Detection of enteric viruses in Hungarian surface waters: first steps towards environmental surveillance
Anita Kern, Mihaly Kadar, Katalin Szomor, György Berencsi, Beatrix Kapusinszky and Marta Vargha

ABSTRACT
Waterborne viruses infect the human population through the consumption of contaminated drinking water and by direct contact with polluted surface water during recreational activity. Although water related viral outbreaks are a major public health concern, virus detection is not a part of the water quality monitoring scheme, mainly due to the absence of routine analysis methods. In the present study, we implemented various approaches for water concentration and virus detection, and tested on Hungarian surface water samples. Eighty samples were collected from 16 sites in Hungary. Samples were concentrated by glass wool and membrane filtration. Human adenoviruses were detected by conventional and quantitative real-time polymerase chain reaction (PCR) methods in 56% (45/80) of the samples; viral titers ranged from $8.60 \times 10^1$ to $3.91 \times 10^4$ genome copies per liter. Noroviruses and enteroviruses were detected in 30% (24/80) and 13% (10/80) of samples, respectively, by reverse transcription-PCR assays. Results indicate a high prevalence of viral human pathogens in surface waters, suggesting the necessity of a detailed survey focusing on the quality of natural bathing waters and drinking water sources.

Key words | enteric viruses, method comparison, PCR, surface water, virus concentration method

INTRODUCTION
Water is the main transport medium for many human pathogens, including bacteria, protozoa and enteric viruses. Infection caused by waterborne pathogenic microorganisms is the most common and widespread health risk associated with water consumption (WHO 2008). Drinking and bathing water related outbreaks, often with unknown etiology, are a serious public health concerns, not only in developing countries but also in countries with a developed infrastructure.

Most of these infections are due to enteric viruses, which spread by the fecal-oral route, such as adenoviruses, noroviruses, enteroviruses, hepatitis A and E viruses, rotaviruses, sapoviruses, Aichi virus, coronaviruses etc. (Wyn-Jones et al. 2011). The general symptoms are nausea, diarrhea and vomiting, but some enteric viruses can cause severe diseases, such as hepatitis, myocarditis, aseptic meningitis, conjunctivitis, respiratory diseases and type I diabetes in children, elderly and immunocompromised persons (Bosch 1998; Griffin et al. 2005; Kapusinszky et al. 2010). Most enteric viruses are excreted into communal sewage, and are only partially eliminated by the current wastewater treatment methods (Fong & Lipp 2005). In Hungary, only about 70% of the wastewater is treated, thus surface water may receive treated and untreated sewage as well.

Despite the well-known risks associated with the presence of human pathogen viruses in surface waters (especially those used for bathing or as a drinking water source), in most countries virus detection is not a part of the monitoring scheme. Until recently, the determination of viruses in natural waters was challenging, mainly due to their low titer. Traditionally, hundreds of liters of water were concentrated by negative or positive charge filtration and/or floculation (EPA 2001), followed by cell culture assays. The nucleic acid based detection method overcame many of the difficulties associated with cell culture, such as
low sensitivity (Kopeczka et al. 1993), high cost and long analysis time (Tsai et al. 1993; Ma et al. 1993), and the toxic effects of water constituents that are concentrated in parallel with the viruses (Abbaszadegan et al. 1993). Genus specific polymerase chain reaction (PCR) allows detection of non-cultivable viruses and the simultaneous detection of multiple serotypes (Girones et al. 2010).

Though PCR-based methods have their limitations in the estimation of viability and infectivity, they are finding their way into natural bathing water quality assessment (e.g. Enterococcus enumeration (Wade et al. 2006)). There are extensive attempts to quantify sewage impact through novel indicators, such as Bacteriodales (Wade et al. 2008). Previously reported studies suggest that human adenoviruses and JC polyomaviruses are also suitable and reliable indicators of human fecal pollution of different water bodies (Puig et al. 1994; Pina et al. 1998; Bofill-Mas et al. 2000). Correlation between molecular and culture based detections was confirmed in the case of bacteria (Converse et al. 2012) and viruses (Donia et al. 2010).

In the present study, we applied various water concentration and virus detection methods and tested their performance and applicability in routine analysis of the surface water in Hungary. To our knowledge, this is the first report on the prevalence of human pathogen viruses in Hungarian surface waters.

