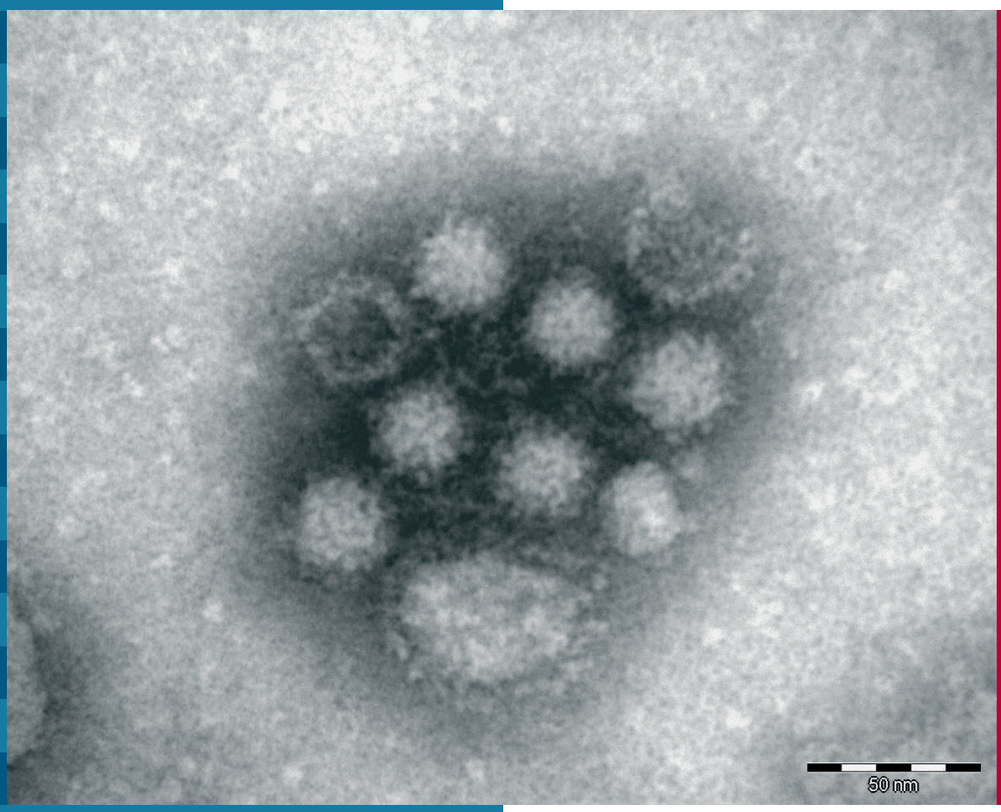
Viruses in the Rhine and source waters for drinking water production

RIWA
Rhine Water Works
The Netherlands



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Asssociation of River Waterworks

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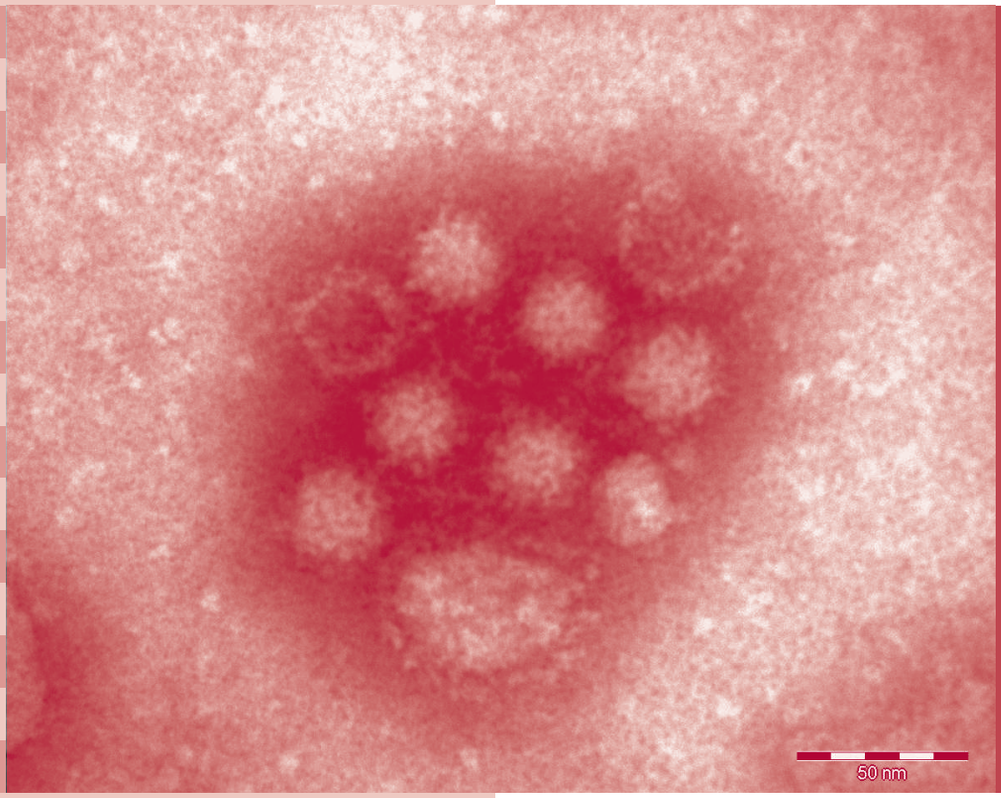

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Contents

Samenvatting

Het in dit rapport beschreven onderzoek, dat is uitgevoerd in opdracht van RIWA-Rijn, richtte zich op het voorkomen van een aantal wateroverdraagbare humane virussen in het Nederlandse deel van het Rijnstroomgebied. De bemonstering vond plaats in de Rijn bij Lobith, in het IJsselmeer bij Andijk en in het PWN bekken bij Andijk. De concentraties van enterovirussen en reovirussen werden met behulp van celkweektechnieken bepaald, terwijl de norovirusen en rotavirussen werden bepaald met behulp van moleculair biologische technieken. Bovendien werd de aanwezigheid van somatische en F-specifieke colifagen onderzocht door gebruik te maken van specifieke gastheerbacteriën. De rapportage begint met een beschrijving van de via wateroverdraagbare virussen. Verder wordt een overzicht van de mogelijke bronnen van dit soort pathogenen gegeven. In de Inleiding wordt ook nog stilgestaan bij de wetgeving, in het bijzonder m.b.t. dit soort pathogenen en hun betekenis voor de drinkwaterbereiding uit oppervlaktewater.

De aantallen entero- en reovirussen werden in variabele aantallen gevonden van niet detecteerbaar tot respectievelijk 0,67 plaque vormende eenheden (pve/l) en 5,6 pve/l. De aantallen virussen in het IJsselmeer en het PWN bekken waren lager dan die in de Rijn. De rota- en de norovirusconcentraties varieerden van niet detecteerbaar tot respectievelijk 18.000 en 4.400 RNA-vormende eenheden per liter en ook voor deze virussen lagen de IJsselmeerwaarden lager dan die van de Rijn bij Lobith. Somatische colifagen werden in alle monsters gedetecteerd, hun aantallen varieerden sterk (9,7 – 92.000 pve/l). De F-specifieke colifagen (nd – 12.000 pve/l) varieerde eveneens zeer sterk, ook voor de fagen geldt dat de lagere aantallen vooral gevonden werden in de meer stagnante wateren.

Uit de verkregen resultaten werd geconcludeerd dat de aanvoer van virussen en fagen vanuit het gebied bovenstrooms van Lobith een belangrijk aandeel van de totale vracht vormt. Op grond van de verzamelde gegevens kon niet worden geconcludeerd wat de feitelijke bronnen van de virussen en fagen zijn. Evenmin kon met deze metingen worden bepaald of de waargenomen fluctuaties in de virusconcentraties het gevolg waren van seizoensinvloeden of niet. Bij de evaluatie van de gegevens werd opgemerkt dat de soms zeer hoge aantallen RNA-deeltjes (PCR bepaalde) vaak veel hoger blijken dat de gekweekte pathogenen. Dit wijst erop dat de aanwezigheid van virus specifiek RNA (of DNA) niet noodzakelijkerwijs aangeeft dat er vergelijkbare aantallen infectieuze virussen in het monster aanwezig zijn. Aanbevolen wordt om een vergelijkend onderzoek uit te voeren waarbij in hetzelfde monster de virussen zowel m.b.v. PCR als kweek bepaald worden.

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Abstract

The present study was carried out on behalf of RIWA-Rhine (Association of River Water works, Netherlands). It reports the results of a survey on the occurrence of human viruses in the Dutch catchment basin of the river Rhine. Samples were taken from the Rhine at Lobith near the German border, the IJsselmeer and a corresponding reservoir near Andijk. The concentrations of entero-, reo-, rota- and noroviruses were determined. Moreover the concentrations of somatic and F-specific coli phages at these sampling points were estimated. For these viruses either culturing methods on cell lines and bacterial hosts or molecular methods (PCR) were applied. In the introductory part of the report a survey of waterborne viruses is given and the possible sources of the viruses was studied. Also, the Dutch drinking water legislation in relation to viruses is discussed.

The observed enterovirus and reovirus concentrations varied from undetectable up to 0.67 and 5.6 pfu/l, respectively. The virus concentrations in the IJsselmeer and the reservoir were lower than the river samples at Lobith. The rota- and norovirus concentrations varied from undetectable to 18,000 and 4,400 PCR detectable units (pdu)/l, respectively. Somatic coliphages were detectable in all samples (9.7 – 92,000 pfu/l) but F-specific phages ranged from undetectable to 12,000 pfu/l. The numbers in stagnant waters tended to be distinctly lower than in the Rhine at Lobith.

