

# Comparison of Two Concentration Methods for the Molecular Detection of Enteroviruses in Raw and Treated Sewage

F. Hmaïed<sup>1</sup> · S. Jebri<sup>1</sup> · M. E. R. Saavedra<sup>2</sup> · M. Yahya<sup>1</sup> · I. Amri<sup>1</sup> ·  
F. Lucena<sup>2</sup> · M. Hamdi<sup>3</sup>

Received: 27 June 2015 / Accepted: 3 August 2015 / Published online: 12 September 2015  
© Springer Science+Business Media New York 2015

**Abstract** Human enteric viruses are a major causative agent of emerging waterborne diseases and constitute a serious public health concern. Environmental contamination occurs through discharge of waste materials from infected persons. Methods for viral detection should be developed to detect low infective dose of enteric viruses in environment. In this study, we aimed at comparing two concentration methods for the detection of naturally occurring enteroviruses in raw and treated sewage. In the first method, polyethylene glycol is used to concentrate viral particles from the collected samples. The second method is based on ultracentrifugation of viral particles at high speed (110,000×g). Genomes of enteroviruses were quantified by the quantitative real-time PCR method in raw and treated sewage samples. PEG-based method yielded higher genomic copies of enteric viruses (with an average of 5.9 log<sub>10</sub> genomic copies/100 mL) when applied to raw sewage samples. While the ultracentrifugation assay in the second method decreases genomic copies number (with an average of 5.4 log<sub>10</sub> genomic copies/100 mL). The recovery differences between the two methods were not significant when applied to clean

samples (treated sewage). This could be explained by the presence of inhibitors, which interfere with qRT-PCR, in less quantity comparatively to raw sewage. PEG-based method would be more accurate for samples with high-organic matter load. This report emphasizes the importance of matrices nature on the recovery of enteroviruses from sewage samples. This should be taken into consideration for establishing standardized virological assays to ensure the virological quality control of discharged water in environment.

## Introduction

The role of water in infectious diseases transmission is well defined; it may act as a reservoir of different types of pathogens, mostly of fecal origin [1–3]. In fact, enteric viruses can survive and persist for a long time in water and sludge, maintaining infectivity in many instances [4]. Enteric viruses are shed in extremely high numbers in the feces of infected individuals, typically between 10<sup>5</sup> and 10<sup>11</sup> virus particles per gram of stool [5, 6]. They have been detected frequently in various types of environmental water samples impacted by fecal contamination, such river, groundwater, seawater, and even in drinking water [7–9]. Raw and treated sewage matrices represent an important source of viruses that show a high stability in environmental conditions [10]. To identify contaminated waters, fecal indicator bacteria such as *Escherichia coli* (*E. coli*) are typically monitored. However, the removal of coliform bacteria does not reflect adequately the removal of pathogenic viruses achieved in a disinfection procedure particularly when indicator bacteria concentrations are low. In fact, some studies have found no relationship between fecal indicator bacteria

---

✉ F. Hmaïed  
hmaiedfatma@yahoo.com

<sup>1</sup> Unité de Microbiologie et de Biologie Moléculaire, CNSTN, Technopôle de Sidi Thabet, 2020 Sidi Thabet, Tunisia

<sup>2</sup> Department of Microbiology, Barcelona University, Diagonal 645, 08028 Barcelona, Spain

<sup>3</sup> Institut National Sciences Appliquées de Tunis, Laboratoire Ecologie Technologie Microbienne, Carthage University, BP 676, 1080 Tunis, Tunisia

occurrence and the presence of enteric viruses or with gastrointestinal illness. This suggests the need to include virus detection in the evaluation of the microbiological quality of waters reused in agriculture.

