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Removal of Enteric Viruses from Surface Water at Eight Waterworks in The Netherlands

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Eight waterworks in The Netherlands, which use surface water as their raw water source, were sampled repeatedly between November 1978 and June 1981. At five waterworks, 30 of 45 samples of raw water contained viruses. Of 55 samples of partially purified water, 11 were virus positive, including 8 after coagulation, sedimentation, and rapid sand filtration, 2 after storage, coagulation, sedimentation, transport chlorination, and rapid sand filtration, and 1 after storage in open reservoirs for 5 months. No viruses were detected in 100 samples of drinking water of 500 liters each from six waterworks. Most isolated viruses were typed, and a great variety of human enteroviruses were found, reflecting both pollution of raw water sources with sewage and vaccination with oral polio vaccine in neighboring countries.

Increasing demands on surface water for the preparation of drinking water are combined with continuing pollution of these resources by sewage. A World Health Organization scientific group, therefore, recommended that "where virological facilities can be provided, it is desirable to monitor raw water sources and drinking water for the presence of viruses" (9).

The health significance of pathogenic viruses in drinking water was recently discussed by an International Association on Water Pollution Research and Control study group (2). Waterborne outbreaks of viral diseases have always been traced to inadequate treatment or contamination by defects in the distribution systems. Hypothetically, waterborne transmission at a low level might become the start of person-to-person spread, but this is difficult to prove. Unfortunately, viruses known as the cause of waterborne diseases cannot be detected by conventional isolation methods (hepatitis A virus, Norwalk[-like] viruses, or rotaviruses). In many reports viruses isolated from water were either not typed or typed as polioviruses, probably nonpathogenic strains excreted by children recently vaccinated with living oral polio vaccine. In The Netherlands only inactivated polio vaccine is used. In this study, a number of waterworks with different treatment processes were repeatedly sampled before and after treatment, and almost all isolated viruses were typed (polioviruses were further distinguished between vaccine-like and wild-type strains), thus "providing baseline data to evaluate the health risk faced by the population," as recommended by the World Health Organization.

MATERIALS AND METHODS

Sampling locations and treatment processes. At six waterworks (Fig. 1; see below, i through vi) which use surface water as their raw water source, 100 samples of finished water of 500 liters each were obtained at two points: at the end of the treatment process (at waterworks 1 and 5 before postchlorination) (indicated by A) and in the distribution system (indicated by B). Each point was sampled seven times between November 1978 and April 1980. At point B, a double sample was taken two or three times for assay by different techniques.

Raw surface water and partially treated water were examined for the presence of viruses at eight waterworks of which the treatment processes are described below. The sampling points for raw or partially treated water are indicated by I, II, and III. The periods in which the sampling took place and the number of samples are given in Fig. 2. Sample volumes varied from 0.25 to 520 liters.

(i) Sampling location 1—river Rhine: I; coagulation and sedimentation; rapid sand filtration; transport chlorination (3 mg/liter at a water temperature ≥ 10°C); II; dune infiltration (90 days, 81 m); aeration, activated carbon, and pH correction; rapid sand filtration; slow sand filtration; A; postchlorination; distribution; B.

(ii) Sampling location 2—Lake IJsselmeer: microstrainers (35 μm); I; breakpoint chlorination; coagulation and sedimentation; rapid sand filtration; activated carbon filtration; postdisinfection (ClO_2 plus Cl_2); A; distribution; B.

(iii) Sampling location 3—river Rhine: riverbank infiltration (±6 months, 700 to 1,100 m); I; aeration; rapid sand filtration; A; distribution; B.

(iv) Sampling location 4—surface water in Westland: I; dune infiltration (60 to 70 days, 60 to 80 m); aeration; rapid sand filtration; slow sand filtration; A; addition of 25 to 50% drinking water from different waterworks; distribution; B.

(v) Sampling location 5—river Drentse Aa: I; coagulation and sedimentation; aeration; and rapid sand filtration; slow sand filtration; A; addition of groundwater (60%); postchlorination; distribution; B.

(vi) Sampling location 6—river Meuse: storage in open reservoirs (±5 months); transport chlorination (1 mg/liter at a water temperature ≥ 10°C); I; coagulation and sedimentation; ozonization; coagulation and sedimentation; rapid sand filtration; activated carbon filtration; postchlorination; A; distribution; B.