From the obtained results it was concluded that the majority of the viruses present in the Dutch catchment basin originated from the upstream part of the Rhine. Based on the present data the actual source of these viruses could not be determined. The results are regarded as insufficient data points to conclude whether the fluctuations were due to seasonal variations or not. It was noticed that PCR counts were often much higher than data obtained from cultures. The presence of virus specific RNA may therefore not necessarily indicate the presence of infectious viruses. It is recommended to plan a comparative study between culture and PCR determinations from the same samples, in order to clarify these differences.

Preface

The river water association (RIWA) was founded some 50 years ago by a number of Dutch drinking water companies, using surface water as the main raw water source. An important RIWA goal is to collect relevant information on water quality. In recent years RIWA has focussed on several studies on water quality in the rivers Rhine and Meuse to estimate possible risks for the production of drinking water from these raw water sources. The presence of various chemical compounds has been evaluated (Genderen et al. 1994; Groshart en Balk 1998) as well as pharmaceuticals (Derksen et al. 2002). Also the possible effects of pollutants in the rivers have been studied using bio-assays and gene-toxicity (Penders en Hoogenboezem 2001; 2003). Several detailed studies have been devoted to the occurrence of Cryptosporidium oocysts and Giardia cysts (Medema et al. 1996; 2001; 2002) and the effects of endocrine disrupting compounds (Ghijsen en Hoogenboezem 2000).

Recently RIWA has been divided into smaller units, covering individual rivers such as the Rhine. RIWA Rhine, financially supported by four drinking water companies (PWN, WLB, Vitens and Hydron) carried out the study on the presence of viruses in the Rhine at Lobith and at some raw water abstraction points.

Relatively nearby and only recently, in some European countries drinking water related outbreaks have been reported. During the summer of 1998 in a small village (3,500 persons) in Switzerland 50% of the population suffered from a viral gastro-enteritis (Norwalk Like Virus, NLV) due to sewage contaminated drinking water (Hafliger et al. 2000). Early in 1994 some 1500–3000 people were infected by Norwalk Like Virus, A and C Rotaviruses and small round viruses. This outbreak is most probably caused by contamination of a groundwater well situated in the embankment of a river (Kukkula et al. 1997). Such cases and other examples illustrate the need of more information of virus loads in our river waters. Moreover, in the Netherlands, one outbreak was reported related with contaminated drinking water in 2001 (Fernandes et al. 2004).

Until some years ago it was only possible to detect (human) viruses in water samples by cultivation techniques on specific monkey kidney cell lines. In the last few years, however, new (molecular biological) techniques have been designed for the detection of human viruses. These detection techniques were adapted for application of large sample volumes, as required for the studies of raw water used for the production of drinking water (Rutjes en de Roda Husman, 2004).

Several reorganisations in the contributing companies make some elucidating remarks necessary. The name of Amsterdam Water Supply (GWA) has changed into WLB, Vitens was formerly known as WMO (Drinking water company Overijssel). The water transport company Rhine Kennermerland (WRK) has been incorporated into both WLB and PWN, (PWN Drinking Water Company of North-Holland, ltd). At the beginning of 2003 the laboratories of four drinking water companies (PWN, WLB, WRK and DZH (Dune Water company South Holland)) were integrated into an independent organisation; Het Waterlaboratorium (HWL).

The supervising committee consisted of E.J.M. Penders (WRK-WCB, Groenendael 6, 3439 LV Nieuwegein, currently HWL), A.P. Krom (WLB, PO Box 8169, 1005 AD Amsterdam, currently HWL), G.L. Bakker (Vitens Water Technology, PO Box 10005, 8000 GA Zwolle) and W. Hoogenboezem (PWN, PO Box 734, 2003 RS Haarlem, currently HWL; chairman/projectleader). We would like to thank R. te Welscher from WLB (currently at the Dutch National Environmental Inspectorate) for his contribution to the design of this project.

The Watervirology group of the Microbiological Laboratory for Health Protection (MGB) of the National Institute for Public Health and the Environment (RIVM) carried out the research described in this report. The authors are therefore indebted to H.A.M. de Bruin and H.H.J.L. van den Berg for water sampling and virus analyses. This report was written by A.M. de Roda Husman of RIVM-MGB (PO Box 1, 3720 BA Bilthoven, tel. +31.30.274 4325, fax. +31.30.274 4434, e-mail am.de.roda.husman@rivm.nl).

Wim Hoogenboezem, Het Waterlaboratorium (chairman)

1 Introduction

1.1 Waterborne pathogenic viruses

Waterborne viruses belong to the large group of enteric viruses that are shed in faeces (Table 1.1; De Roda Husman 2001). Commonly, these are small viruses (about 30 nanometres) without envelope. They bind to a specific receptor on the epithelial cells of the host intestines after which the virus genome can replicate in the presence of host factors. The newly formed virus particles are packaged into the virus capsid and released from the host cell. The virus replication cycle is host specific and host dependent implicating that these viruses can not multiply in water. Enteroviruses, though more prevalent in the population in summer, are detected at higher concentrations in water in winter because of reduced inactivation at lower temperatures and with decreased sun hours. The enteric viruses described here infect and may cause disease in humans.

Table 1.1. Waterborne viruses

Family	Genus	Virus
Picornaviridae	Enterovirus	Poliomyelitis virus 1-3
		Coxsackie virus A/ B
		ECHO virus
		Enterovirus 70-71
		Human enterovirus 68-71
	Hepatovirus	Hepatitis A virus (HAV)
Reoviridae	Orthoreovirus	Reovirus 1-3
	Rotavirus	Rotavirus A-C
Caliciviridae	Norovirus	GGI prototype Norwalk
		GGII prototype Lordsdale
Hepatitis E –like viruses		Hepatitis E virus (HEV)
Astroviridae	Astrovirus	Human astrovirus (HAstV) 1-5
Adenoviridae	Mastadenovirus	Human adenovirus (HAdV) 1-47
Coronaviridae	Coronavirus	SARS virus

The source of human pathogenic viruses in water is faeces from infected individuals independent of their disease status. In contrast with bacteria, enteric viruses may be relatively persistent to inactivation by variations in pH and temperature of the water. Due to these characteristics enteric viruses survive well in water and on food especially when stored at intermediate humidity and at low temperatures. In the United States, noroviruses (previously Norwalk-like viruses (NLV), small round-structured viruses (SRSV)), rotaviruses, astroviruses and hepatitis A viruses were recognised as the most important emerging viral food pathogens of the past thirty years (Tauxe 2002). The majority of the human enteric viruses causing gastro-enteritis symptoms such as diarrhoea, vomiting and fever, belong to the virus families *Caliciviridae* (noro- and sapoviruses), the *Astroviridae* (astroviruses) and the *Reoviridae* (reo- and rotaviruses) (Table 1.2). Other waterborne viruses cause the more severe diseases such as meningitis associated with enteroviruses (Muir et al. 1998) and reoviruses (Johansson et al. 1996) or hepatitis caused by the enteric hepatitis A viruses (HAV) (Cuthbert 2001), hepatitis E viruses (HEV) or hepatitis F viruses. The enterovirus types poliovirus 1-3 may cause poliomyelitis. Infection with adenoviruses may also lead to gastro-enteritis, and alternatively to conjunctivitis and/ or pharyngitis.

Recently, severe acute respiratory syndrome (SARS) was found to be caused by a newly emerged coronavirus that also caused (watery) diarrhoea in half the patients and was shown to be transmitted via the faecal-oral route (Leung et al. 2003).

Table 1.2. Waterborne viral diseases and their causative agents.

Meningitis	Poliomyelitis	Gastro-enteritis	Conjuctivitis	Hepatitis
Coxsackieviruses	Polioviruses 1-3	Caliciviruses	Adenoviruses	Hepatitis A viruses
ECHO viruses		Rotaviruses	ECHO viruses	Hepatitis E-like viruses
Polioviruses 1-3		Enteroviruses		Hepatitis F viruses
Reoviruses		Adenoviruses		
		Astroviruses		
		Coronaviruses		

Enteric viruses occur worldwide, although not all of these viruses are endemic in all regions. Epidemic occurrence is often observed for caliciviruses as a result of gastro-enteritis outbreaks transmitted from person-to-person or via food. In the Netherlands, hepatitis A viruses circulate among homosexuals or are introduced after the return of individuals who got infected abroad (Bruisten et al. 2001). Enteroviruses circulate throughout the year with peaks in more severe disease in summer (Schijven et al. 1995). In contrast with the winter peak seen for rotavirus infection, no striking seasonal variation has been observed for enteric adenovirus associated gastro-enteritis.

Numerous virus associated gastro-enteritis and hepatitis outbreaks have been described related to sewage-contaminated shellfish, recreational water and drinking water (Hejkal et al. 1982; Kaplan et al. 1982; Beller et al. 1997; Brugha et al. 1999; Xu et al. 1992; Kukkula et al. 1999; Hafliger et al. 2000; Sundkvist et al. 2000; Hoebe et al. 2004). In Finland, the share of food- and waterborne associated gastro-enteritis outbreaks is estimated to be 25% (Lopman et al. 2003). In the Netherlands, this share has been estimated to be 17%. However, in both and other European countries it is yet unknown which part of these gastro-enteritis outbreaks may be solely attributed to exposure of the population to human pathogens in water. We do know that each year 4 million episodes of gastro-enteritis occur transmitted from person-to-person, by zoonotic (animal-to-person) transmission or transmission via food or water (De Wit et al. 2001a). Approximately 200,000 persons visited their general practitioner for complaints of gastro-enteritis (De Wit et al. 2001b). Caliciviruses (both noroviruses and sapoviruses), *Campylobacter* and rotaviruses belong to the top three of most important causative agents of gastro-enteritis in the Netherlands.