Detection of viruses in sewage and drinking water should be sensitive, resistant for false-positive results, and enable full automation. Moreover, applied methods should be fast and inexpensive. Nowadays, techniques based on plaque-forming tests are not popular in monitoring of water environment [11]. The high analysis cost, time consumption, and the difficulties associated with permissive systems to some types of non-cultivable enteric viruses in vitro constitute the disadvantages of cell culture-based methods [12]. Molecular detection techniques, like quantitative reverse transcription polymerase reaction (qRT-PCR), can overcome these disadvantages with their high sensitivity and detection time [13]. An effective concentration of samples is necessary to evaluate virological quality of wastewaters, since matrices are highly contaminated with inhibitory substances that will interfere with viral detection [11]. Numerous methods have been described for concentrating and extracting enteric viruses from sewage [14–16]. All of them studied on spiked viral particles in decontaminated samples. Typical environmental sample concentrate ion procedures developed to purify virions are not always compatible with qRT-PCR. Despite the great sensitivity of qRT-PCR to detect enteroviruses in sewage or sludge samples extracted with adequate viral concentration technique, it still has serious limitations for routinely viral detection dealing with high numbers of processed samples. These difficulties lead to the concept of indicator microorganisms. Phages share many properties with human enteric viruses, notably composition, morphology, and structure, which imply that they may reflect the behavior and resistance to treatment process of viruses [17, 18]. The investigation of their fate, by using simple techniques, and their removal by the activated sludge process gives an overview of their usefulness as viral indicators presence and resistance to biological process.

In order to define a concentration method with high level of cost-efficiency and applicability, two viral concentration methods from raw and treated sewage were assayed, in this study, on naturally occurring enteroviruses. We aimed at investigating the effect of ultracentrifugation-based method described and applied in several studies and PEG-based method described by Lewis and Metcalf (1988) [19] as viral concentration methods for the recovery of Enteroviruses from raw and treated sewage. As model viruses, somatic coliphages and F-specific RNA phages were enumerated directly from raw and treated sewage using the double-layer agar technique.

## Methods

### Raw and Treated Sewage Samples

Twelve raw and treated sewage samples were collected from an urban medium charge (wastewater treatment plant) WWTP in North Tunisia. Treatment is based on activated sludge process; its influents are mainly domestic sewage. Treated sewage is drained off from the plant to a river which irrigates nearby lands. Viral extraction was performed within 48 h of sample collection.

### Viral Concentration Methods

Viruses were extracted from samples using the two methods. Each extraction experiment was done in triplicate.

#### *Ultracentrifugation-Based Method*

42 mL of sewage samples were concentrated as described by Puig et al. (1994) [20]. Briefly, samples were centrifuged at high speed ( $110,000\times g$  for 1 h at  $4\text{ }^{\circ}\text{C}$ ) to pellet viral particles together with suspended material. The pellet was eluted with 0.25 N glycine buffer (pH 9.5), and suspended solids were separated by centrifugation at  $12,000\times g$  for 20 min. Viruses were concentrated finally by ultracentrifugation ( $110,000\times g$  for 1 h at  $4\text{ }^{\circ}\text{C}$ ), re-suspended in 1 mL of  $0.1\text{ mol l}^{-1}$  phosphate buffer (pH = 7.2), and stored at  $-80\text{ }^{\circ}\text{C}$ .

#### *Polyethylene Glycol-Based Method*

The applied method for the elution of viruses from sewage samples is adapted from the one reported by the United States Environmental Protection Agency standards [21]. Conditioning of sewage samples binds un-adsorbed viruses present in the liquid matrix to the sewage solids using  $\text{AlCl}_3$  and varying samples pH. The eluate was concentrated by precipitation with polyethylene glycol 6000 (PEG) as described by Lewis and Metcalf (1988) [19], in proportions (1/5) 50 % PEG (w/v). After an overnight incubation at  $4\text{ }^{\circ}\text{C}$ , the extract was centrifuged at  $8000\times g$  for 45 min, and the pellet was re-suspended in 2 ml phosphate buffer, pH 7.2 ( $0.1\text{ mol l}^{-1}$ ). This suspension was filtered through a  $0.22\text{-}\mu\text{m}$  pore size membrane (Millex-GS, SLGS0250S, Molsheim, France), collected as virus concentrate, and stored at  $-80\text{ }^{\circ}\text{C}$ .

### Viral Genome Detection

#### *Viral RNA Extraction*

RNA was extracted from  $140\text{ }\mu\text{L}$  concentrate with QiAamp viral RNA kit (Qiagen) according to the manufacturer's instructions.

