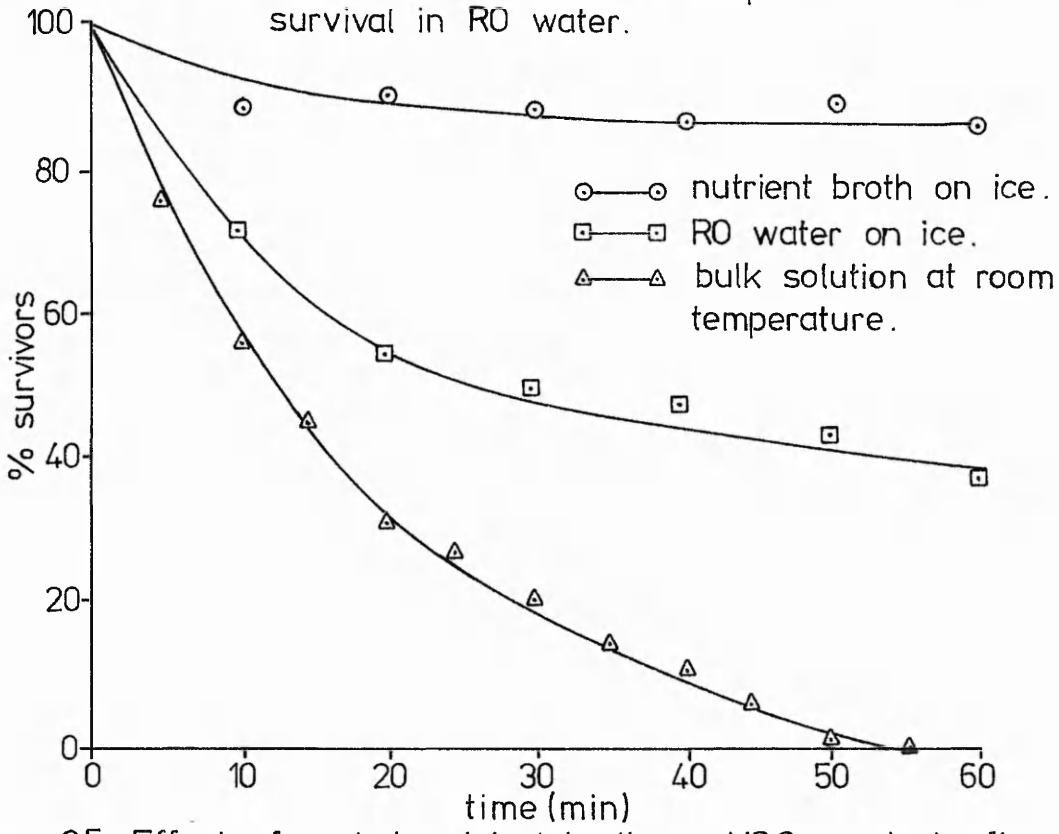
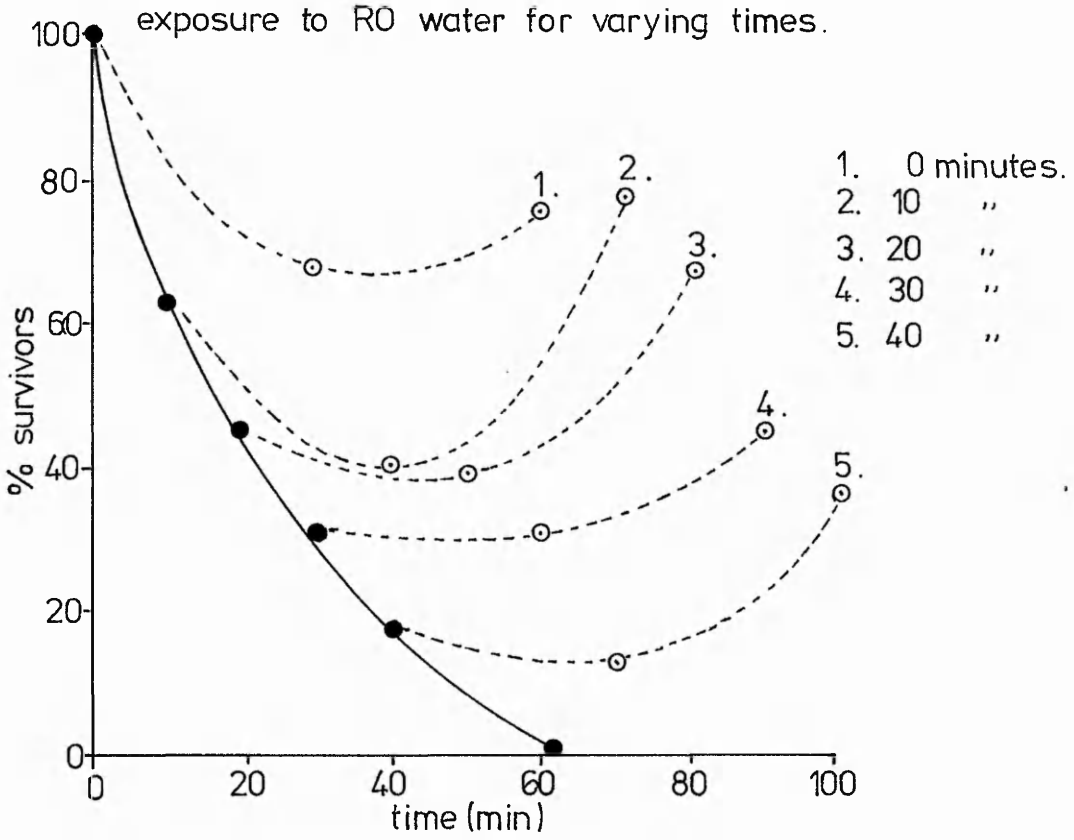


Figure 25. Effect of cooled nutrient broth on MS2 survival after exposure to RO water for varying times.



nutrient broth held on ice in a large capacity vacuum flask prior to assay.

Thus it was seen that protein solutions were capable of protecting both viruses and assay tissues from the inactivating effects of RO concentrate water. This was probably achieved by a combination of mechanisms, including desorption of solids bound viruses, deaggregation of viruses by dilution, neutralisation of soluble toxicants by adsorption to the protein molecules and reduction of toxicant effectiveness by their dilution.

Complementary to these studies on the treatment of river water by RO, studies of virus removal were undertaken using an 'R type' spaghetti module RO unit receiving clarified, tertiary filtered, activated sludge treated sewage. The natural virus load was employed at the header tank feeding the tertiary filters, the outlets and the RO concentrate stream. Two assay hosts were used, E. coli K12 Hfr H (EMG 23) and E.coli K12 f^- (EMG 1). It was realised that EMG 23 would detect all coliphage present in the water and so EMG 1 was employed to detect non-male specific types. It was reasoned that subtraction of results would reveal male-specific MS 2 like viruses. Samples were taken at 15 minute intervals, using different recycle rates to achieve 12.5, 40.0 and 75.0 % water recovery. Physical data was displayed in Table 28 and viral results in Table 29.

Table 28. Physical data from RO unit (Budd's Farm) for natural coliphage detection

Parameter	Sample	Run 1	Run 2	Run 3
Flow ($l\ min^{-1}$)	Concentrate	33.6	7.2	1.2
	Permeate	4.8	4.8	4.8
	Recycle	12.5%	40.0%	75.0%
pH	Concentrate	6.3	6.2	6.3
	Permeate	5.1	5.1	5.15
Conductivity (μ mhos $^{-1}cm^{-1}$)	Feed	900.0	912.5	910.0
	Concentrate	1054.0	1460.0	2400.0
	Permeate	92.5	121.5	180.0
Temperature ($^{\circ}C$)	Feed	19.0	19.0	19.0
	Concentrate	22.0	24.0	25.0
	Permeate	20.0	25.0	25.0
Pressure (Bar)		24.0	24.0	24.0
Flux ($l\ day^{-1}\ cm^{-2}$)		6.44	6.44	6.44

Results showed that the anthracite filters had little effect on virus numbers, removal being erratic and low. The RO unit however effectively removed all the input viruses at low water recoveries, but at higher rates phage particles were detected in the permeate water, using *E. coli* K12 Hfr H. This would have been expected because of the increased re-circulation and flow that would occur with higher recovery

Table 29. Results of Natural coliphage loading of RO unit

Recovery %	time (min)	sample site	E. coli K12 f⁻			E. coli HfrH			male specific coliphage (pfu ml⁻¹)
			Virus pfu ml⁻¹	filter removal %	rejection ratio %	virus pfu ml⁻¹	filter removal%	rejection ratio %	
12.5	0	inlet	8.5×10^2			7.6×10^2			-
		filter outlet	7.9×10^2	6.7		9.4×10^2	0.0		1.5×10^2
		RO concentrate	7.8×10^2			9.0×10^2			-
	15	inlet	7.5×10^2			6.9×10^2			-
		filter outlet	6.9×10^2	7.63		6.8×10^2	2.44		-
		RO concentrate	7.8×10^2			6.4×10^2			-
	30	inlet	8.0×10^2			8.2×10^2			1.4×10^1
		filter outlet	8.7×10^2	0.0		8.3×10^2	0.0		-
		RO concentrate	8.5×10^2			1.1×10^3			2.5×10^2
	RO Permeate		0.0		100.0	0.0		100.0	
40.0	0	inlet	-			9.6×10^2			-
		filter outlet	9.1×10^2	-		7.0×10^2	27.08		-
		RO concentrate	6.2×10^2			7.9×10^2			1.8×10^2

Table 29 - continued

Recovery %	time (min)	sample site	E. coli K12 f⁻			E. coli HfrH			male specific coliphage (pfu ml⁻¹)
			virus pfu ml⁻¹	filter removal %	rejection ratio %	virus pfu ml⁻¹	filter removal %	rejection ratio %	
40.0	15	inlet	1.2×10^3			1.1×10^3			-
		filter outlet	7.7×10^2	35.4		8.6×10^2	19.38		8.4×10^1
		RO concentrate	8.7×10^2			9.0×10^2			3.0×10^1
	30	inlet	1.05×10^3			9.03×10^2			-
		filter outlet	7.8×10^2	25.1		7.4×10^2	18.05		-
		RO concentrate	7.9×10^2			9.2×10^2			1.3×10^2
	RO Permeate		0.0		100.0	10.0		98.65	
75.0	0	inlet	9.4×10^2			7.6×10^2			-
		filter	8.5×10^2	9.25		7.4×10^2	2.87		-
		RO concentrate	5.4×10^2			3.8×10^2			-

Table 29 - continued

Recovery %	time (min)	sample site	E. coli K12 f^-			E. coli HfrH			male specific coliphage (pfu ml^{-1})
			virus pfu ml^{-1}	filter removal %	rejection ratio %	virus pfu ml^{-1}	filter removal %	rejection ratio %	
75.0	15	inlet	-			-			-
		filter outlet	-	-		-	-		-
		RO concentrate	-			-			-
	30	inlet	9.7×10^2			1.0×10^3			2.7×10^1
		filter outlet	9.7×10^2	0.0		9.9×10^2	0.4		2.3×10^1
		RO concentrate	5.6×10^2			-			
	RO Permeate		0.0		100.0	3.0		99.7	

overall filter removal E. coli f^- = 14.03 %

overall filter removal E. coli HfrH = 8.77 %

rates, but in both cases the numbers were very low, even after concentration by iron oxide adsorption.

Because of the low numbers of viruses detected, it was necessary to add viruses in order to assess the efficiency of the RO unit. Two 1.0 ml, syringe inoculated, slugs of 10^{10} ml^{-1} MS 2 were therefore injected into this unit as described for the Colwick unit, and 5.0 ml samples of concentrate water taken at 30 second intervals for 10 minutes. These produced the same characteristic diminishing cyclic peaks of MS 2 recovery and losses were calculated as for Colwick (Table 30).

Table 30 indicated that the same phenomenon of virus loss was occuring as at Colwick, and as before, the loss of viruses was seen to be higher than that calculated on dilution alone. Unlike the Colwick plant, however, this RO unit was acid dosed to maintain a pH of 6.0 to 6.5 in the concentrate stream, and, as predicted from Colwick data, produced multiple peak runs, again showing the influence of pH on virus survival.

Table 30. Physical data, theoretical and actual cumulative losses from RO unit (Budd's Farm)

		Run 1	Run 2
Virus titre (pfu ml^{-1})		1×10^{10}	1×10^{10}
Flux (l day^{-1} cm^{-2})		4.83	4.83
Rejection ratio (%)		100.0	100.0
Concentrate pH		6.5	6.5
Cycle 1:	Calculated loss (%)	54.1	54.1
	Actual loss (%)	99.70	99.48
	Load for cycle 2 (pfu ml^{-1})	1.34×10^{7}	4.4×10^{7}
Cycle 2:	Calculated loss (%)	79.0	79.0
	Actual loss (%)	99.75	99.49
	Load for cycle 3 (pfu ml^{-1})	5.09×10^{7}	5.09×10^{7}
Cycle 3:	Calculated loss (%)	90.36	90.36
	Actual loss (%)	99.89	99.53
	Load for cycle 4 (pfu ml^{-1})	1.02×10^{7}	4.7×10^{7}
Cycle 4:	Calculated loss (%)	95.58	95.58
	Actual loss (%)	99.89	99.63
	Load for cycle 5 (pfu ml^{-1})	1.02×10^{7}	3.73×10^{7}

It had long been felt, however, that the artificially introduced problems connected with the introduction of viruses as a discrete inoculum could be overcome by continual dosing at a lower titre into the RO machine. This was possible at Budd's Farm, and so to prepare a continuous feed of viruses for the unit 1.0 ml of phage suspension at 10^{11} ml^{-1} was added

to 500 litres of water and mixed vigourously for one minute using a Stuart-Turner 18 recirculating pump. This water was fed to the unit, set for 75 % recovery, and concentrate samples were taken with time. The operating pressure was initially set at 20 bar, and increased to 40 bar to note the effect of pressure on virus exclusion. The experiment was performed in duplicate, results being illustrated in Table 31.

Table 31. Continual loading of an R type RO unit with phage MS 2 and the effect of altering operational pressure

Parameter	Sample	Run 1		Run 2	
		20 bar	40 bar	20 bar	40 bar
Flow ($l\ min^{-1}$)	Concentrate	1.02	1.98	1.20	1.98
	Permeate	3.0	6.0	3.6	6.0
	Recycle	75.0%	75.0%	75.0%	75.0%
pH	Concentrate	6.6	6.5	6.45	6.5
	Permeate	5.6	5.5	5.4	5.4
Conductivity (μ mhos $^{-1}cm^{-1}$)	Feed	850.0	905.0	1000.0	1000.0
	Concentrate	2850.0	3010.0	2950.0	3010.0
	Permeate	195.0	172.0	210.0	170.0
Temperature (^{o}C)	Feed	18.5	18.5	18.5	18.5
	Concentrate	23.0	23.0	24.0	24.5
	Permeate	24.0	24.5	23.0	24.0
Rejection (%)		100.0	99.98	100.0	99.99
Mixing Tank: 0 minutes		$6.1x10^{4}$	$6.2x10^{4}$	$1.1x10^{5}$	$7.3x10^{4}$
Concentrate: 5 minutes		$3.2x10^{4}$	$7.3x10^{4}$	$1.4x10^{5}$	$2.5x10^{5}$
Concentrate: 15 minutes		$3.1x10^{4}$	$8.3x10^{4}$	$1.5x10^{5}$	$2.3x10^{5}$
Permeate		0.0	$2.7x10^{1}$	0.0	$1.0x10^{1}$

It could be seen that the increased pressure in both cases forced some viruses through the membrane into the permeate water, although the number was low. The increased pressure also increased the concentrate flow rates, enhancing the possibility of virus penetration by increasing the virus concentration at the membrane. However, in both sets of experiments the percentage rejection remained very high, and so, with a system of water sterilisation to supplement RO removal, membrane breakthrough was not considered to be a potential problem.

Because of the evidence of virus penetration of the membrane, two experiments were undertaken to determine the virus removal efficiencies of activated carbon and ozone, these being the two systems employed in the event of RO membrane failure.

After mixing MS 2 at 10^9 ml^{-1} in 250 litres of permeate water as described, samples of 10 ml were collected from the inlet and four sampling ports of the activated carbon column at 0, 15 and 30 minutes and assayed for phage using E. coli K12 Hfr H (Table 32).

As could be seen, the whole column was capable of removing only 56.95 % of the viruses added and could not therefore be relied upon to provide a safe and adequate means of sterilising RO permeate water, especially in the event of membrane failure.

It was thought possible that virus elution was responsible for the negative results demonstrated after 30 minutes, this in fact being 100 minutes after the initial loading of the column with a high virus titre.

Examination of ozonisation was performed, as described in the methods section, 20 ml samples being taken every three minutes over 15 minutes. The results (Figure 26) demonstrated the dramatic action of ozone on viruses, with a 99.9 % titre reduction in 14 minutes. It was therefore clear that ozonisation would act as a much more effective permeate water sterilant than activated carbon, if such was required after RO treatment.

The results from all RO units suggested that certain factors were contributing to the reduction of the influent virus titre, thus enhancing virus exclusion during the RO treatment of polluted waters, except at low pH's where the acidity of the water was the major factor. The evidence seemed to suggest that toxic inactivation aggregation, and adsorption were the most likely causes and further experiments were undertaken to examine these aspects.

Samples of both Colwick and Budd's Farm RO concentrate waters were treated by either membrane filtration through a Millipore 47 mm, 22 µm cellulose acetate filter to remove suspended solids whilst leaving soluble toxicants, or by autoclaving at 121°C for 15 minutes to remove or destroy

Figure 26. Effect of ozonisation on MS 2.

