

Surveillance of Influenza A and the pandemic influenza A (H1N1) 2009 in sewage and surface water in the Netherlands

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ABSTRACT

The role of the water cycle in spreading human pathogenic influenza viruses is poorly studied and is not considered to be significant. However, gastrointestinal symptoms developed in a large proportion of influenza A (H1N1) 2009 virus infected people during the pandemic in 2009 and fecal shedding was reported. This fecal route could potentially play a role in the entry of human pathogenic influenza viruses in to the water cycle. Monitoring of influenza viruses in sewage and surface water during the pandemic in 2009 showed that influenza A viruses were detected in sewage and surface water. However, the pandemic influenza A (H1N1) 2009 virus was not detected. These findings imply that the water cycle did not play a relevant role in spreading the pandemic influenza virus during the epidemic in the Netherlands in 2009. Analyses of deliberately contaminated water samples confirmed the ability of quantitative RT-PCR to detect influenza viruses in sewage samples whereas the analysis of large volumes of surface water was strongly hampered by the presence of PCR-inhibiting substances.

Key words | influenza virus, quantitative RT-PCR, sewage, surface water, RNA

INTRODUCTION

A novel influenza A (H1N1) virus emerged among humans in California and Mexico in early April 2009. The virus spread quickly worldwide through human-to-human transmission resulting in an influenza pandemic. In the Netherlands, the first case of infection with this pandemic influenza A (H1N1) 2009 virus was reported on 30 April 2009 and the first patient was hospitalized on 5 June 2009 (van 't Klooster *et al.* 2010). In the Netherlands, this pandemic resulted in 2181 hospitalized patients, 219 of them were treated in an intensive care unit and 53 died (van 't Klooster *et al.* 2010). Typical flu-like clinical symptoms (fever, cough, sore throat, myalgia, malaise, chills, rhinorrhea, conjunctivitis, headache, and shortness of breath) develop after infection with pandemic influenza A (H1N1) 2009 virus (Girard *et al.* 2010). But, gastrointestinal symptoms (vomiting, diarrhea, and abdominal pain) are also observed frequently in patients infected with the pandemic

influenza A (H1N1) 2009 virus (Dawood *et al.* 2009; Health Protection Agency *et al.* 2009; Riquelme *et al.* 2009). Diarrhea manifested in 29.4% of confirmed pandemic influenza A (H1N1) 2009 cases among Chilean patients (Riquelme *et al.* 2009), in 25% of the confirmed cases in a study in the USA (Dawood *et al.* 2009), and in 26% of cases in a study in the UK (Health Protection Agency *et al.* 2009) demonstrating the high prevalence of diarrhea in patients infected with the pandemic influenza A (H1N1) 2009 virus. Pandemic influenza A (H1N1) 2009 viral RNA could also be detected in stool from 16 of 65 (24.6%) hospitalized infected patients in Korea (Yoo *et al.* 2010). These findings suggest that infected patients shed this virus via the fecal route implying that contact with fecal material from influenza virus-infected persons might be a potential mode of transmission for this influenza virus. This could also mean that fecal shedding of influenza

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virus would result in contamination of sewage and discharge of contaminated sewage might contaminate surface water. This would have potential impact on the safety of surface water for the use of recreational purposes. And, although removal and inactivation processes used for the production of drinking water have been shown to be effective (Lenes *et al.* 2010) for influenza viruses, the presence of human pathogenic influenza viruses may have implications for water companies that use surface water for the production of drinking water. However, this mode of virus spreading is only poorly studied and more research is needed to evaluate the relevance of this potential transmission route. The influenza A (H1N1) 2009 pandemic gave an opportunity to study spreading of influenza viruses across the water cycle. The expected high number of infected people in combination with a relatively high number of people with gastrointestinal symptoms due to infection with the pandemic influenza A (H1N1) 2009 virus would expect to result in high concentrations of influenza virus in sewage during the peak of the epidemic. Thus methods were developed and used to study the presence of influenza A (general) and the pandemic influenza A (H1N1) 2009 virus in sewage and surface water samples during the influenza A (H1N1) 2009 epidemic in the Netherlands in this study. Samples from a sewage treatment plant that was treating sewage highly contaminated with human fecal waste were analyzed. Sewage influent and effluent were analyzed separately to obtain information about the removal of influenza viruses in the sewage treatment plant. Discharge of influenza virus in surface water was monitored in samples taken from the river which is used to discharge the treated sewage at a location downstream from the treatment plant. This approach would make it possible to determine if influenza viruses are still detectable after (short) transport and dilution in surface water. Viruses from the water samples were concentrated using ultrafiltration methods to enable sensitive detection and two different real-time quantitative reverse transcriptase PCR (qRT-PCR) assays were used to detect influenza A and the pandemic influenza A (H1N1) 2009 separately. The application of these methods was evaluated on RNA extracted from water concentrates which were artificially contaminated with the pandemic influenza A (H1N1) 2009 virus.