### Real-Time PCR Amplification and qRT-PCR Standard

The primers and probes sequences used to amplify and detect viral genomes targeted the conserved sequences at 5' un-transcribed region of Enteroviral genome (5'UTR) are shown in Table 1. The quantitative RT-PCR reaction was performed in a final volume of 25 ml by using the RNA Ultrasense™ One-step Quantitative RT-PCR System (Invitrogen) kit. Reaction mix containing 1 µg of RNA template, 0.2 µM primers, and 0.1 µM probe was submitted to the following cycling conditions: reverse transcription step at 50 °C for 30' followed by a hot start denaturing step at 94 °C for 15', and then 45 cycles of denaturation at 94 °C for 15'', annealing at 60 °C for 30'', and elongation at 72 °C for 30''. All reactions were performed on the Step One™ Real-Time PCR System (Applied Bio system). Amplification data were collected and analyzed using Sequence Detection Software version 1.0 (Applied Biosystems).

To avoid false-positive results, quality control measures such as using separate rooms were adopted and each set of amplifications included two negative controls: double-distilled sterile water after RNA extraction procedure, and a negative sample (mineral water) and one positive control Coxsackievirus CB3 RNA.

The detection method was validated with standard curves (11-fold successive dilutions of standard viral stock,  $n = 10$ ). The numbers of RNA copies present in each sample are estimated by comparing the sample cycle threshold (CT) value to standard curve. The final concentration was then adjusted based on the volume of nucleic acids analyzed and was expressed as genome copies (GC) per mL of analyzed sample. The number of GC is defined as the average of the data in triplicate obtained. The GC values are extrapolated to the number of enterovirus GC in each sample.

### Somatic Coliphages and F-RNA Phages Enumeration

Bacteriophages were enumerated directly from raw and treated sewage samples. Plaque-forming units (PFU) of somatic coliphages were counted by the double agar layer technique on strain WG5 of *E. coli* following the ISO

10705-1 standard [23]. F-specific RNA bacteriophage's PFU numbers were determined on strain WG49 of *Salmonella typhimurium*, following the ISO 10705-1 standard [24]. Antibiotics were added to soft agar to prevent growth of background bacteria present in the sewage samples. The Petri plates were incubated at 37 °C for up to 18 h prior to plaque enumeration.

### Statistical Analysis

Experiments were carried out in triplicate with 12 collected raw and treated sewage samples. Statistical analysis was performed using the Statgraphics statistical analysis software package (Statgraphics Plus 5.1; StatPoint, Inc.). Some data were plotted as boxes and whiskers. This plotting provides a summary statistics using five numbers: the minimum, the maximum, the median, the 25th percentile, and the 75th percentile.

## Results

### Enterovirus qRT-PCR Standard Curve

Briefly, *E. coli* JM109 cells (Promega) were transformed with a pGEM-T Easy plasmid (Promega) containing a 155 bp sequence of enteroviral 5'UTR insert. Transformed colonies were selected and screened by conventional PCR to evaluate the presence of the vector containing the insert. The vector was purified from the positive colonies using the Qiagen plasmid purification midikit (Qiagen) following the manufacturer's instructions. The concentration of the vector construct was quantified by spectrophotometer. The number of GC/mL of the stock prepared for each gene was calculated. Serial decimal dilutions are made in TE buffer to prepare the standard curve for qRT-PCR. The standard dilutions were then aliquoted and stored at -80 °C until use. Three replicates of each dilution were added to each qRT-PCR reaction. The standard curve was created by plotting the log number of enteroviral particles versus their corresponding cycle threshold (CT) value to create a best fit line through these points (Fig. 1). The CT value was

**Table 1** Enteroviruses primers and probe for qRT-PCR

Primer/probe name	Sequence (5'-3')	%GC	$T_m$ (°C)	References
Entero F	TCCTCCGGCCCTGAATGCG	70	64.8	[16]
Entero2R	ATGTCACCATAAGCAGCCA	48	54.2	
Entero S	6FAMCGGAACCGACTACTTTGGGTGTC-BHQ-1	57	65-70	[22]

$T_m$  melting temperature

inversely related to the viral particles. Run acceptability was defined as a correlation coefficient ( $R^2$ ) > 0.99.

## Concentration Methods

### *Comparison of Methods for Concentrating Enteroviruses from Raw Sewage*

Evaluated samples were positive for enteroviruses using both concentration methods. Raw sewage samples showed higher enteroviral GC occurrences after proceeding with viral concentration by PEG-based method (Table 2). Results obtained with UC-based method were tended to be different from the other methods ( $P$  value = 0.081) (Fig. 2).

