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Characterization of the efficiency and uncertainty of skimmed milk flocculation for the simultaneous concentration and quantification of water-borne viruses, bacteria and protozoa

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ABSTRACT

In this study, the use of skimmed milk flocculation (SMF) to simultaneously concentrate viruses, bacteria and protozoa was evaluated. We selected strains of faecal indicator bacteria and pathogens, such as *Escherichia coli* and *Helicobacter pylori*. The viruses selected were adenovirus (HAdV 35), rotavirus (RoV SA-11), the bacteriophage MS2 and bovine viral diarrhoea virus (BVDV). The protozoa tested were *Acanthamoeba*, *Giardia* and *Cryptosporidium*. The mean recoveries with q(RT)PCR were 66% (HAdV 35), 24% (MS2), 28% (RoV SA-11), 15% (BVDV), 60% (*E. coli*), 30% (*H. pylori*) and 21% (*Acanthamoeba castellanii*). When testing the infectivity, the mean recoveries were 59% (HAdV 35), 12% (MS2), 26% (RoV SA-11) and 0.7% (BVDV). The protozoa *Giardia lamblia* and *Cryptosporidium parvum* were studied by immunofluorescence with recoveries of 18% and 13%, respectively. Although q(RT)PCR consistently showed higher quantification values (as expected), q(RT)PCR and the infectivity assays showed similar recoveries for HAdV 35 and RoV SA-11. Additionally, we investigated modelling the variability and uncertainty of the recovery with this method to extrapolate the quantification obtained by q(RT)PCR and estimate the real concentration. The 95% prediction intervals of the real concentration of the microorganisms inoculated were calculated using a general non-parametric bootstrap procedure adapted in our context to estimate the technical error of the measurements. SMF shows recoveries with a low variability that permits the use of a mathematical approximation to predict the concentration of the pathogen and indicator with acceptable low intervals. The values of uncertainty may be used for a quantitative microbial risk analysis or diagnostic purposes.

1. Introduction

Diseases related to water contamination constitute a major human health issue. Inadequate drinking water and poor sanitation are estimated to cause 842,000 diarrhoeal disease-related deaths per year (World Health Organization, 2014). They are related to a broad range of health problems and cause impacts on productivity due to water-borne diseases (Amini and Kraatz, 2014). Moreover, the creation of protocols to measure water quality, considering the diversity of pathogens that may be present, is one of the major problems that must be solved for improving the control of water quality and Quantitative Microbial Risk Assessment (QMRA) studies.

The following four main critical steps in the process of evaluating the microbiological quality of water need to be considered: (1) which pathogens may be present; (2) which microorganisms are used as indicators of contamination; (3) which method is used to concentrate the particular indicator or indicators; and (4) which technique is used to detect them.

Indicator organisms are used for a range of purposes as follows: indicators of faecal pollution and to evaluate the effectiveness of processes such as filtration or disinfection. The most popular indicator organisms are thermotolerant coliforms, *E. coli* and intestinal enterococci. However, the suitability of *E. coli* as an indicator has been questioned, because its survival in water and sensitivity to treatment and disinfection processes differ substantially from those of excreted viruses and protozoa.

Abbreviations: BVDV, Bovine viral diarrhoea virus; HAdV, Human adenovirus; IFA, Immunofluorescence assays; JCPyV, JC polyomavirus; NoV, Norovirus; PI, Prediction intervals; QMRA, Quantitative microbial risk assessment; q(RT)PCR, Quantitative (reverse transcriptase) PCR; RoV, Rotavirus; SMF, Skimmed milk flocculation; TCID50, 50% Tissue culture infective dose.

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E. coli is far more sensitive, and the consequence is a low correlation with the presence of other pathogens (Amini and Kraatz, 2014; Bofill-Mas et al., 2013; Gorchev and Ozolins, 2011).