METHODS

Sample locations

Sewage samples were collected from the sewage treatment plant of the city of Utrecht in the Netherlands. This plant treats sewage from a maximum of 529,000 people and processes between 2.8×10^6 and 1.5×10^7 L/h sewage by using sedimentation followed by activated sludge treatment. Treated water (effluent) is discharged in the river De Vecht. Sewage influent and effluent samples with a volume of approximately 1 L and surface water (approximate volume: 60 L) from the river at a location 5 km downstream of the discharge point of the treatment plant were collected between 15 October 2009 and 17 February 2010. Water samples were transported to the laboratory and processed within 24 hours.

Sample processing

Ultrafiltration-based methods were used to concentrate particles (including viruses) from sewage influent, effluent, and surface water. Surface water was processed by a modification of a method described by Hill *et al.* (2005), using a hollow-fiber (polysulfone) dialysis Fresenius F80S ultrafilter (Fresenius Medical Care, Nieuwkuijk, the Netherlands). Hemoflow F80S filters were used untreated. A Masterflex (Cole-palmer, USA) peristaltic pump in combination with Masterflex tubes (type 96400-73) were used to pump water through the filter at a pump speed of 4 to 5 and a maximum pressure of 0.6 bar. The flow-rate through the filter was adjusted to approximately 900 mL/min. This method has been described as an efficient concentration method for different organisms including viruses (Hill *et al.* 2005, 2007) and has been shown to concentrate viruses with high recovery efficiencies in our hands also. The particles in the samples of 60 L surface water were concentrated to an average volume of 445 mL (range 322–556 mL) using this procedure. Concentrated surface water samples and sewage influent and effluent samples were centrifuged at $1500 \times g$ in 250 mL conical centrifuge tubes (Corning, Fisher Scientific, Landsmeer, the Netherlands) to pellet large particles. The supernatant was collected and centrifuged through 30-kDa Centricon Plus-70 (Millipore,

Amsterdam, the Netherlands) ultrafilters according to the manufacturer's instructions, and as described before (Hill *et al.* 2007), to concentrate viral particles to an average volume of 21 mL (range 10–45 mL) from sewage influent, 4 mL (range 1–8 mL) from sewage effluent and 28 mL (range 12–64 mL) from surface water.

RNA extraction

The magnetic extraction reagents of the Biomerieux Nuclisens kit (Biomerieux, Boxtel, the Netherlands) were used to extract nucleic acids from the concentrated water samples. One millilitre of every concentrated water sample was added to a nuclisens tube containing 2 mL of nuclisens lysis buffer and incubated for 10 min at 20 °C. A mixture containing 60 µL of magnetic silica and 940 µL nuclisens buffer was added to the lysate and mixed briefly by vortexing and incubated for 10 min at 20 °C. The samples were centrifuged for 2 min at 1500 *g* to pellet the magnetic silica particles, the supernatant was removed, the silica particles were suspended in 500 µL wash buffer 1 and transferred to the first tube of a KingFisher ml magnetic particle processor (Thermo scientific, Breda, the Netherlands), and this device was used to automatically perform the washing steps. The magnetic silica particles were subsequently washed in wash buffer 1 (for 1 min with fast dual mixing), transferred to the second tube containing 500 µL wash buffer 2 (wash step for 1 min with fast dual mixing), transferred to the third tube containing 500 µL wash buffer 2 (wash step for 1 min with fast dual mixing), transferred to the fourth tube containing 800 µL wash buffer 3 (wash step for 10 s with slow mixing) and then suspended in 100 µL elution buffer. The magnetic bead suspension is transferred to 1.5 mL eppendorf tubes and nucleic acids are released from the beads by incubating the magnetic bead suspension at 60 °C for 5 min. The magnetic beads are removed by incubating the tube on a magnetic separation stand (Promega, Leiden, the Netherlands) for 1 min and transferring the supernatant to a new tube.