### *Comparison of Methods for Concentrating Enteroviruses from Treated Sewage*

Figure 3 is a box plot of results obtained from the two viral concentration methods from treated sewage ready to be discharged. Unlike the raw sewage results, the two assayed methods showed similar viral titers. Concentration methods were not significantly different ( $P$  value > 0.5) (Table 3).

## Phages Enumeration

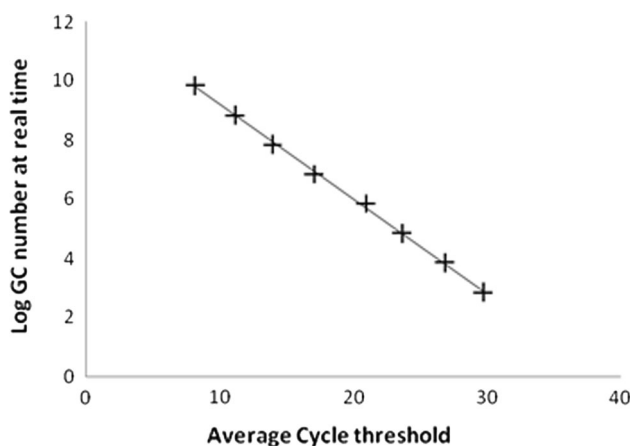
Raw sewage samples contain high amounts of somatic coliphages ranging between 6.1  $\log_{10}$  PFU/100 mL and 6.8  $\log_{10}$  PFU/100 mL. F-specific RNA phages titers were about 1log lower than those of somatic coliphages, ranging between 4.9  $\log_{10}$  PFU/100 mL and 5.8  $\log_{10}$  PFU/100 mL. After the biological treatment by activated sludge, titers of both phages decrease in all samples. While somatic coliphages concentrations decrease by 1.6  $\log_{10}$  PFU/

100 mL, F-RNA specific phages amounts decrease by 2.2  $\log_{10}$  PFU/100 mL (Fig. 4).

## Discussion

Human enteric viruses may be introduced into environmental waters through various routes like the discharge of untreated or inadequately treated wastewater. There are many challenges related to sampling studies to determine the virus presence due to both differences and limitations in recovery and concentration methods for the detection of viruses in natural samples. Concentration of viral particles after elution and extraction is a crucial step for viral detection. In order to establish a good concentration method for viral detection by qRT-PCR in raw and treated sewage samples, we aimed at comparing two viral concentration methods using these two matrices. We have chosen enteroviruses as representative indicators of viral and fecal contamination of water used in agriculture to do experiments on viral concentration. In fact, enteric viruses such as Hepatitis A virus, caliciviruses, adenoviruses, rotavirus, and enteroviruses have the greatest effect on public health [9, 25]. They have common characteristics such as morphology, persistence in environment, and contamination cycle. They have the same behavior and resistance to the treatment process.