(vii) Sampling location 7—river Meuse: I; storage in open reservoir (±3 months); coagulation and sedimentation; transport chlorination (0.5 to 1.0 mg/liter); rapid sand filtration; transport chlorination (1.5 mg/liter at a water temperature ≥ 7°C); II; dune infiltration (70 days, ±65 m); III; activated carbon; aeration; rapid sand filtration; slow sand filtration; distribution.

(viii) Sampling location 8—Lake Haringvliet: I; dune infiltration (135 days, 100 m); II; rapid sand filtration; ozonization; activated carbon; postchlorination; distribution.

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FIG. 1. Situation of the sampling locations of waterworks 1 through 8. Symbols: ●, raw or partially purified water; ×, distribution network; - - - -, transport pipelines.

Concentration procedures. Viruses were recovered from water samples by a modified adsorption-elution technique (1), followed by organic flocculation (6). Hydrochloric acid, magnesium chloride, and sodium thiosulfate (chlorinated water only) were added to the water samples with a fluid proportioner (Johanson and Sons, Machine Corp., Clifton, N.Y.) at a dilution of 1 to 100. The final concentrations of magnesium chloride and sodium thiosulfate were 0.05 and 0.001 M, respectively, and the final pH was 3.5 to 3.9. Volumes of 500 liters of drinking water and 10 to 520 liters of raw or partially treated water were filtered through a fiber glass-epoxy filter cartridge with a pore size of 8 μm (25 by 178 mm; grade C; Balston, Ltd., Maidstone, England). Flow rates were maintained at 10 liters/min or less. After filtration, the viruses were eluted from the filters with 300 ml of 3% Lab Lemco (Oxoid Ltd., London, England) solution in 0.05 M Tris buffer (pH 9.3) by recirculating the beef extract through the filter for 30 min at room temperature. This procedure was repeated, and the final volume of the eluate was 600 ml. This eluate was reconcentrated by the organic flocculation technique. By adding 2 M hydrochloric acid until pH 3.5 to pH 3.7 was reached, a floc was formed. The proceedings mentioned so far were done in the field at the sampling locations. The formed floc was transported at low temperature to the laboratory, where it was stored at −70°C.

On the day of the inoculation of water concentrates onto cells, the flocculated eluate was thawed and centrifuged at 3,000 × *g* for 10 min. The supernatant was discarded, and the sediment was dissolved in 3.6 ml of sodium monohydrogen phosphate (0.15 M and pH 9). Antibiotics (penicillin, 54,400 U/ml; streptomycin, 14,400 μg/ml; amphotericin B, 47 μg/ml; kanamycin, 5,300 μg/ml; gentamicin, 3,300 μg/ml; and neomycin, 1,300 μg/ml) were added to the concentrate. After neutralization, the final concentrate was inoculated onto BGM cells.

A number of samples of raw water were treated by a one-step concentration method. For this purpose, volumes of 4 liters or less were acidified with 2 M hydrochloric acid to pH 3.5 to pH 3.8, and after addition of magnesium chloride to a final concentration of 0.05 M, the sample was filtered through a membrane filter (filter type HA; pore size, 0.45 μm; diameter, 47 mm; Millipore Corp., Bedford, Mass.). The adsorbed viruses were recovered from the filters by passing two successive volumes of 3 ml of a 3% solution of Lab Lemco in Tris buffer (pH 9.3) through the filters after a contact time of 30 min for each elution. Antibiotics (penicil-