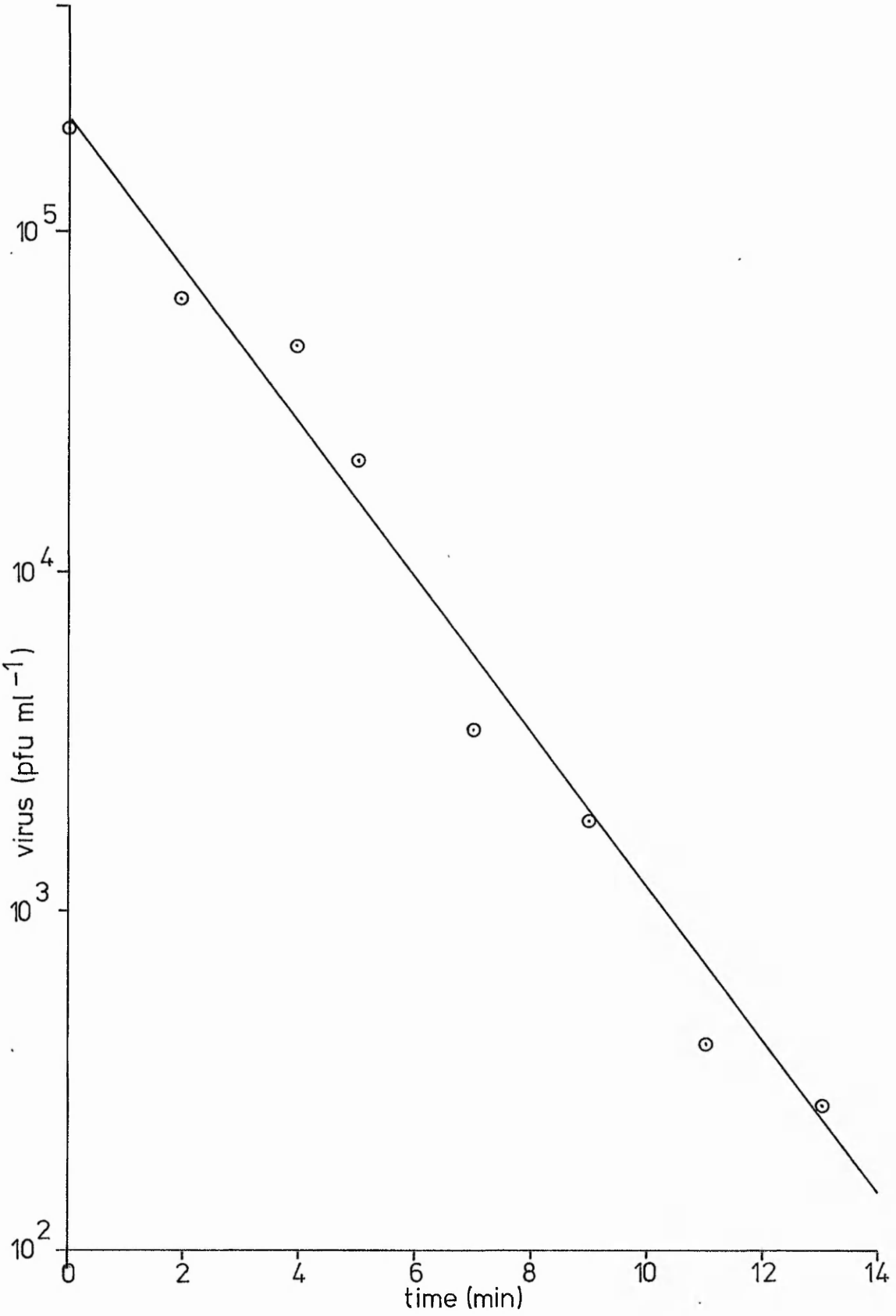


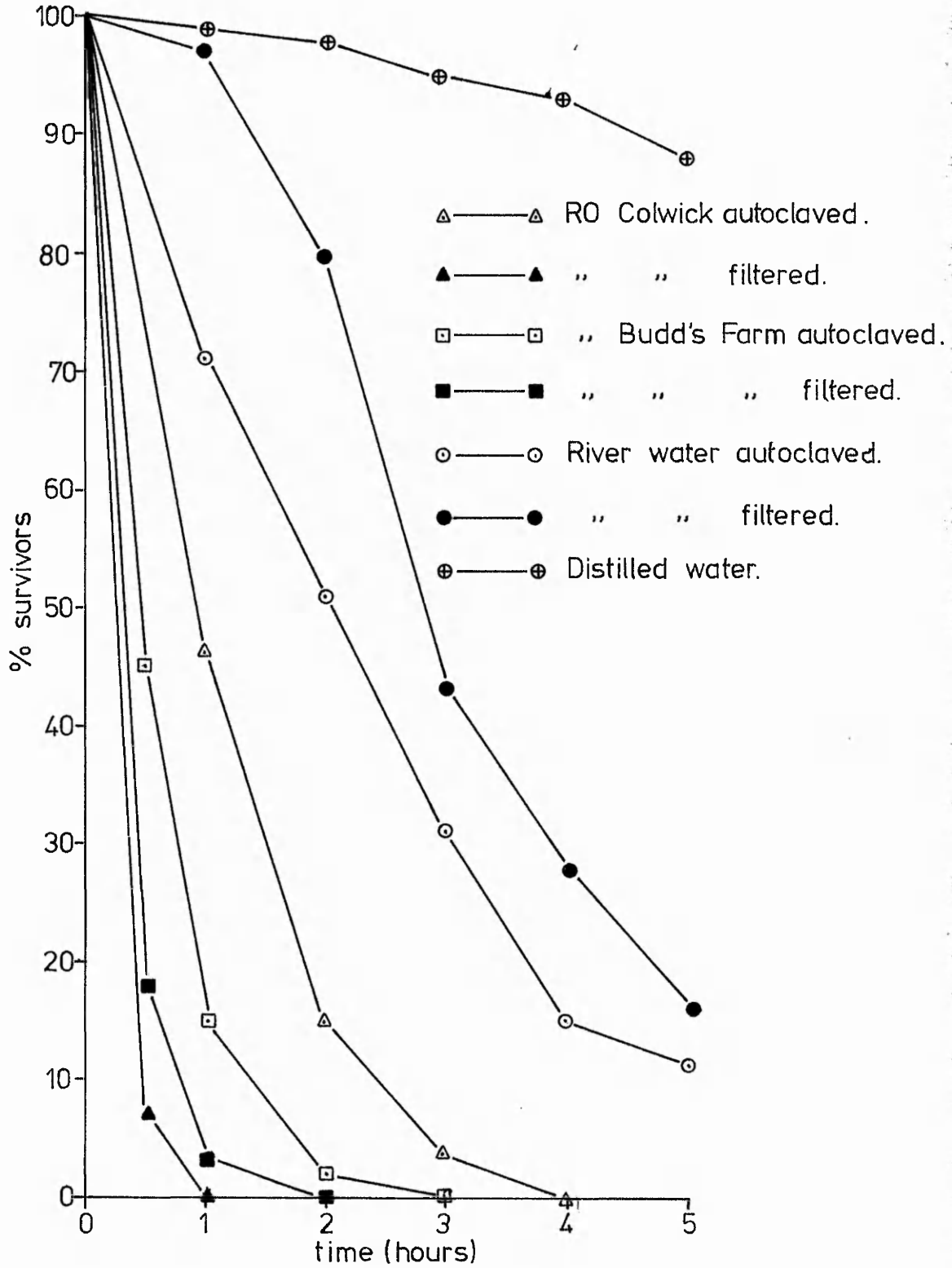
Table 32. Efficiency of MS 2 removal by activated carbon

Time (min)	Water pH	Temperature (^{o}C)	Depth (cm)	Titre	% removal
0	5.5	20.0	Inlet	1.78×10^{4}	0.0
			14.0	1.66×10^{4}	3.97
			39.4	1.31×10^{4}	26.3
			66.0	1.19×10^{4}	33.02
			86.5	8.27×10^{3}	53.16
15	5.5	20.0	Inlet	1.63×10^{4}	0.0
			14.0	1.37×10^{4}	16.11
			39.4	1.36×10^{4}	16.53
			66.0	1.21×10^{4}	25.72
			86.5	7.03×10^{3}	56.95
30	5.5	20.0	Inlet	1.38×10^{4}	0.0
			14.0	1.52×10^{4}	-10.45
			39.4	1.62×10^{4}	-17.39
			66.0	1.38×19^{4}	0.0
			86.5	6.80×10^{3}	50.72

toxicants whilst leaving solids for adsorption. River water from the River Trent at Colwick was treated by the same processes and samples from all three sources were then seeded with MS 2, stirred at $20^{o}C$ and sampled hourly (Figure 27).

Filtration and autoclaving both caused rapid loss of virus titre in both RO waters, with greater loss occuring in samples from which the suspended solids had been removed. The experiments also showed that RO water, as had been predicted, was more toxic to the viruses than river water, but all waters

Figure 27. Survival of MS 2 in river and RO water.



were shown to be more toxic than the distilled water control. In river water samples virus loss was greater after water pretreatment by autoclaving, the opposite effect to that noted for RO water. This suggested that in river water adsorption seemed to be the predominant means of virus removal from suspension, whereas in RO waters toxic inactivation was more likely to occur. This was supported by the evidence that autoclaving, known to remove soluble toxicants by thermal chelations, resulted in greater virus survival in the RO waters. Filtration, however, lead to greater loss, and so it was postulated that the solids were to some extent protecting the viruses from inactivation.

Bitton and Mitchell (1974, c) had shown that colloidal montmorillonite and non-host bacterial cells protected the bacteriophage T 7 from the viricidal action of sea water by adsorption of the virus to their surfaces, and/or coadsorption and inactivation of anti-viral toxins. The above evidence suggested that the same mechanism was occurring in the RO concentrate samples, although the level of toxicants was such that the viruses were subjected to their action both before and after adsorption. The cytotoxicity of RO concentrate water and the protection of viruses by the addition of nutrient broth, which would release solids-bound viruses as well as neutralising soluble toxins, was seen as further evidence of this theory.

Further investigations were made into the nature of RO toxicity by its centrifugal separation at 3000 RPM (4000c.) in a MSE 4/64 Mark II Magnum centrifuge for 30 minutes, into a sediment free supernatant, which was also membrane filtered, and a distilled water resuspended sediment fraction. Using these two materials pH studies were repeated over the range of 2.3 to 7.4 (Figure 28) again revealing the toxic nature of the water, and the high losses incurred with high acidity. At higher pH's, however, the sediment containing fraction demonstrated greater loss of virus titre than the sediment free sample, indicating the accumulative effect of toxic inactivation and adsorption by the sediment.

It was apparent from iron oxide experiments that adsorption was only slightly temperature dependent, whereas the temperature dependence of chemical reactions was well known, and so the effect of temperature on virus inactivation in RO water was assessed at 25°C and 37°C. The two RO concentrate fractions, at pH 5.8, were incubated with MS 2 at the temperature described and the results (Figure 29) showed that increased temperature greatly increased virus loss, suggesting that chemical reaction, rather than physical adsorption, were involved in virus inactivation in RO waters.

The Budd's Farm RO concentrate water was also examined for temperature effects on added viruses (Figure 30). In these experiments the slopes at 20°C and 37°C were very similar for all RO waters, but steeper than those at 5°C, or those of the distilled water control. This further evidence

Figure 28. Effect of pH on MS 2 in separated RO water fractions.

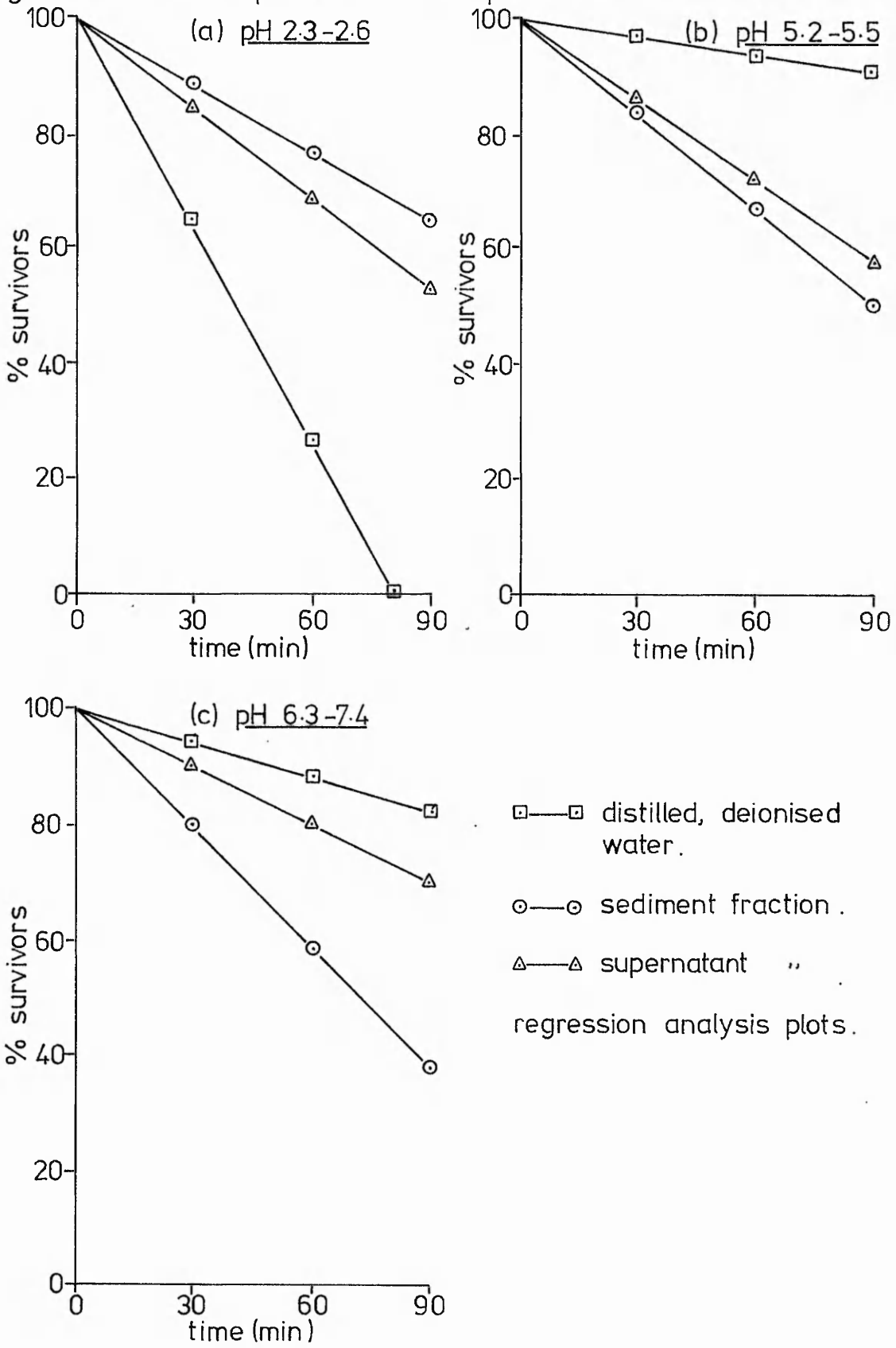
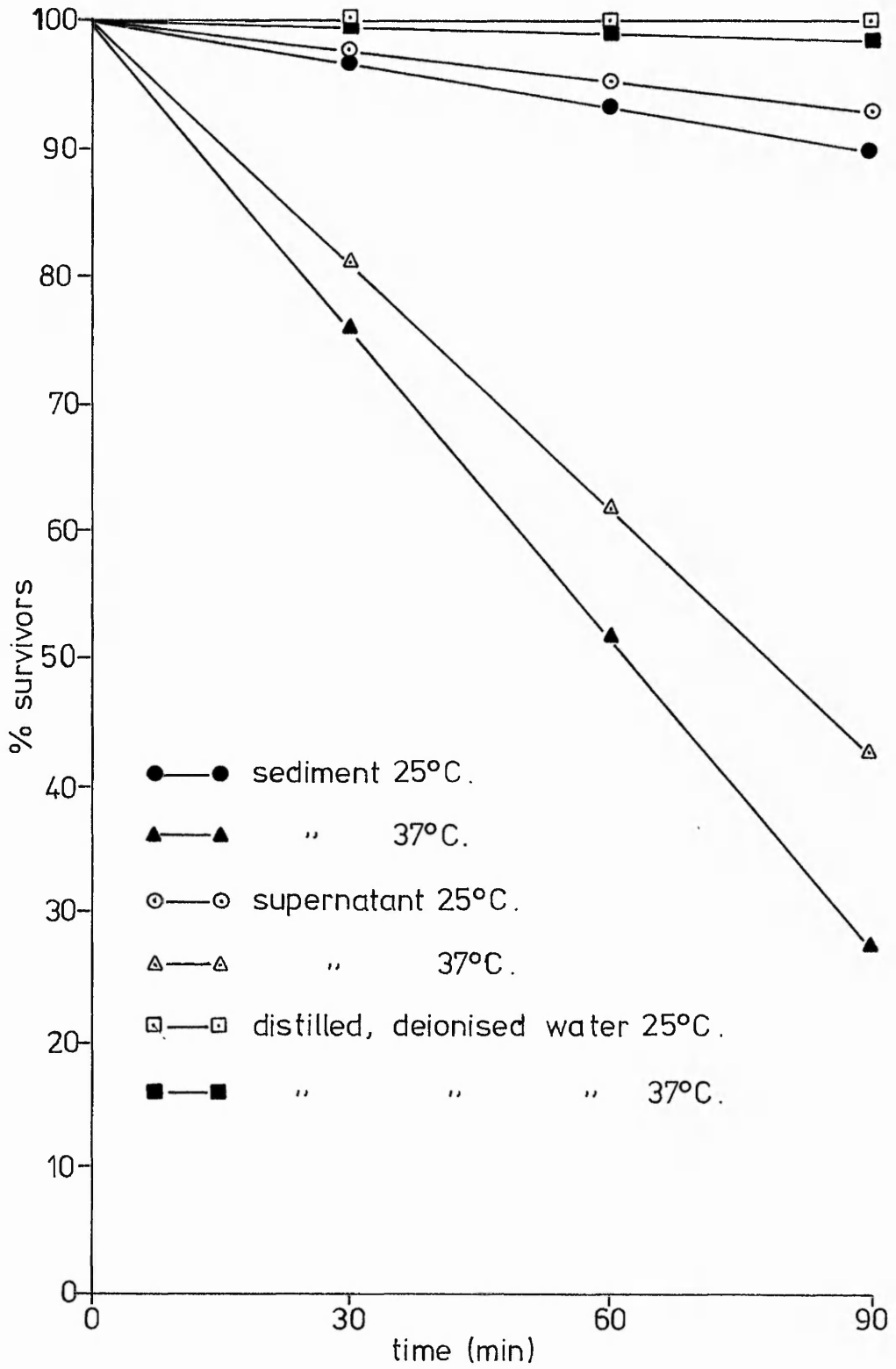


Figure 29. Effect of temperature on MS 2 in separated RO water fractions (Colwick) - regression plots.



strongly supported the hypothesis of chemical toxicity rather than adsorption being responsible for virus loss.

If toxins adsorbed to solids were responsible for virus losses from RO water, then the soluble fractions would be removed by washing, resulting in increased virus survival. A sediment sample was therefore washed several times over three days and the supernatant samples were tested after 2 hours, 24 hours and 72 hours. The final sediment was resuspended and also tested, all being examined at 20°C (Figure 31). Interpretation of these results suggested that toxic agents were being removed from the sediment fraction with time, the 72 hour supernatant giving the greatest toxicity to MS 2, whilst the final sediment resuspension produced the least virus loss.

Whilst it was not possible to obtain material adhering to membranes from the Colwick plant in order to test this phenomenon, this was achieved with the Budd's Farm unit, a two metre section of bundle being carefully washed with 100 ml of distilled water, and the washings collected. These were homogenised and samples plated with E. coli K12 f^- and E. coli K12 Hfr H on blood agar base agar plates. No plaques were found with either host and so 10 ml volumes of whole washings and centrifuge-cleared supernatant were seeded with MS 2 (Figure 32). The untreated suspension of formerly membrane bound material was found to have less of an effect on MS 2, although both solutions rapidly reduced virus titre,

Figure 30. Effect of temperature on MS 2 in RO water (Budd's Farm)

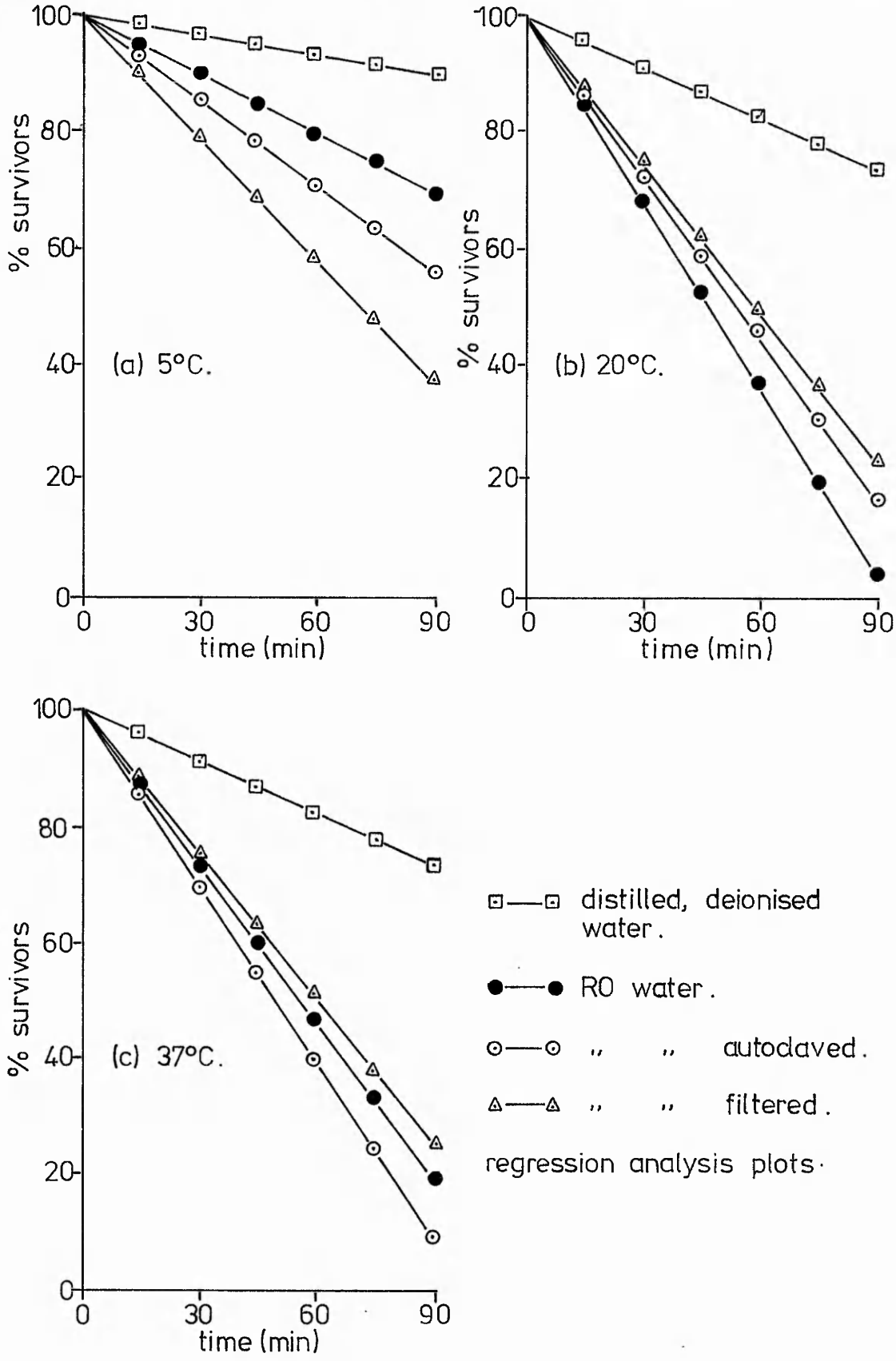


Figure 31. Effect of sediment washing on MS 2 survival in RO water.