### MATERIALS AND METHODS

#### Samples and sampling sites

A total of 80 surface water samples were collected between August 2006 and July 2010 in Hungary (Figure 1 and Table 1). Grab samples were taken at 120 cm water depth, 30 cm below surface in sterile 10 L containers from 16 different sampling sites along Hungarian rivers and streams. Sampling sites were selected to represent rivers of different size and flow rate, and geographically distant areas. The two largest rivers in Hungary, the Danube and the Tisza, were characterized in more detail (four and six sampling sites, respectively). Three sampling points were located on the tributaries of the Tisza (Berettyó, Hármas-Körös, Sebes-Körös). Two streams running into Lake Balaton (Kéki, Koloska), and an oxbow lake of a former Danube branch were also sampled. Seven of the sites were used for recreational water activities \((n = 42)\), nine sampling sites had no recreational use \((n = 38)\). Recreational water samples were collected during the bathing season (for sampling dates, see Table 1).

Wastewater treatment plants, releasing secondary (activated sludge) or tertiary (phosphorus removal or chlorination) treated effluent, were located close to several sampling points, but there was generally no direct contact with the effluent, as the point of inflow was downstream.
to all sampling sites except Danube2–4. Danube2 is close to the efflux of a tertiary treated wastewater, Danube3 is near a discharge point of primary treated (mechanically filtered) wastewater and Danube4 is located 2 river km downstream from Danube3. Tisza4 is the only natural bathing water sampling site directly affected by secondary treated communal sewage. There was no drinking water abstraction from surface water at any of the sampling sites, though bank filtered wells are located along the Danube. Drinking water in the sampled areas is mainly from groundwater.