1.2 Detection of viruses in water

At low concentrations, viruses may still be infectious and pathogenic. The low virus concentrations present in water are relevant for public health and at the same time difficult to assess. Raw sewage contains high numbers of virus particles sufficient for detection in one litre or even less. In contrast, detection of lower numbers of viruses as observed in treated sewage effluents and surface waters requires concentration of hundreds of litres of waters. Reduction of water volumes to manageable water concentrates may be achieved using chemical precipitation, (ultra-) centrifugation, glass wool filtration, ultra-filtration, positive or negative membrane filtration or a combination of these methods. The precipitation, centrifugation, glass wool filtration and ultra-filtration methods are restricted to volumes of up to 10 litres. Membrane filtration can be applied to very large volumes of thousands of litres of water. Since virus particles are negatively charged in water, they will bind very well to positive membranes but elute with great difficulty. Virus particles, therefore, will bind less efficient to negative membranes and chemicals have to be used to aid this process. Desorption of virus particles is facilitated by the negative charge of these membranes.

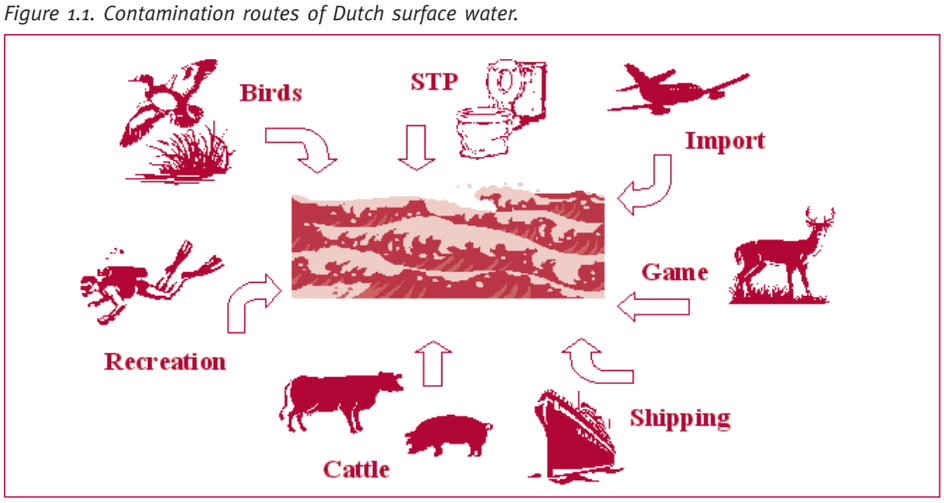
Following virus concentration the subsequent virus detection methods will preferably rely on quantitative cell culture to selectively identify infectious virus particles. Enteric viruses, such as poliovirus, can be cultured on cell lines that are sensitive to infection because these cells express the virus specific receptor on their cell surface. A liquid cell culture assay will give only qualitative data on the presence of infectious viruses in the water concentrate. Infection of a monolayer of cells allows enumeration of auras of lysed cells which were assumed to have initiated from one infectious virus (so-called plaque assay) (Dahling and Wright 1986). However, for some of the most important causative agents of viral gastro-enteritis, such as noroviruses, no sensitive cell lines have been identified so far. In the eighties molecular methods were developed which could not be employed to detect infectious virus particles but that could detect the virus genomes (Saiki et al. 1985). Most waterborne viruses carry either single or double RNA strands, which can be transcribed into copy DNA by reverse transcriptase (RT) enzyme. Copy DNA is then used as a template for exponential amplification by the polymerase chain reaction (PCR) that can be visualised. This so-called RT-PCR method generates qualitative or semi-quantitative data. Typing of the virus genomes will define whether the virus is pathogenic for humans and may aid tracing to possible sources of contamination of surface waters. Viruses such as noroviruses are specifically useful for molecular tracing because of the high genomic variation and year-to-year change of predominant strains.

Alternatively, infectious bacteriophages are used as indicators for human viruses. Detection of phages is much faster, easier and cheaper. Bacteriophages are useful for the evaluation of the efficiency of virus removal and/ or inactivation by water treatment processes. They are less adequate as indicators of source water quality because of their different, non-human, host (Havelaar 1993).

1.3 Possible sources of human viruses

Surface waters can become contaminated with enteric viruses originating from infected humans or infected animals (Figure 1.1). Recently, it was shown that import from neighbouring countries via the large rivers Rhine and Meuse is the most important source for contamination with the protozoan parasites *Cryptosporidium* and *Giardia* (Hoogenboezem et al. 2001). This could also be shown for enteroviruses (Schijven et al. 1995; Hovi et al. 1996), but is yet unknown for other viral pathogens. Other sources of virus contamination that may be important are discharge of untreated sewage by sewage treatment plants with heavy rainfall, by professional and recreational ships and houseboats. Since sewage treatment plants are not set up for specific removal of human viruses and treated sewage is largely discharged into the surface waters in a heavily populated area as the Netherlands, treated sewage contributes significantly to the virus load in surface waters (van den Berg et al. 2005; Lodder and de Roda Husman 2005).

Figure 1.1. Contamination routes of Dutch surface water.



Animal manure run-off is another possible source of virus contamination as was shown previously for *Cryptosporidium* and *Giardia* (Hoogenboezem et al. 2001). This is still not known for human viruses though recently husbandry animals were found to be infected with viruses that were very similar to the human viruses. The virus genome of noroviruses detected in cattle manure and to a greater extent in pig manure at Dutch farms was closely related to human norovirus variants (Van der Poel et al. 2000). RNA sequences of hepatitis E virus (HEV) detected in pig manure was also found to resemble human HEV RNA sequences (Van der Poel et al. 2001). Zoonotic transmission (from animal to man) though not proven is much more likely when RNA sequences are more similar. Moreover, these viruses are very prevalent at Dutch pig farms with 22% of farms positive for the presence of HEV (Van der Poel et al. 2001). By run-off from manure into surface waters these potentially zoonotic viruses could contaminate surface waters. Besides husbandry animals, pets and game, such as fowl and deer, could faecally contaminate our surface waters.

1.4 Legislation

Recently, the European guideline for the quality of water designated for human consumption has been renewed (98/83/EG). One of the most important threats for the quality of drinking water is the presence of pathogenic micro-organisms. The quality of the drinking water is to be monitored using faecal indicators. In the European guideline, it is explicitly mentioned that pathogens should not be present in the drinking water in counts that are hazardous for the public health.

In the Dutch drinking water guideline enforced on the 9th of February 2001 (Anonymous 2001) the counts of E. coli and Enterococci should not be detectable in 100 millilitres tap water samples. In addition, a quantitative risk assessment should be undertaken for the pathogenic micro-organisms C*ryptosporidium*, *Giardia* and (entero)viruses when surface waters are used for drinking water production and for vulnerable groundwater sources. To this purpose, the micro-organisms have to be measured in raw water and the reduction by treatment should be assessed. The health burden should be less than 1 infection per 10,000 persons per year.

1.5 Research goals

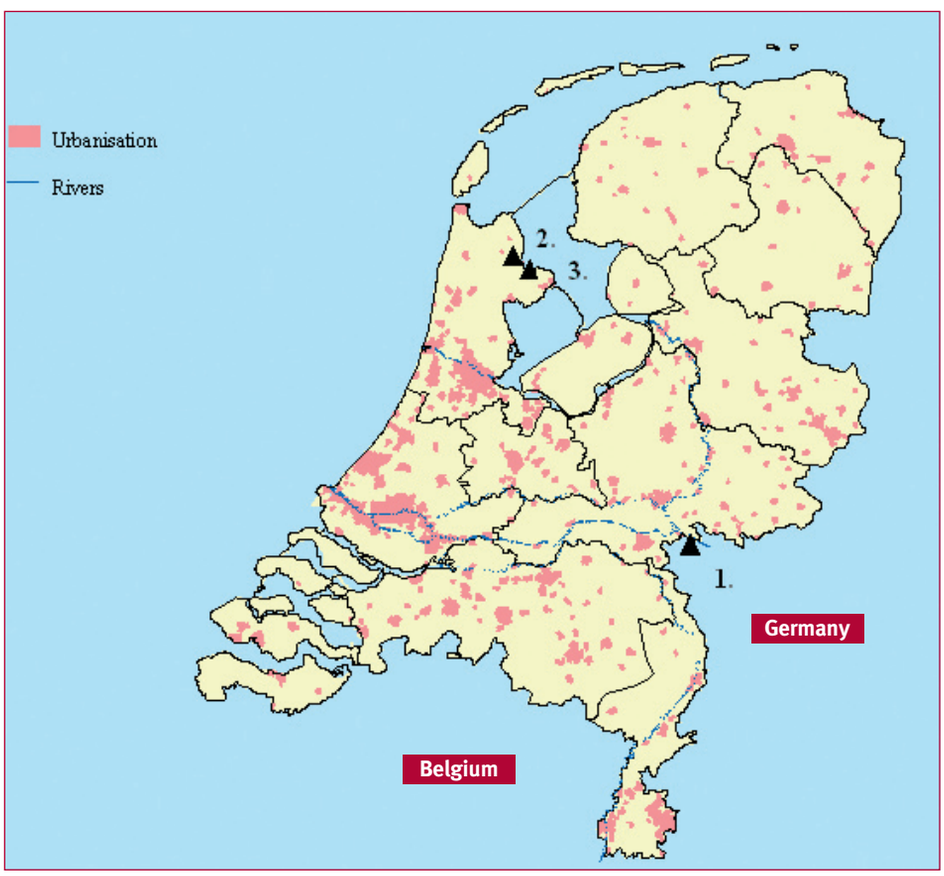
The Rhine encompasses the largest catchment area in the Netherlands. These surface waters are used for transport, recreational purposes and for the production of drinking water. The Dutch drinking water guideline demands companies that produce drinking water from surface waters, such as the Rhine and the IJsselmeer, to perform a quantitative risk assessment for *Cryptosporidium*, *Giardia* and (entero)viruses. This requires knowledge of the surface water quality with respect to these micro-organisms and their removal and/ or inactivation by applied treatment processes. In the past ten years research into the protozoan parasites has been extensive but knowledge on viruses is restricted. With the development of molecular methods, virus research in the Rhine catchment area became feasible and was started by the RIVM on behalf of the RIWA Rhine that represents the Dutch companies that use Rhine water for drinking water production. This report describes the results of measurements performed to determine the surface water quality with respect to the presence of human pathogenic viruses. In addition, the required efficiency of virus removal by water treatment processes was assessed for the production of drinking water complying with the Dutch drinking water guidelines. Recommendations are made for future research accommodating a reliable quantitative risk assessment for exposure to human pathogenic viruses in drinking water.