Primers and probes

Primers and probes, specific for two different quantitative RT-PCR methods (qRT-PCR) were used in this study. One

has a general specificity for all influenza A viruses (Flu-A), the other (panFlu-A) specifically detects the pandemic influenza A (H1N1) 2009 virus.

The primers used for the Flu-A qRT-PCR were based on a previously described method (Ward *et al.* 2004). The primers target a conserved 106 bp fragment of the matrix gene of influenza A viruses. Based on sequence comparison of influenza A matrix genes from public databases, the sequence of the forward primer was modified at one position to optimize detection of all influenza A viruses including the pandemic influenza A (H1N1) 2009 virus: 5'-AAGACCAATCYTGTCACCTCTGA-3' (modification in bold). The sequence of the reverse primer was as published (Ward *et al.* 2004) and the modified probe sequence was as previously described (Munster *et al.* 2007).

Previously described primers (panN1-sense/panN1-anti-sense) and probe (panN1-probe), targeting a 104 bp fragment of the neuraminidase gene, were used for the panFlu-A qRT-PCR (van der Vries *et al.* 2010).

Probes were labeled with a FAM (6-carboxyfluorescein) label at the 5' end and a BHQ1 (Black Hole Quencher) quencher at the 3' end of the probe. Primers and probes were obtained from Biologio (Nijmegen, the Netherlands).

qRT-PCR reaction conditions

Separate qRT-PCR reactions (Flu-A and panFlu-A), using the same reaction conditions, were performed for both targets. QRT-PCR reactions were performed in duplicate in a reaction volume of 50 µL containing 10 µL of RNA extract, 25 µL reagent from the iScript One-Step RT-PCR kit (BioRad, Veenendaal, the Netherlands), primers and probe to a concentration of 0.2 µM, and bovine serum albumin (PCR grade: Roche Diagnostics, Almere, the Netherlands) to a concentration of 0.4 mg/mL. The BioRad CFX system (BioRad) was used to perform qRT-PCR reactions with the following conditions for both reactions: 10 min 50 °C (reverse transcription), 5 min 95 °C, followed by 40 cycles of 30 s at 95 °C, and 1 min at 60 °C.

qRT-PCR quantification using in vitro transcribed RNA

RT-PCR reactions were performed with both methods (Flu-A and panFlu-A) on a laboratory-cultured isolate

(A/NL/602/2009) of the pandemic influenza (H1N1) 2009 virus; this virus was a kind gift of Prof. R. Fouchier (National Influenza Center, Erasmus Medical Center, Rotterdam, the Netherlands). The RT-PCR fragments were ligated into the pGEM-TEasy vector system and cloned in *E. coli* cells using the manufacturer's procedure (Promega). Plasmids which contained the correct RT-PCR fragments were used to generate in vitro transcripts. T7 RNA polymerase based in vitro transcription using the Riboprobe system (Promega) was used to generate RNA transcripts; DNA was digested using RQ1 DNase (Promega). RNA-transcripts were purified with the RNA clean & concentrator kit (Zymo Research, Orange, USA) and fluorescently stained with the Quant-iT RNA assay kit (Invitrogen, Breda, the Netherlands) and quantified with the Qubit fluorometer (Invitrogen). Serial dilutions of these quantified RNA suspensions were used to generate cycle thresholds from known quantities of RNA. These cycle thresholds values were used by the software of the BioRad CFX to generate Flu-A and panFlu-A calibration curves, and the calibration curves were used to determine the concentration of genome copies (GC) in the RNA-extracts.