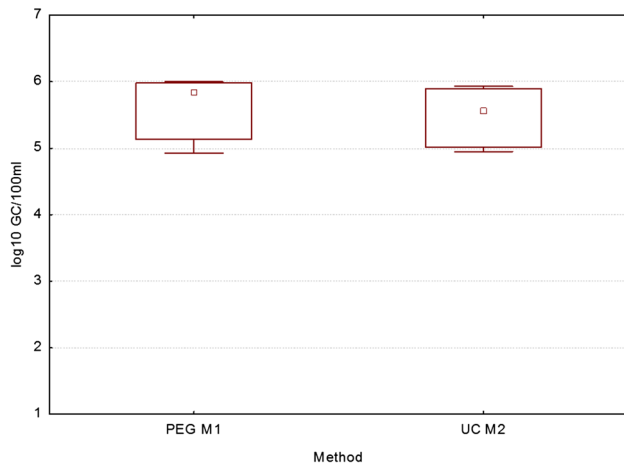
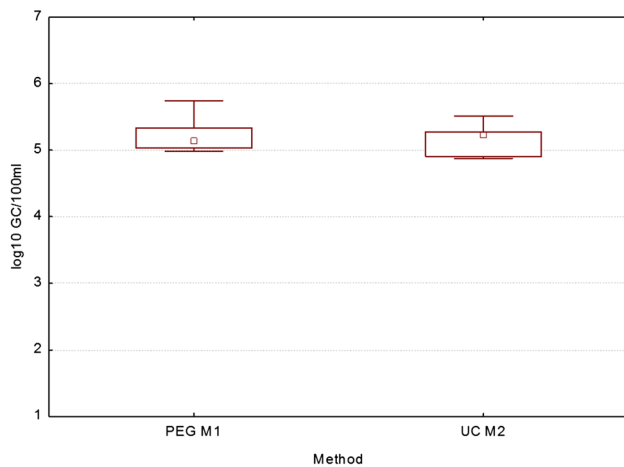
In this study, the percentages of raw and treated sewage samples testing positive for the enteroviruses genome using both concentration methods were 100 %. These results are in concordance with other studies raising concerns about viral contamination risk in treated water [15, 26, 27]. Enteroviral genomes concentration in raw and treated sewage were high in comparison with studies made on Human adenoviruses and polyomaviruses ranging between  $10^2$  and  $10^3$  (GC/mL), respectively, in sewage samples [15, 28]. Statistical analysis shows that PEG-based method generated higher recovery rates of viral genomes (with an average of 5.9  $\log_{10}$  genomic copies/100 mL) than the UC-based method in raw sewage (with an average of 5.4  $\log_{10}$  genomic copies/100 mL). Meanwhile, the two assayed methods were not significantly different when applied to treated sewage samples. These findings proved that concentration methods recovery depend on samples nature and the studied matrix. The UC-based method seems not to be adequate for enterovirus detection by qRT-PCR in natural samples with high load of suspended materials and organic matter. To our knowledge, this report is the first to prove the inhibitory effect of ultracentrifugation on the concentration of naturally occurring viruses in raw sewage. These results could have been due to the great sensitivity of the qRT-PCR for inhibitors, which are more concentrated by ultracentrifugation, existing in raw sewage and sludge



**Fig. 1** Standard curve generated with successive dilutions of standard viral stock.  $R^2 = 0.999$

**Table 2** Descriptive statistics of results obtained in raw sewage

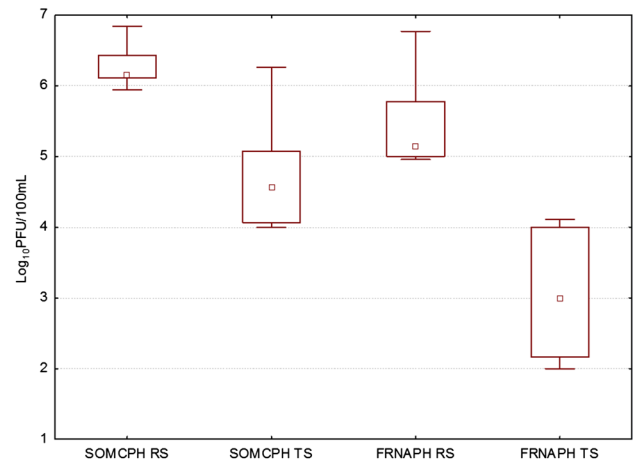
	Mean	S.E. Mean	SD	Variance	Skewness	Kurtosis
PEG-based method	5.9	0.02	0.10	0.01	-0.59	-1.57
UC-based method	5.44	0.09	0.41	0.16	0.01	-1.91

**Fig. 2** Box plot of  $\text{Log}_{10}$  enteroviral GC in raw sewage samples using the two concentration methods ( $P$  value = 0.0818)**Fig. 3** Box plot of  $\text{Log}_{10}$  Enteroviral GC in treated sewage samples using the two concentration methods ( $P$  value = 0.974)

samples. In fact, proteins and carbohydrates can bind to nucleotides and magnesium ions, making them unavailable to the polymerase in molecular techniques [29]. These inhibitors might be in less quantity in treated sewage considered as clean samples with low concentration of suspended materials and organic matter PEG is well known

**Table 3** Descriptive statistics of results obtained in treated sewage

	Mean	S.E. Mean	SD	Variance	Skewness	Kurtosis
PEG-based method	5.10	0.11	0.45	0.39	-0.56	0.03
UC-based method	5.07	0.08	0.39	0.15	-1.46	1.50