2 Materials and methods

2.1 Surface waters

The Rhine enters the Netherlands near Lobith (Figure 2.1). The Rhine continues as the Nederrijn-Lek and Waal towards the West to eventually flow into the North Sea. The IJssel, which branches of the Nederrijn, flows to the North past Zwolle where it discharges into the IJsselmeer.

Figure 2.1. Sampling locations (=) in the Rhine catchment area in the Netherlands; 1. Lobith, 2. Andijk at PWN, 3. Andijk at WRK.



The drinking water production companies Rijn-Kennemerland (WRK) and Noord-Holland (PWN) take in surface water from the West of the IJsselmeer for the production of drinking water (Figure 2.1). The WRK intake station Prinses Juliana (WPJ) in Andijk has a capacity of 109 million m^3 per year. The PWN station situated next to the WRK station in Andijk has a capacity of 24 million m^3 per year. The residence time of the water at the WRK station is approximately two hours. The maximum flow at WRK-WPJ is 14,500 m^3 per hour.

At the WRK intake station in Andijk the water from the IJsselmeer is predominantly treated by coagulation, rapid sand filtration and activated coal filtration before it is transported to the West Coast for infiltration into the dunes. The reclaimed water is subsequently subjected to rapid sand filtration, savety disinfection (0.1 ClO_2 mg/l) at PWN production plants. The WLB plants apply ozonisation, softening and activated coal filtration and slow sand filtration to the reclaimed water.

2.2 Sampling, concentration and physical analyses

The Rhine water was sampled ten times from December 2001 until December 2002 at Lobith (Annex 1A) according to RIVM SOP MGB/ M154 (Figure 2.1). At Andijk, the IJsselmeer was sampled four-weekly alternately at the PWN station and at the WRK station starting at the PWN station on six days from January to May 2002 (Annex 1B). On three of these six sampling days (February 20th, April 3rd and May 15th) treated water samples were taken after activated coal filtration at the WRK station.

In total 300 litres of untreated surface water and/ or 600 litres of treated surface waters were concentrated by use of a conventional filter adsorption-elution method (Lodder et al. 1999). Magnesiumchloride was used for binding of the viruses to the filter at low pH (3.8). Virus elution from the filter after filtration was done was achieved at high pH (9.0). The precipitate was dissolved and neutralised. One-third of the eluate was stored prior to two-phase separation for PCR purposes. Two-thirds of the eluate was subjected to ultrafiltration for cell culture. At Andijk, 300 litres of the water from the IJsselmeer was sampled alternately at the station of PWN and WRK, starting in January 2002 at PWN and finishing after six sampling dates at WRK in May 2002. Samples derived from the IJsselmeer were analysed for the presence of pathogenic viruses by cell culture or RT-PCR. In addition, the concentrations bacteriophages were determined in the water from the IJsselmeer and after treatment by activated coal filtration prior to transport at WRK.

Turbidity was determined using the Hanna 193703 microprocessor turbidity meter (range 0.00-1000 FTU) according to RIVM SOP MGB/A160. The pH and the temperature records of the water were read out retrospectively from the virus filtration unit according to the manufacturer's instructions.

2.3 Detection of cultivable viruses

After membrane filtration, 600 ml of the eluates was ultrafiltered for detection of viruses such as bacteriophages, entero- and reoviruses that can be cultured on either host bacteria or on sensitive cell lines. Elution of the viruses was accomplished using beefextract. The appropriate host bacteria were exposed to the concentrates to culture the F-specific and somatic bacteriophages according to RIVM SOP MGB/ M173 and ISO/ DIS 10705-02: 1999, respectively. In the presence of bacteriophages, plaques could be enumerated after overnight culture.

To be able to demonstrate enteroviruses and reoviruses buffalo green monkey kidney cells were inoculated with part of the concentrate. Infections of the cells could be visualised by neutral red staining of virus plaques in the monolayer. The plaques were enumerated and the virus concentration in the original water sample was calculated from the test volume and the virus count.

2.4 Detection of non-cultivable viruses

Noroviruses for which no sensitive cell line is known as yet and rotaviruses for which cell culture is difficult are detected using molecular methods such as PCR. Instead of ultrafiltration further concentration of eluates is realised with two-phase separation. This method is based on the selective separation of viruses and inhibitory factors into two phases, which can not be mixed. To this purpose 1% (w/v) Dextrane T40, 10% (w/v) PEG 6000, 0.2 M NaCl en 10 mM phosphate buffer (pH 7.2) were added to the watery phase. This mixture was shaken for 1 hour at 4°C. The sample is transferred to a funnel to settle

overnight at 4 °C. After separation the lower as well as the interphase were collected. The samples were purified by spin column gel chromatography and ultrafiltration. Viral RNA was isolated by binding to silica beads followed by wash steps to remove inhibitors. The isolated RNA was ten-fold diluted until 10-5. The RNA was transcribed into copy DNA with the enzyme reverse transcriptase, which could be amplified using a heat resistant polymerase. The RT-PCR products could be visualised by fluorescent gel electrophoresis and hybridisation with fluorescent-labelled, virus-specific probes.

2.5 Statistical methods

The numbers of virus particles present in water were estimated quantitatively by culture or semi-quantitatively by RT-PCR on ten-fold serially diluted RNA (end point dilution). Virus concentrations in the undiluted samples were estimated as most probable numbers by the use of the number of plaque forming units or the presence-absence of virus genomes in the ten-fold RNA dilutions under the assumption that negative samples do not contain virus or viral RNA. Application of the Poisson distribution was justified by the assumption that the infectious virus particles or viral RNA was dispersed randomly in the sample. The maximum likelihood method was used to estimate the number of virus particles in the undiluted sample (Mood et al. 1974). A negative binomial model gives the best fit for the distribution of virus particles in the original and diluted samples. The 95% confidence interval was estimated for each virus concentration. Possible correlations between parameters were determined by regression analyses.

The decimal reduction achieved by the treatment processes applied at the WRK station in Andijk was estimated by use of a simple equation and by application of a binomial model to be able to determine the 95% confidence interval. First, the weighed arithmetic means of the virus concentrations in the source (S) and treated (T) waters were estimated by division of the total virus counts (n) and the total test volumes (V) (equation 1 and 2, respectively).

$$S = \frac{(n_{s1} + n_{s2} + n_{sn})}{(V_{s1} + V_{s2} + V_{sn})} \quad \text{equation (1)}$$

$$T = \frac{(n_{T1} + n_{T2} + n_{Tn})}{(V_{T1} + V_{T2} + V_{Tn})} \quad \text{equation (2)}$$

Subsequently, the weighed arithmetic means of the virus concentrations in the source (S) and treated (T) waters were divided and logtransformed to form the decimal reduction (DR) by treatment (equation 3).

$$DR = \log_{10}\left(\frac{S}{T}\right) \quad \text{equation (3)}$$

A binomial model for unpaired data was also applied to the same dataset S and T. The resulting arithmetic means from this model were compared with the arithmetic means resulting from equation (1 and 2).

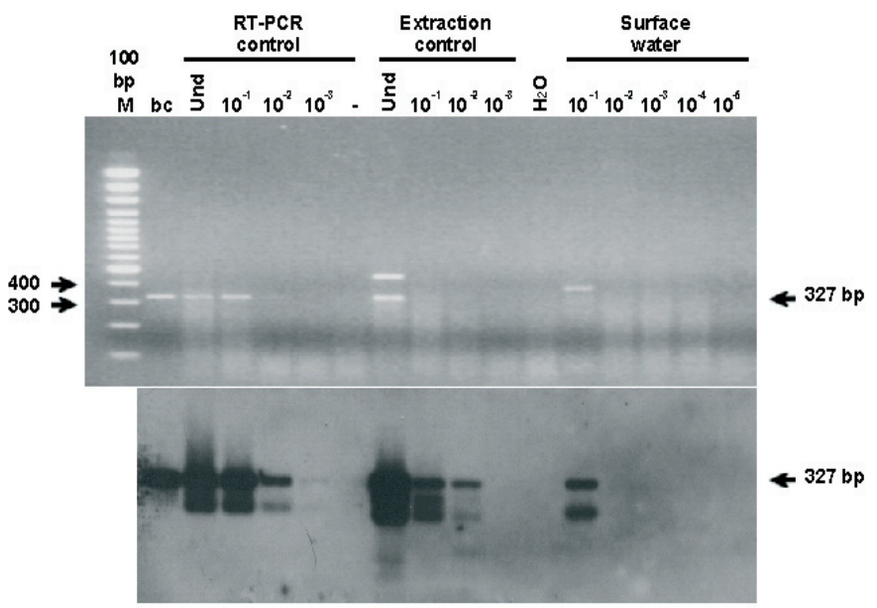
3 Results

Source waters for the production of drinking water were analysed for the presence of human pathogenic viruses. Treatment processes were evaluated with respect to their efficiency of virus removal by using indicator bacteriophages.