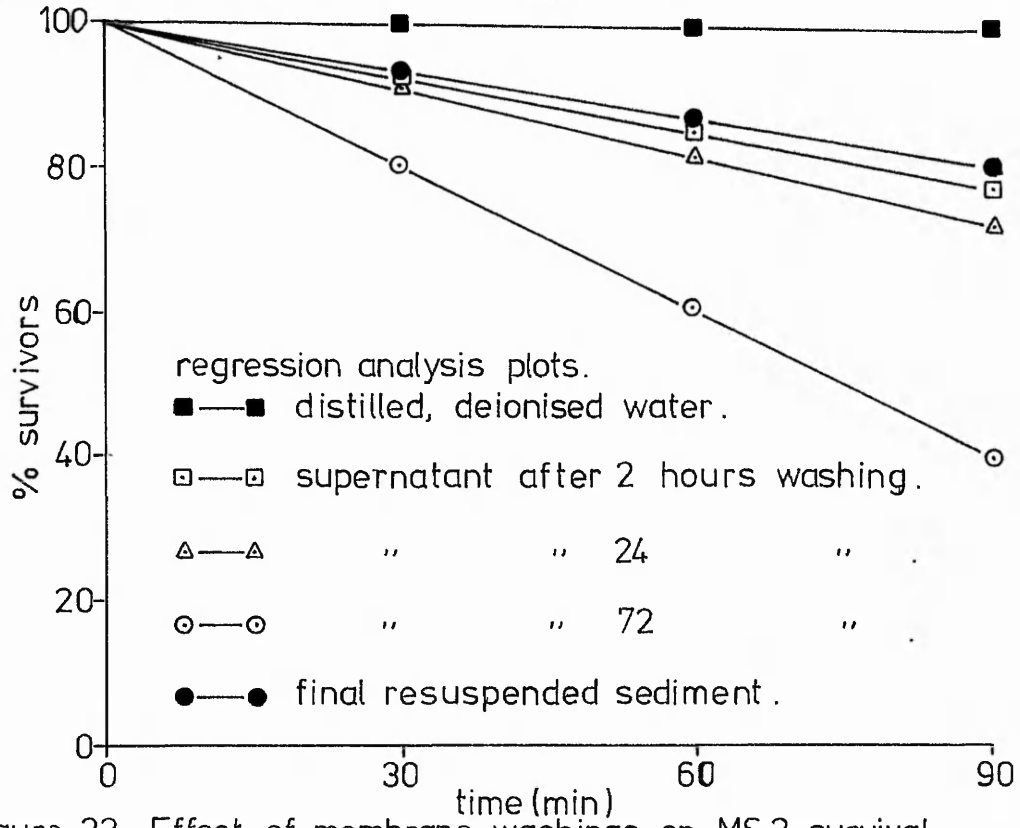
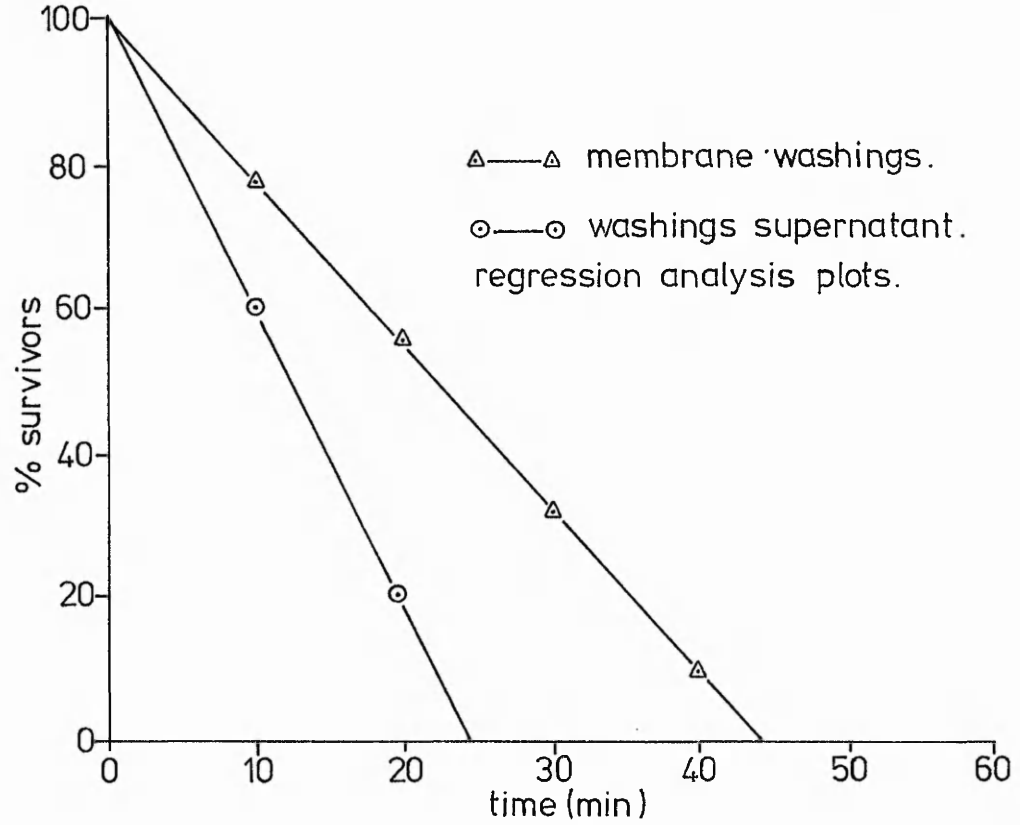


Figure 32. Effect of membrane washings on MS 2 survival.

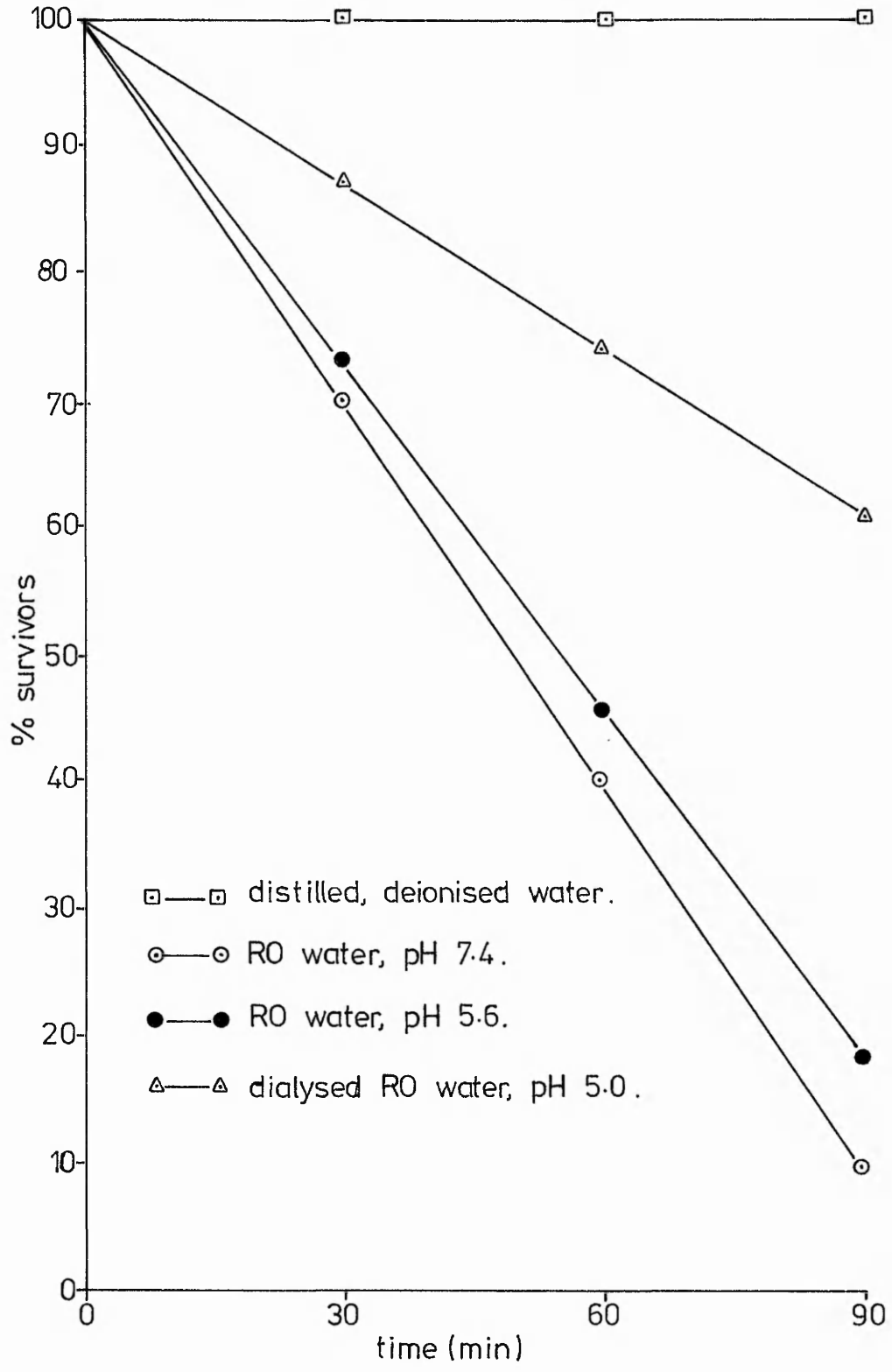


explaining the lack of detection of naturally occuring phage particles. The enhanced viricidal effect of particle free supernatant again suggested the toxic nature of these waters. As the suspension liquid was distilled water, shown to have only a minor detrimental effect on MS 2, the high toxicity could only have originated from membrane particle leachates.

It was therefore seen that toxic inactivation was the major factor in virus loss and in order to examine the nature of these substances in RO water, 50 ml of Colwick concentrate water was dialysed at $4^{o}C$ against distilled, deionised water in 'Visking' dialysis tubing, size 36/32, for six days. The dialysate, and whole RO samples at pH 5.6 and 7.4 were inoculated with MS 2 and the results expressed in Figure 33. The sample for dialysis had a pH of 7.4 before dialysis and 5.0 after treatment, and produced a reduced toxic effect when compared with whole RO water at a similar pH.

Dialysis is normally employed to remove small ions or molecules from aqueous solutions of proteins and other high molecular weight solutes, molecular size being the main factor determining escape rate through the membrane. Although there is an initial influx of solvent due to osmosis, the difference in chemical potentials of the solute on both sides of the dialysis membrane provides the force impelling solute molecules through this barrier to equilibrium.

Figure 33. Effect of dialysis on MS 2 inactivation by RO water.
(regression analysis plot)



Therefore the increased virus survival must have been due to the removal, by dialysis, of small ionic or molecular toxic species. The suspended solids responsible for adsorption would have remained in the dialysis sack, affording additional protection to the viruses.

It was therefore clear from the experiments with RO water that although adsorption was one of the causes of virus reduction, toxic agents were responsible for the major portion of their loss. This loss of viruses by chemical inactivation should therefore reduce the risk of virus penetration of the membrane, and the importance of this necessitated an examination of the mechanisms involved in virus/chemical interactions.

Heavy metal inactivation of viruses

Results from river surveys and RO studies produced strong evidence to show that heavy metal toxicity was the cause of virus inactivation and it was therefore decided to pursue this aspect further. In order to avoid the complication of preassay viral aggregation influencing results, virus suspensions were diluted ten fold from stock, this having been shown to actively deaggregate viruses at $4^{o}C$ over 24 hours.

Studies were initiated with an examination of 18 of the commonest metals likely to be found in polluted waters, these being dissolved as 0.2 mM solutions of their chlorides in 10 ml of distilled water in Universal bottles. To each was added MS 2 or Poliovirus 1 and the bottles rotary mixed for 60 minutes at 10 rev min^{-1}. One ml aliquots of each MS 2-containing solution were then pipetted into 9.0 ml of 1.0 % peptone, thoroughly mixed and plated onto triplicate predried blood agar base plates as described. From Poliovirus-containing solutions 1.0 ml aliquots were taken to 9.0 ml volumes of maintenance medium, mixed and 5.0 ml of this distributed equally between five replicate monolayers of HEp 2 cells in 125 ml medical flat bottles. After incubation of the two viruses, plaques were counted and percentage losses determined, together with the final pH values of the metal solutions, and displayed as a modified form of the Periodic table of elements (Table 38).

Table 33. Percent loss of two viruses in 0.2 mM solutions of metal chloride

Li		Be													B	C	
21.1	5.9	-	-												- -	- -	
27.0	5.9	-	-												- -	- -	
Na		Mg													Al	Si	
28.6	5.9	7.2	6.2												100 4.5	- -	
35.4	5.7	46.0	6.1												100 4.3	- -	
K		Ca		Sc		Mn		Fe		Co		Ni		Cu		Zn		Ga		Ge	
30.2	6.0	8.59	5.8	-	-	15.0	5.7	68.2	3.4	6.3	6.0	16.8	6.3	79.1	5.6	25.2	5.8	-	-	-	-
60.0	5.9	68.0	5.9	-	-	48.0	5.8	91.4	3.2	48.0	6.1	44.0	6.1	84.0	5.4	52.0	5.8	-	-	-	-
Rb		Sr		Y		Tc		Ru		Rh		Pd		Ag		Cd		In		Sn	
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	49.7	6.1	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	62.0	6.0	-	-	-	-
Cs		Ba		La		Re		Os		Ir		Pt		Au		Hg		Tl		Pb	
39.3	6.1	22.7	6.2	100	5.7	-	-	-	-	-	-	-	-	-	-	99.3	4.9	-	-	100	5.1
74.0	5.9	91.0	6.0	100	5.8	-	-	-	-	-	-	-	-	-	-	100	5.1			100	5.2
Fr		Ra		Ac																	
-	-	-	-	-	-																
-	-	-	-	-	-																

M	
A	B
C	D

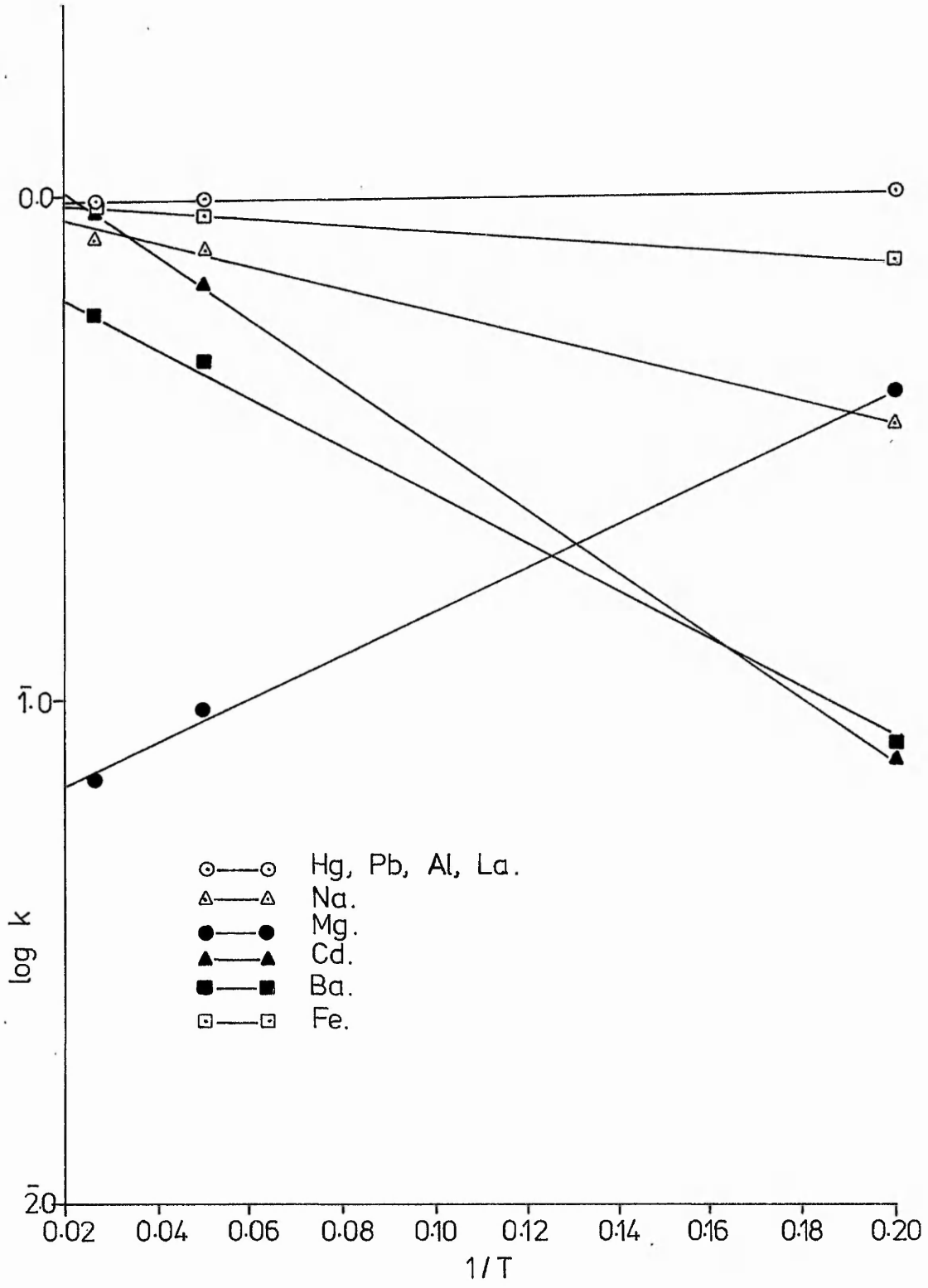
A: Percentage loss (MS 2)
B: Solution pH (MS 2)
C: Percent loss (Poliovirus)
D: Solution pH (Poliovirus)

It was obvious from Table 33 that Poliovirus sustained greater losses of titre than MS 2 with all metal salts, and that for a given periodic groups the percentage loss with each virus increased with increasing atomic size. As a metal ion in solution could only exist as an acidic aquo-ion, the atomic size would affect the degree of hydration and hence the extent of attraction between the ion and a charged surface, i.e. the virus particle, in a Hofmeister series. Thus increasing atomic size, as in the series Li, Na, K, Cs, caused greater attraction between viruses and cations and therefore greater loss of titre.

It was also clear that trivalent metal cations were very effective in virus removal, whereas monovalent and divalent cations were of mixed ability, with the exceptions of mercury and lead, which each caused almost total loss within 60 minutes. The greater effect of divalent cations over monovalents, noted during the enhancement of iron oxide adsorption (Table 16), was here apparent only with Poliovirus. Finally, it was noted that for each set of data, the solution pH values were approximately equal, showing that pH did not influence comparative results.