**Fig. 4** Box plot of  $\text{Log}_{10}$  PFU/100 mL of somatic coliphages in raw sewage (SOMCPH RS) and treated sewage (SOMCPH TS) and of F-specific RNA phages in raw sewage (FRNAPH RS) and treated sewage (FRNAPH TS)

as good inductor of attractive interactions that crystallize viruses in the inter-polymer spaces between PEG molecules. It is a neutral macromolecule precipitating viral particles in any type of solution even when it contains proteins. It is also known to remove toxicity from assayed samples. Furthermore, precipitation of viruses after elution is frequently used to increase the concentration of the virus extract for successful molecular detection. PEG is often used for this purpose, as it easily allows the precipitation of these viruses at neutral pH and at high ionic concentrations without precipitation of other organic material existing in raw sewage samples [30, 31] or even in other complex matrices rich with organic compounds like fruits [32]. While UC-based method has the disadvantage of precipitating inhibitors and organic compounds that interfere with molecular detection in samples with high load of suspended materials, PEG-based method would be more adequate for viral concentration in both matrices (raw and treated sewage) for further samples processing to detect naturally occurring enteroviruses by qRT-PCR. Furthermore, due to the lack in standardized methods for viral detection and enumeration in waters with high load of

organic matter (like raw sewage), there is a need for easily standardizable virus concentration methods design to be used in routine laboratories. PEG-based method might be a good alternative for this purpose. Besides, it does not require heavy equipments and can be applied in most laboratories.

In this study, Somatic coliphages and F-specific RNA phages were also enumerated. In fact, Somatic coliphages [33], F-specific RNA bacteriophages [34], and bacteriophages infecting *Bacteroides fragilis* [35] have been suggested as model microorganisms for water quality assessment. Phages have been successfully used in a variety of environmental applications. They are considered as fecal indicators. The environmental occurrence and persistence of some groups relate to health risks associated with fecal pollution and the potential occurrence of enteric pathogens in aquatic environments [36–39]. They are also considered as process indicators employed as enterovirus surrogates in evaluating the effectiveness of water treatment processes [40–43]. Results of this study show that both types of phages were present in all samples before and after biological treatment like enteroviruses but with higher titers. Somatic coliphages (reduction by 1.6 Log<sub>10</sub> PFU/100 mL) were more resistant to biological treatment by activated sludge than F-specific RNA phages (reduction by 2.2 Log<sub>10</sub> PFU/100 mL). Moreover, somatic coliphages occurrence in raw and treated sewage shows similar variations to enteroviruses GC numbers. Hence, somatic coliphages might be used as enteroviruses surrogates in terms of biological process resistance. In fact, some somatic phages such as T-4, T-7, ΦX174, and PRD-1 have proven useful as viral surrogates of fate and transport in laboratory investigations, pilot trials, and validation testing [25, 39].

## Conclusion

This study is attempting to evaluate viral concentration methods from WWTP residues to determine the best viral concentration techniques for naturally occurring enteroviruses detection by qRT-PCR in collected samples. This report highlights the importance of matrices nature on the recovery of enteroviruses from sewage samples. Standardized virological assays will enable the formulation of guidelines to ensure the virological quality control of discharged water and sludge in environment.

**Acknowledgments** This research was carried out within the framework of the European Union (ROUTES-FP7-ENV-2010-265156), the Generalitat de Catalunya (2009-SGR-01043), and 3-PCI-Tunisia Spanish Agency for International Cooperation for Development (AECID).

## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

## References

1. Craun GF (1984) Health aspects of groundwater pollution. In: Bitton G, Gerba CP (eds) Groundwater pollution microbiology. John Wiley & Sons Inc, New York, pp 135–179
2. Stevenson ME, Blaschke AP, Schauer S, Zessner M, Sommer R, Farnleitner AH, Kirschner AKT (2014) Enumerating microorganism surrogates for groundwater transport studies using solid-phase cytometry. *Water Air Soil Pollut* 225:1827
3. WHO (2004) The Global Burden of Disease. 2004 updated. World Health Organization, Geneva
4. Canepari P, Pruzzo C (2008) Human pathogens in water: insights into their biology and detection. *Curr Opin Biotechnol* 19: 241–243
5. Fong TT, Lipp EK (2005) Enteric viruses of humans and animals in aquatic environments: health risks, detection, and potential water quality assessment tools. *Microbiol Mol Biol Rev* 69: 357–371
6. Gerba CP (2000) Assessment of enteric pathogen shedding during recreational activity and its impact on water quality. *Quant Microbiol* 2:55–68
7. Borchardt MA, Bertz PD, Spencer S, Battigelli DA (2003) Incidence of enteric viruses in groundwater from household wells in Wisconsin. *Appl Environ Microb* 69:172–1180
8. Lucena F, Finance C, Jofre J, Sancho J, Schwartzbrod L (1982) Viral pollution of superficial waters (river water and seawater) from the urban area of Barcelona (Spain). *Water Res* 16:173–177
9. Okoh AI, Sibanda T, Gusha SS (2010) Inadequately treated wastewater as a source of human enteric viruses in the environment. *Int J Environ Res Public Health* 7:2620–2637
10. Crockett CS (2007) The role of wastewater treatment in protecting water supplies against emerging pathogens. *Water Environ Res* 79:221–232
11. Li W, Wang X, Yuan CQ, Zheng JL, Jin M, Song N (2002) Detection of enteroviruses and hepatitis a virus in water by consensus primer multiplex RT-PCR. *World J Gastroenterol* 8:699–702
12. Yadav R, Dwivedi S (2010) Trends and perspectives of biosensors for food and environmental virology. *Food Environ Virol* 2:53–63
13. Kim J, Lim J, Lee C (2013) Quantitative real-time PCR approaches for microbial community studies in wastewater treatment systems: applications and considerations. *Biotechnol Adv* 31(8):1358–1373
14. Amdiouni H, Maunula L, Ajjami K, Faouzi A, Soukri A, Nourilil J (2012) Recovery comparison of two virus concentration methods from wastewater using cell culture and real-time PCR. *Curr Microbiol* 65:432–437
15. Calgua B, Rodriguez-Manzano J, Hundesa A, Sunen E, Calvo M, Bofill-Mas S, Girones R (2013) New methods for the concentration of viruses from urban sewage using quantitative PCR. *J Virol Methods* 187:215–221
16. Hwang YC, Leong OM, Chen W, Yates MV (2007) Comparison of a reporter assay and immunomagnetic separation real-time reverse transcription-PCR for the detection of enteroviruses in seeded environmental water samples. *Appl Environ Microbiol* 73:2338–2340