3.1 Viruses in the Rhine near Lobith

The quality of the Rhine water flowing into the Netherlands near Lobith was determined with respect to the presence of human pathogenic viruses. Norovirus RNA was detected in 4 out of ten samples (40%) whereas three samples (30%) were positive for rotavirus RNA (Table 3.1). Once the 10-fold RNA dilution was positive and three times the 100-fold RNA dilutions by norovirus RT-PCR (Figure 3.1). Two samples were rotavirus PCR positive in the 100-fold RNA dilutions and one sample up to the 1000-fold diluted RNA. Two of the samples taken in April and December 2002 contained noro- and rotavirus RNA.

Figure 3.1. Photograph of norovirus specific PCR products derived from ten-fold RNA dilutions visualised by fluorescent agarose gel electrophoresis and hybridisation.



Infectious enteroviruses were found in 9 out of ten samples (90%) ranging from 0.0033 to 0.46 plaque forming particles per litre (pfu/l) water in April and December, respectively (Table 3.1). Infectious reoviruses were detected in each of the ten samples at concentrations ranging from 0.0030 to 5.9 particles per litre in September and December, respectively. Both F-specific phages and somatic phages were detected in 100% of the samples at high concentrations of up to 11,636 and 21,522 particles per litre, respectively, in the samples taken in February (Table 3.1).

Table 3.1. Virus concentrations in Rhine water at Lobith.

Sampling date	Concentration (95% confidence interval)					
Virus	F-specific phages (pfu/l)	Somatic phages (pfu/l)	Enteroviruses (pfu/l)	Reoviruses (pfu/l)	Noroviruses (pfu/l)	Rotaviruses (pfu/l)
5 Dec	514 (447-587)	10592 (96217-11626)	0.46 (0.33-0.61)	1.7 (1.4-2.0)	0 (0-2.3)	0 (0-1.5)
9 Jan	1110 (921-1322)	8999 (7238-11025)	0.27 (0.19-0.36)	1.1 (0.92-1.3)	317 (15-2833)	0 (0-1.6)
6 Feb	11636 (9621-13916)	21522 (18735-24573)	0.24 (0.18-0.33)	0.83 (0.70-0.97)	0 (0-23)	0 (0-16)
6 Mar	841 (683-1023)	4037 (2924-5402)	0.049 (0.025-0.086)	0.16 (0.11-0.22)	0 (0-29)	0 (0-20)
10 Apr	169.0 (151-188)	2689 (2023-3488)	0.0033 (0.00019-0.014)	0.17 (0.13-0.22)	20 (0.93-175)	131 (6.2-1156)
28 Aug	1.4 (0.83-2.1)	91 (80-103)	0 (0-0.0058)	0.0060 (0.0010-0.019)	0 (0-15)	0 (0-10)
25 Sep	0.18 (0.085-0.32)	33 (27-40)	0.0061 (0.0010-0.019)	0.0030 (0.00017-0.013)	0 (0-16)	0 (0-10)
23 Oct	10 (9.3-12)	6382 (5506-7346)	0.19 (0.14-0.25)	0.68 (0.58-0.79)	0 (0-19)	166 (7.8-1486)
20 Nov	194 (174-216)	3393 (2567-4381)	0.38 (0.30-0.47)	2.2 (2.0-2.4)	341 (16-3053)	0 (0-17)
18 Dec	837 (721-966)	9357 (5832-14078)	0.19 (0.13-0.27)	5.9 (5.5-6.3)	210 (9.9-1880)	1450 (90-13643)

Pfu/l: plaque forming units per litre; pdu/l: PCR detectable units per litre.

The temperature of the water varied from the lowest measurement of 3 °C in January to the highest measurement of 22 °C in August of 2002. The pH was very stable around 8 with one low figure of pH 7.3 in December 2002 and the highest record in April of 2002 (pH 8.5). More variation was seen for the turbidity ranging from 14 FTU in April 2002 to 120 in December 2001.

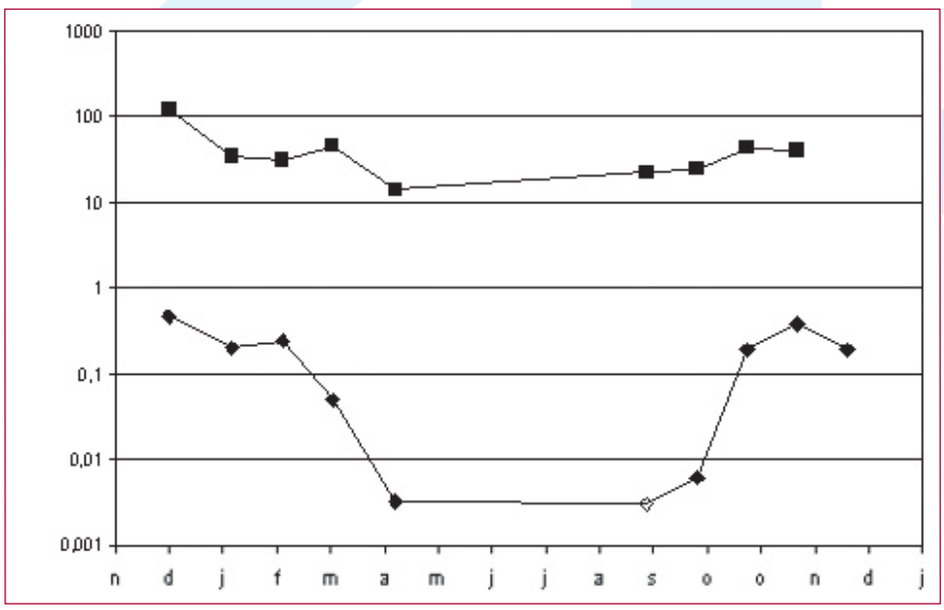
Table 3.2. Correlations between virus concentrations and physico-chemical parameters in the Rhine.

	NoV	HRV	EV	ReoV	FSPECPH	SOMCPH	T	pH	Turbidity
NoV	1								
HRV	0.26	1							
EV	0.45	0.0034	1						
ReoV	0.53	0.89***	0.41	1					
FSPECPH	0.17	0.10	0.17	0.038	1				
SOMCPH	0.0090	0.12	0.53	0.27	0.85**	1			
T	0.57	0.32	0.50	0.49	0.87**	0.76*	1		
PH	0.26	0.64*	0.016	0.65*	0.23	0.20	0.21	1	
Turbidity	0.093	0.21	0.73*	0.56	0.10	0.29	0.31	0.0045	1

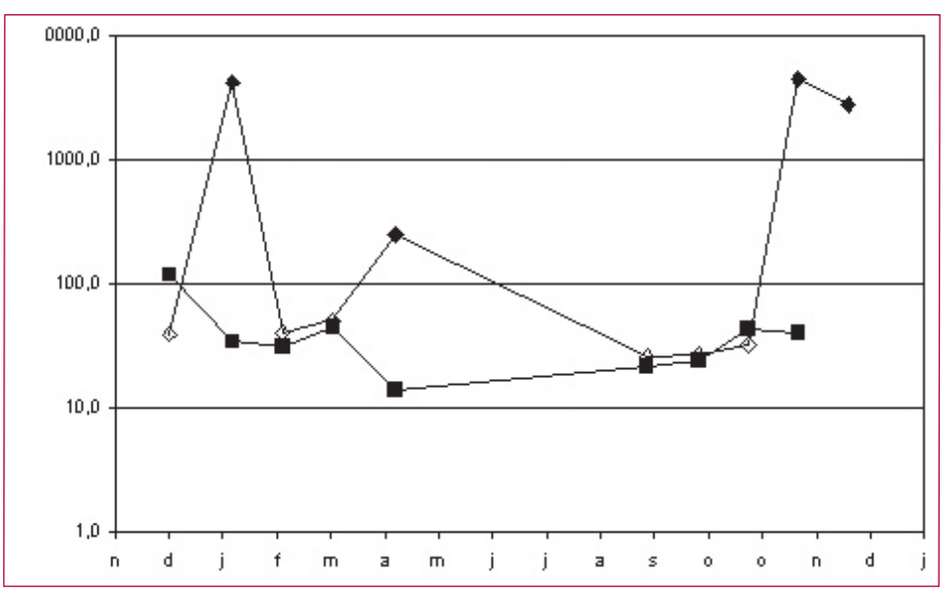
NoV: Noroviruses; HRV: Rotaviruses; EV: Enteroviruses; ReoV: Reoviruses; FSPECPH: F specific phages; SOMCPH: Somatic coliphages; T: Temperature; *p<0.05; **p<0.005; ***p=0.0005.

Significant correlations between virus concentrations were recorded for rotavirus RNA and infectious reoviruses (p=0.0005) and for F-specific phages and somatic phages (0.005; Table 3.2). Furthermore, concentrations of F-specific phages were significantly correlated with temperature (p=0.002). The presence of rotavirus RNA was significantly correlated with pH (p=0.05) as were the concentrations reovirus with pH (p=0.04).

Figure 3.2. Comparison of enterovirus (A) or norovirus concentrations (diamonds: closed symbols at positive detection, open symbols at negative detection) (B) and turbidity (closed squares) in the Rhine near Lobith.



A



B

Recently, a clear correlation between Cryptosporidium and turbidity was observed that was also suggested for viruses and turbidity (Hoogenboezem et al. 2001). In our study, enteroviruses but none of the other viruses were found to correlate significantly (p=0.03) with turbidity of the water (Table 3.2). In figure 3.2A it is shown that increase in turbidity coincides with increase in enterovirus concentration whereas decrease in turbidity of the water corresponds with reduced enterovirus concentrations. Notably, rotavirus and norovirus RNA were absent at the highest turbidity peak (120 FTU) and at the lowest turbidity level (14 FTU) both rotavirus and norovirus RNA could be detected in the 10-fold and 100-fold RNA dilution, respectively (Figure 3.2B).