Coliphages share many properties with human viruses and are used as models to assess the behaviour of excreted viruses in the water environment. In this regard, they are superior to faecal bacteria. However, there is no direct correlation between the numbers of coliphages and the numbers of excreted viruses (Gorchev and Ozolins, 2011). The use of excreted viruses as microbial indicators is based on the shortcomings of the existing choices. Human adenovirus (HAdV) has been proposed as a viral indicator of contamination (Gorchev and Ozolins, 2011; Pina et al., 1998) and has been used in various studies as a viral indicator of human faecal contamination and a microbial source tracking tool (Bofill-Mas et al., 2011, 2013; Rusiñol et al., 2014).

Most HAdVs are associated with respiratory disease, but types 40 and 41 are responsible for gastroenteritis outbreaks in children (Wold and Horwitz, 2013). Rotavirus (RoV) is also associated with gastroenteritis; RoV-A is the most common cause of severe vomiting and diarrhoea among children up to 30 months old (Estes and Greenberg, 2013). The coliphage MS2 is commonly used as a surrogate and process control in microbiological food and water analyses (van Duin and Olsthoorn, 2012). BVDV is an important cause of morbidity, mortality, and economic loss in dairy and beef cattle worldwide (MacLachlan and Dubovi, 2011).

E. coli is commonly found in the lower intestine of warm-blooded organisms. Most strains are harmless, but others can cause serious food poisoning and are responsible for product recalls due to food contamination (Madigan et al., 2014). *H. pylori* is an acid-tolerant bacterium usually found in the stomach and is related to gastric cancer (Johnson et al., 1997). *H. pylori* has been detected in wastewater (Moreno and Ferrús, 2012), surface water and other environmental samples all over the world (Eusebi et al., 2014) and has even demonstrated the capacity to survive in chlorinated water when the enumeration of coliforms indicates that the water is potable (Santiago et al., 2015). *G. lamblia* and *C. parvum* are responsible for outbreaks of gastroenteritis related to the consumption of contaminated water (Gascón, 2006). *Acanthamoeba* spp., free-living protozoa, are considered to be opportunistic pathogens (Marciano-Cabral and Cabral, 2003) and are known to have a role in the persistence of some bacterial pathogens, such as *Legionella*, in water environments (Lambrecht et al., 2015).

The direct examination of water is difficult due to low and fluctuating concentrations of microorganisms and because concentration procedures are usually organism and/or matrix-specific and most techniques have high or unknown variability parameters. One-step skimmed milk flocculation (SMF) has been proposed as an efficient low-cost method to concentrate viruses in all types of water samples. This method has been used in environmental water matrices such as river water (Calgua et al., 2013a), seawater (Calgua et al., 2008), ground water (Bofill-Mas et al., 2011) and wastewater (Calgua et al., 2013b). However, the efficacy of the recovery in controlled conditions has not been properly described until now.

Quantitative Microbial Risk Analysis (QMRA) is a scientific tool used to assess the microbial safety of water and is needed for developing a strategy of risk management models. QMRA models each variable using a probability distribution. The advantage is that the result is represented by a probability distribution function instead of a single value. The objective of QMRA is the ability to calculate the combined impact of the uncertainty in the model's parameters to determine an uncertainty distribution of the possible model outcomes (Vose, 2008).

The aim of the present study was to determine the efficacy of the SMF recovery to simultaneously concentrate viruses, bacteria and protozoa and then compare q(RT)PCR and infectivity assays to detect and quantify the number of viruses recovered. Finally, an extrapolation method was evaluated with the q(RT)PCR quantification using the prediction interval (PI) based on the known recoveries to correctly achieve the actual concentration of the spiked microorganisms and define the uncertainty values of the method.