Table 1  Sampling sites and sampling events

<table>
<thead>
<tr>
<th>Sampling site ID</th>
<th>Sampling site</th>
<th>Wastewater Impact</th>
<th>Collection date (dd/mm/yyyy)</th>
<th>Number of samples</th>
<th>Sample type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ber1</td>
<td>Berettyó River, Szeghalom</td>
<td>Diffuse&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.08.2008</td>
<td>2</td>
<td>Surface water</td>
</tr>
<tr>
<td>Danube1</td>
<td>Danube River, Györ</td>
<td>Diffuse&lt;sup&gt;a&lt;/sup&gt;</td>
<td>09.09.2008</td>
<td>1</td>
<td>Surface water</td>
</tr>
<tr>
<td>Danube2</td>
<td>Danube River, Budapest</td>
<td>Tertiary treated effluent&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.03.2008</td>
<td>2</td>
<td>Surface water</td>
</tr>
<tr>
<td>Danube2</td>
<td>Danube River, Budapest</td>
<td>Tertiary treated effluent&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.11.2008</td>
<td>2</td>
<td>Surface water</td>
</tr>
<tr>
<td>Danube2</td>
<td>Danube River, Budapest</td>
<td>Tertiary treated effluent&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.02.2009</td>
<td>2</td>
<td>Surface water</td>
</tr>
<tr>
<td>Danube2</td>
<td>Danube River, Budapest</td>
<td>Tertiary treated effluent&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.05.2009</td>
<td>2</td>
<td>Surface water</td>
</tr>
<tr>
<td>Danube2</td>
<td>Danube River, Budapest</td>
<td>Tertiary treated effluent&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.08.2009</td>
<td>2</td>
<td>Surface water</td>
</tr>
<tr>
<td>Danube3</td>
<td>Danube River, Budapest</td>
<td>Primary treated effluent&lt;sup&gt;c&lt;/sup&gt;</td>
<td>20.11.2006</td>
<td>1</td>
<td>Surface water</td>
</tr>
<tr>
<td>Danube3</td>
<td>Danube River, Budapest</td>
<td>Primary treated effluent&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19.11.2008</td>
<td>2</td>
<td>Surface water</td>
</tr>
<tr>
<td>Danube3</td>
<td>Danube River, Budapest</td>
<td>Primary treated effluent&lt;sup&gt;c&lt;/sup&gt;</td>
<td>16.02.2009</td>
<td>2</td>
<td>Surface water</td>
</tr>
<tr>
<td>Danube3</td>
<td>Danube River, Budapest</td>
<td>Primary treated effluent&lt;sup&gt;c&lt;/sup&gt;</td>
<td>13.05.2009</td>
<td>2</td>
<td>Surface water</td>
</tr>
<tr>
<td>Danube4</td>
<td>Danube River, Budapest</td>
<td>Primary treated effluent&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19.11.2008</td>
<td>2</td>
<td>Surface water</td>
</tr>
<tr>
<td>Danube4</td>
<td>Danube River, Budapest</td>
<td>Primary treated effluent&lt;sup&gt;c&lt;/sup&gt;</td>
<td>16.02.2009</td>
<td>2</td>
<td>Surface water</td>
</tr>
<tr>
<td>Danube4</td>
<td>Danube River, Budapest</td>
<td>Primary treated effluent&lt;sup&gt;c&lt;/sup&gt;</td>
<td>13.05.2009</td>
<td>2</td>
<td>Surface water</td>
</tr>
<tr>
<td>Danube4</td>
<td>Danube River, Budapest</td>
<td>Primary treated effluent&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.08.2009</td>
<td>2</td>
<td>Surface water</td>
</tr>
<tr>
<td>Fadd1</td>
<td>Danube River oxbow, Fadd</td>
<td>Na</td>
<td>13.08.2006</td>
<td>4</td>
<td>Natural bathing water</td>
</tr>
<tr>
<td>Koros1</td>
<td>Hármas Körös River, Gyomaendröd</td>
<td>Diffuse&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.08.2008</td>
<td>2</td>
<td>Surface water</td>
</tr>
<tr>
<td>Koros2</td>
<td>Sebes Körös River, Körössladány</td>
<td>Diffuse&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.08.2008</td>
<td>2</td>
<td>Surface water</td>
</tr>
<tr>
<td>Stream1</td>
<td>Koloska Rivulet, Balatonfüred</td>
<td>Diffuse&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.09.2008</td>
<td>2</td>
<td>Surface water</td>
</tr>
<tr>
<td>Stream2</td>
<td>Kéki Rivulet, Balatonfüred</td>
<td>Diffuse&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.09.2008</td>
<td>2</td>
<td>Surface water</td>
</tr>
<tr>
<td>Tisza1</td>
<td>Csongrád, Tisza River</td>
<td>Na</td>
<td>05.08.2007</td>
<td>12</td>
<td>Natural bathing water</td>
</tr>
<tr>
<td>Tisza1</td>
<td>Csongrád, Tisza River</td>
<td>Na</td>
<td>06.08.2008</td>
<td>3</td>
<td>Natural bathing water</td>
</tr>
<tr>
<td>Tisza2</td>
<td>Tiszakécske, Tisza River</td>
<td>Na</td>
<td>01.07.2007</td>
<td>12</td>
<td>Natural bathing water</td>
</tr>
<tr>
<td>Tisza2</td>
<td>Tiszakécske, Tisza River</td>
<td>Na</td>
<td>06.08.2008</td>
<td>3</td>
<td>Natural bathing water</td>
</tr>
<tr>
<td>Tisza3</td>
<td>Rákócziútjárfalu, Tisza River</td>
<td>Na</td>
<td>20.07.2009</td>
<td>2</td>
<td>Natural bathing water</td>
</tr>
<tr>
<td>Tisza4</td>
<td>Rákócziújfalva, Tisza River</td>
<td>Secondary treated effluent&lt;sup&gt;d&lt;/sup&gt;</td>
<td>20.07.2009</td>
<td>2</td>
<td>Natural bathing water</td>
</tr>
<tr>
<td>Tisza5</td>
<td>Tiszaptúspöki, Tisza River</td>
<td>Na</td>
<td>27.07.2010</td>
<td>2</td>
<td>Natural bathing water</td>
</tr>
<tr>
<td>Tisza6</td>
<td>Nagykőrösi, Tisza River</td>
<td>Na</td>
<td>15.07.2009</td>
<td>2</td>
<td>Natural bathing water</td>
</tr>
</tbody>
</table>

Na, not applicable.
<sup>a</sup>Illicit discharge from septic tanks or holiday homes.
<sup>b</sup>FeCl<sub>3</sub> treatment for phosphorus removal.
<sup>c</sup>Mechanical filtration.
<sup>d</sup>Activated sludge treatment.

Samples were collected from eight riverine locations between 2006 and 2010, five sites were sampled on multiple occasions. Sites are specified by the investigated river and the nearest municipality.
Samples were stored at +2–8 °C until processing (<24 h).