Sequence analysis of Flu-A positive samples

The Flu-A specific PCR fragments were cloned in the pGEM-T Easy II vector system using procedures described by the manufacturer of the kit (Promega). Sequence analysis of the 95 bp long qPCR fragment inserts were determined (Macrogen, USA) using standard T7 and Sp6 specific primers. Sequences of four individual clones were determined from every Flu-A positive sample. The sequences were compared with sequences in the Genbank database using the Blast program (Altschul *et al.* 1990) at the NCBI website.

Analysis of spiked samples

All samples were analyzed after the addition of laboratory-cultured pandemic influenza A (H1N1) 2009 virus (spiked samples) to study the influence of the matrix of the concentrated water samples on the performance of the qRT-PCR

methods. Approximately 2,000 GC of qRT-PCR quantified pandemic 2009 influenza A (H1N1) virus (A/NL/602/2009) were mixed with 1 mL of water concentrate or 1 mL of fresh tap water and added to nuclisens lysis buffer to extract RNA. The ratio between the qRT-PCR values measured in extracts from spiked concentrated water samples, and the qRT-PCR value of the extract from the spiked suspension in 1 ml of tap water was called the detection efficiency.

RESULTS

Efficiency and lower limit of detection of qRT-PCR

Flu-A and panFlu-A specific qRT-PCR reactions, performed on 10-fold serial dilutions of quantified in vitro transcribed RNA fragments, demonstrated a linear range between the log starting copy number and the threshold cycle from 2.5 RNA copies/ μL to at least 2.5×10^5 RNA/ μL for both Flu-A and panFlu-A specific qRT-PCR reactions. The slope was calculated by the Biorad CFX software as 3.53 and 3.31 for Flu-A and panFlu-A qRT-PCR reactions resulting in PCR efficiencies of 100 and 92%, respectively. Comparable reaction efficiencies were obtained from extracts from serially diluted laboratory-cultured pandemic influenza A (H1N1) 2009 virus.

The lower limit of detection of the qRT-PCR methods was tested on RNA extracted from serial dilutions of a quantified (with qRT-PCR) laboratory-cultured isolate of the pandemic influenza A (H1N1) 2009 virus. Dilutions containing a concentration of 2.5 influenza GC/ μL (25 influenza virus GC/qRT-PCR reaction) tested positive with both qRT-PCR methods in all cases ($n = 6$) and dilutions containing 0.25 influenza GC/ μL (2.5 influenza virus GC/qRT-PCR reaction) were positive in three Flu-A reactions ($n = 6$) and two panFlu-A reactions ($n = 6$).

Detection efficiency in spiked concentrated water samples

Spiked water samples were analyzed to monitor the performance of the methods in concentrated sewage and surface water. Extracts of the spiked suspensions were

analyzed separately; equivalent spiked suspensions were added to concentrated water samples and the extracts were analyzed with qRT-PCR. The differences between the qRT-PCR values obtained from the separate spiked suspensions and the spiked sample-concentrates were used to determine the detection efficiency. The detection efficiency gives insight in to the influence of the matrix of the concentrated water samples on the qRT-PCR values obtained. Detection efficiencies were also determined on undiluted RNA-extracts and on 10-fold diluted RNA-extracts. The detection efficiencies are shown in Table 1.

The average detection efficiencies vary between the different matrices. The highest average detection efficiencies are obtained in extracts from sewage influent (53.8% for Flu-A; 35.5% for panFlu-A); detection efficiencies are lower in concentrated sewage effluent (42.7% for Flu-A and 27.2% for panFlu-A) and lowest in concentrated river water samples (5.4% for Flu-A and 4.3% for panFlu-A) when undiluted RNA-extracts are analyzed. Detection efficiencies vary also between the different qRT-PCR methods, efficiencies are higher for the general Flu-A qRT-PCR. Higher detection efficiencies are obtained on RNA extracts which are diluted 10-fold.