2. Materials and methods

2.1. Microorganism stocks and cell lines

The following viruses were analysed and spiked into the water samples: HAdV-35 (ATCC, LGC Standards AB, Borås, Sweden) cultured in cell line A549 (ATCC CCL-185), MS2 (ATCC 23631) cultured in *Salmonella typhimurium* strain WG49 (NCTC 12484), RoV SA-11 (ATCC VR-1565) cultured in MA104 (ATCC CRL-2378) and Bovine viral diarrhoea virus (BVDV) strain NADL kindly donated by the EU and OIE Reference Laboratory for Classical Swine Fever, Institute of Virology, University of Veterinary Medicine, Hannover, Germany, and cultured in NDBK (ATCC CCL-22). The analysed bacteria were *E. coli* (ATCC 23725) and *H. pylori* (NCTC11637). The protozoa tested in the study were *A. castellanii* (CCAP 1534/2), *G. lamblia* H3 isolate (Waterborne Inc., New Orleans, LA) and a *C. parvum* Iowa isolate (Waterborne Inc., New Orleans, LA).

2.2. Water samples

This experiment was conducted with tap water from the metropolitan area of Barcelona; the volume of water evaluated in each bucket was 10 L. The number of buckets inoculated with each of the microorganisms and their respective inoculated concentration are specified in Table 1. The tap water was previously treated with 100 mL of sodium thiosulfate (10% (*w*/*v*)) to eliminate chloride residues. Four additional buckets with the same volume of water were analysed as negative control samples.

2.3. Skimmed milk flocculation concentration

The skimmed milk flocculation concentration protocol has been described in previous studies (Bofill-Mas et al., 2011; Calgua et al., 2008). In summary, a pre-flocculated skimmed milk solution (1% (*w*/*v*)) was prepared by dissolving 10 g of skimmed milk powder (Difco-France) in 1 L of artificial seawater and carefully adjusting the pH to 3.5 with 1 N HCl. One hundred millilitres of this solution was added to each of the previously acidified (pH 3.5) 10 L water samples (the final concentration of skimmed milk was 0.01% (*w*/*v*)). The conductivity was also measured and adjusted with artificial sea salt (Sigma, Aldrich Chemie GMBH, Steinheim, Germany) to achieve a minimum conductivity of 1.5 mS/cm^2. The samples were stirred for 8 h at room temperature, and the flocs were allowed to settle by gravity for another 8 h. The supernatants were removed, and the sediment was collected and transferred to a 500 mL centrifuge container and centrifuged at 8000 × *g* for 30 min at 4 °C. The obtained pellet was resuspended in 8 mL of

Table 1
The number of microorganisms inoculated in each of the ten litre water buckets used for the skimmed milk flocculation concentration process.

Microorganisms (number of samples)	Molecular quantification		Quantification by infectious assays	
HAdV (10)	2.88E + 07	GC	4.60E + 06	IFA
MS2 (13)	2.92E + 09	GC	2.07E + 09	PFU
	2.92E + 07	GC	5.03E + 06	
RoV (19)	6.31E + 08	GC		
	2.09E + 07	GC	4.08E + 05	TCID50
BVDV (3)	2.10E + 08	GC	6.31E + 05	TCID50
E. coli (10)	2.37E + 06	GC		
H. pylori (9)	1.97E + 08	GC		
A. castellanii (9)	7.27E + 04	GC		
C. parvum (8)	1.46E + 04	IFA		
G. lamblia (8)	1.56E + 04	IFA		

GC: genomic copies; IFA: immunofluorescence assay; PFU: plaque-forming units; TCID50: 50% tissue culture infective dose.

0.2 M phosphate buffer at pH 7.5 (1:2, *v*/*v* of 0.2 M Na_2HPO_4 and 0.2 M NaH_2PO_4). Once the pellet was completely dissolved, the phosphate buffer was added to a final volume of 10 mL. The concentrates were kept at −20 °C after the SMF method was performed. The quantification was then performed within five days.

2.4. Nucleic acid extraction

Viral nucleic acids (NA) were extracted using the QIAmp Viral RNA kit (Qiagen, Inc., Valencia, CA). Bacterial and protozoan DNA was extracted using the UNEX method (Hill et al., 2015). The volumes of the concentrates used for the extraction were 140 and 300 μL, and the elutions were 80 and 100 μL, for viruses/bacteria and protozoa, respectively. Immediately after extraction, q(RT)PCR analyses were performed.