Sample processing

Virus concentration was performed by the methods developed in the European Union funded project Virobathe (Wyn-Jones et al. 2011). Ten liters of water samples were concentrated using two different adsorbents: glass wool, type 725 (Rantigny, Saint-Gobain, France) and mixed cellulose ester membrane with 0.45 mm pore size, and 142 mm diameter (Whatman International Ltd) with a glass wool pre-filter, diameter 125 mm (Sartorius, Germany). Briefly, pH of the samples was reduced to 3.5 by adding 1 N HCl (Molar Chemicals, Hungary), and filtered. The positively charged viral particles were adsorbed to the negatively charged filters. Viruses were eluted from the filter with 200 mL of 3% glycine beef extract solution, pH 9.5 (Biolab, Hungary). The pH of the beef extracted elute was adjusted to 3.5 by 1 N HCl, thus locking the protein fraction including the viral particles. The locked elute was centrifuged at 8000 × g for 30 min and the pellet was resuspended in phosphate buffer to 10 mL final volume (Figure 2).

Twenty-four samples (six recreational and 18 other surface water) were processed by both membrane- and by glass wool concentration methods in order to compare the virus binding efficiency.

Escherichia coli and intestinal enterococci counts were determined from all surface water samples by the microtiter most probable number (MPN) method (ISO 9308-3:1998 and ISO 7899-1:1998, respectively) on MUG/EC and MUD/SF microplates (Bio-Rad, France).

Virus detection

Viral nucleic acids were extracted from 5 mL sample concentrates using the magnetic silica bead kit NucliSens® miniMAG™ (Biomérieux, France) according to manufacturer’s instructions. The final volume of the purified nucleic acid was 100 μL. Nucleic acid extract was stored at −20 °C until further analysis.

Human adenoviruses (HAdV) were detected by nested PCR as previously reported (Allard et al. 2001). The reaction parameters were based on the protocol of Wyn-Jones et al. (2011) with modifications. The PCR reactions included 1× Platinum® Taq buffer, 1.5 mM MgCl2 (Applied Biosystems), 250 μM of each dNTP (Thermo Scientific (Fermentas), Lithuania), 500–500 nM forward and reverse primer (Table 2) (Integrated DNA Technologies Inc.), 0.4 M bovine serum albumin (Thermo Scientific (Fermentas), Lithuania), 1 U/μL Platinum® Taq (Applied Biosystems), 10 or 2 μL nucleic acid (first and second round, respectively) in 50 μL total volume. The quantity of HAdV genome copies were determined by quantitative real-time PCR (qPCR) (Hernroth et al. 2002). The qPCR was performed in a total volume of 25 μL using the corresponding primers at 0.9 M and TaqMan® FAM-TAMRA-labeled probe at 0.225 M and 1× TaqMan® Environmental Master Mix (Applied Biosystems) and 10 μL DNA. The reactions were performed with an ABI7300 sequence detector system (Applied Biosystems). Linearized pBR322 plasmid containing the adenovirus hexon region was used as a standard (106–108 GC/10 μL range). The detection limit of the assay was 1 genome copy per 10 μL of nucleic acid. Each dilution of the standard curve, undiluted DNA and 10-fold dilutions of the extracted DNA were run in duplicate for each reaction. Human enteroviruses were detected by RT-nested PCR described by Puig et al. (1994) (Table 2). Human noroviruses were detected by RT-seminested PCR with oligonucleotide primers by Vennema et al. (2002) (Table 2). The parameters of the reaction were previously reported by Wyn-Jones et al. (2011). Bovine serum albumin (Thermo Scientific (Fermentas), Lithuania) was added to every reaction mixture in a final concentration of 0.4 M. Conventional PCR products were detected by electrophoresis in a 2% agarose ~ 1× TAE gel stained with ethidium bromide.