The effect of temperature on virus inactivation was then established, using MS 2, incubated in 0.2 mM solutions of nine metal chlorides at 5°C, 20°C and 37°C. For each set of results, obtained over 60 minutes, the inactivation slope was plotted on semi-logarithmic paper against the reciprocal of the temperature, producing a straight line (Figure 34), as defined by the Arrhenius equation:

Figure 34. Arrhenius plots for MS 2 inactivation by metal cations.



$$\log k = \frac{-Ea}{2.303\ R} .1/T + C$$

Where k = rate constant

R = gas constant

Ea = activation energy

T = temperature (absolute)

C = integration constant

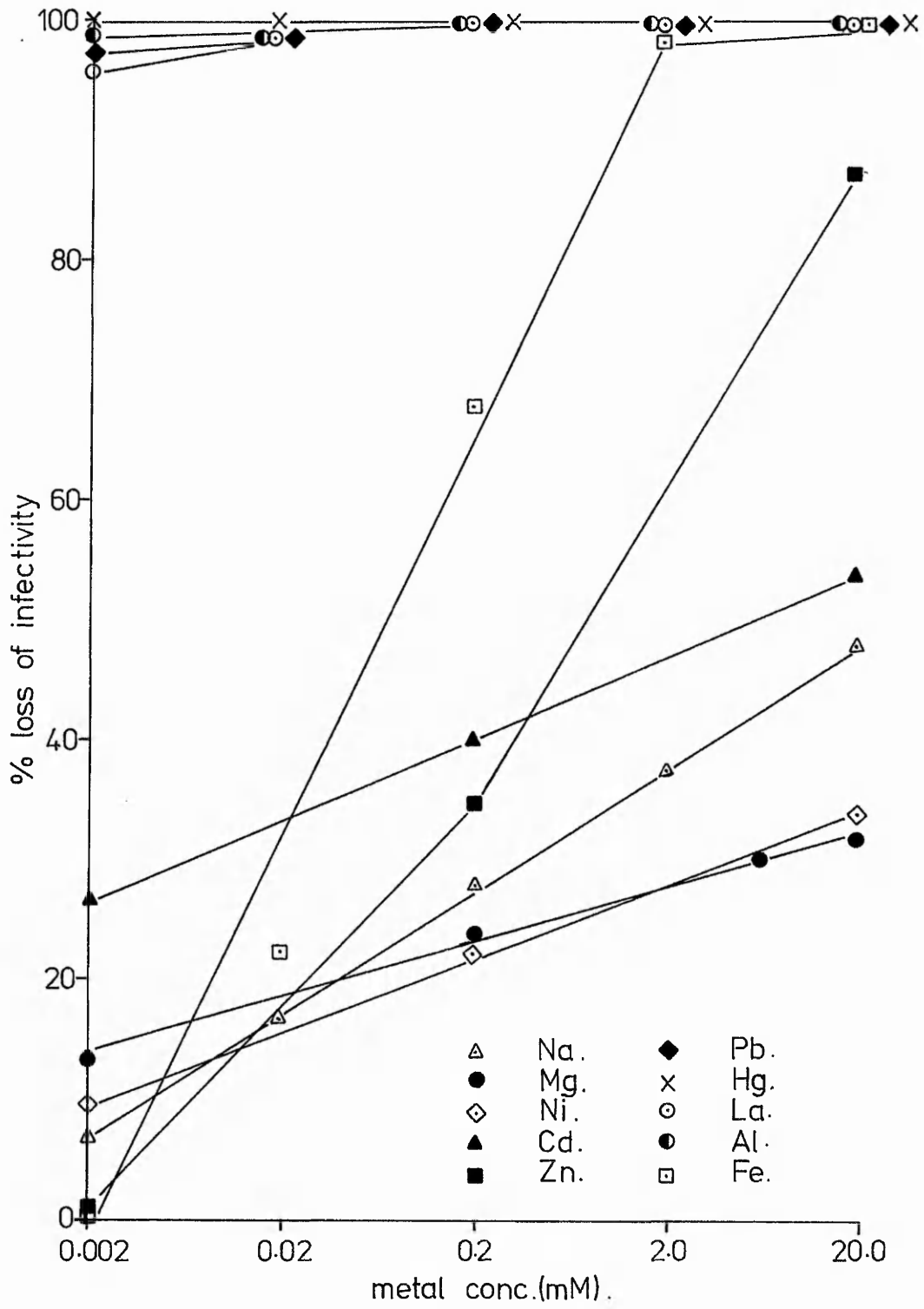
As the rates of most chemical reactions are strongly temperature dependent, any increase in temperature favours the formation of 'active' molecules capable of reaction. These 'active' molecules have sufficient energy of activation to permit them to form activated complexes from which conversion to reaction products occurs. Therefore, in the temperature region in which the reactants are not destroyed by heat, the Arrhenius equation would describe a straight line, and such lines were demonstrated, with slopes directly proportional to the activation energy. As a greater slope indicated increased temperature sensitivity of the inactivation, it was seen that the cationic inactivations fell into two groups.

In the first group (Na, Cd, Ba and Mg) a fairly steep slope was recorded, suggesting a temperature sensitive chemical reaction, causing greater loss of infectivity at higher temperatures. Within the second group (Hg, Pb, Al, La and Fe) the loss of viruses was almost temperature independent, the metals being so toxic as to override any influence of temperature.

Both the above groups, however, confirmed the inhibiting effect of metal cations on viruses, and clearly such a reaction should be concentration dependent. It was therefore necessary to ascertain the degree of inactivation to be expected at the increased levels of metal cations detected during virus concentration procedures, and at the high levels that were to be expected in RO concentrate water during the treatment of polluted waters. Therefore 10 ml solutions of ten metal chlorides, each over a range from 0.002 mM to 20.0 mM, were inoculated with MS 2, rotary mixed at room temperature for 60 minutes, and then diluted 1.0 ml into 9.0 ml of 1.0 % peptone to reduce the excess metal concentration. Assay revealed that loss of infectivity increased with increasing concentration for all cations examined (Figure 35), and that as before, trivalent ions and divalent lead very effectively inactivated the added viruses. Mercury was amongst those cations examined, but above 0.2 mM it effectively inactivated the bacterial host, making assay by this method impossible. However, at and below 0.2 mM, it allowed a bacterial lawn to develop, but without virus plaques. Thus, it was assumed that complete loss of virus infectivity occured at all concentrations tested, within 60 minutes.

The metals in Figure 35 were seen to fall into the same groups as in Figure 34 and so, with the possible exception of ferric iron, trivalent cations and divalent ions such as lead and mercury must mask temperature and concentration effects, within the regions assessed.

Figure 35. Effect of metal concentration on MS 2 inactivation.



Because of the repeated, and unexpected, expression of inactivation graphs as curves rather than straight lines, inactivation by 0.2 mM cadmium, ferric iron, mercury and lanthanum as chloride salts was reexamined in greater detail at 20°C, with sampling every three minutes. The results (Figure 36) were clearly biphasic, showing that two populations of the phage MS 2 coexisted within the suspension. The initial, steeper slope was caused by the inactivation of the sensitive particles in the population, leaving those viruses that were more resistant to be detected as the flatter, second portion of each curve.

Evidence for the existence of two populations of MS 2 has been conclusively derived in this laboratory by studies of electrophoretic mobility (Irwin, PhD thesis, 1977) and density gradient centrifugation and ultraviolet inactivation (Jones, D., PhD thesis, 1977), and the results confirmed the suggestion of two populations observed during pH inactivation studies using RO water (Figures 21 and 22).

In order to determine whether the anion radical influenced virus inactivation, three metals were reexamined as different anionic species. Magnesium, lanthanum and sodium were used at final concentrations of 20.0, 2.0 and 0.2 mM, the percentage inactivation being recorded after 60 minutes. Table 34 showed, as previously determined, the trend for increased valency to increase virus loss, but there was no detectable difference due to the anions employed, for a given cation and valency, this therefore enforcing the Hofmeister series concept for the cationic species.

Figure 36. Demonstration of biphasic curves obtained during MS 2 inactivation by metal cations.

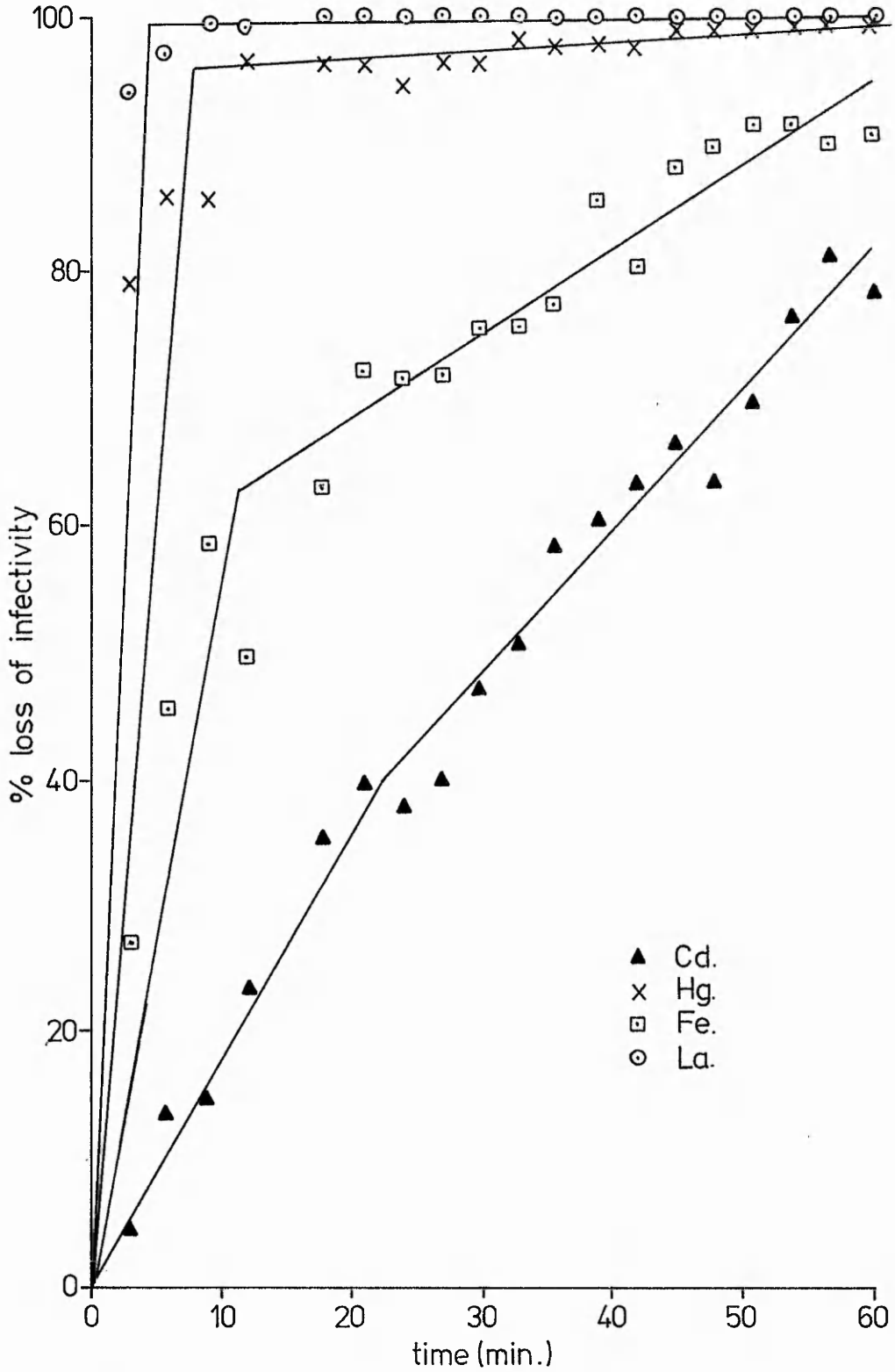


Table 34. MS 2 inactivation in salt solutions of differing anions

Cation concn (mM)		Chloride	Sulphate	Nitrate	Iodide
Na	20.0	17.09	7.72	20.23	10.66
	2.0	64.41	13.88	3.15	10.66
	0.2	27.55	16.61	12.03	16.61
Mg	20.0	47.72	35.53	30.46	-
	2.0	10.15	10.15	32.49	-
	0.2	8.63	15.23	42.26	-
La	20.0	100.00	-	99.93	-
	2.0	100.00	-	99.98	-
	0.2	100.00	-	100.00	-

As both adsorption and toxicity were thought to be responsible for virus losses during normal RO operation, they were modelled in order to determine the extent of their influence on virus titre. Because of the experience gained in the use of iron oxide this was chosen as a model adsorbent, MS 2 being allowed to mix with oxide particles in the presence of a number of metal cations for 30 minutes at room temperature. Magnesium, calcium and aluminium were selected as they had previously been shown to be enhancers of virus adsorption, as Table 35 reaffirmed. Nickel and zinc salts also acted as effective enhancers of adsorption but lead, already shown to totally inactivate MS 2, caused an apparent 100 % adsorption and failure to recover any plaque forming units. Lanthanum

Table 35. Effect of various metals, as chlorides or sulphates, on the adsorption of MS 2 to iron oxide

Cation concn. (mM)	% adsorption	% overall recovery	% recovery from no. adsorbed
Ca (2.0)	95.70	65.25	68.35
Mg (2.0)	97.56	53.86	55.28
Ni (2.0)	94.07	74.18	79.14
Zn (2.0)	94.31	47.94	50.96
Pb (2.0)	100.00	0.00	0.00
La (2.0)	100.00	0.97	0.97
Al (2.0)	100.00	1.65	1.65

yielded results similar to those of aluminium, as might have been expected from previous evidence, with excellent virus adsorption and minimal recovery. Magnesium and calcium were used at ten fold lower concentrations than those for effective virus concentration, explaining the lower recovery efficiencies, and aluminium was used at a 100 fold greater concentration. It would therefore appear that, in this adsorption system, two alternative mechanisms were possible. The cations could have enhanced the adsorption of the viruses to the iron oxide, with subsequent and variable recovery by proteinaceous lab-lemco. In this case the failure to recover viruses, especially with lead and the trivalent cations, would be due to strong binding of the viruses to the particles of oxide. Alternatively, it was possible that the metals might have caused aggregation/inactivation before or during

adsorption. As the percentage adsorption recorded was derived from the lack of viruses in the supernatant after particle removal, it was therefore possible that the lack of viruses was actually due to their destruction, rather than their removal onto the oxide.

However, the evidence for virus recovery from iron oxide (Table 17), and the observation that the addition of MS 2, treated with magnesium, cadmium or mercury, to 1.0 % lab-lemco failed to reverse the inactivation, even after 24 hours, supported the first hypothesis of strong binding and probable co-adsorption of virus and metal ions.

A series of experiments were therefore undertaken in an attempt to recover metal inactivated viruses, firstly by dialysis. Phage dilutions were added to several metal chloride solutions at 0.2 mM, rotary mixed at 20°C for 60 minutes, and then 2.0 ml of each pipetted into dialysis sacks made from 8/32 grade 'Visking' tubing. These were tied and placed into separate 250 ml conical flasks full of distilled water and allowed to dialyse for 24 hours at room temperature.

The dialysis tubing was prepared by soaking lengths of tubing in a 1.0 mM solution of 8-hydroxy-quinoline sulphate at 5°C for 24 hours in order to chelate any metals within the tubing. The tubes were then soaked in three changes of distilled water, each at 5°C for 24 hours, before use in the above tests.

Table 36. Effect of dialysis on MS 2 inactivation by metal ions

Cation concn. (mM)	% survival after 60 minutes at 20°C	% survival after 24 hours dialysis at room temp.
Pb 0.2	0.00	60.90
Hg 0.2	0.69	62.22
La 0.2	0.00	17.65
Al 0.2	0.00	14.45
Fe 0.2	31.72	47.75
Distilled water	81.55	33.34

From Table 36 it was clear that dialysis was capable of facilitating a recovery of some virus infectivity, by the reduction of the cationic concentration through the dialysis membrane. The percentage survival rather than loss of infectivity was calculated as it was felt that this more clearly demonstrated the recovery of titre, and from this the divalent and trivalent metals were seen to fall into two separate groups. Lead and mercury both caused 100 % inactivation after 60 minutes at room temperature and 60+ % recovery of infectivity was obtained by dialysis. However, in the same times only 14 to 17 % of the virus titres lost by the action of trivalent lanthanum, aluminium or ferric iron were recovered.

On the basis of dialysis, therefore, heavy metal inactivation, like adsorption, was deemed to be reversible, within the confines of the valency of the metal employed. It was therefore very likely that the removal of toxicity from RO water by

dialysis (Figure 33), was the result of the removal of heavy metal ions.

It was noted that the distilled water, devoid of metals and employed as a control, caused a decrease in virus survival during dialysis. A possible reason for this was an excess removal of ions from the virus particles, making them unstable. This would not occur with the metal treated viruses because of the high ionic concentration.

It was thought possible that metal treated viruses were bound into aggregates and therefore recovery of titre was attempted by ultrasonication. This required that the effect of sonication on deaggregated MS 2 be firstly determined using a MSE sonicator at a maximum amplitude of 7.0 microns, peak to peak, and at 20 kc s^{-1}. The stainless steel probe, measuring 14.6 cm long and tapering from 2.5 cm to 0.3 cm at the tip, was immersed in 10.0 ml of virus dilution in sterile distilled water in an open Universal bottle in the sonicator, so that the probe just penetrated the liquid surface. The bottle had previously been placed in a 250 ml plastic beaker packed with crushed ice, to prevent viral inactivation by heat associated with ultrasonication and the viruses were sonicated in six 10 second bursts for a total of 60 seconds, removing 0.1 ml into 0.9 ml of 1.0 % peptone after each burst. The experiment was repeated with three 20 second and two 30 second bursts, each for 60 seconds total, and all samples were assayed and percentage losses of viruses calculated. As seen in Figure 37, the loss of titre over 60 seconds was similar

Figure 37. Effect of sonication on MS 2 survival.