17. Contreras-Coll N, Lucena F, Mooijman K, Havelaar A, Pierz V, Boque M, Gawler A, Höller C, Lambiri M, Mirolo G, Moreno B, Niemi M, Sommer R, Valentin B, Wiedenmann A, Young V, Jofre J (2002) Occurrence and levels of indicator bacteriophages in bathing waters throughout Europe. *Water Res* 36:4963–4974
18. Skraber S, Gassilloud B, Gantzer C (2004) Comparison of coliforms and coliphages as tools for assessment of viral contamination in river water. *Appl Environ Microbiol* 70:3644–3649
19. Lewis GD, Metcalf TG (1988) Polyethylene glycol precipitation for recovery of pathogenic viruses, including hepatitis A virus and human rotavirus, from oyster, water, and sediment samples. *Appl Environ Microb* 54:1983–1988
20. Puig M, Jofre J, Lucena F, Allard A, Wadell G, Girones R (1994) Detection of adenoviruses and enteroviruses in polluted waters by nested PCR amplification. *Appl Environ Microbiol* 60:2963–2970
21. USEPA 625/R92/013 (2003) Control of pathogens and vector attraction in sewage sludge, Cincinnati, OH
22. Nijhuis M, Van Maarseveen N, Schuurman R, Verkuijlen S, De Vos M, Hendriksen K, Van Loon AM (2002) Rapid and sensitive routine detection of all members of the genus enterovirus in different clinical specimens by real-time PCR. *J Clin Microbiol* 40:3666–3670
23. ISO (2000). ISO 10705-2: water quality. Detection and enumeration of bacteriophages. Part 2: Enumeration of somatic coliphages. International Organization for Standardization, Geneva
24. ISO (1995) ISO 10705-1: water quality. Detection and enumeration of bacteriophages. Part 1: Enumeration of F-specific RNA bacteriophages. International Organization for Standardization, Geneva
25. WHO (2004) Water, sanitation and hygiene links to health: Facts and figures. World Health Organization, Geneva, Switzerland
26. Fumian TM, Guimarães FR, Pereira Vaz BJ, da Silva MT, Muylaert FF, Boffill-Mas S, Girones R, Leite JP, Miagostovich MP (2010) Molecular detection, quantification and characterization of human polyomavirus JC from waste water in Rio De Janeiro, Brazil. *J Water Health* 8:438–445
27. Gantzer C, Maul A, Audic JM, Schwartzbrod L (1998) Detection of infectious enteroviruses, enterovirus genomes, somatic coliphages, and *Bacteroides fragilis* phages in treated wastewater. *Appl Environ Microbiol* 64:4307–4312
28. Rodríguez-Manzano J, Alonso JL, Ferrús MA, Moreno Y, Amorós I, Calgua B, Hundesa A, Guerrero-Latorre L, Carratala A, Rusiñol M, Girones R (2012) Standard and new faecal indicators and pathogens in sewage treatment plants, microbiological parameters for improving the control of reclaimed water. *Water Sci Technol* 66:2517–2523
29. Schwabb KJ, De Leon R, Sobsey MD (1995) Concentration and purification of beef extract mock eluates from water samples for the detection of enteroviruses, hepatitis A and Norwalk virus by reverse transcription PCR. *Appl Environ Microbiol* 61:531–537
30. Atha DH, Ingham KC (1981) Mechanism of precipitation of proteins by polyethylene glycols. *J Biol Chem* 256:12108–12117
31. Lee JC, Lee LLY (1981) Preferential solvent interactions between proteins and polyethylene glycols. *J Biol Chem* 256:625–631
32. Stals A, Baert L, Van Coillie E, Uyttendaele M (2011) Evaluation of a norovirus detection methodology for soft red fruits. *Food Microbiol* 28:52–58
33. Kott Y, Roze N, Sperber S, Betzer N (1974) Bacteriophages as viral pollution indicators. *Water Res* 8:165–171
34. Havelaar AH, Hogeboom WM, Pot R (1984) F-specific RNA bacteriophages in sewage; methodology and occurrence. *Water Sci Technol* 17:645–655
35. Jofre J, Bosch A, Lucena F, Girones R, Tartera C (1986) Evaluation of *Bacteroides fragilis* bacteriophages as indicators of the virological quality of water. *Water Sci Technol* 18:167–173
36. IAWPRC (1991) Bacteriophages as model viruses in water quality control. *Water Res* 25:529–545
37. Leclerc H, Edberg S, Pierzo V, Delattre JM (2000) Bacteriophages as indicators of enteric viruses and public health risk in groundwaters. *J Appl Microbiol* 88:5–21
38. Lucena F, Ribas F, Duran AE, Skraber S, Gantzer C, Campos C, Morón A, Calderón E, Jofre J (2006) Occurrence of bacterial indicators and bacteriophages infecting enteric bacteria in groundwater in different geographical areas. *J Appl Microbiol* 101:96–102
39. Lucena F, Jofre J (2010) Potential use of bacteriophages as indicators of water quality and wastewater treatment processes. In: Sabour PM, Griffiths MW (eds) *Bacteriophages in the control of food and waterborne pathogen*. ASM Press, Washington, pp 103–118
40. Durán AE, Muniesa M, Mocé-Llivina L, Campos C, Jofre J, Lucena F (2003) Usefulness of different groups of bacteriophages as model microorganisms for evaluating chlorination. *J Appl Microbiol* 95:29–37
41. Havelaar AH (1993) Bacteriophages as models of enteric viruses in water treatment processes. *Am Soc Microbiol News* 59:614–619
42. Payment P, Trudel M, Plante R (1985) Elimination of viruses and indicator bacteria each step of treatment during preparation of drinking water at seven water treatment plants. *Appl Environ Microbiol* 49:1418–1428
43. Persson F, Langmark J, Heinicke G, Hedberg T, Tobiasson J, Stenstro T, Hermansson M (2005) Characterization of the behavior of particles in biofilters for pre-treatment of drinking water. *Water Res* 39(16):3791–3800