Figure 2 | Summary of the virus concentration procedure.
The sequencing reaction was done by the DYEnamic ET Dye Terminator Cycle kit (Amersham Biosciences Inc.) following the manufacturer's instructions. Sequencing was performed in MegaBACE™ 1000 DNS sequencer (Amersham Biosciences Inc.). Acquired sequences were analyzed for nucleotide homology by BLAST program in NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

**Quality control**

For quality control purposes, 10 L of dechlorinated, sterile tap water was spiked with echovirus serotype 11 (ECHO-11) or human adenovirus type 2 (HAdV-2) and processed in parallel with the surface water samples (positive process control). Dechlorinated, sterile tap water was used as a negative process control with every batch. In addition, reaction control was included in every PCR run; a negative (reagent) control and purified viral nucleic acid (HAdV-2, ECHO-11 and human norovirus genogroup II genotype 13) as positive control. Internal amplification control was omitted to increase sensitivity, but all PCRs were run using three dilutions (using undiluted, 10-fold and 100-fold diluted nucleic acids) to overcome inhibition effects.

**RESULTS**

**Virus detection and quantification**

Over 56% (45/80) of the samples were positive for adenovirus by nested PCR. Norovirus and enterovirus RNA were
detected in 24 (30.0%) and 10 samples (12.5%), respectively (Table 3). Approximately one-third of the samples contained at least two of the targeted viral groups; four samples were positive for both HAdV and human enteroviruses (5.0%), 17 samples contained HAdV and human noroviruses (21.3%), and all three virus groups were detected in five samples (6.3%). A total of 62.5% (50/80) of the samples contained at least one of the examined viruses.

The HAdV quantity was determined in 30 out of 45 (66.7%) PCR positive samples, viral load varied from $8.60 \times 10^1$ to $3.91 \times 10^4$ genome copies per liter (GC/L) with a geometric mean value of $1.84 \times 10^3$ GC/L. Nineteen of the 45 samples that contained HAdV DNA originated from natural bathing waters ($8.60 \times 10^1$–$1.02 \times 10^3$ GC/L, with a mean value of $3.33 \times 10^2$ GC/L), the other 26 HAdV-positive samples were collected from rivers, where bathing is forbidden or not possible ($1.91 \times 10^2$–$3.91 \times 10^4$ GC/L, with a mean value of $2.88 \times 10^3$ GC/L).

The detection rate of human enteroviruses in surface water for recreational and non-recreational use was 11.9% (5/42) and 13.2% (5/38), respectively. Noroviruses were detected in six out of 42 (14.3%) natural bathing water samples and in 18 out of 38 (47.4%) water samples with no recreational use.

Nine of the 45 HAdV positive samples were further analyzed to determine virus serotype. The detected HAdV serotypes were as follows: type 41 ($n = 5$), type 40 ($n = 2$), type 12 ($n = 1$) and type 2 ($n = 1$). Human norovirus genogroup II was confirmed from four samples and human enteroviruses from two samples by sequence analysis.

All negative process controls reagent controls (15/15) and PCR reagent controls were negative. Spiked virus was successfully recovered from positive process controls in every case (15/15).

**Comparison of membrane and glass wool filtration methods**

In order to compare the efficiency of two ubiquitously applied adsorbents, 24 samples were concentrated by both membrane filtration and glass wool adsorbent (Figure 3). Eighteen of the 24 samples (75.0%) were positive for at least one of the examined viruses after glass wool filtration and 15 samples (62.5%) after membrane filtration. Adenoviruses were detected in 16 (66.7%) and 14 samples (58.3%) by glass wool and membrane filtration, respectively. A similar trend was observed for the concentration of

![Figure 3](https://iwaponline.com/jwh/article-pdf/11/4/772/395643/772.pdf)
enteroviruses: seven (29.2%) and three positive samples (12.5%), respectively after glass wool and membrane filtration. The same number (though not identical set) of the samples (10/24; 41.7%) was positive for norovirus by both concentration methods.

The average titer of HAdV was higher after the glass wool concentration method than after the membrane filtration (geometric mean titer 1.97 × 10³ GC/L and 1.58 × 10² GC/L, respectively).

**Fecal indicator counts**

In the investigated natural bathing waters _E. coli_ counts ranged from 3.0 × 10¹ to 7.3 × 10² MPN/100 mL, with a mean value of 2.0 × 10² MPN/100 mL, and intestinal enterococci counts from 1.5 × 10¹ to 1.0 × 10³ MPN/100 mL, with a mean value of 1.0 × 10² MPN/100 mL. In other surface water samples _E. coli_ were detected between 6.1 × 10¹ MPN/100 mL and 9.5 × 10⁴ MPN/100 mL with a mean value of 8.9 × 10² MPN/100 mL and intestinal enterococci between 1.5 × 10¹ MPN/100 mL and 2.0 × 10⁴ MPN/100 mL with a mean value of 5.7 × 10² MPN/100.