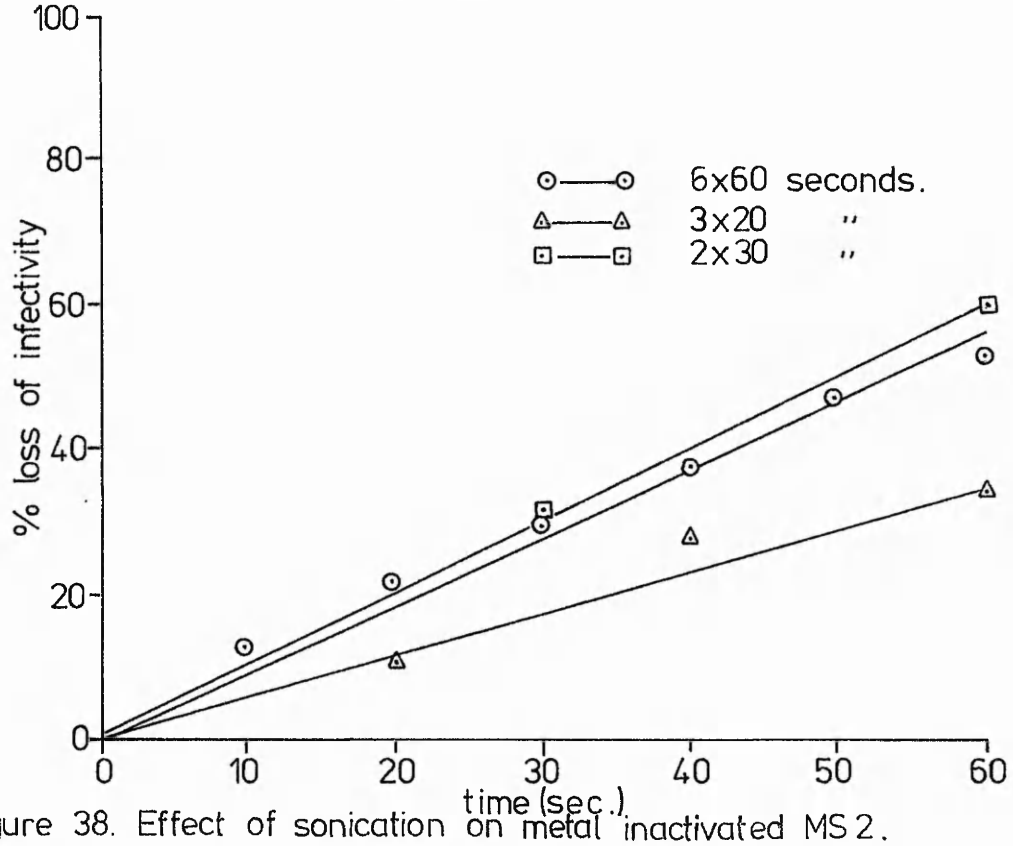
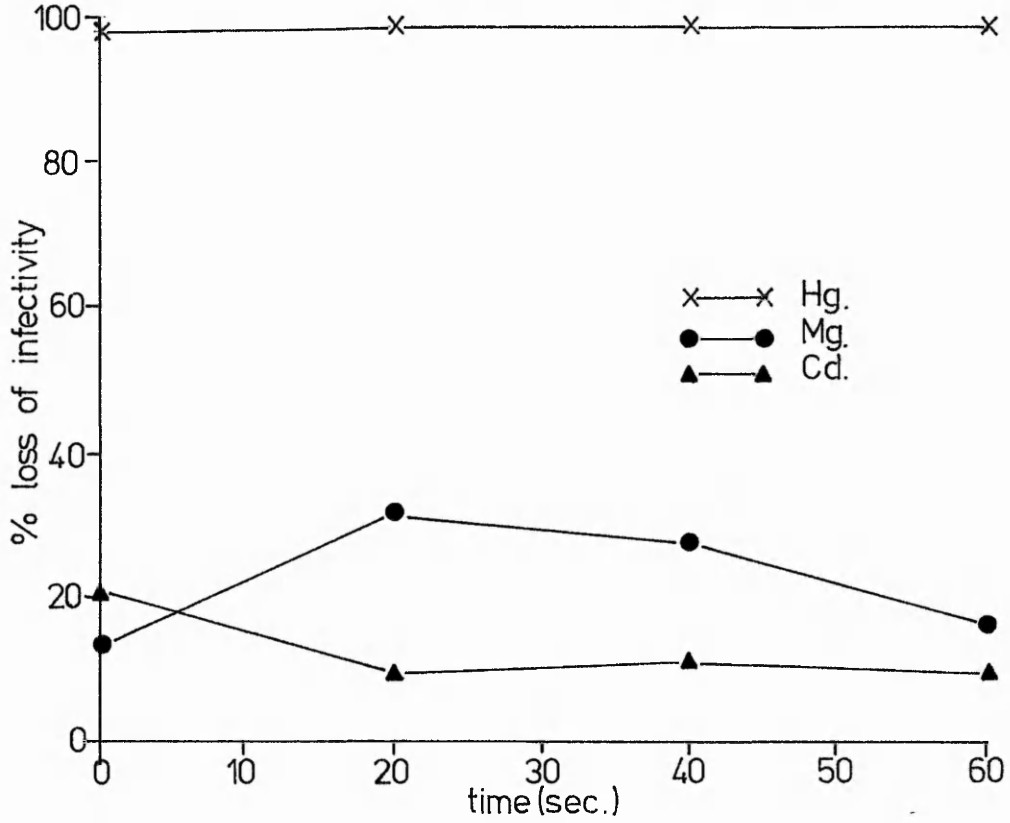


Figure 38. Effect of sonication on metal inactivated MS 2.



irrespective of the length of time of each burst of ultrasound, but as three 20 second bursts gave slightly lower losses, this was used in a second experiment to determine the effect of sonication on metal treated virus particles. Ms 2 was mixed with magnesium, cadmium and mercury, all at final concentrations of 0.2 mM, for 60 minutes at 20°C and then each was sonicated for three 20 seconds of ultrasound, each sample being plated out and the loss or recovery determined. Figure 38 showed that sonication had no effect on mercury inactivation, this being 98.2 % after 60 minutes mixing and 98.6 % after a total of 60 seconds sonication. The loss of cadmium and magnesium reacted viruses after 60 minutes was 21.5 % and 13.4 % respectively and, as could be seen, after 60 seconds sonication a small percentage of viruses had been recovered from those previously in cadmium. Sonication of magnesium treated viruses initially lead to increased loss of infectivity at a rate similar to untreated viruses, but this was reversed so that after 60 seconds total, the loss was 16.3 %.

If the viruses had been inactivated by the metal cations, sonication would be expected to disrupt the remainder at a rate similar to those in Figure 37. However, no overall change in the percentage loss was seen for any of the three viruses, and it was therefore postulated that they could have been protected against sonication by aggregation due to the cations. Alternatively, cation induced aggregates could be deaggregating at the same rate as sonication disrupted viable

particles, resulting in an overall stabilisation of titre. However, the experiments had been conducted in the metal solutions, dilution into peptone occuring after sonication, and so cationic stabilisation was seen as the most likely explanation.

The bacteriostatic ability of mercury, known for many years, was reported to be due to its association with -SH containing enzymes (Lawrence and Block, 1971), and to be reversible by the addition of -SH containing compounds, such as glutathione, cysteine and thioglycollate. Mercuric poisoning of bacteriophage was also reported to be reversible by the addition of H_2S (Kreuger and Baldwin, 1934), or other -SH containing compounds, and so an attempt was made, using sodium thioglycollate, to recover mercury inactivated coliphages.

Initially the toxicity of thioglycollate to MS 2 was determined by reacting phage dilutions with thioglycollate at 20, 2.0, 0.2 and 0.02 mM for 30 and 60 minutes, without metal addition. From the histograms in Figure 39 it was immediately obvious that high concentrations of thioglycollate were very toxic, but that 0.2 mM had no effect on the virus titre over 60 minutes. Therefore phage and 0.2 mM mercuric chloride were reacted as described for 5, 15, 30 and 60 minutes, at each time 1.0 ml being removed to 9.0 ml of thioglycollate. These suspensions were mixed for 30 minutes and then assayed, the results being plotted in Figure 40.

Figure 39. Toxicity of thioglycollate to MS 2.

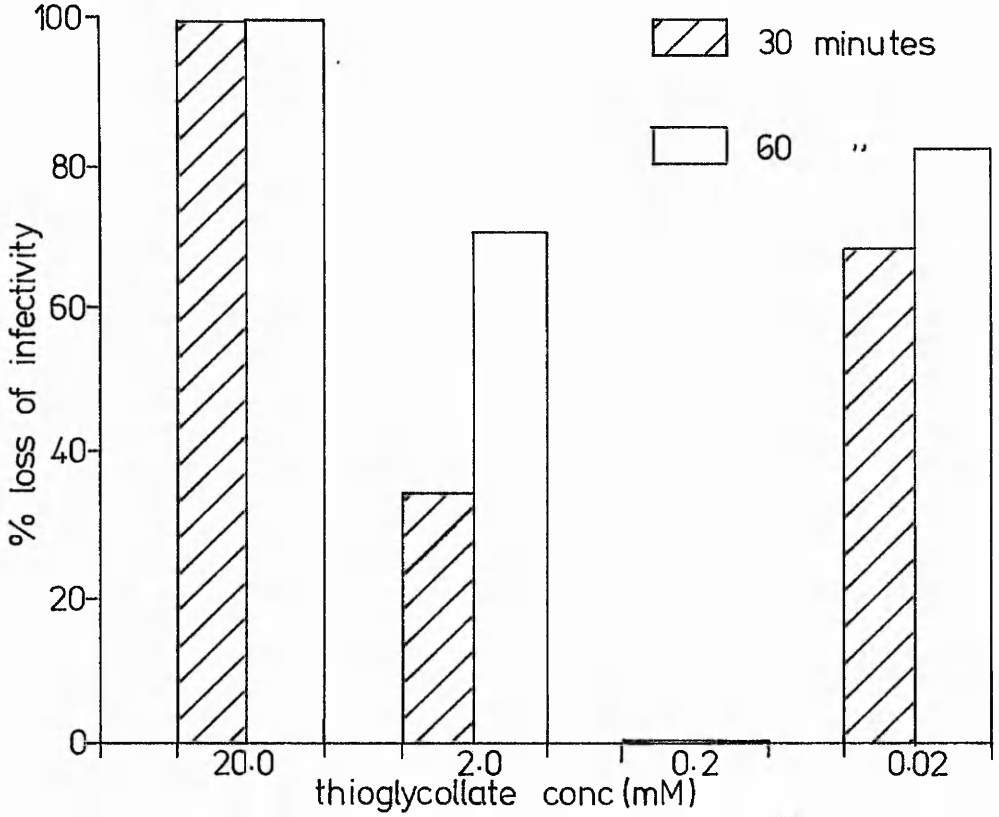
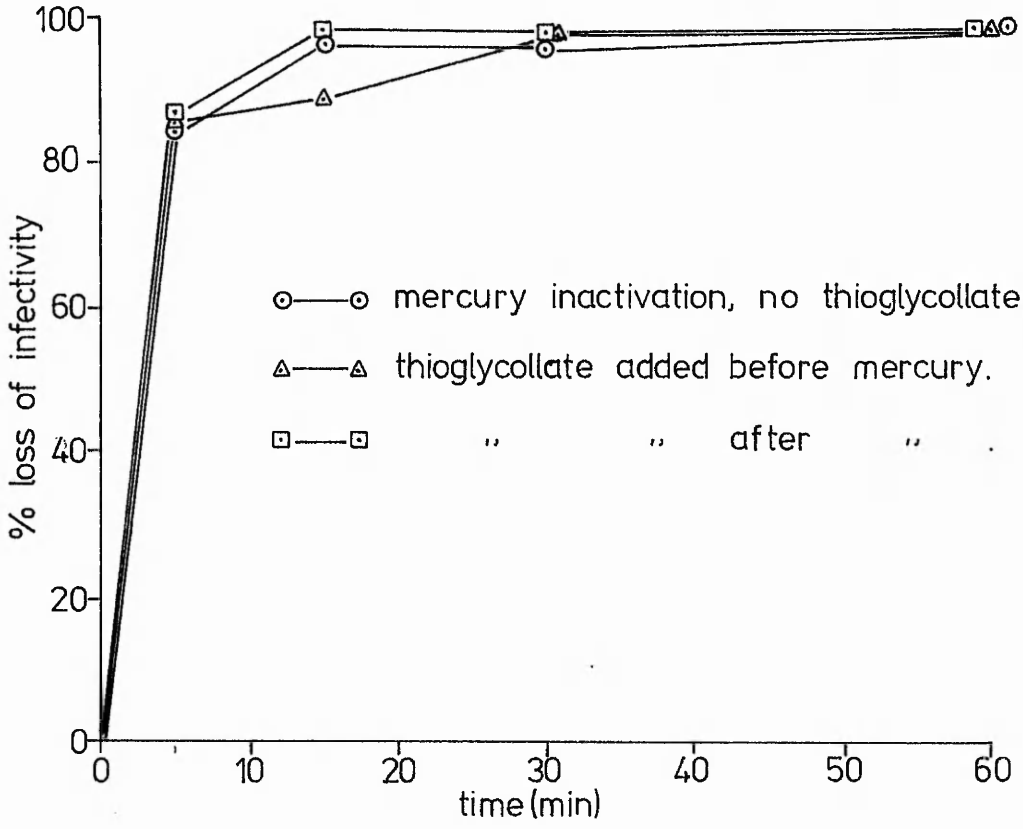


Figure 40. Effect of thioglycollate on mercuric inactivation of MS 2.



As it had been reported (Topley and Wilson, 1975) that thioglycollate could also protect enzymic -SH groupings from heavy metal 'poisoning' by binding with and maintaining the -SH in a reduced state, and by forming mercaptides with the heavy metals, an attempt was made to protect MS 2 before mercuric inactivation. Coliphage and 0.2 mM thioglycollate were therefore mixed together for 30 minutes, after which time 0.1 ml of suspension was added to 9.9 ml of 0.2 mM mercuric chloride, mixed and sampled at 5, 15, 30 and 60 minutes as above. The results of these experiments (Figure 40) showed that neither pre- or post- addition of thioglycollate had any effect on mercuric inactivation of MS 2, in conflict with evidence from bacterial and early phage studies. A fundamental alteration in the factors affecting virus infectivity must therefore have occured as a result of the addition of mercury, these alterations being so radical as to prevent the regeneration of infectivity.

In order to examine further the effect of heavy metals on MS 2 electron microscopic examinations were undertaken, first by the dip grid method using an E. coli high titre MS 2 mixed suspension. Host bacteria were added to determine whether metal treated viruses would still adsorb to the 'f' pilus, the bacteria also assisting in virus location during microscopic examination. In a 0.2 mM lanthanum treated mixture, pili were found to be very clearly resolved, with no phage attached to them. Phage particles detected were, however, aggregated, this also being found with mercury and cadmium treated

mixtures, although some pili attached particles were noted with cadmium. Lanthanum also caused all the bacterial cells observed to be ruptured at one end, a phenomenon not noted with mercury and cadmium.

High titre MS 2 was also deposited onto formvar eroded grids with a Fullam nebuliser/sprayer after reaction with the above three metal chlorides. This method of application was known to spread virus particles evenly over an electron microscope grid without the surface tension effects found with the dip grid. Therefore if the aggregates observed above were a result of surface tension forces formed during air drying they would not be seen after spray application. However, viral aggregates were again observed showing that they were probably the result of metal action, although it must be noted that the high titre of viruses required for detection by electron microscopy could by itself result in some aggregation prior to metal addition. Of greater significance, however, was the lack of viral adsorption to sex pili in the virus/bacteria mixture when the viruses were mixed with lanthanum or mercury, in excellent agreement with previous experiments where lack of titre was the result.

Unfortunately the three metals caused a great loss of resolution under electron microscopic examination, making the taking of electron micrographs unprofitable. It was assumed that this phenomenon was a result of the electron opacity of the metal salts.

Evidence from the literature and many previous experiments lead to the conclusion that viruses were acting as lyophilic colloidal suspensions in water. As such they would have a sphere of hydration, the degree of which would affect the action of any electrolyte, and which would in turn be affected by the amount and type of electrolyte. Further the addition of a dehydrating molecule, such as an alcohol would reduce the hydration sphere of a lyophilic colloid, making it 'lyophobic' and therefore much more sensitive to the addition of electrolytes. In order to test this hypothesis the influence of alcohols on MS 2 was first determined. Pure ethanol and isopropanol were diluted with distilled water from 100 % to produce 70 %, 50 %, 30 % and 10 % solutions, and to 10 ml of each in Universal bottles on ice was added MS 2. Bottles were mixed to distribute the viruses and samples were assayed at 5, 10, 15, 25, 30, 45 and 60 minutes. The results, plotted as loss of infectivity against time (Figures 41 and 42), clearly showed that pure ethanol and isopropanol quickly inactivated MS 2, but that for a given concentration below 100 % ethanol was more viricidal than isopropanol. This was undoubtedly due to the fact that MS 2, like the Enteroviruses, consisted of nucleic acid and an outer protein coat only, making the particles hydrophilic and consequently susceptible to ethanol, but very resistant to isopropanol, because of the latters larger size and resulting lower reactivity.

Figure 41. Effect of ethanol on MS 2 inactivation.

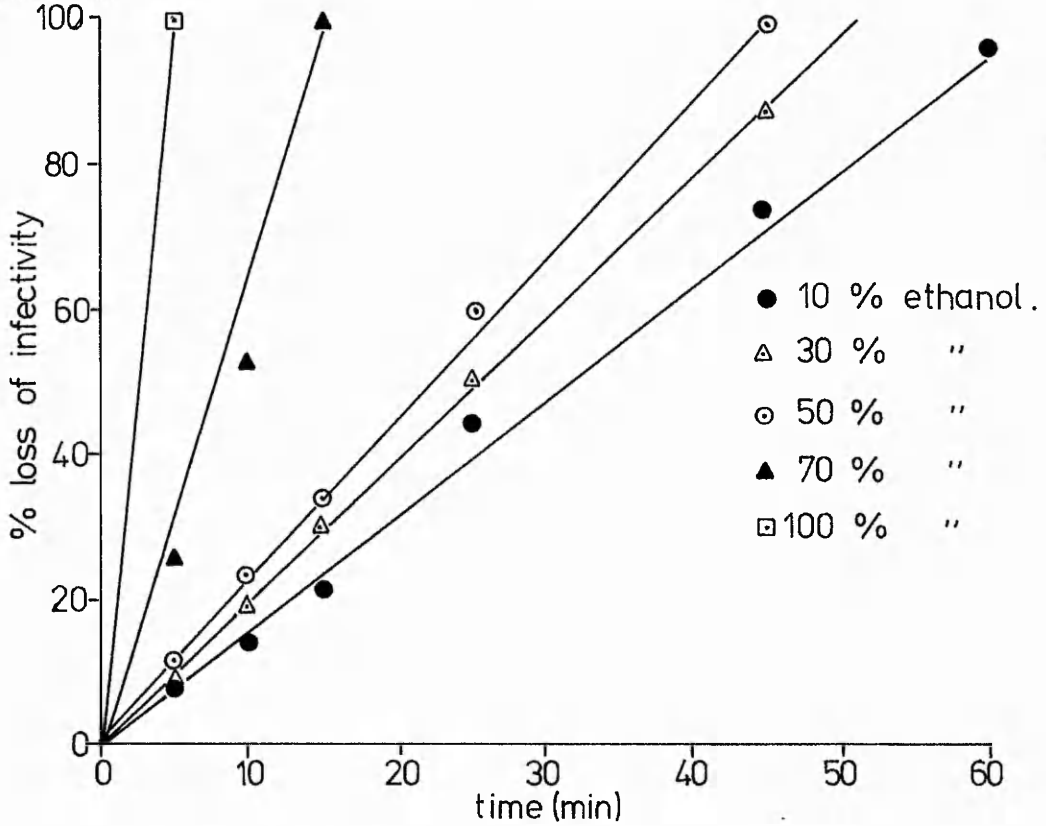
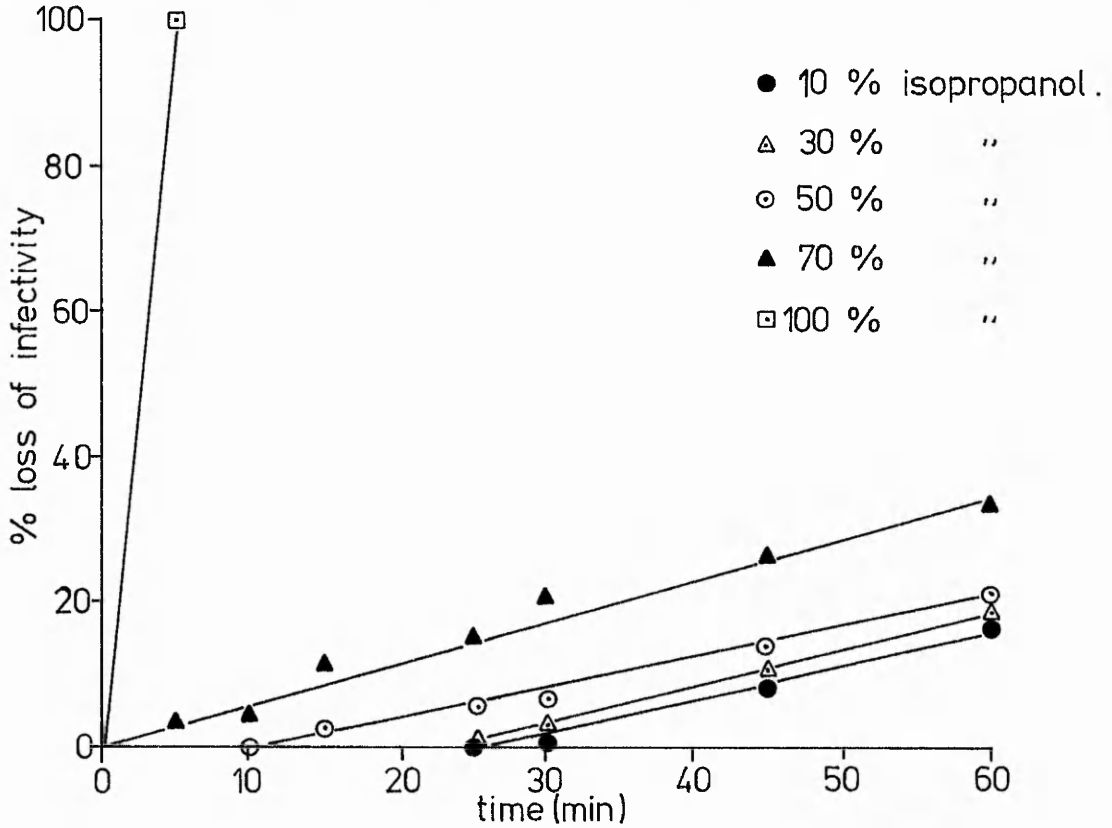