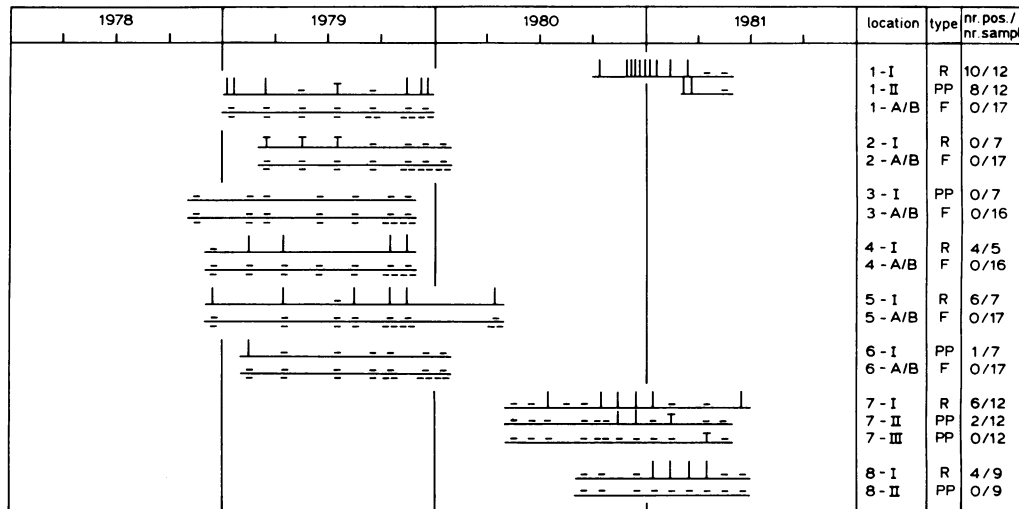

FIG. 2. Survey of sampling period, location, and results of the virological examination. Symbols for type of water: R, raw; PP, partially purified; and F, finished. Vertical bars, positive samples; dashes, negative samples; T, toxic for BGM cells.

TABLE 1. Virological examination of raw surface water

Sample no.[a]	Mo and yr	Sample vol (liters)	No. of plaques	No. of virus types[b]	Fecal coliforms per liter
1-I	October 1980	75	6	4 CB5; 2 reo	8.0×10^3
	December 1980	1	5	3 CB3; 2 E 7	
		3	8	6 P2-SL; 1 P3-NSL; 1 E-NT	
		11	8	1 P2-SL; 1 P3-SL; 1 CB2; 1 CB3; 1 reo; 3 E-NT	3.0×10^3
		2.7	4	1 P2-SL; 1 CB4; 2 CB5	
		10	2	1 P3-SL; 1 CB4	
	January 1981	0.6	1	ND	2.0×10^3
		0.7	1	1 P3-SL	
	February 1981	60	2	1 P3-SL; 1 E 7	4.0×10^3
	March 1981	0.8	1	1 E 7	2.0×10^3
	April 1981	3	0		2.0×10^3
	May 1981	3	0		3.8×10^3
4-I	December 1978	3.5	0		53
	February 1979	4	2	1 NT-A; 1 NT-B	1.5×10^5
	April 1979	0.9	20	14 NT-A; 5 NT-B; 1 E 31	1.6×10^4
	October 1979	100	2	2 NT-B	8.2×10^2
	November 1979	35	5	1 NT-A; 4 CB3	5.3×10^2
5-I	December 1978	46	114	1 CB3 (others ND)	7.1×10^4
	April 1979	100	4	1 CB2; 3 reo 2	5.3×10^4
	July 1979	50	0		2.8×10^3
	August 1979	50	1	1 CB3	1.9×10^4
	October 1979	100	1	1 CB4	1.0×10^3
	November 1979	1.4	11	4 CB4; 1 E 9; 6 E 11	1.0×10^6
	April 1980	2.1	7	7 E 11	1.0×10^4
7-I	May 1980	1.5	0		1.5×10^4
	June 1980	1	0		2.5×10^4
	July 1980	1.7	10	10 CB4	4.9×10^4
	August 1980	1	0		4.3×10^4
	September 1980	1.2	0		3.3×10^5
	October 1980	1.4	3	1 CB5; 2 CA9	1.0×10^5
	November 1980	1	3	3 CB4	3.8×10^4
	December 1980	0.7	4	1 P2-SL; 1 P3-SL; 1 CB5; 1 E 1	2.0×10^4
	January 1981	0.5	3	1 CB4; 2 E-NT	8.0×10^4
	February 1981	0.8	0		3.0×10^4
	April 1981	4	0		2.6×10^4
	June 1981	41	4	1 CB2; 3 CB5	1.6×10^3
8-I	September 1980	330	0		<10
	October 1980	200	0		<10
	December 1980	70	0		ND
	January 1981	200	10	3 P2-SL; 1 CB2; 2 CB4; 2 E 1; 2 E7	2.5×10^2
	February 1981	90	22	9 P2-SL; 12 CB5; 1 CB6	9.5×10^2
	March 1981	260	8	6 P2-SL; 1 CB5; 1 E 1	50
	April 1981	230	11	10 CB5; 1 E 7	7.7×10^3
	May 1981	100	0		8.5×10^2
	June 1981	320	0		<40

[a] See Materials and Methods for location.