**DISCUSSION**

A high prevalence of enteric viruses was detected in the eight investigated Hungarian rivers and streams (Table 3). Smaller streams and rivers were usually less contaminated (though the average sample number was also lower), while the samples from large rivers (Tisza and Danube) were almost 70% positive for at least one of the targeted viruses. There is no previous systematic prevalence information available on enteric viruses in Hungarian surface waters. Recent information on viruses in Hungarian water environments is limited to reports on raw and treated sewage (Daniel & Dömök 1962; Meleg et al. 2006, 2008). The presence of rotavirus (group C) and human astrovirus (genotype I) was confirmed in the majority of raw sewage samples, and secondary treatment only reduced the rate of positive samples by 50%. Other studies also indicate that secondary wastewater is a main source of enteric virus load in surface water (Bosch 1998; Pina et al. 1998; Okoh et al. 2010).

Wastewater impact was clearly reflected both by the fecal indicator counts and the viral prevalence in the Danube2, 3 and 4 sampling sites (all located in Budapest). Danube2 was near a tertiary treated (phosphorus removal by FeCl₃) effluent inflow, while Danube3 was receiving practically untreated sewage (mechanical filtration only). Danube4 is located 2 km downstream from Danube3, without further sewage impact in between. Accordingly, bacterial counts, HAdV titers and the rate of positivity for the other viruses were lowest at Danube2, highest at Danube3 and showed the effect of dilution (a decrease of at least an order of magnitude in bacterial counts and HAdV titers) at Danube4 (Table 2, Figure 4).

The Tisza river is also known to receive (mostly biologically treated) effluent from wastewater treatment plants. However, only one of the sampling points (Tisza4) was directly impacted by effluent (within 5 river km), the others were at least 5 river km downstream from the nearest sewage inlet. Secondary treated wastewater in the area is not disinfected continuously, though there is usually a possibility to perform emergency disinfection by chlorine-based disinfectants in the case of outbreaks or sewage overflw. In the case of the Tisza river, the effect of sewage impact was less obvious. Tisza4 samples were positive for all viruses, but other Tisza sites also showed high prevalence, and adenovirus titers were even higher at some points. The bacterial water quality was similar for all sites. The results suggest other sources of contamination, such as diffuse discharge from septic tanks, surface run-off and streams with wastewater impact, or holiday houses near the river illicitly releasing untreated sewage to the river. Though there is a known input from industrial wastewater near some Tisza sampling sites, from chemical industries, food and energy industry, these are not considered significant sources of human viruses. The natural bathing water sites were sampled during the bathing season, often when bathers were also present, thus the viruses might have originated directly from the bathers.

Sites used for bathing or other recreational activities, such as kayaking or canoeing, were a special focus of the study, as the presence of enteric viruses only poses a risk if there are also potential exposure routes. Of the seven natural bathing sites, adenoviruses were detected at five locations, while norovirus and enterovirus were detected at three locations.
Water quality assessment of natural bathing waters is based on the fecal indicator bacteria, usually determined by culture, though some regulations recently incorporated real-time PCR method for Enterococcus (EPA 2012). The currently most widely accepted regulation for bathing water quality assessment is the European Union Bathing Water Directive 2006/7/EC and the USEPA (2012). However, both are using a risk based approach, rather than a single quality threshold, and thus cannot be used in the context of the current study to evaluate water quality based on the observed fecal indicator counts. The former EU Bathing Water Directive, 76/160/EEC (CEU 2006) defined limit values of ‘good’ quality for E. coli and Enterococcus (1000 CFU/100 mL and 200 CFU/100 mL, respectively). All natural bathing water samples complied with the E. coli criteria and only four single samples (in the case of Tisza1 and Tisza2) exceeded the limit value for Enterococcus, which were positive for HAdV. All other virus positive samples were compliant with good quality limit values. Most of the investigated natural bathing sites also qualify as good according to the current EU Bathing Water Directive, with the exception of Tisza 1 and Tisza2.

The results show that the good quality of the natural bathing water does not mean the absence of viruses and potentially human pathogen viruses were detected in recreational waters of excellent quality according to the bacterial hygiene parameters. Though the disease burden of waterborne viruses in association with bathing is still being assessed, this is a further implication for introducing enteric viruses as a water quality parameter.