Influenza A and pandemic influenza A (H1N1) 2009 virus in sewage and river water

The two qRT-PCR methods were used to detect influenza A and pandemic influenza A (H1N1) 2009 in extracts from sewage influent and effluent, and river water between 15 October 2009 and 17 February 2010 (Table 2). Influenza-A

was detected in one sewage influent sample and in four river water samples. The pandemic influenza A (H1N1) 2009 virus was not detected in sewage or river water.

Sequence analysis of cloned Flu-A specific RT-PCR fragments revealed that five different sequence types were observed in Flu-A positive samples (Table 3). The sequences of these small fragments (95 bp) were identical to previously described sequence types of the matrix gene of influenza A. Although more identical sequences are present in the database, the accession number of only one example of an identical sequence is given in the table.

DISCUSSION

The method used in this study combines highly efficient concentration methods with qRT-PCR approaches to detect influenza viruses in water samples with high sensitivity. The application of these ultrafiltration-based procedures has been shown to be able to concentrate viruses and other microbes from water with high recovery efficiencies in other studies (Hill *et al.* 2007) and has also shown to be efficient in our lab.

The efficiency of Flu-A and panFlu-A specific qRT-PCR methods were close to 100% when tested on serial dilutions of quantified in vitro transcribed RNA fragments and also on extracts from serial diluted influenza virus. This means that the specific cDNA fragments are duplicated during every PCR cycle demonstrating that the combination of these primers with the chosen reaction conditions results in optimal amplification of the specific fragments.

Table 1 | Average detection efficiency of spiked pandemic influenza A (H1N1) 2009 virus in concentrated sewage and river-water samples using qRT-PCR for detection of influenza-A (Flu-A) in general and specific detection of pandemic influenza A (H1N1) 2009 virus (panFlu-A)

Matrix	Volume ^a Average examined water volume/qRT-PCR reaction in mL (range)	Detection efficiency							
		Flu-A				panFlu-A			
		Undiluted RNA extract		10 × diluted RNA extract		Undiluted RNA extract		10 × diluted RNA extract	
%	Range (%)	%	Range (%)	%	Range (%)	%	Range (%)		
Sewage influent	4.9 (2.0–9.3)	53.8	30–126	77.6	36–160	35.5	12–89	67.7	40–130
Sewage effluent	30.2 (11.8–92.8)	42.7	11–105	46.3	0–105	27.2	16–39	62.1	0–113
River water	291.7 (97.8–490.8)	5.4	0–13	22.4	0–107	4.3	0–14	14.2	4–49

^aThe examined water volume/qRT-PCR reaction is the result of the concentration and extraction procedure.

Table 2 | Detection of influenza-A (Flu-A) virus and the pandemic influenza A (H1N1) 2009 virus in sewage influent and effluent, and river water using qRT-PCR

Date	Sewage influent			Sewage effluent			River water		
	Volume (ml) ^a	Flu-A GC/L	panFlu-A	Volume (ml)	Flu-A GC/L	panFlu-A	Volume (ml)	Flu-A GC/L	panFlu-A
15 Oct 2009	3.4	ND	ND	23.3	ND	ND	318.0	ND	ND
26 Oct 2009	5.4	ND	ND	23.8	ND	ND	206.7	ND	ND
09 Nov 2009	4.1	2.6 × 10 ⁵	ND	92.8	ND	ND	157.0	ND	ND
02 Dec 2009	6.2	ND	ND	18.9	ND	ND	386.3	2.8 × 10 ³	ND
07 Dec 2009	9.2	ND	ND	31.4	ND	ND	135.6	ND	ND
23 Dec 2009	9.3	ND	ND	37.5	ND	ND	490.8	2.0 × 10 ³	ND
07 Jan 2010	3.1	ND	ND	15.6	ND	ND	414.0	ND	ND
18 Jan 2010	2.5	ND	ND	23.6	ND	ND	290.5	1.6 × 10 ⁵	ND
02 Feb 2010	2.0	ND	ND	23.3	ND	ND	97.8	ND	ND
17 Feb 2010	3.3	ND	ND	11.8	ND	ND	429.9	2.8 × 10 ²	ND

GC: Genome copies; ND: Not Detected.