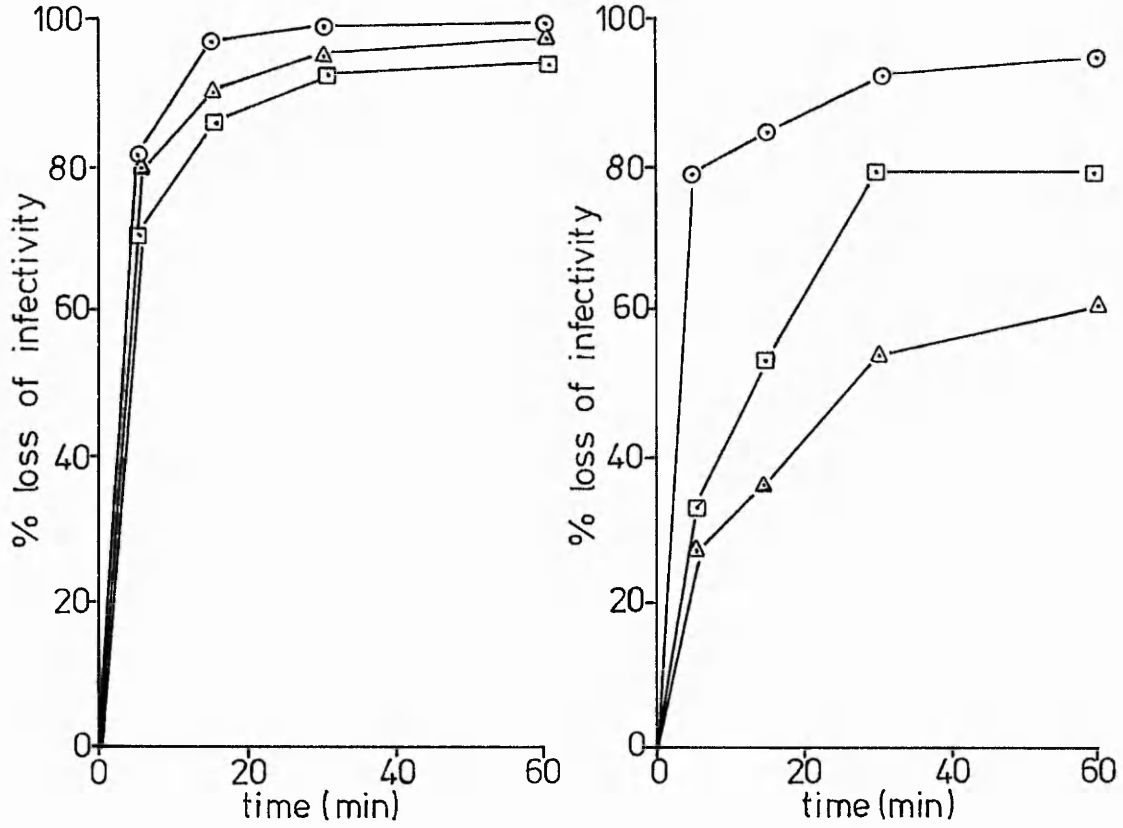
Figure 42. Effect of isopropanol on MS 2 inactivation.



The influence of the two alcohols on the hydration sphere of the virus was then examined by mixing phage with 50 % ethanol or isopropanol for 10 minutes at 5°C, and then pipetting 0.1 ml aliquots of this into 0.2 mM solutions of NaCl, $MgCl_2$, $CdCl_2$, $HgCl_2$ and $FeCl_3$, all at 5°C. These were mixed and sampled after 5, 15, 30 and 60 minutes, 0.1 ml of each being pipetted into 0.9 ml of 1.0 % peptone on ice. Plotting percentage loss against time, as in Figure 43, showed the influence of ethanol and isopropanol. It was clear that 50 % ethanol alone produced a 23 % loss in titre after 10 minutes, but with NaCl, $MgCl_2$ or $CdCl_2$ added the difference in inactivation rate between ethanol treated and untreated viruses quickly widened, so that after 60 minutes it was 34 %, 50 %, and 45 % respectively. As the ethanol was effectively diluted out of the reaction mixture, it could not have itself contributed to the increased loss. Therefore the theory of reduction in hydration sphere and conversion to a hydrophobic, sensitised particle was proven. This theory did not, however, appear to hold for $HgCl_2$ or the trivalent $FeCl_3$, but because of the formers proven toxicity and the latters increased valency this was to be expected.

Isopropanol, as Figure 43 showed, had exactly the opposite effect on metal inactivation as had ethanol. A 50 % solution over 10 minutes had no toxic influence on MS 2 alone, and for NaCl, $MgCl_2$ and $CdCl_2$ a greatly reduced loss of virus infectivity. As the molecular weight and configuration of alcohols increased, van der Waal forces between the hydro-

Figure 43. Effect of alcohol sensitisation on MS 2 inactivation by metal cations.

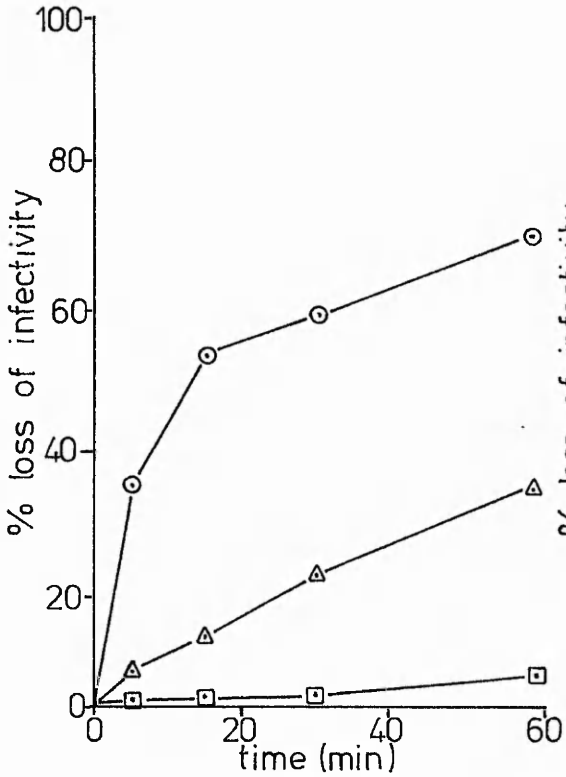


△—△ inactivation without alcohol pretreatment.

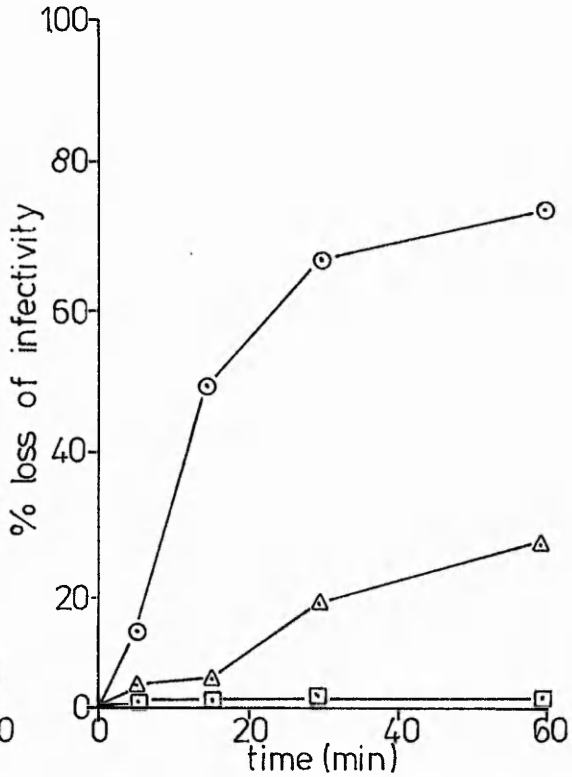
⊙—⊙ " after 50 % ethanol treatment.

⊡—⊡ " " " isopropanol "

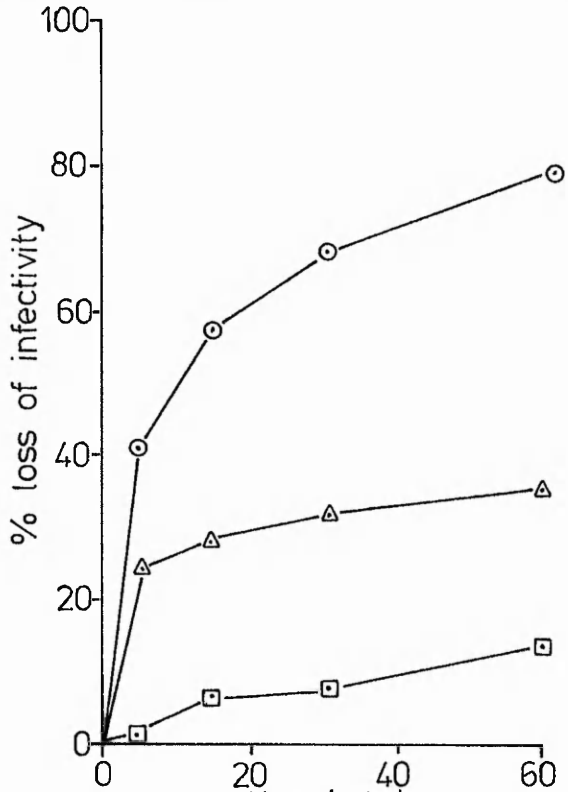
Figure 43. cont.



(c). sodium chloride



(d). magnesium chloride



(e). cadmium chloride

carbon portions became more effective in attracting the alcohol molecules to each other, rather than towards water molecules. Therefore the isopropanol, rather than depleting the sphere of hydration around each virus particle, would act to enhance it, thereby reducing the effect of the metal cations. This effect was also apparent for $HgCl_2$, although the proven toxicity of mercury was also seen in the high percentage loss of virus infectivity of isopropanol pretreated viruses. In the case of ferric iron, however, both alcohols resulted in a cation mediated loss of viruses greater than that achieved by metal ions alone, although ethanol pretreatment still caused increased inactivation over that in isopropanol. This was a result of the trivalent nature of the iron radical, as demonstrated in all previous experiments.

In summary, these studies showed that viruses in water exhibited many of the characteristics of classical colloids with particular respect to electrolyte action. Heavy metal inactivation followed the Hofmeister series for any given elemental group of cations, although anions did not have the same influence. Further, an increase in inactivation with increasing metal valence was noted, especially with Poliovirus 1. Two populations of phage were discovered from inactivation curves, with differing rates of resistance to metal action. These virus inactivations were found to be metal-concentration and temperature-dependent, and irreversible except by dialysis. Electron microscopy revealed that the

most probable cause of loss of virus titre was an inability to adsorb to the sex pilus of the host bacterium, as well as aggregation of the virus particles in solution.

DISCUSSION

To establish the waterborne virus exclusion efficiency of RO the virus load likely to serve as feed had to be determined. Thus a survey of the River Trent and some of its major tributaries was undertaken and the number of viruses estimated. However, the original combination of aluminium hydroxide concentration and subsequent $TCID_{50}$ assay used in this study proved to be most unsuitable for a number of reasons. The most significant of these was the concentration by $Al(OH)_3$ of non-viral cytotoxic agents from some of the waters, an effect not mentioned by any of the advocates of chemical flocculants for virus concentration. Information was however found on the use of $Al(OH)_3$ as a co-precipitator of heavy metals from water prior to their analysis. This showed that of the metals subsequently detected by STWA in aluminium hydroxide concentrated samples, iron, chromium and zinc were effectively concentrated to very high levels. Further, the STWA results demonstrated that lead, copper and manganese were also concentrated by this flocculant, so that both the cell lines employed for virus assay, and the viruses themselves, were subjected to greatly increased concentrations of six toxic heavy metals.

These findings explained the high cytotoxicity observed, which had made interpretation of the results extremely difficult. This meant that only qualitative demonstration of viruses was possible because of chemically induced damage and probably virus titre loss. However, evidence did suggest

their presence in waters from Colwick, Kegworth, Shardlow and Egginton during May to September 1973. No isolations were made from the River Derwent at Church Wilne during this period, suggesting either the viruses were absent, or that they were being removed or destroyed after their entry into the water.

The difficulties experienced in trying to ascertain the fate of viruses under these conditions, and also during RO treatment, necessitated the adoption of a term encompassing water toxicity, virus adsorption and inactivation, whether temporary or permanent. 'Loss of infectivity' (LOI) was chosen to cover all cases when the virus titre was reduced or lost, during laboratory studies or in field work.

The LOI of viruses in these waters was assessed by adding attenuated Poliovirus 1 to water from each main river sampling site , at a titre great enough to be detected without concentration. Virus loss in 24 hours varied from 33.3% in River Dove water to 97.2% in River Trent water from Colwick, the LOI following a close correlation with increasing BOD and suspended solids, and decreasing biotic index. Thus virus survival in the Trent system was seen to be closely related to water quality, most probably as a result of the levels of suspended solids and heavy metals found in these waters.

These results tended to confirm the findings of others (e.g. Wellington, et al, 1955), where coliform bacterial counts bore little relationship to viral contamination. This was concluded because bacterial survival was known to be proportional to the degree of pollution, whereas our results showed virus survival to be inversely proportional to that parameter.

Poynter, et al, 1973

Therefore in the Trent basin, at least, coliform counts should not serve as sole microbiological indicators of water quality.

Thus it was clearly demonstrated that enteric viruses were present in the Trent system and that their survival depended on water quality. It was also shown that a concentration technique devised for cleaner waters could not be successfully adapted to polluted conditions, and that greater effort was required to develop an alternative method. Similarly, although the $TCID_{50}$ assay was found to indicate the presence of chemical toxicants, it was inadequate for the assessment of viruses in river and RO concentrate waters, and it too was replaced. Thus the combination of aluminium hydroxide and $TCID_{50}$ was superceded by iron oxide and Dulbecco's plaque assay (Dulbecco and Vogt, 1954).

However, in the course of application of this latter assay several modifications were made. Wallis, et al (1966) had demonstrated improved plating efficiency by the addition of divalent cations, and so calcium chloride was added to a final concentration of 1.0%. The addition of $CaCl_2$ had also been shown to reduce virus inhibition and to encourage ECHOvirus plaque formation and so was added to sample dilution media prior to assay.

Secondly, Difco Bacto-agar was substituted for the Noble agar originally suggested by Dulbecco, because Wallis, et al (1966) showed that the latter contained virus inhibiting polysaccharides.

Furthermore, as both cells and enteric viruses were known to be photosensitive to neutral red (Wilson and Cooper, 1963: Wallis, et al, 1962) application of this vital stain was delayed until after virus/cell incubation, in contrast with the original technique. Incubation with the stain for a maximum of five hours in the dark at 37°C allowed for good penetration of living cells and the visualisation of the virus plaques.

As an alternative assay technique the plaque overlay method proposed by Cooper (1967) was attempted, but with little success. This method involved the incorporation of a virus-cell suspension into nutrient rich molten agar, this then being pipetted into a flat bottle or Petri-plate containing a second, preset , nutrient rich agar base layer. No cell monolayer was involved, the assay being analogous to that for bacteriophage, and a higher plating efficiency than Dulbecco's technique was claimed. It was found, however, that the high cell concentration required could not be economically or easily obtained for routine use.

Cooper also advocated the replacement of neutral red with tetrazolium salts, particularly 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT), but this compound failed to adequately stain either cell suspensions or monolayers under the conditions imposed in this laboratory.

The replacement of aluminium hydroxide by iron oxide in the virus concentration stage required considerable laboratory study to establish the parameters for processing large volumes

of water. The work centred around the use of an iron oxide suspension, prepared from a concentrated slurry, rather than an oxide packed column because the latter was prone to blockage and even under optimum conditions would permit the passage of a small volume of water only.

Adsorption of viruses to iron oxide was found to be very rapid, with a 60 to 90 % reduction in titre from the assayed supernatant in five minutes, and maximum reduction within twenty to thirty minutes. This finding was in total agreement with reports on virus adsorption to other inorganic surfaces, as reviewed by Bitton (1975). Temperature was found to have little influence on the process over the range of 6°C to 37°C, but pH clearly affected adsorption, greater adsorption occuring with increasing pH, up to the maximum examined pH of 7.6, where 98% removal was obtained. This was in agreement with Larin and Gallimore (1971), who showed that a pH range of 6.8 to 7.3 was most favourable for Myxovirus adsorption, but disagreed with the findings of Bitton, et al (1976), who declared that Poliovirus adsorption did not vary significantly when the pH varied from 5.0 to 9.0. However, as all the rivers had pH's in the ranges 7.5 to 8.0, it was felt that the system would concentrate viruses from these waters without the need for pH adjustment.

The extent of virus adsorption was shown to be oxide dependent, and to conform to the Freundlich isotherm, which suggested that adsorption was physical in nature, relying on van der Waal type bonding. Adsorption was also greatly

enhanced by the addition of metallic cations, presumably because both MS 2 and the iron oxide originally had isoelectric points that made them negatively charged at or near neutrality (Irwin (1977) for the isoelectric point of MS 2 and Iwasaki, et al (1962) for that of iron oxide). Each would therefore tend to repel the other unless positive counterions were added to neutralise the repulsion. As will be explained later, the concentration and valency of these ions greatly influenced adsorption efficiency and it was determined that aluminium chloride at 0.02 mM would be the most effective, particularly as this also proved to be the best salt for virus elution.

Elution from the iron oxide was facilitated by the use of the soluble meat hydrolysate, lab-lemco, at a concentration of 1.0 % and a pH of 9.0. This proteinaceous solution successfully competed with viruses for the adsorption sites on the oxide surface, whilst also reducing readsorption efficiency by removing any excess aluminium ions from solution.

Thus a reliable virus concentration method was established, with 99 % adsorption and 85 % recovery but only from small volumes with relatively large numbers of viruses. In order to scale up to a process capable of detecting small numbers of viruses in much larger volumes of water, it was necessary to devise new methods for harvesting the virus-cation-iron oxide complex.

The first of these involved the filtration of oxide-containing water through Whatman GF/A filter papers, but neither scraping of surface-deposited solids nor maceration of whole filters in eluent allowed efficient virus recovery. It was also clear that larger volumes of eluent were required when maceration was employed, thus reducing the recovery efficiency.

Electromagnetic separation was found to be a preferable method of harvesting iron oxide and with a magnetic depth filter and increased elution time, recoveries comparable with initial small scale experiments were obtained. The original spiral coil collector was found to be very ineffective because of the amount of oxide retained within it, and its inability to collect oxide at flow rates greater than 50 to 100 ml min^{-1}. The depth filter, however, consisting of a magnetised steel wool matrix, not only allowed up to 88 % recovery of influent viruses, it also lowered river suspended solids and turbidity. As viruses were known to be adsorbed to suspended solids, this obviously improved the chances of collecting both free and bound virus particles.

Using iron oxide adsorption and monolayer plaque assay, quantitative and reliable determination of the viral conditions at the river sampling sites could be made, and these clearly showed a seasonal variation in virus numbers during 1975, with peaks in June and July. Unlike the previous assessment period, viruses were isolated more frequently from all river sites, at numbers up to 2.4 pfu l^{-1}, and a correlation was

noted with improved river quality between 1973 and 1975. (STWA, 1976) This indicated that although desirable, the steady improvement in water quality could result in increased virus survival.

During 1975 the effluent from the main Nottingham sewage works, at Stoke Bardolph, was also sampled, with maximum recorded recovery titres of 26 pfu l^{-1}. The ten fold difference in virus numbers between this and river values could be explained on the basis of dilution alone, the flow of the River Trent in this region being 5206 Ml d^{-1}, whilst that of the effluent stream entering the river was 153 Ml d^{-1} (STWA, 1976). Differences in flows of these magnitudes were also noted for other sewage works effluents throughout the sampling area, suggesting that the same conclusions would apply. These observations, and the known survival rates of enteric viruses in river water, especially in warmer months, supported the idea that there was probably little viral LOI in river water over the distance involved within the sampling area, but rather that virus-solids association occured, with retention of an assayable titre.