In the present study, human adenoviruses had the highest prevalence of the three examined virus groups in accordance with previous observations (Puig et al. 1994; Pina et al. 1998; Wyn-Jones et al. 2011). It was detected in the majority of the Danube and Tisza sites (3/4 and 4/6, respectively), and two additional points (Fadd Oxbow and Berettyó). Norovirus was present at three Danube, two Tisza sampling points and in the Fadd oxbow, while human enterovirus was only detected from the two major rivers. The higher stability of adenovirus particles and its
genetic material might have also contributed to its higher detection rate (Enriquez et al. 1995; Pina et al. 1998). Both the stability and its ubiquitous presence in communal sewage were arguments for proposing human adenoviruses as indicators of fecal contamination (Pina et al. 1998); and the results of the present study also support their applicability.

The sequence analyses confirmed that potentially human pathogen viruses were detected during this study. Predominantly enteric adenoviruses were present as it was previously reported (Hamza et al. 2009). In the case of enterovirus typing, the molecular analysis of the virus protein 1 (VP1) region would be more appropriate instead of the highly conserved 5’NTR region and needs further analysis (Oberste et al. 1999).

Comparison of the concentration methods on the same samples set demonstrated a slightly better efficiency of glass wool filtration, in accordance with previous results of method characterization for freshwater (Albinana-Gimenez et al. 2009; Maunula et al. 2012). The ratio of positive samples was generally higher for glass wool filtered samples, and adenovirus titers, where determined, were also higher, though the difference was not statistically significant (data not shown). In freshwater, suspended and dissolved solids are the main limiting factors of the adsorption-elution methods. Suspended solids clog the pores of the filter material, while dissolved or colloidal organic material competes with viral particles for the active binding sites (Sobsey 1976). Glass wool was less sensitive to clogging, and its larger active surface may explain the better concentration efficiency. It was also easier to process multiple samples by glass wool filtration in parallel, thus the overall time-demand of the process was considerably lower than that of membrane filtration.

Not only the choice of the concentration method, but other steps of the analysis steps may influence the detection results. Suspended solids sediment during centrifugation and may hinder nucleic acid extraction. Certain materials in the water, such as metal ions or organic acids, can be concentrated during samples processing, and interfere with the molecular detection through the inhibition of the polymerase enzyme (Tsai & Olson 1991; Abbaszadegan et al. 1995). In the present study, inhibition was not investigated in detail, but samples were analyzed in three dilutions to overcome the effect. Around 16% of the positive samples (10 adenovirus and three norovirus detections) only gave a positive reaction in dilution.

CONCLUSIONS

A high prevalence of human enteric viruses in Hungarian surface waters was demonstrated using a combination of negatively charged filtration and flocculation with molecular detection methods. The results demonstrate that even natural bathing waters, which comply with the quality criteria based on bacterial fecal indicators, are often contaminated with potentially pathogenic viruses. This indicates the necessity of a more detailed survey extended to other types of surface waters, such as lakes or reservoirs, and targeting issues of seasonality and viability. The ubiquitous presence of adenoviruses in sewage impacted surface waters and the relation of its titer to the sewage load were observed even in this preliminary study. The findings support the proposals of adenovirus as an indicator of fecal contamination from human sources.

ACKNOWLEDGEMENTS

This work was funded by EU: FP6 SSP 022618 (EpiBathe), GVOP-3.1.1-2004-05-0517/3.0 and FP7 SSP 243923 (VIROCLIME). We would like to thank Jane Sellwood at the Royal Berkshire Hospital, Reading University, UK and Mária Takács at the National Center for Epidemiology, Budapest, Hungary and Annika Allard at University of Umeå for kindly providing HAdV-2, ECHO-11 and human norovirus isolates, respectively. We are grateful to Peter Wyn-Jones, to David Kay and to the staff of the Department of Microbiology at the University of Barcelona for their scientific support and providing the HAdV qPCR standard.

REFERENCES

Abbaszadegan, M., Huber, M. S., Gerba, C. P. & Pepper, I. L. 1995
Detection of enteroviruses in groundwater with the


First received 20 December 2012; accepted in revised form 3 July 2013. Available online 12 August 2013