3.2 Viruses in the IJsselmeer near Andijk

The presence of human pathogenic viruses was also studied in water of the IJsselmeer near Andijk, alternately sampled from the PWN station and the WRK station, approximately 500 metres apart. In one of the six samples taken in April 2002 from the IJsselmeer norovirus RNA could be detected up to the 100-fold RNA dilution at a concentration of 683 (32-6,094) PCR detectable units (pdu) per litre (Table 3.3). In none of the samples the presence of rotavirus RNA could be demonstrated (Table 3.3).

Table 3.3. Virus concentrations in IJsselmeer water at Andijk.

Sampling date	Concentration (95% confidence interval)					
Virus	F-specific phages (pfu/l)	Somatic phages (pfu/l)	Enteroviruses (pfu/l)	Reoviruses (pfu/l)	Noroviruses (pdu/l)	Rotaviruses (pdu/l)
21 Jan	12 (10-13)	2342 (2036-2677)	0.067 (0.033-0.12)	0.28 (0.20-0.37)	0 (0-31)	0 (0-20)
20 Feb	0.96 (0.65-1.4)	371 (322-424)	0 (0-0.021)	0.022 (0.0037-0.068)	0 (0-59)	0 (0-39)
13 Mar	0.12 (0.056-0.23)	99 (77-125)	0 (0-0.029)	0.015 (0.00086-0.066)	0 (0-43)	0 (0-29)
03 Apr	0.75 (0.53-1.0)	146 (115-182)	0 (0-0.027)	0 (0-0.027)	683 (32-6094)	0 (0-34)
24 Apr	0.13 (0.040-0.30)	9.7 (8.1-12)	0 (0-0.0080)	0.0041 (0.00024-0.018)	0 (0-21)	0 (0-14)
15 May	0 (0-0.062)	11 (7.7-15)	0.0052 (0.00030-0.023)	0 (0-0.010)	0 (0-26)	0 (0-17)

Pfu/l: plaque forming units per litre; pdu/l: PCR detectable units per litre.

Infectious enteroviruses were present in two out of six samples at a concentration of 0.067 (0.033-0.12) particles per litre in January 2002 at PWN and 0.0052 (0.00030-0.023) particles per litre in May 2002 at WRK. In four out of six samples reoviruses were found with the highest concentration at PWN in January at 0.28 (0.20-0.37) plaque forming particles per litre and decreasing concentrations in February (0.022 (0.0037-0.068) pfu/l; WRK), March (0.015 (0.00086-0.066) pfu/l; PWN) and April (0.0041 (0.00024-0.018) pfu/l; PWN). Five out of six samples contained F-specific phages with a peak concentration of 12 (10-13) particles per litre at PWN in January and the lowest concentration of 100 times less phages per litre two months later, also at PWN. The maximum concentration of somatic phages was much higher at 2,342 (2,036-2,677) pfu/l in January and the lowest phage concentrations of 9.7 (8.1-12) pfu/l in April of 2002.

Table 3.4. Correlations between virus concentrations in the IJsselmeer.

	NoV	EV	ReoV	FSPECPH	SOMCPH	Temp	pH	Turbidity
NoV	1							
EV	0.22	1						
ReoV	0.24	0.99***	1					
FSPECPH	0.16	0.99***	1.0****	1				
SOMCPH	0.19	0.99***	1.0****	1.0****	1			
Temp	0.091	0.68	0.75	0.74	0.79	1		
PH	0.49	0.53	0.49	0.54	0.48	0.040	1	
Turbidity	0.46	0.46	0.35	0.33	0.30	0.23	0.17	1

NoV: Noroviruses; EV: Enteroviruses; ReoV: Reoviruses; FSPECPH: F specific phages; SOMCPH: Somatic coliphages; ***p<0.0005; ****p<0.00005.

The presence of norovirus RNA was not significantly correlated to the presence of any of the cultivable viruses as shown in Table 3.4. Rotavirus RNA was not detected in any of the samples, so correlations were not determined. The phage concentrations were significantly correlated to each other and to the entero- and reoviruses, so was the correlation between entero- and reoviruses (each p-value below 0.0005). No significant correlation was found between virus concentrations and the parameters temperature, pH and turbidity.

3.3 Virus concentrations in treated water and virus removal at WRK, Andijk

The bacteriophage concentrations were determined at the WRK station in Andijk to estimate the decimal reduction (Table 3.5). In all three samples of treated waters somatic phages were detected at 0.13-21 pfu/l. Only in one of the samples of treated waters 0.17 pfu/l F-specific phages were detected.

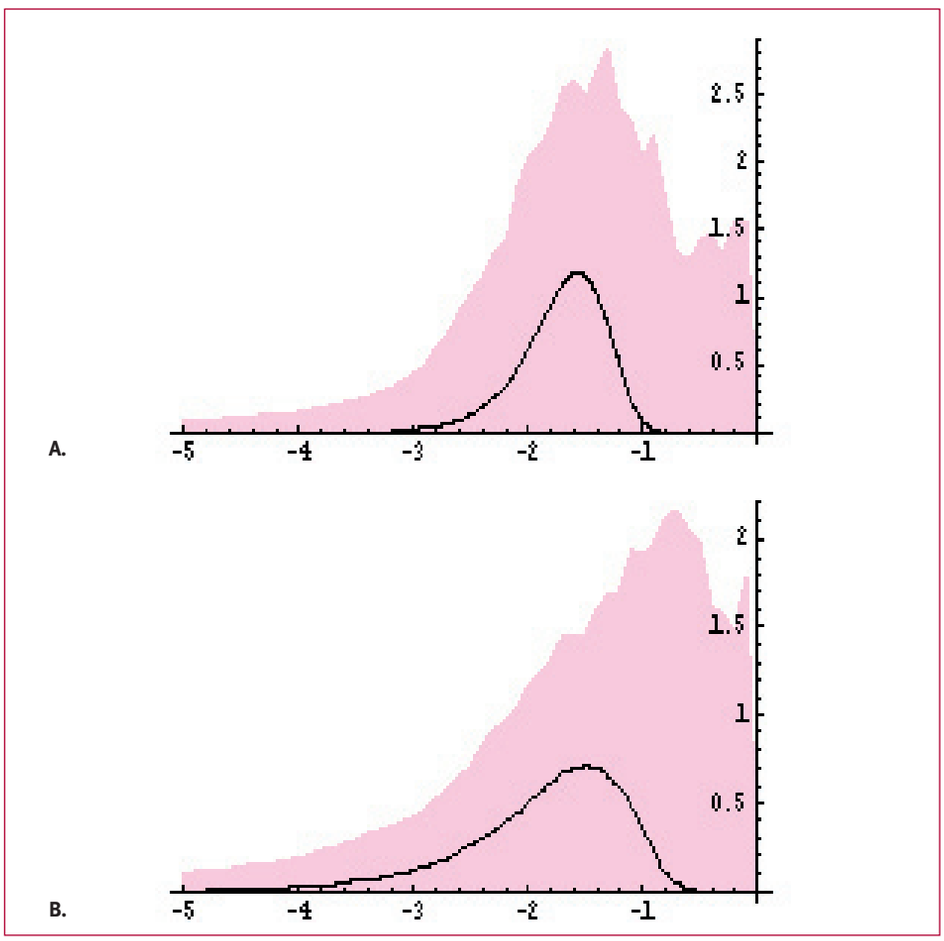
Table 3.5. Bacteriophage concentrations in source and treated waters at the WRK station in Andijk.

Sampling date	Concentration (pfu/l) (95% confidence interval)			
Virus	Somatic phages		F-specific phages	
Location	S (source)	T (treated)	S (source)	T (treated)
21 Jan	2342 (2036-2677)		12 (10-13)	
20 Feb	371 (322-424)	21 (16-27)	0.96 (0.65-1.4)	0.17 (0.11-0.25)
13 Mar	99 (77-125)		0.12 (0.056-0.23)	
03 Apr	146 (115-182)	6.0 (3.8-9.0)	0.75 (0.53-1.0)	0 (0-0.035)
24 Apr	9.7 (8.1-12)		0.13 (0.040-0.30)	
15 May	11 (7.7-15)	0.13 (0.022-0.41)	0 (0-0.062)	0 (0-0.032)

Pfu/l: plaque forming units per litre.

The efficiency of removal and/ or inactivation of viruses through coagulation, followed by rapid sand filtration and activated coal filtration, was assessed by measuring bacteriophage concentrations before and after treatment in Andijk at the WRK station. The weighed arithmetic mean concentrations in the source waters were 127 and 0.39 pfu per litre for the somatic and F-specific phages, respectively. The weighed arithmetic mean concentrations in treated waters were 9.1 somatic phages per litre and 0.058 F-specific phages per litre. The decimal reduction by coagulation, rapid sand filtration and activated coal filtration at the WRK station in Andijk following equation (3) as shown in paragraph 2.6 is 1.05 log10-units for somatic phages and 1.1 log10-units for the F-specific phages. Using the binomial model for unpaired data the respective decimal reductions and 95%-confidence intervals for somatic and F-specific phages are 1.6 (1.1-2.6) and 1.5 (0.9-3.6) log10-units, respectively (Figure 3.3).

Figure 3.3. Decimal reductions (black line) of F-specific phages (A) and somatic phages (B) at the WRK station in Andijk as estimated by use of the binomial model for unpaired data with 95% confidence intervals (grey area).



The initial treatment processes applied at the WRK intake station in Andijk to the water derived from the IJsselmeer include coagulation, rapid sand filtration and activated coal filtration. A decimal reduction of 1.6 and 1.5 log10-units for somatic and F-specific phages, respectively, was achieved by these processes, which may be extrapolated to the reduction of human pathogenic viruses. Subsequently, the water is transported to the West Coast where it is infiltrated into the dunes. Human pathogenic viruses may be removed up to 8 log10-units by (30 m) dune infiltration (Schijven et al. 1999), the actual infiltration distance is usually even larger. The reclaimed water is subsequently subjected to rapid sand filtration, safety disinfection (0.1 ClO_2 mg/l) at PWN production plants. The WLB plant applies ozonation, softening and activated coal filtration and slow sand filtration to the reclaimed water.