^aThe volume shows the equivalent volume of water analyzed in every qRT-PCR reaction.

All samples were analyzed after artificial contamination of the concentrates with pandemic influenza A (H1N1) 2009 virus (spiked samples) to study the effect of the matrix on the qRT-PCR analyses. The analysis of these spiked samples showed that only part of the added pandemic influenza A (H1N1) 2009 virus was detected. This demonstrates that the matrix had significant impact on the quantitative readings of the qRT-PCR assays. Dilution of

the RNA-extracts resulted in higher detection efficiencies suggesting that the presence of qRT-PCR inhibiting substances in these extracts is the main reason for the low detection efficiencies. The detection efficiencies were especially low in concentrated samples from river water implying that the concentration of qRT-PCR inhibitors is high in these concentrates. This could be due to the different nature of the samples or the result of the extra concentration steps applied to surface water samples resulting in the analysis of larger volumes of surface water in every qRT-PCR reaction. Detection efficiencies for Flu-A qRT-PCR were higher than for the panFlu-A qRT-PCR assays suggesting that inhibiting substances have more impact on the performance of the panFlu-A qRT-PCR assay. Low detection efficiencies will result in underestimation of the amount of influenza virus in the samples. Inhibition of qRT-PCR reactions will also result in a higher limit of detection, especially in cases where dilution of the extract is needed to obtain reasonable detection efficiencies. The development of extraction procedures that are able to extract RNA with high efficiency and low concentration of qRT-PCR inhibiting substances would lower the limit of detection in concentrated water samples. Research is currently underway to optimize RNA-extraction procedures to make it possible to quantitatively measure influenza viruses in concentrated water samples with high sensitivity. However, the Flu-A and panFlu-A detection efficiencies in sewage

Table 3 | Sequence analysis of cloned Flu-A specific qRT-PCR fragments

Sample	Date	Identical sequence in Genbank database Genbank accession number: source
Sewage influent	09 Nov 2009	CY078628: flu A (H3N6)
	09 Nov 2009	HM849004: flu A (H7N3)
River water	02 Dec 2009	CY078628: flu A (H3N6)
	02 Dec 2009	CY067920: flu A (H1N1)
	23 Dec 2009	CY021678: flu A (H6N8)
	23 Dec 2009	GQ907351: flu A (H12N3)
	18 Jan 2010	HQ897967: flu A (H1N1)
	18 Jan 2010	GQ907351: flu A (H12N3)
Spiked suspension	17 Feb 2010	HQ897967: flu A (H1N1)
	17 Feb 2010	GQ907351: flu A (H12N3)
		CY046944: flu A / Netherlands/602/2009 (H1N1)

influent (53.8 and 35.5%, respectively) and sewage effluent (42.7 and 27.2%, respectively) were sufficient to allow sensitive detection of these viruses in sewage. However, the low detection efficiency of Flu-A and panFlu-A in concentrated surface water (5.4 and 4.3%, respectively) implies that it is problematic to follow influenza viruses in surface water.

The development of the influenza epidemic in the Netherlands, caused by the pandemic influenza A (H1N1) 2009 virus, was described on the basis of confirmed hospitalized cases (van 't Klooster *et al.* 2010). The first hospitalized case was reported on 30 April 2009. From May 2009 to October 2009 only sporadic cases were reported; then an exponential rise in the number of cases of pandemic influenza was observed in from week 40 to week 46. The incidence of hospitalization decreased rapidly after week 46 and the epidemic ended during the last weeks of December 2009. The days when water samples were collected included the period of the epidemic. The first two sample days were during the period where there was an exponential rise in the number of pandemic influenza cases (15 and 26 October 2009). The third sample day was at the peak of epidemic in the Netherlands (9 November 2009) followed by two sample days (2 and 7 December 2009) in the period when the number of pandemic influenza virus cases were decreasing. The last samples were collected on five days in the period after the epidemic in the Netherlands (23 December 2009, 7 and 18 January 2010, 2 and 17 February 2010). A relatively large number of samples were collected in the period after the epidemic as it had been expected that the epidemic would last at least until February 2010.