[b] P, Poliovirus; NSL, non-Sabin-like; SL, Sabin-like; CA, coxsackievirus A; CB, coxsackievirus B; reo, reovirus; E, echovirus; E1, echovirus type 1 or the related type 8; NT, untypable, see text for NT-A and NT-B; ND, not done.

lin, 10,000 U/ml; streptomycin, 2,700 μg/ml; kanamycin, 10,000 μg/ml; neomycin, 25 μg/ml; gentamicin, 600 μg/ml; and amphotericin B, 44 μg/ml) were added, and after neutralization the concentrate was inoculated onto BGM cells.

Cell cultures. BGM cells, a continuous cell line derived from African green monkey kidney (Flow Laboratories, Ltd., Irvine, United Kingdom), were used at passages 80 through 140. The line was maintained in plastic bottles (75 and 150 cm^2; Costar Data Packaging, Cambridge, Mass.) on Eagle minimal essential medium (Hanks) as growth medium, supplemented with 0.09% bicarbonate, 10% fetal bovine serum, and antibiotics (penicillin, 100 U/ml; streptomycin, 100 μg/ml; and amphotericin B, 2.5 μg/ml). Three days after seeding, the medium was replaced by Eagle minimal essential medium (Earle) as maintenance medium, supplemented with 0.15% bicarbonate and 0.02 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 7% fetal bovine serum, and the same concentration of antibiotics.

Viral assay procedures. A Sabin vaccine strain of poliovirus 1 (LSc 2ab) was used as a model for the evaluation of the recovery efficiency of the concentration procedures. After propagation on BGM cells, the virus was stored at −20°C.

TABLE 2. Virological examination of partially treated river water

Sample no.[a]	Mo and yr	Vol (liters)	No. of plaques	No. of virus types[b]	Fecal coliforms per liter	Chlorine (mg/liter)	
						Free	Total
1-II	January 1979	100	7	2 P1-NSL; 2 P2-SL; 1 P2-i; 1 CB2; 1 E-NT	6.3×10^3	<0.02	<0.02
		3	10	3 CB5; 4 reo 1; 2 reo 2; 1 reo 3		<0.02	<0.02
	March 1979	100	1	1 reo	60	<0.02	<0.02
	May 1979	130	0		<10	<0.02	0.3
	July 1979	100	Toxic		<10	0.07	0.3
	September 1979	100	0		<10	0.2	0.5
	November 1979	3	1	1 CB5	ND	<0.02	<0.02
	December 1979	116	3	2 P1-SL; 1 E-NT	2.8×10^2	<0.02	<0.02
		4.1	5	1 P2-SL; 4 reo		<0.02	<0.02
	March 1981	300	2	1 CB3; 1 CB4	40	<0.02	<0.02
		520	16	3 P2-SL; 3 CB5; 2 E 1; 8 reo		<0.02	<0.02
	May 1981	520	0		<10	0.02	0.2
6-I	February 1979	100	1	1 reo	<10	<0.02	<0.02
	April 1979	100	0		ND	0.6	0.7
	July 1979	50	0		<10	ND	ND
	September 1979	26	0		<10	<0.02	0.3
	October 1979	100	0		23	<0.02	0.3
	December 1979	400	0		<10	0.02	0.3
	January 1980	350	0		50	<0.02	<0.02
7-II	May 1980	500	0		<2	ND	0.1
	June 1980	600	0		<2	ND	0.1
	July 1980	500	0		<2	ND	0.06
	September 1980	500	0		<2	ND	0.08
	October 1980	350	0		<2	ND	0.08
		500	0		<2	ND	0.1
	November 1980	500	12	3 P2-SL; 9 P3-SL	<2	ND	<0.02
	December 1980	450	1	1 P3-SL	<2	ND	<0.02
	January 1981	400	0		<2	ND	<0.02
	February 1981	500	Toxic		<2	ND	<0.02
	April 1981	500	0		<2	ND	0.1
	May 1981	500	0		<2	ND	0.1

[a] See Materials and Methods for location and treatment.
[b] P, Poliovirus; NSL, non-Sabin-like; SL, Sabin-like; i, intermediate; CA, coxsackievirus A; CB, coxsackievirus B; reo, reovirus; E, echovirus; E 1, echovirus type 1 or the related type 8; NT, untypable; ND, not done.

Approximately 100 PFU was added to 500 liters of finished water or 50 PFU to 1 liter of sterilized raw water.