4

Discussion

Discussion and conclusions

4.1 Surface water quality

The water quality of the river Rhine is closely monitored because of, among other purposes, its use for drinking water production. However, parameters that are difficult to assess such as human pathogenic viruses are not included in the regular monitoring programmes. In this study, the Rhine was sampled at two locations in the catchment that is at Lobith near the border and at Andijk in the IJsselmeer in which the Rhine ends. The last location was selected for its use as a source for drinking water production. In all surface water samples viruses were present, either entero-, reo-, rota- or noroviruses or a combination. Human noroviruses and rotaviruses are the most important gastro-enteritis agents in the Netherlands. Norovirus RNA was detected in four out of ten samples taken from the Rhine. Similarly, in the Meuse catchment area 41% of the samples taken from 1999-2001 were positive for norovirus RNA (de Roda Husman en Ketelaars, 2004). In the samples taken from the IJsselmeer one out of six samples was positive for the presence of norovirus RNA. In comparison, rotavirus RNA was found in three out of ten Rhine samples, but none of the six samples taken from the IJsselmeer. One out of 29 Meuse samples was positive for the presence of rotavirus RNA. Differences may be explained by the fact that these viruses have different sources, inactivation rates and seasonal distribution. Though some viruses such as some enteroviruses may occur more frequently in the population in summer, their inactivation rates will be higher in summer by the higher temperatures and more sun hours resulting in higher enterovirus concentrations in winter than summer. An explanation for the lower number of rotavirus positive samples as compared with the norovirus positive samples may be that rotaviruses mainly cause infections in small children whereas norovirus infections regard all age groups. Rotaviruses disappear mostly into the solid waste along with the diapers, noroviruses however contaminate the sewage and subsequently the surface waters. Moreover, the numbers of surface water samples analysed per location were low either six or ten samples from the IJsselmeer and the Rhine, respectively. Statistical analyses of more data will reduce the uncertainty of the outcome, which will result in a narrower 95% confidence interval of the estimated virus concentration. And, with the development of more rapid and simple virus detection methods, studies analysing larger numbers of samples will become feasible.

The presence of rotavirus RNA correlated with infectious reoviruses in the Rhine and also with pH but this was not the case for the IJsselmeer. Turbidity did correlate with infectious enteroviruses but none of the other viruses. Previous research has shown that the presence of Cryptosporidium is correlated with turbidity (Hoogenboezem et al. 2001). More measurements are needed to determine possible correlations between viruses and physico-chemical parameters such as turbidity and pH.

In previous studies, the concentrations RNA containing particles were estimated taking into account the presence or absence of viral RNA in tenfold dilution series of the extracted RNA and the detection limit of the method (Medema 2000; Hoeijmakers en De Roda Husman 2001). Application of this method, however, introduces uncertainty in the estimation of the virus concentration. In 1995, the detection limit for the RT-PCR as applied for the noroviruses (previously designated Norwalk-like viruses or NLV's) was estimated at 30 intact virus particles by enumeration of virus particles in a fecal sample by electron microscopy en RT-PCR (Koopmans et al. 2000). However, norovirus replication is prone to changes in the genomic RNA of the virus leading to the evolution of novel variants that may displace other variants as the predominant type in the population. The previously estimated detection limit was based on the detection of Girlington but in 2002 the norovirus variant GGII.4

Lordsdale was the most prevalent type (Lopman et al. 2004). Since noroviruses mutate and the detection limits may differ from type to type it may be better to assume a chance for virus detection necessitating at least one RNA containing particle present in the sample to obtain a positive signal (Evers en Groennou 1999). Samples that do not result in a positive signal were assumed not to contain viral RNA. The maximum likelihood method was used to assess the numbers of virus particles in the original samples. The numbers of PCR detectable units in the original sample were calculated by dividing this number and the test volume. A Poisson distribution was used under the assumption that the viral RNA was randomly distributed in the sample. However, viruses may aggregate during the applied concentration steps (Floyd 1979). Also, viruses may be present as aggregates in surface waters prior to concentration thereby sheltered from inactivation. It is known that viruses are aggregated and/ or associated with fecal matter when excreted by their host. To which extent viruses are aggregated or bound to other than viral particles before or after concentration is unknown but should be studied in order to exclude that a positive signal is derived from more than one virus particle causing underestimation of the virus concentration.

Noroviruses can not be cultured and it is not reasonable to assume that this will be possible in the near future (Duizer et al. 2004). Therefore quantitative PCR methods should be developed for the detection of noroviruses in water (Rutjes en De Roda Husman 2004). Recently, quantitative PCR methods were developed for the detection of HIV and HCV in blood. The numbers of virus RNA containing particles in the original sample are estimated through comparison of the time it takes for the target RNA and an internal standard to be amplified above the threshold level of detection. The specificity of the obtained PCR products is confirmed by determining the melting temperatures of the controls and the target. Estimation of the concentration RNA or DNA containing particles by quantitative PCR no longer necessitates the use of RNA dilutions. The main challenge to develop a truly quantitative method for viruses in water, however, lies in the preceding concentration steps. Moreover, with concentration of viruses from large volume water samples inhibitors of the PCR may be co-extracted. An internal control indicates possible inhibition of the RT-PCR that gives a false negative signal.

The infectivity of any micro-organism should be known to be able to perform a reliable risk assessment as required by the Dutch drinking water guidelines (Anonymous 2001). Ideally, this should be established in a susceptible host such as humans but this would be unethical. Sensitive cell lines may also be used in order to estimate the numbers of infectious virus particles in source waters for drinking water production. A stated above this is not yet possible for noroviruses and difficult for rotaviruses, but in case of enteroviruses a well-established but time-consuming monolayer plaque assay is in use (Rutjes en de Roda Husman 2004). By use of this assay the virus concentrations in the Rhine and IJsselmeer were determined. The highest concentrations of entero- and reoviruses were determined in the Rhine as compared to the IJsselmeer. The concentrations in the Rhine were similar to those found in different parts of the Meuse (Hoogenboezem et al. 2001; De Roda Husman en Ketelaars 2004). The BGM cell line used here is well-characterized and can be infected by coxsackieviruses A7, A9, A16 and B1-5, echoviruses 2, 4, 5, 7, 11-15, 17, 19, 21, 24, 25, 27 and polioviruses 1-3, and reoviruses 1-3 (Dahling and Wright 1986). These enteroviruses may cause gastro-enteritis, meningitis, encephalitis or poliomyelitis. Though well-established the monolayer plaque assay applied here is very time-consuming taking ten days before the first result which then has to be confirmed and extended to a larger test volume. A cell culture method in which one virus replication cycle is followed by PCR detection may shorten the procedure to 2-5 days (Greening et al. 2002). Prerequisites are high sensitivity and specificity of such a cell culture-PCR method and control for the presence of substances that are toxic for the cells or inhibit the PCR (Rutjes et al. 2005).

For one sample taken from the Rhine in October 2002 the recovery of the virus concentration procedure involving two subsequent filtration steps was determined. Comparison of direct detection of somatic phages in water resulted in a count of 309 particles per 20 ml, 468 in 54 ml after negative membrane filtration and 185 particles in 29 ml after subsequent ultra-filtration. The estimated recovery of the first concentration step was 56% and after the second concentration was 74%. The recovery of the entire concentration procedure was estimated at 41%. To perform a risk assessment that does not unnecessarily overestimate the risk of infection, recovery rates should be determined with every analysis. It may be possible to determine recovery rates per water type to reduce hands-on time, however, this requires further research.

The concentrations of norovirus PCR detectable units may be up to 4 log10-units (10,000 times) higher than the concentrations of infectious enteroviruses depending on the location. In part this may be due to the fact that besides infectious also non-infectious RNA containing noroviruses can be detected by molecular methods. The lack of norovirus culture methods renders it impossible to determine the numbers of infectious noroviruses. Cell culture methods for feline and canine caliciviruses that belong to the same virus family as the noroviruses (human caliciviruses) have been described (Mochizuki et al. 2002; Slomka and Appleton 1998). The numbers of PCR detectable units and plaque forming units of these animal caliciviruses could be compared as determined by PCR and cell culture, respectively. However, the mechanism of infection by animal caliciviruses may differ from that of human caliciviruses but this is unknown to date.

Other viruses such hepatitis A and E viruses causing liver failure may also be present in Dutch surface waters (Van der Poel et al. 2002). Hepatitis A viruses are imported into the Netherlands by children at the end of the holidays from countries in which the disease is endemic (Richardus et al. 2004). These viruses may be asymptomatic in children but cause severe hepatitis in susceptible adults. Hepatitis E virus was recently detected in over 20% of swine farms in the Netherlands (Van der Poel et al. 2001). Moreover, a cluster of hepatitis E cases in humans was determined in the North of the Netherlands (Widdowson et al. 2003). The cause of the outbreak was not found but the infected patients had not travelled abroad. At that time period no faecal contamination was seen at the drinking water company as determined with bacterial indicators making drinking water as a common source less likely. In addition, sapoviruses, belonging to the Caliciviridae, have also been shown to be important gastro-enteritis agents in the Netherlands (De Wit et al. 2001b). These virus types especially hepatitis viruses should be included in the monitoring of surface waters because of the serious illness they may cause in unvaccinated individuals.