It was unfortunate that the Stoke Bardolph effluent stream entered the River Trent downstream of the furthest sampling site (Colwick), but it was felt that this water reclamation works produced effluents typical of the region. Indeed, when compared with other sewage works in the STWA region, Stoke Bardolph produced a good quality effluent, officially described as 'satisfactory' (STWA, 1976), with,

presumably, fewer virus numbers. Therefore it could be assumed that the numbers of viruses entering the river system above sampling sites, and the dilution rates, would be similar.

The above explanation seemed to be contradicted by the laboratory findings on virus survival, conducted on each river water type. However, whilst these tests were useful in determining relative water quality, they were to some extent artificial, using high virus loads and a single inoculum, instead of the low titre semi-continuous loading experienced in the rivers. In addition, with the exception of Colwick, LOI's were only in the region of 30 to 60 % over 24 hours, and these would not be noticeable at the low initial virus numbers experienced. These figures would, however, partially explain the failure to isolate viruses on some occasions, when they might have been expected.

Viruses were known to become solids bound in water and the electromagnet was shown to be capable of removing river suspended solids as well as added iron oxide. Therefore it was clear that the higher numbers of viruses demonstrated during 1975 were a result of the collection of these particle bound viruses, as well as the improved water quality.

Thus it was shown that iron oxide adsorption was capable of recovering viruses from polluted surface waters, and that the earlier failture to quantitatively assess the viral status of the River Trent system was due to the use of an inadequate concentration technique, designed for better quality waters.

This suggested that the physio-chemical state of a water body should be adequately determined before selecting a virus isolation procedure, and that the ability of the concentration method to recover waterborne chemicals should be known or ascertained.

The river survey results showed that with a maximum expected number of naturally occuring enteric viruses of 2.4 pfu l^{-1}, assays of the efficiency of virus removal by any water treatment process would be extremely difficult, if not impossible. Therefore for the work involving all three RO units, artificially high loadings, either as low volume/high titre 'slugs', or as larger volume/lower titre continuous feeds were used. The resmlts from early studies, contemporary to the original river surveys, reinforced the need for a concentration and assay technique change and the plaque assay was quickly substituted for $TCID_{50}$ analysis. However, the iron oxide concentration procedure was not at this time sufficiently developed for application to larger water volumes. Therefore, although water concentrate samples were assayed without virus concentration, influent water and RO permeate were subjected to $Al(OH)_3$ treatment. Permeate water, free from chemical contaminants, failed to produce CPE on tissues, and this was taken as an indication that virus penetration of the membrane had not occured. Concentrate water samples from the B type tubular rod unit, after 'slug' virus inoculation, showed the diminishing cyclic recovery of viruses, peaking at 5.0 to 5.5 minute intervals, characteristic of the water recirculation pattern associated with the machine's operational mode.

Experiments were repeated with the two spaghetti rod, R type units and again cyclic virus recovery from concentrate was demonstrated. However, in these cases, peaks of recovery were obtained at approximately two minute intervals, reflecting the faster passage time and recirculation rates of these machines. The virus rejection ratio, that is the measure of the membrane exclusion efficiency, of the R type units was shown to be in excess of 99.999+ %, at an applied pressure of 20 bar. It was demonstrated, however, that this ratio was pressure dependent, because an applied pressure of 40 bar resulted in virus penetration of the membrane with their subsequent detection in permeate water. The resulting rejection ratio of 99.985 % illustrated, however, that even at elevated pressures the system was still extremely efficient at virus exclusion.

Continuous loading of viruses was performed because it was felt that this would remove the anomalies associated with single high titre virus inoculations, in addition to more accurately simulating virus epidemic conditions. Therefore MS 2 was added to a RO unit at a continuous dose rate of 8.5×10^4 pfu ml^{-1} of influent water. They were subsequently detected in the concentrate stream at a constant mean titre of 9.0×10^4 pfu ml^{-1}, suggesting that all the added viruses were recovered. However, the virus titre should have increased because they were all concentrated into one quarter of the inlet flow volume, the other three quarters being virus free permeate. Therefore, with only 26.83 % of the added viruses

recovered, the evidence for physiochemical viral LOI suggested by single inoculum runs was enhanced.

At Budd's Farm the two emergency units, to be used in the event of membrane failure, were also briefly examined for virus removal efficiency. Maximum adsorption onto activated carbon was 57.0 % at pH 5.5 and 20°C, the lack of effective removal being due to the surface configuration on the carbon, which allowed virus penetration of only the macroporous structure, leading to weak van der Waal type bonding. The viruses could not penetrate into the 10 to 30 Å diameter micropores that made activated carbon such an efficient adsorbent for small molecules and therefore effective virus removal was not possible. There was a similar low efficiency of removal with the two anthracite depth filters, where only 35.0 % of the influent phage were adsorbed.

Ozonisation, the other water sterilisation technique assessed proved to be very efficient at virus inactivation, producing a 99.9+ % reduction of titre within 14 minutes. This suggested that if a method of virus removal were required to assure permeate water sterility, then ozone treatment would be far superior to activated carbon.

The data obtained from the experiments conducted with the three RO units suggested that a probable combination of viral aggregation and/or adsorption, leading to LOI, were occuring when high titres were employed. The LOI would yield an apparently lower titre than the actual number present and thus artificially raise the calculation removal efficiency.

It was noted that low pH, coupled with high salt concentration and an absence of chelating agents, as would be found in RO concentrate water during normal machine operation, favoured viral aggregation and capsid protein solubility. At higher pH's, however, especially in the presence of metal cations, virus adsorption to suspended solids would be more probable. In either case, the fate of viruses during RO treatment would be dependent on water pH.

With correct acid dosing to pH 5.0 to 5.5, for optimum membrane performance, only one peak of rejected viruses was produced, suggesting total loss of titre within four minutes. With inadequate acid dosing, however, resulting in higher pH's closer to those of the raw water, multiple peaks of virus survival resulted, even though MS 2, the test virus, was stable across the range of pH 4.0 to 8.0+. These results therefore supported the hypothesis of viral aggregation at pH's close to 5.0, aided by the presence of heavy metal cations concentrated by the process.

Metals are employed in a number of industries (Table 37) and many are commonly found in the River Trent, the major source of them being from the River Tame (Garland, 1972). In water they exist in the form of multinuclear hydroxo and oxo complexes which are readily adsorbed to solid-liquid surfaces, such as muds and clays (Gardiner, 1974: Helz, et al, 1975) and sewage solids (Swanwick, et al, 1973). They are not appreciably complexed in natural waters by inorganic ligands other than H_2O and -OH because most of these are

Table 37. Industrial Distribution of Heavy Metals (Modified after Burrell, 1974)

	Ag	Al	As	Au	Be	Bi	Cd	Co	Cr	Cu	Fe	Hg	In	Mg	Mn	Mo	Ni	Pb	Sb	Se	Sn	Ti	Zn
General Industrial		x			x	x	x	x	x	x	x		x	x	x	x	x	x			x		x
Plating							x		x	x							x	x			x		x
Paint Products									x						x			x	x			x	
Fertilisers							x		x	x	x	x			x		x	x					x
Pesticides			x							x		x											
Tanning			x						x														
Paper Products									x	x		x					x	x				x	x
Photographic	x								x														
Painting/Dyeing									x			x						x					
Electrical/Electronics	x			x						x			x	x		x				x			
Cooling Water									x														
Pipes										x								x					
Glass/Ceramics			x									x											x
Batteries					x		x											x	x				
Medical				x	x	x	x										x	x					

unidentate, with only one atom responsible for binding. Organic chelating ligands, on the other hand, form very stable complexes with a wide range of heavy metals, even when only present in trace amounts.

The enhancing role of metal cations, and in particular that of divalent calcium and magnesium, is well documented in the cases of virus adsorption to host cells, membranes and all types of particulates, as well as in the stabilisation of Enterovirus to heat inactivation. Virus adsorption is usually reversible, but this was found not to be the case with RO water containing solids. It was therefore reasoned that virus aggregation as a result of heavy metal action, was the cause of this irreversible LOI. Evidence for the toxic nature of RO water was accumulated from a number of sources, including a comparison of water from both R type units with River Trent water from Colwick. All these waters were pretreated by two alternative methods, either by autoclaving at 121^{o}C for 15 minutes, or by filtration through 45 µm porosity Millipore membrane filters. Autoclaving was thought to chelate, and hence remove, metals and soluble organic species, whilst leaving suspended solids for adsorption. Membrane filtration, on the other hand, would remove all particulates, leaving soluble toxicants in solution. It was discovered that viruses survived longer in filtered river water than in autoclaved water indicating that they were normally adsorbed to particulates in suspension, . In RO water from both units, however, a greater loss of virus titre occured in filtered

water, suggesting that the soluble fraction of the water was responsible for the LOI.

It was found that RO water exerted a toxic influence over tissue culture cells, and that this cytotoxicity could be prevented by the addition of soluble protein, suggesting protein-toxicant interaction and neutralisation. Further, the addition of cooled nutrient broth to MS 2 in RO water arrested, and partially reversed the rate of inactivation, although this could equally have been due to toxicant dilution rather than removal by complexation.

Finally it was found that repeated washing of sediment from the Colwick unit over 72 hours, resulted in the leaching of toxicants into solution. As a result the supernatant from the longest washed sample showed the greatest antiviral activity, whilst the final sediment possessed the least.

These findings were confirmed by examination of the viricidal action of membrane adhering particles from the Budd's Farm unit. When resuspended in distilled water these particles produced total viral LOI in less than 50 minutes. The separate supernatant fraction, however, caused the same percentage loss in only half the time, showing that particle bound toxicants had leached into solution and that, as above, particle free solutions from RO waters possessed a greater viricidal action.

The action of selected heavy metals on virus infectivity was further investigated and it was found that the form of the inactivation curves were due to the presence of two virus populations, an observation confirmed by electrophoresis and

density gradient centrifugal separation. These two populations, also noted during pH inactivation studies in buffers and RO concentrate, had different sedimentation rates but the same amounts of RNA and capsid protein. They must therefore have had different structural configuration, the lighter particle having an expanded structure when compared with the heavier one. Similar observations have been made on Poliovirus (Rowlands, et al, 1975), explaining why all these viruses had two isoelectric points. It was therefore concluded that the lighter particles, with an open structure, would be more susceptible to heavy metal interactions. Consequently they would be quickly inactivated and form the first portion of the inactivation curve. The more compact, denser particles would be more resistant and, with a lower rate of inactivation would form the second portion of each curve.

It was also found that LOI was concentration and temperature dependent for monovalent and most divalent cations added, suggesting chemical interactions. However, for divalent mercury and lead, and all trivalent cations, virus LOI was largely independent of temperature and concentration effects, illustrating the greater aggregative ability of the latter metals.

Furthermore it was noted that percentage virus losses, in addition to following the series of increasing effectiveness of monovalent < divalent < trivalent cations, could also be arranged into series of increasing loss corresponding to the groups of the periodic table.

Finally, attempts to recover this chemically induced LOI met with limited and varying success, although dialysis did yield a reduction of virus inhibiting factors in both RO concentrates and metal cation solutions.

These results, it was found, could best be explained by reference to classical colloid chemistry, and in order to understand further the mechanisms involved, it was necessary to appreciate first the nature of the surface phenomena involved.

The Enteroviruses and coliphages of the MS 2 type could be considered as hydrophilic (lyophilic) colloidal sols, because their capsid structure had a high affinity for water, and contained $-COO^-$, $-NH_3^+$, $-(RO)_2POO^-$, secondary alcoholic -OH, -SH and phenolic -OH groupings which would be fully ionised, and usually overall negative, at neutrality. Similarly, water suspended solids, clays, organic pollutants and insoluble oxides such as iron oxide, would possess a surface charge and a negative surface potential at or near neutral pH. Viruses would therefore tend to repel each other and, in turn, be repelled by suspended species in water.

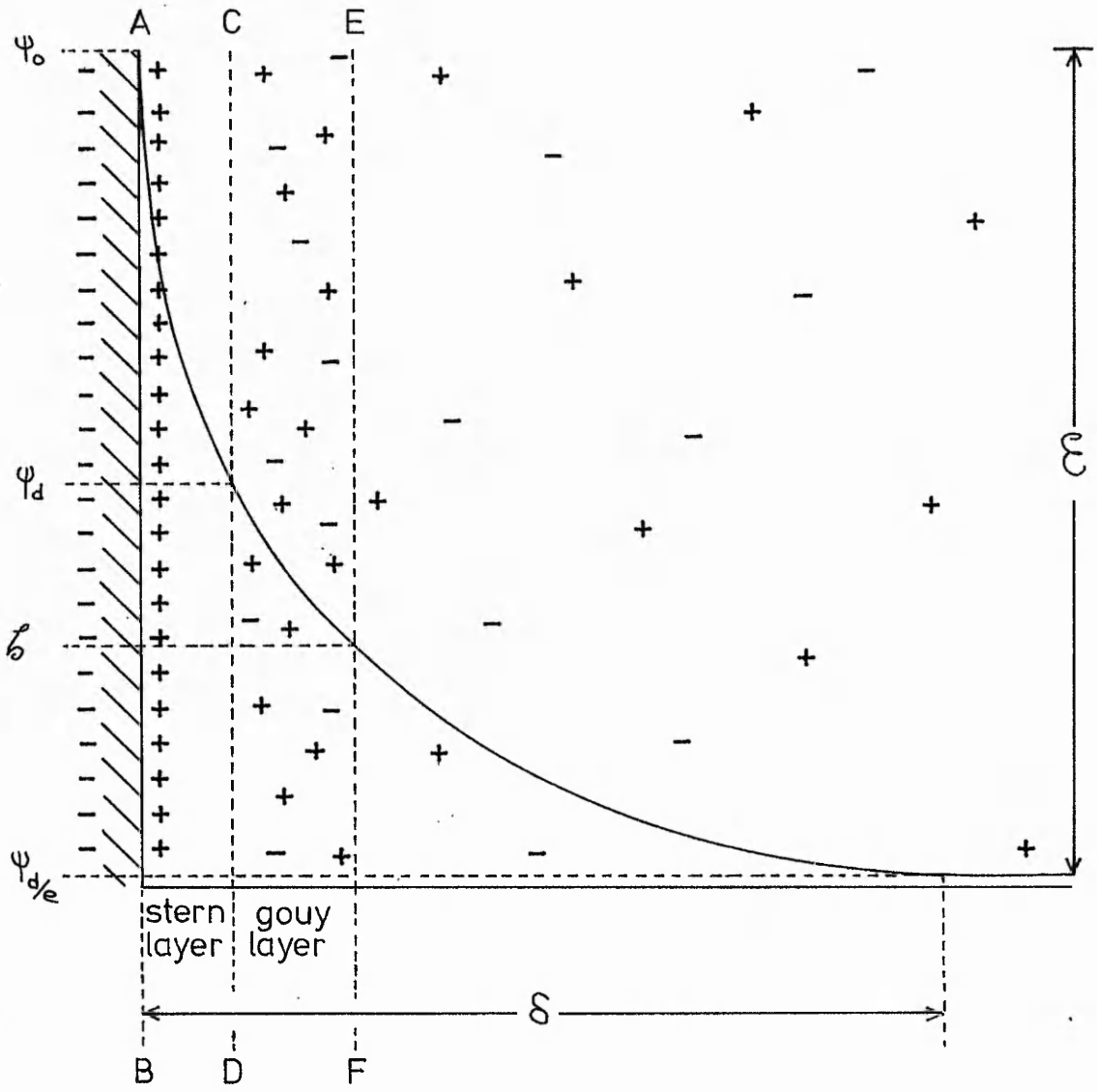
The electrical composition of a colloid surface is determined by two potentials, the electrochemical potential (ε) and the electrokinetic potential (ζ). The charge at the surface, and random thermal agitation of the particle in suspension creates a diffuse double electrical layer, the Gouy-Chapman double layer, the potential within which decreases gradually from the surface to infinity. Decreasing potential is due to decreasing ion concentration and falls at first

rapidly and then more slowly, intensity decreasing with the square of the distance from the centre of the sphere. There is no defined end point to this decreasing potential, but rather it slowly becomes negligible.

The actual composition of this potential is very complex, but has been simplified to present a double layer structure with a definite thickness (δ) and with all the counterions neutralising the surface charging ions in one plane, this being the Helmholtz double layer model (Figure 44). This model therefore represents the state of a lyophilic virus particle in distilled water, in the absence of metal cations.

The capsid of MS 2 is a highly polarised single protein species with negative charges on the outer surface and positive ones on the inside neutralising the acidic phosphate backbone of the RNA (Matthews and Cole, 1972). There are no covalent bonds holding the protein capsomeres together, only hydrophobic forces, and the two cysteine and two methionine residues in each coat monomer are exposed on the surface, resulting in a lack of sulphydryl bonds between coat protein molecules (Thomas, et al, 1976). Poliovirus has a similar general structure, with the important differences that the virion has four distinct coat protein types, and no A protein, responsible in MS 2 for initiating replication. However, two of the surface proteins of Poliovirus carry the attachment sites for binding to the receptors of susceptible cells, and so are analogous to the A protein (Crowell and Philipson, 1971).

Figure 44. Representation of Helmholtz double layer model.



ψ_o = total potential at surface of particle (A-B).

ψ_d = potential at boundary of Stern layer (C-D).

ζ = electrokinetic potential at boundary between solvent adhering to particle and that moving with respect to it.

E-F = boundary of Gouy layer.

$\mathcal{E}$ = electrochemical potential = ψ_o

δ = defined thickness of Helmholtz model.

As each amino acid residue of the coat protein would have a characteristic isoelectric point, with most between 4.3 and 6.3, the solvent pH would play an important role in the action of any counterion (that is one having an opposite charge to that of the surface layer). The overall isoelectric points of MS 2 and Poliovirus have been shown to be 4.5 and 7.0 for MS 2 and 4.5 and 7.1 for Poliovirus. Therefore at the pH's close to neutrality the surface charges of both viruses would be overall negative.

Any two colloidal particles plus their double layers would be electrically neutral at greater distances, but as they approached the double layers would interact and the surface layers would tend to repel each other. On the addition of an electrolyte no specific interaction would result just an increase in the number of counterions and similiions in the bulk solution. As a consequence the thickness of the double layer (δ) would be decreased, ψ_0 would remain constant, but ψ_δ would decrease with a reduction in ζ , resulting in aggregation of the particles by short range electrostatic forces. Increasing the number of counterions would lead to a greater compaction of δ , and greater aggregation, as occured during iron oxide concentration of viruses. In this example an increase in metal chloride concentration resulted in increased virus adsorption. Similarly, an increase in metal concentration caused greater loss of virus titre in a particle free solution, with a range of cations, by reduction of repulsive forces and consequent virus aggregation.

Secondly, increasing the counterion charge would cause a double layer condensation, allowing attractive forces to greatly increase. Adsorption under these conditions would therefore be greatly favoured, as shown by the increased virus adsorption efficiency of trivalent aluminium chloride over divalent magnesium or calcium chloride, for equivalent concentrations of salts. This trend was also noted for the two test viruses in metal cation solutions, with the establishment of an ascending series of virus infectivity inhibition of monovalent < divalent < trivalent cations as described.

The finding that ions of the same sign and valency often produced different viral LOI, resulted from difference in the depression of ζ, proportional to the ionic specific volume, due to increased polarizability and decreased hydration with increased ionic volume. Therefore, ions could be arranged into groups of increasing effectiveness, as in the Hofmeister or lyotropic series. For a given elemental group the degree of effectiveness increased with increasing atomic size, and this was noted for both viruses, in the ascending series Li < Na < K < Cs, Mg < Ca < Ba and Zn < Cd < Hg, as described. Similar series had been noted during shell formation of f 2, a close seriological relation of MS 2 (Matthews and Cole, 1972), although in this case the distinctions within each group were not as clear.