There was no pandemic influenza A (H1N1) 2009 virus detected in RNA-extracts from concentrated sewage influent, concentrated sewage effluent or concentrated surface water samples in the period between 15 October 2009 and 17 February 2010. This means that the concentration of this virus was below the limit of detection even in the period with the highest incidence of pandemic influenza A (H1N1) 2009 virus infections in November 2009. There are only reliable data available about the incidence of hospitalized confirmed pandemic influenza A (H1N1) 2009 virus infections in the Netherlands (van 't Klooster *et al.* 2010). These data showed 367 hospitalized cases during the peak of the epidemic between 15 and 21 November 2009 (week

46). But, this gives no indication about the true incidence of infection since only a small proportion of cases needed hospitalization. Data from the USA and Canada reported that 2–5% of the confirmed cases required hospitalization (Girard *et al.* 2010). This would mean that between 7,340 and 18,350 (or between 0.04 and 0.11% of the total population in the Netherlands) cases occurred if the same proportion applied for the Netherlands. An incidence of 0.04–0.11% would imply that a maximum of 200 to 600 pandemic influenza A (H1N1) 2009 virus-infected persons were delivering their (fecal) waste to the sampled sewage plant of the city of Utrecht.

Assuming that 25% of these diseased persons suffer from diarrhea (as was the case in a study in the USA (Girard *et al.* 2010)) would suggest that between 50 and 150 persons were shedding pandemic influenza A (H1N1) 2009 virus into the Utrecht sewage system. The fact that it was not possible to detect pandemic influenza A (H1N1) 2009 virus in sewage influent implies that the combination of the low number of excreting people and the concentration of influenza virus in feces resulted in an influenza virus concentration below the limit of detection in sewage influent. It may also mean that influenza viruses are already degraded during transport through the sewage system. This suggests that it is unlikely that spreading of the pandemic influenza A (H1N1) 2009 through sewage was relevant during this epidemic in the Netherlands.

Influenza A was detected in four river water samples and in one sewage influent sample using the general Flu-A qRT-PCR. This confirms the suitability of the applied methods to concentrate influenza viruses from sewage and surface water and demonstrates the applicability of the qRT-PCR method to detect influenza viruses in these environmental water samples. Sequence analyses of the qRT-PCR fragments showed that the sequences of all obtained fragments were specific for the matrix gene of influenza A demonstrating the specificity of this qRT-PCR assay. However, the sequences obtained from sewage and surface water all showed minor differences compared to the sequence of the influenza strain (pandemic influenza A/NL/602/2009) which was used in spiking experiments, demonstrating that positive qRT-PCR reactions were not the result of contamination with traces of virus, viral RNA or qRT-PCR products from the spike strain (data not

shown). Frequent detection of influenza A virus in river water and detection of influenza A virus in only one sewage water sample suggests that discharged treated sewage is not the primary source of the influenza A viruses which are detected in river water. It is more likely that aquatic birds are the main source of these viruses. Aquatic birds (like ducks, geese, swans, gulls) are common in the river studied and these birds are recognized as the natural reservoirs of avian influenza viruses (Webster *et al.* 1992). It has been shown that high concentrations of influenza A viruses are shed in environmental water through discharged feces from infected ducks (Webster *et al.* 1978; Markwell & Shortridge 1982). Avian influenza A viruses have also shown to be very persistent in river water, especially at the low temperatures (Domanska-Blicharz *et al.* 2010) as was the case during period of sampling. These arguments make water birds a more likely source of the detected influenza A viruses.

CONCLUSIONS

This study demonstrates the applicability of the combination of ultrafiltration methods, to concentrate viruses from sewage and river water samples, and qRT-PCR methods to specifically detect influenza A and the pandemic influenza A (H1N1) 2009 virus in extracts from the water concentrates. The pandemic influenza A (H1N1) 2009 was not detected in sewage or surface water indicating that spreading of this virus through this part of the water cycle was not significant during the epidemic in the Netherlands.

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