4.2 Treatment efficiency

The drinking water quality may be determined by analysing the source waters for the production of drinking water for the presence of human pathogenic viruses and evaluating the treatment processes with respect to their efficiency of virus removal by using indicator bacteriophages. Only six samples taken from the IJsselmeer and three samples after treatment at WRK were analysed for the presence of somatic and F-specific phages. Approximately 100 times higher concentrations were found for somatic versus F-specific phages in concordance with previous observations in Dutch surface waters (Hoogenboezem et al. 2001; De Roda Husman en Ketelaars 2004). These data were all included in the statistical analyses using a binomial model for unpaired data since paired analyses would limit the data sets further to only three instead of six measurements before treatment leading to increased uncertainty in the estimated virus concentrations. The only criteria for not including data in the statistical analyses should be on the basis of failure of the methods for concentration, extraction or detection of the viruses. Requirements for methods used for the detection of viruses in water should be established (Rutjes en De Roda Husman 2004).

The weighed arithmetic means could be calculated before and after treatment since phage concentrations were determined in large volume water samples over a six month period. Division of the weighed arithmetic means of the virus concentrations in the source (S) and treated (T) waters resulted in 1 log10-units removal of either phage group. Using the binomial model for unpaired data 95% confidence intervals could be estimated indicating the relative error in the measurements. The decimal reductions for somatic and F-specific phages were somewhat higher using the binomial model namely 1.6 (1.1-2.6) and 1.5 (0.9-3.6) log10-units, respectively.

The initial treatment processes applied at the WRK intake station in Andijk to the water derived from the IJsselmeer include coagulation, rapid sand filtration and activated coal filtration. A decimal reduction of 1.6 and 1.5 log10-units for somatic and F-specific phages, respectively, was achieved by these processes, which may be extrapolated to the reduction of human pathogenic viruses. Subsequently, the pretreated water is transported to the West Coast where it is infiltrated into the dunes. Human pathogenic viruses may be removed up to 8 log10-units by (30 m) dune infiltration (Schijven et al. 1999), the actual infiltration distance is usually even larger. The reclaimed water is subsequently subjected to rapid sand filtration, safety disinfection (0.1 ClO_2 mg/l) at PWN production plants. The WLB plants apply ozonation, softening and activated coal filtration and slow sand filtration to the reclaimed water. The multiple barrier principle applied here is prerequisite for the assurance of safe drinking water production (WHO 2003).

The concentrations F-specific phages correlated with the concentrations somatic coliphages determined in the Rhine and IJsselmeer. The bacteriophage concentrations also significantly correlated with the concentrations of reo- and enteroviruses in the IJsselmeer but not the Rhine. The bacteriophage concentrations did not correlate with those of noroviruses or rotaviruses. Apparently, bacteriophages in surface waters not always correlate with human viruses. However they are useful indicators for assessing the efficiency of water treatment processes and much more appropriate than bacterial indicators. Animal caliciviruses may also be useful to approximate the efficiency of treatment processes with regard to human noroviruses (De Roda Husman et al. 2004). Prior to approving the use of indicators for treatment efficiency their reduction should be assessed as compared with the reduction of human pathogenic micro-organisms.

The Dutch drinking water act, enforced in February 2001, prescribes a quantitative microbial risk assessment for *Cryptosporidium*, *Giardia* and (entero)viruses (Anonymous 2001). The realisation of this requirement necessitates large data sets on the source water quality and the efficiency of treatment processes. In this study the collection of such data sets was initiated and limitations to methods and tools were established. Quantitative microbial risk assessment is a useful tool to determine the risk of infection through drinking water consumption especially for newly emerging pathogenic viruses and in countries with a low disease burden such as the Netherlands (Schijven et al. 2003; 2004). However, outbreaks and sporadic cases of disease through tap water may happen on bad days when possible peak virus concentrations occur in the source waters for drinking water production and treatment processes fail. One Dutch drinking water associated outbreak that was identified took place in the vicinity of Utrecht when tap water accidentally was contaminated with partially treated surface water for use in washing machines and for flushing the toilet (Fernandes et al. 2004). The individuals exposed to tap water had a two to five times higher risk of contracting gastro-enteritis than inhabitants of neighbouring estates who were not exposed to the contaminated drinking water. These epidemiological studies are essential to our understanding of waterborne disease and the development of preventive measures.

Recommendations

1) To reduce the uncertainty of the estimated virus concentrations, more rapid and simple detection methods need to be developed for viruses in water. More reliable virus concentrations in surface waters will directly result in less uncertainty of the assessed annual infectious risk for drinking water consumption.
2) Some physico-chemical parameters may be determined on-line without the delay in response to time consuming virus detection methods, however, the correlation between viruses and parameters such as turbidity and pH needs to be established.
3) It is unknown to which extent viruses are aggregated or attached before or after concentration. Since this could result in underestimation of the virus concentration it should be studied whether a positive signal is derived from one virus particle or from a virus aggregate.
4) Since noroviruses can not be cultured a real-time quantitative PCR method should be developed for the detection of identified and newly emerging norovirus strains with appropriate internal controls and standards.
5) Cell culture is the preferred assay for virus detection in water samples since these data provide more accurate input in an infection risk assessment. For rotaviruses, astroviruses, adenoviruses, reoviruses, hepatitis A and E viruses cell culture assays should be developed followed by PCR detection to make these assays more sensitive and rapid.
6) The recovery of the virus concentration methods needs to be determined with every sample. If this is not possible the virus recovery should at least be determined per location or alternatively per water type. In case of recovery per water type this should be defined by specific determinants that first need to be identified.
7) The numbers of PCR detectable units and plaque forming units of these animal caliciviruses should be compared for different virus types which can be determined by PCR and cell culture. For noroviruses which can not be cultured this comparison could be made by use of similar caliciviruses such as feline and canine caliciviruses for which PCR and cell culture methods are available.
8) Requirements for methods used for the detection of viruses in water should be established.
9) The use of indicators as a measure for reduction of human pathogenic micro-organisms should be evaluated.
10) Epidemiological studies on waterborne disease need to be conducted in order to assess the burden in the Netherlands.

Abbreviations

ARK	Amsterdam Rijn kanaal
DNA	Desoxyribo Nucleic Acid
FSPECPH	F-specific phages
FTU	Formazine turbidity unit
HAdV	Human adenovirus
HAstV	Human astrovirus
HAV	Hepatitis A virus
HEV	Hepatitis E virus
HWL	Het Waterlaboratorium
l	Litre
ml	millilitre
m3	cubic metre
NLV	Norwalk-like virus
NV	Norovirus
Pdu	PCR detectable unit
pfu	Plaque forming unit
PWN	Waterleidingbedrijf Noord-Holland
RIVM	Rijksinstituut voor Volksgezondheid en Milieu
RIWA	Samenwerkende rivierwaterleidingbedrijven
RNA	Ribo nucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
SOMCPH	Somatic coliphages
SRSV	Small round-structured virus
TK	Twentekanaal
WLB	Waterleidingbedrijf Amsterdam
WPJ	Waterwinstation Prinses Juliana
WRK	Watertransportmaatschappij Rijn-Kennemerland
Wwg	Weerseloseweg

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A. Numbers of bacteriophages and human pathogenic viruses in concentrated water at Lobith.

Parameter	F-specific phages		Somatic phages		Enteroviruses		Reoviruses		Noroviruses		Rotaviruses	
Laboratory	RIVM		RIVM		RIVM		RIVM		RIVM		RIVM	
Characteristic	Count	Volume	Count	Volume	Count	Volume	Count	Volume	Dilution	Volume	Dilution	Volume
Entity	Number	Litre	Number	Litre	Number	Litre	Number	Litre	Positive	Litre	Positive	Litre
Date												
5-Dec-01	208	0.41	429	0.041	42	91	153	91	Negative	0.83	Negative	1.2
9-Jan-02	118	0.11	87	0.0097	40	151	162	151	1E-2	0.81	Negative	1.2
6-Feb-02	113	0.0097	209	0.0097	40	164	136	164	Negative	0.082	Negative	0.12
6-Mar-02	94	0.11	41	0.010	10	203	33	203	Negative	0.066	Negative	0.098
10-Apr-02	330	2.0	52	0.019	1	307	52	307	1E-1	0.12	1E-2	0.19
28-Aug-02	18	13	246	2.7	0	331	2	331	Negative	0.13	Negative	0.19
25-Sep-02	9	51	99	3.0	2	331	1	331	Negative	0.12	Negative	0.19
23-Oct-02	325	31	185	0.029	44	235	160	235	Negative	0.10	1E-2	0.15
20-Nov-02	340	1.8	54	0.016	72	191	413	191	1E-2	0.075	Negative	0.11
18-Dec-02	179	0.21	20	0.0021	28	146	864	146	1E-2	0.12	1E-3	0.18

B. Numbers of bacteriophages and human pathogenic viruses in concentrated water at PWN and WRK (IJsselmeer).

Parameter	F-specific phages		Somatic phages		Enteroviruses		Reoviruses		Noroviruses		Rotaviruses	
Laboratory	RIVM		RIVM		RIVM		RIVM		RIVM		RIVM	
Characteristic	Count	Volume	Count	Volume	Count	Volume	Count	Volume	Dilution	Volume	Dilution	Volume
Entity	Number	Litre	Number	Litre	Number	Litre	Number	Litre	Positive	Litre	Positive	Litre
Date												
21-Jan-02	181	16	205	0.088	10	150	42	150	Negative	0.063	Negative	0.094
20-Feb-02	29	30	205	0.55	0	91	2	91	Negative	0.032	Negative	0.049
13-Mar-02	8	65	67	0.68	0	67	1	67	Negative	0.044	Negative	0.067
3-Apr-02	37	50	73	0.50	0	72	0	72	1E-2	0.037	Negative	0.056
24-Apr-02	4	31	116	12	0	241	1	241	Negative	0.093	Negative	0.14
15-May-02	0	31	37	3.4	1	192	0	192	Negative	0.074	Negative	0.11

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