The concentrated samples were inoculated on 3-day-old monolayers (1.5 ml/75 cm^2). After 1 h of adsorption at room temperature, an agar medium was added. This overlay consisted of Eagle minimal essential medium (Earle), 0.15% bicarbonate, 0.02 M HEPES, 7% fetal bovine serum, antibiotics (penicillin, 100 U/ml; streptomycin, 100 μg/ml; and amphotericin B, 5 μg/ml), and 0.9% agar (Bacto-Agar; Difco Laboratories, Detroit, Mich.). The cultures were incubated inverted at 37°C. After 10 days, a second overlay was added consisting of 0.9% agar in phosphate-buffered saline with 0.03% neutral red, and visible plaques were counted. For confirmation, plaques were picked and inoculated onto BGM cells for the development of cytopathic effect. The cultures were observed for 12 days at most, at which time one further blind passage was performed in case no cytopathic effect was observed.

A number of concentrates from the distribution systems (sampling point B) were examined for the presence of viruses without an agar overlay. In these cases, the cultures were observed for 10 days for cytopathic effect. A passage was performed and observed for another 10 days.

Virus identification. Viral isolates were tested for growth in the cells which were in routine use in the diagnostic enterovirus department, i.e., primary or secondary cynomolgus monkey kidney (MK) cells and a diploid human embryonic lung fibroblast strain, or on BGM cells only. Reoviruses were identified by their peculiar cytopathic effect and typed by neutralization in MK cells in tubes. Some strains were further typed by hemagglutination inhibition. The other viruses were typed by routine neutralization tests in microtitration plates (Greiner) by using Titertek equipment (Flow Laboratories). Typing sera were prepared in previous years by immunization of horses with pools of enterovirus, designed so that each of 21 of the most common virus types was present in two pools. Monovalent antisera were prepared in cynomolgus monkeys as previously described (5).

Intratypic serodifferentiation of poliovirus isolates was done by neutralization with adsorbed sera by the method of van Wezel and Hazendonk (8), which distinguishes Sabin vaccine-like strains from non-Sabin-like, wild-type strains.

When typing results were unsatisfactory, the virus was treated with chloroform (5), titrated, and tested with 100 50% tissue culture infective doses against all available enterovirus antisera and if necessary against the preimmunization sera.

Bacteriological examination. For the detection of fecal coliform bacteria, volumes of up to 500 ml were filtered

through membrane filters of cellulose nitrate with a pore size of 0.45 μm, which were cultured on Membrane-Enriched Teepol agar (Oxoid MM 369; Teepol 610 Serva). After incubation for 4 h at 30°C, followed by 14 h at 37°C, yellow colonies were counted, and a number was confirmed in brilliant green lactose bile broth by incubation for 48 h at 44°C.

RESULTS

Recovery efficiency of attenuated poliovirus 1. The recovery efficiency of attenuated poliovirus 1 of the two-step procedure used for the concentration of volumes larger than 10 liters was 50% (mean value) with a standard deviation of 13% (20 experiments with finished water). The recovery percentage of the single-step technique used for the concentration of raw water (samples of 4 liters or less) was 65% (mean value) with a standard deviation of 11% (12 experiments).

Drinking water. A total of 100 samples of drinking water (500 liters each) were examined for the presence of viruses (see Materials and Methods sampling points A and B; Fig. 2); 42 samples of water were taken at the end of the purification process (A), and 58 samples were taken from the distribution network (B). Viruses could not be detected in any of these samples. Fecal coliforms were also absent (less than two per liter).

Surface water and partially treated water. Three of the seven concentrates of the water of Lake IJsselmeer (2-I) were toxic for BGM cells (see Materials and Methods; Tables 1 and 2; Fig. 2). No reliable virological results could be obtained, although the presence of coliforms indicated that the water was contaminated.

From the untreated surface waters of the river Rhine (1-I), the Westland (4-I), the Rivers Drentse Aa (5-I) and Meuse (7-I), and Lake Haringvliet (8-I), 30 of 45 samples contained viruses as well as high numbers of bacterial indicators of fecal pollution.

Partially treated water was monitored at five waterworks (see Materials and Methods, sampling points 1-II, 3-I, 6-I, 7-II, 7-III, and 8-II). The treatment as given in Materials and Methods appeared to not completely remove the viruses present in both the Rivers Rhine and Meuse in the winter (1-II and 7-II). Of 24 samples, 10 contained viruses.

After infiltration of water of the river Rhine into riverbanks (3-I), no viruses could be isolated from volumes of 28 to 400 liters. Fecal coliforms were also absent.

Infiltration of virus-containing water into the dunes was effective in removing viruses and fecal coliforms (7-III and 8-II). No viruses were grown from volumes of 200 to 500 liters).