The anionic species added with each cation had no influence over viral aggregation, although it would have been expected to produce an initial rise in ζ, due to anionic adsorption at low concentrations. This adsorption was caused by an

unsaturated immobile surface layer of ions, and resulted in a transfer of counterions into the diffuse layer, thereby increasing ζ and δ, and expanding the hydration sphere. However, once the adsorbed ionic layer was saturated, an increase in the concentration of electrolyte would cause double layer contraction, a lowering of ζ, and the previously recorded cationic effects. Thus it was shown that chloride, sulphate, nitrate and iodide radicals had no effect on the loss of virus titre induced by metal cations.

The predictable effects of metal cations on viruses were subsequently used to demonstrate the hydrophilic nature of the phage MS 2, using the two alcohols ethanol and isopropanol. The solvation of a hydrophilic virus particle would play an important role in particle stability, and the removal of this stabilising hydration sphere by a dehydrating agent, such as alcohol or acetone, would make the system sensitive to small amounts of electrolyte. Ethanol pretreatment of the viruses resulted in an increase in metal cation mediated antiviral activity, indicating that hydrophilic MS 2 particles could be sensitised and made hydrophobic. On the other hand, isopropanol pretreatment resulted in increased virus survival, when compared with both ethanol pretreatment, and non pretreated virus experiments. This was due to the increased inert alkyl group on each isopropanol molecule, which would act as a drag on the active hydroxyl group participating in hydrogen bonding with water molecules. These alkyl groups would also tend to attract the alcohol molecules towards each

other, therefore offsetting the effects of hydrogen bonding.

Electron microscopic examination of heavy metal treated viruses showed that viral aggregation occured, but that there was little or no virus adsorption to bacterial f pili. This explained the lack of virus titre during assay and showed that, as well as surface protein aggregation A protein function must also have been impaired.

The structure of R 17, a close serological relative of MS 2 has been reported to consist of a network of protein arising from twenty doughnut shaped nonagons, with the A protein ionically linked to the RNA, and protruding through the capsid surface at one of the empty spaces at each of the twelve vertices of the icosahedron (O'Callaghan, et al, 1973). This structure, if assumed for MS 2, would allow not only surface adsorption of metal cations but also penetration of metals into the virus in order to inactivate both the A protein and RNA. This structural model would explain the LOI demonstrated for MS 2 and also the observed lack of f pilus adsorption during electron microscopic examination.

Thus it was concluded that the loss of hydrophilic viruses during RO treatment of polluted surface waters was due to a pH dependent, heavy metal induced, interviral aggregation with possible nucleic acid inactivation. The resulting complex from such an action might or might not enter into adsorptive partnership with suspended solids present in the same concentrate water. This was less likely to occur in rivers, however, because of the lower concentrations of

both viruses and metal ions, whereas adsorption to the abundant suspended solids was known to be common.

The majority of metal ions, under the conditions imposed by RO, would act as astringents, precipitating coat proteins, whilst mercury would act more specifically with sulphydryl groups present only on the surface. However all actions would result in coat and A protein aggregation leading to loss of viral infectivity, although this loss was shown to be partially reversible by dialysis.

Although the majority of the investigations were performed using coliphage MS 2, sufficient duplications of experiments were performed with Poliovirus 1 to predict that the above observations and conclusions would hold for the Enterovirus group during RO treatment of polluted surface water. Therefore, although very efficient exclusion of Enteroviruses by RO could be predicted, it was clear that concentrate water would still contain increased levels of potentially pathogenic viruses. Therefore further disinfection of concentrates from virus contaminated surface waters might be necessary, prior to utilisation or disposal.

CONCLUSIONS

Aluminium hydroxide flocculation of samples from surface waters was found to concentrate not only viruses but also toxic compounds, most notably metal cations, making it a most unsuitable method for virus recovery from such waters.

We would conclude therefore that prior to a full virus survey being conducted the physicochemical state of the water should be determined. Additionally the ability of the concentration method to assimilate chemicals that might influence either the viruses or their assay should be measured.

Iron oxide adsorption was found to be a superior virus concentration technique, with up to 99.0 % adsorption of Enteroviruses and coliphage MS 2 under controlled conditions. Adsorption conformed to the Freundlich adsorption isotherm, was pH dependent, but only marginally temperature dependent, and was enhanced by the addition of trivalent aluminium cations. It was also shown that the degree of toxic metal concentration was considerably less than with an alum floc.

Elution of viruses from iron oxide was best achieved with a 1.0 % solution of lab-lemco, pH 9.0, resulting in a maximum recovery of 87.46 % of the added viruses, and a volume concentration from 20 litres of 1000 fold.

Electromagnetic separation using a steel wool depth filter was the most successful method of oxide recovery, being safe, clean and efficient. This method proved capable of recovering water borne suspended solids as well as iron

oxide particles, and must therefore have aided overall recovery by collecting both free and bound viruses.

The $TCID_{50}$ virus assay method was shown to be inadequate for enteric virus assay, after $Al(OH)_3$ concentration, and was therefore replaced by a modified plaque assay.

The iron oxide/plaque assay combination showed that viruses could be detected in all rivers examined on a seasonal basis. One sewage effluent stream was also examined at this time and shown to contain up to ten times as many viruses as river water. Examination of the effluent and river flows showed that this ten fold difference in virus numbers could be accounted for by dilution alone, and it was suggested that adsorption to suspended solids was the main fate of viruses under these conditions.

The overall increase in isolations between 1973 and 1975 suggested that improvements in river quality could lead to increased virus survival. The recovery patterns also made it evident that conclusions drawn from bacterial coliform counts could not be extrapolated to include viruses. Thus it would be advised that coliform counts should not be used as the sole microbiological indicator of water quality, certainly in the River Trent system.

The low numbers of viruses present in river water, detected at up to 2.4 pfu l^{-1}, necessitated the artificial loading of RO units with high titres of viruses, to increase the likelihood of virus penetration of the membranes and to ensure adequate numbers for assay. Single dose, high titre

inocula resulted in cyclic recovery of viruses in concentrate water, in accordance with recirculation rates, and in artificially high levels of pretreatment virus aggregation that produced anomalous results. It was therefore concluded that a more realistic evaluation of the behaviour of virus particles during RO would be achieved by a lower titre, continuous dosing regime and this was also performed.

Under normal operational conditions, no viruses were detected in permeate water, and an exclusion efficiency in excess of 99.999 % was determined, calculated within the limitations of the concentration and assay techniques. At an elevated applied pressure of 40 bar viruses were detected in the permeate water, although it was not clear whether this was due to membrane penetration or leakage around seals. However, even under these conditions 99.99 % exclusion was achieved, and so RO was seen to be an effective method of virus exclusion during treatment of surface waters.

It was noted that not all of the applied virus load could be recovered in the concentrate stream and it was shown that the recirculation water pH played an important role in viral LOI, virus survival decreasing with increasing acidity. This did not account for all of the observed LOI however, and it was thought possible that adsorption, aggregation and/or inactivation could also be responsible. The evidence from all of the experiments involving RO concentrate water suggested that a chemically mediated virus inactivation, probably as a result of particle aggregation, had occurred. Adsorption,

because of its relative temperature independence, was not thought to be a major influence on LOI although it was recognised that it would account for some virus loss. Virus titre was also found to be recoverable after adsorption as in the case of iron oxide, but this was not shown to be true for RO water or solids.

It was therefore proposed that during normal RO operation aggregation by low pH and high electrolyte (metal) concentration was the principal cause of viral LOI in the treatment of polluted surface waters. Electron microscopic examination showed this to be true for metal cations, resulting in partial or complete failure to adsorb to bacterial f pili, in the case of MS 2.

Virus-metal interaction was best explained in terms of colloid chemistry, LOI being concentration and valency dependent and following the Hofmeister concept for periodic series. Partial recovery of titre was possible by dialysis and the anionic species was shown to have little influence on virus loss.

The suggested structural configuration for MS 2-like phage would allow metal interference with coat protein, A protein and RNA, and it was suggested that the results could be extrapolated to include enteric human viruses.

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APPENDIX 1

Media and biochemical preparations

"Analar" grade chemicals were used wherever possible in all preparations.

Tissue culture media

(a) Growth medium for Green Monkey Kidney (Vero) cells

Sterile distilled, deionised water	90.0 ml
Medium 199, x 10, with Earles salts	10.0 ml
Foetal calf serum	5.0 ml
Penicillin/Streptomycin (1000 units ml^{-1})	1.0 ml
Sodium bicarbonate (7.5 % solution)	2.0 ml

(b) Maintenance medium for Vero cells

As above except 0.5 ml instead of 5.0 ml of foetal calf serum.

(c) Growth medium for HeLa and HEp 2 cells

Sterile distilled, deionised water	90.0 ml
Medium MEM, x 10, with Earles salts	10.0 ml
Newborn calf serum	10.0 ml
Glutamine (200 mM)	1.0 ml
Penicillin/Streptomycin (1000 units ml^{-1})	1.0 ml
Sodium bicarbonate (7.5 % solution)	2.0 ml

(d) Maintenance medium for HeLa and HEp 2 cells

As above except 2.0 ml instead of 10.0 ml of newborn calf serum.

(e) Trypsin solution

Sterile distilled, deionised water	90.0 ml
Hank's basal salts solution, x 10	10.0 ml
Trypsin (2.5 % solution)	10.0 ml
Sodium bicarbonate (7.5 % solution)	2.0 ml

(f) Phosphate buffered saline (Dulbecco's A)

This was purchased from Oxoid Ltd. in tablet form (BR 14A). One tablet was dissolved in 100 ml distilled, deionised water and autoclaved at 121°C for 15 minutes.

Monolayer plaque assay media

(a) Agar

Difco Bacto-agar was purchased from Difco Ltd. in 500 g amounts, washed three times in distilled water and then twice with acetone. It was then dried at 37°C on filter paper and ground in a pestle and mortar. The resulting powder was stored in an air tight tin until required, at which time 1.5 g amounts were dissolved in 50 ml aliquots of distilled, deionised water and sterilised by autoclaving at 121°C for 15 minutes.

(b) Vero assay media

(i) Sample dilution medium

Distilled, deionised water	90.0 ml
Medium 199, x 10, with Earles salts	10.0 ml
Foetal calf serum	0.5 ml
Bovine serum	1.0 ml
Penicillin/Streptomycin (1000 units ml^{-1})	1.0 ml
Sodium bicarbonate (7.5 % solution)	2.0 ml
$CaCl_2.6H_2O$ (10.0 %, membrane filtered)	10.0 ml

(ii) Overlay medium

Distilled, deionised water	40.0 ml
Medium 199, x 10, with Earles salts	10.0 ml
Foetal calf serum	0.5 ml
Bovine serum	1.5 ml
Penicillin/Streptomycin (1000 units ml^{-1})	1.0 ml
Sodium bicarbonate (7.5 % solution)	2.0 ml

(iii) Neutral red stain

Distilled, deionised water	90.0 ml
Hank's basal salts solution, x 10	10.0 ml
Neutral red (0.1 %, membrane filtered)	10.0 ml
Sodium bicarbonate (7.5 % solution)	0.5 ml

Stain was prepared on the day of use and stored at $4^{\circ}C$ until required.

(c) Media for HEp 2 assay

Media used were the same as for Vero cells except for substituting medium MEM, x 10, with Earles salts for medium 199, x 10, with Earles salts, and newborn calf serum for foetal calf serum.

Bacteria and bacteriophage media

Blood agar base was purchased from Oxoid Ltd. as dehydrated medium (CM 55) and prepared as directed, in glass distilled water, as were nutrient broth (CM 1) and Ringer's solution (Br 52). Soft agar was prepared as a 0.6 % suspension of Oxoid Ion agar No. 3 (L 13) in distilled water with 1.0 % Oxoid bacteriological peptone (L 37) added, pH adjusted to 7.0, and sterilised by autoclaving at $121^{\circ}C$ for 15 minutes.

(a) Medium A for liquid culture of coliphage MS 2

(i) Basal salts solution

Na_2HPO_4	7.0 g
KH_2PO_4	3.0 g
NH_4Cl	0.5 g
Distilled water	1000.0 ml

(ii) Supplements

30.0 % glycerol in 0.5 M $MgSO_4$,	10.0 ml
12.0% Casamino-acids (Difco)	50.0 ml
0.5 M $CaCl_2$	2.0 ml

Salts and supplements were prepared and autoclaved at 121°C for 15 minutes, with the exceptions of glycerol and $CaCl_2$ which were filter sterilised. All ingredients were stored at 4°C and mixed when required.

(b) Lysing solution for coliphage concentration

Lysozyme (Sigma)	10.0 mg
1.0 M EDTA, pH 8.0 (BDH)	1.0 ml
1.0 M Tris, pH 8.0 (Sigma)	96.5 ml
Distilled water to make a total volume of	100.0 ml

Preparation of aluminium hydroxide

To 1.0 litre of distilled water in a beaker in a fume cupboard was added 3.334 g anhydrous aluminium chloride, with stirring at high speed on a Chilton magnetic stirrer.

To 30.0 ml distilled water was added 7.0 g sodium carbonate, this being added to the chloride solution and the reaction mixture stirred for one hour. After this time the resulting suspension was transferred to four 250 ml centrifuge

bottles and deposited by centrifugation in a MSE Magnum II centrifuge at 1000 rev min^{-1} (450 g) for 15 minutes. The supernatants were discarded and the sediments resuspended by pooling in 1.0 litre of 0.9 % sodium chloride in a two litre conical flask. This was autoclaved at 121°C for 15 minutes and when the resulting floc had settled, the supernatant was decanted and the sediment resuspended in a fresh, sterile 1.0 litre of 0.9 % sodium chloride. This was stored at 4.0°C until required.

Preparation of iron oxide

Pfizer iron oxide powder was measured into a 600 ml beaker until full and then transferred into a two litre Pascall porcelain Ball mill. Distilled water was added until the mill was full and then the rubber seal placed into position and the top sealed. The mill was run for 24 hours on a Pascall milling machine to achieve a particle size of 1.0 to 5.0 µm, and then decanted into two one litre beakers.

The milled oxide was allowed to settle for one hour and then the water and finer particles decanted to leave a thick slurry. This slurry was resuspended in an equal volume of fresh distilled water and allowed to settle a second time. This operation was repeated a third time, the final washed slurry being stored in a large screw-capped bottle to prevent evaporation. A 10.0 ml aliquot of the iron oxide was pipetted into a lidless and preweighed Petri plate and dried overnight at 100.0°C in order to determine the oxide dry weight, and therefore the slurry concentration.

Addresses

Flow Laboratories Ltd., Victoria Park, Heatherhouse Road, Irvine KA 12 & NB, Ayrshire.

Gibco Ltd., Washington Road, Sandford Industrial Estate, Paisley, PA 3 4 EP, Renfrewshire.

Difco Laboratories Inc., Detroit 1, Michigan, USA.

Oxoid Ltd., Wade Road, Basingstoke, Hampshire.

BDH Chemicals Ltd., Atherstone, Warwickshire.

Sigma London Chemical Co. Ltd., Norbiton Station Yard, Kingston-on-Thames, Surrey.

Fisons Scientific Apparatus Ltd., Bishop Meadow Road, Loughborough, Leicestershire.

National Collection of Industrial Bacteria, Torrey Research Station, Ministry of Agriculture, Fisheries and Food, 135 Abbey Road, Aberdeen, Aberdeenshire.

APPENDIX 2

Lagrange Interpolation computer programme

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1. JOB NAME, NUMBER,FORTRAN  (orange job card).
2. MT
3. ****
4. DOC SOURCE
5.         MASTER NAME
6.         DIMENSION X(20),Y(20),W(20)
7.         READ(1,5)L
8.         DO25LRUNS=1,L
9.         READ(1,10)X
10.        READ(1,10)Y
11.        N=20
12.        XSTEP=0.25
13.        XVAL=0.0
14.        DO 120 I=1,60
15.   100  CAL F4LAGRNG
16.     1  (X,Y,XVAL,N,W,YVAL)
17.   110  WRITE(2,25)XVAL,YVAL
18.   120  XVAL=XVAL+XSTEP
19.     5  FORMAT(10)
20.    10  FORMAT(20(F0.0))
21.    25  FORMAT('0XVAL =',F5.2,6X,'YVAL = ',F5.2)
22.    11  STOP
23.        END
24.        FINISH
25. ****
26. DOC DATA
27. 2
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28. et al. These are data cards, recording the first set of X values, then the first set of Y values, followed by the second set of X's, and so on; in the format, eg. 1.0 2.0 3.0 etc.

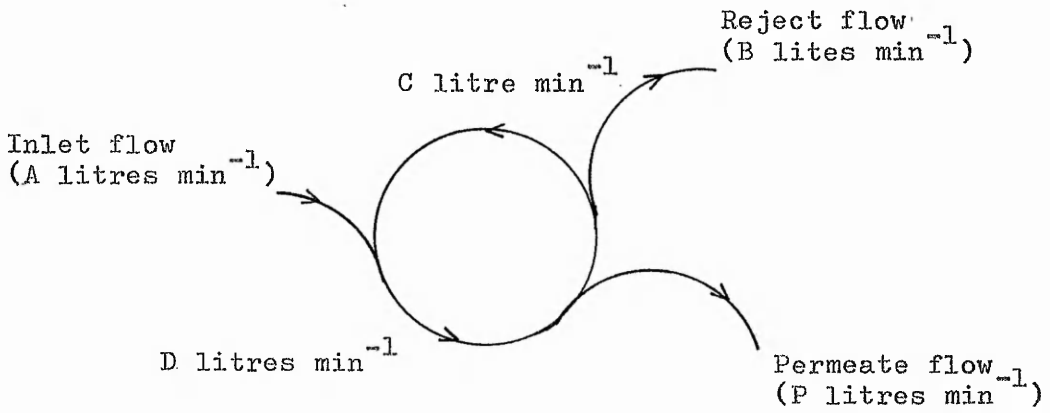
The figure 20 in card numbers 6, 11 and 20 refers to the number of X and Y points in each set.

The figure 2 in card 27 refers to the number of sets of data.

APPENDIX 3

Calculation of percentage losses of viruses during RO machine operation

Given a unit with inlet flow A litres min^{-1}, reject flow B lites min^{-1} and recirculation flow C litres min^{-1}, the combined inlet-recirculation flow would be A+C, or D litres min^{-1}, as in the following diagram:



The apparent increased flow D would be balanced by the removal of water as concentrate and permeate flow. However, as no viruses were detected in Colwick permeate and only a few under increased pressure conditions at Budd's Farm, this could be excluded from calculations. Following assay of concentrate samples and plotting of results as in Figure 20 the number of graph paper squares under each graph peak was counted, and the number of viruses passing the sampling port per minute calculated by multiplication. This was termed virus concentration V2, the initial concentration of viruses being V1.

Thus, $\frac{V2}{B}$ = mean flow ml^{-1} = X pfu ml^{-1} min^{-1}

The same mean would occur in the recirculation water giving:

X x C = Y pfu ml^{-1} min^{-1}

The percentage recovery of detected viruses was therefore calculated as:

$\frac{Y + V2}{V1}$ x 100 = % remaining

The percentage removal therefore equals:

100- % remaining

The calculated load for estimating the removal during the next cycle was derived from:

$\frac{Y \times C}{D}$ = virus concentration V3

Calculation of theoretical loss during RO machine operation

Assuming no virus loss due to death or leakage into the permeate water the theoretical loss during one cycle was calculated as follows.

Given an inoculum of V1 pfu ml^{-1} of virus, an inlet flow of A litres min^{-1} and an assumed inoculation of the total virus titre into the inlet stream within one second, the V1 pfu ml^{-1} were inoculated into:

$\frac{A}{60}$ x 1000 ml inlet water s^{-1}

The concentration in the inlet water was therefore:

$\frac{V1}{A(1000/60)}$ = $\propto$ pfu $ml^{-1}s^{-1}$

Similarly, if the concentrate flow was B litres min^{-1}, this would become:

$\frac{1000B}{60}$ ml water s^{-1}

The number of viruses expected in the concentrate water after one machine cycle would therefore be:

$$\frac{1000B}{60} \times \alpha = \beta \text{ pfu } ml^{-1}s^{-1}$$

The calculated percentage loss would then be:

$$100 - \left(\frac{\beta}{VI} \times 100 \right) = \% \text{ loss}$$