The effect of storage of river water in open reservoirs was measured at waterwork 6 (6-I). One PFU in 100 liters was isolated only once from seven samples. This virus was a reovirus. Fecal coliforms were absent, although coliform bacteria (12 per liter) and fecal streptococci (30 per liter) were observed.

Virus identification. The plaques obtained from the various samples were formed by a great variety of viruses, even in small samples (Tables 1, 2, and 3). An outstanding example is the sample of only 0.7 liter (December 1980) from the river Meuse (7-I), which contained four different virus types.

Most of the isolates (77.6%) were human enteroviruses. The origins of the reoviruses and the untypable viruses NT-A and NT-B are unknown.

The Rivers Rhine and Meuse contained Sabin-like polioviruses of all three types, only a few of type 1 and many of types 2 and 3. The vaccine viruses were still detected in Lake Haringvliet, where water from both rivers comes together. To test the hypothesis that the vaccine viruses originated from pollution in the neighboring countries, we sampled the Rhine in Lobith, where the river enters the Netherlands (Fig. 1), from January through March 1981. From samples of about 0.5 to 1 liter, Sabin-like polioviruses 2 and 3 were regularly isolated.

In water from the Rhine, non-Sabin-like polioviruses were also found; type 1 in January 1979 (point 1-II) and type 3 in December 1980 (point 1-I). Sampling location 5-I (Drentse Aa) is polluted by sewage from a provincial Dutch town. Here only nonpolio human enteroviruses were detected.

The surface water of Westland (4-I) contained untypable viruses of 28-nm diameter which grew only in BGM cells. A group of 16 isolates (NT-A) was neutralized by some pre- and postimmunization sera of horses; 8 other isolates (NT-B) were neutralized by a pre- and postimmunization serum of a monkey. Both viruses were not pathogenic for suckling mice.

TABLE 3. Identification of 228 viruses isolated from 41 virus-positive samples

Virus type[a]	No.
Poliovirus 1	
NSL	2
SL	2
Poliovirus 2	
Int	1
SL	36
Poliovirus 3	
NSL	1
SL	15
Coxsackievirus	
A9	2
B2	5
B3	11
B4	24
B5	41
B6	1
Echovirus	
1	6
7	7
9	1
11	13
31	1
Enterovirus (NT)	8
Reovirus 1, 2, and 3	27
NT	
A	16
B	8

[a] NSL, Non-Sabin-like; SL, Sabin-like; Int, intermediate; NT, untypable; NT-A and NT-B, see text.

DISCUSSION

In this study, attenuated poliovirus was used as a model in recovery experiments with two concentration methods; no data for the recovery of other enteric viruses were obtained. The BGM cell line is very sensitive for the detection of

poliovirus and coxsackievirus B but not useful for the detection of adenovirus (3, 7). Many samples of raw and partially treated water had a certain turbidity, which had a negative influence on the volume that could be concentrated. Nevertheless, a great variety of human enteroviruses was detected. More than 17 enterovirus types reflect virus excretion after inapparent infection or by patients. Every year in the Netherlands, about 40 different enterovirus types are isolated from patients (4). The few wild-type polioviruses might originate from infections imported from abroad. But the many attenuated (Sabin-like) virus strains are certainly not derived from vaccination in the Netherlands, because only inactivated polio vaccine is used there. Poliovirus was isolated from sampling locations 1, 7, and 8 and from Lobith, i.e., from water of the international Rivers Rhine and Meuse. The neighboring countries of the Federal Republic of Germany and Belgium use living polio vaccine. The raw water of the river Drentse Aa, which is polluted by a small Dutch town, contained no poliovirus in the same period but did contain many other enterovirus types (5-I).

Reoviruses are infrequently isolated from patients. These viruses and the untypable viruses A and B isolated from surface water are probably not of human origin.

In this study, several treatment processes were evaluated. Riverbank (3-I) and dune infiltration (7-III and 8-II) treatments appeared effective in the removal of viruses. The influence of storage in open reservoirs (6-I) is obvious. Treatment by coagulation and sedimentation, followed by rapid sand filtration (1-II and 7-II), is insufficient, especially during the winter. Viruses were not found in any of the 100 samples of drinking water (some tested by two detection methods). The currently practiced water treatment methods seem to be adequate for the removal of viruses. This study is being continued with improved methods and emphasis on virus removal in various stages of water treatment.

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