2.5. q(RT)PCR quantification

Specific real-time q(RT)PCR assays were used to quantify the microorganisms following the specifications previously described for HAdV (Hernroth et al., 2002), RoV (Zeng et al., 2008), MS2 (Calgua et al., 2014), BVDV (Losurdo et al., 2015), *E. coli* (Khan et al., 2007), *H. pylori* (Santiago et al., 2015) and *A. castellanii* (Qvarnstrom et al., 2006). Undiluted and 10-fold dilutions of the nucleic acid extracts were analysed in duplicate, including the concentrates from negative control buckets. All of the q(RT)PCR assays included four non-template controls to demonstrate that the mix did not produce fluorescence. The standards for viruses were prepared using synthetic gBlocks® Gene Fragments (IDT) and quantified with a Qubit® fluorometer (Thermo Fisher Scientific). For bacteria and *A. castellanii* standards, the DNA was extracted from cultures of known concentration and quantified using a Nanodrop 1000. For all of the standards, ten-fold dilutions were prepared from 10^0 to 10^7 copies per reaction.

2.6. Infectivity and immunofluorescence assays

Specific infectivity assays were performed using previously described methods for viruses as follows: IFA for HAdV-35 (Calgua et al., 2011), TCID50 for RoV (Otto et al., 2015) and BVDV (OIE, 2015) and plaque assays for MS2 (Anonymous, 1995).

For the quantification of *G. lamblia* and *C. parvum*, we used an immunofluorescence method previously described for the staining of cysts and oocysts with 4′,6-diamidino-2-phenylindole (DAPI) and observed the staining using differential interference contrast microscopy (USEPA, 2005).

2.7. Recovery, concentration and PI estimates

In our assays, in every replicate, the true concentration, Y_K, was known, allowing us to compute the recovery mean $\overline{r_K}$. In a future non-controlled experiment, the only information available will be the q(RT)PCR-measured concentration, m_U, while the concentration Y_U and the recovery r_U will be unknown. In this section, we introduce a new PI, which estimates this unknown concentration Y_U of new observations, guaranteeing an $1-\alpha$% confidence level.

Our approach starts by considering the distribution of our controlled recoveries as a valid model for the future measures. In a new experiment (with one replicate), the three quantities are related by the equation $m_U = Y_U r_U$. A *point estimate* of Y_U can be obtained by simply substituting r_U with $\overline{r_K}$:

$$\hat{Y}_U = \frac{m_U}{\overline{r_K}} \tag{1}$$

The *relative error* of this estimation is:

$$e_R = \frac{\hat{Y}_U}{Y_U} = \frac{r_U}{\overline{r_K}} \tag{2}$$

We consider it essential to improve Eqs. (1) and (2) by also measuring their confidence: the accuracy of $\overline{r_K}$ depends on the sample size and variability of our current experiments.

The PIs described in the statistical literature are built specifically to predict new observations when the parameters of the distribution are estimated with a sample. The purpose and formulas of the PIs are different from the more commonly used confidence intervals. In the Gaussian case, the PI has a closed simple expression. However, the normality assumption for the recoveries is not supported in practice, and other probabilistic models are often used; for instance, Petterson et al. (2015) assumed the beta distribution to study the variability in the recovery of a virus in water.

The absence of closed expressions for the PI plus the difficulty in ensuring a correct goodness of fit of any probabilistic model has finally impelled us to find a free-distribution method. Among the different approaches previously described (see Bai et al., 1990; Mojirsheibani, 1998, for a comparison of several methods), we chose the non-parametric bootstrap-t technique. In brief, this standard computational method defines a bootstrap statistic T^*, which combines the distributions of the past and the future samples. Given a confidence level of $1-\alpha$%, the resampling procedure lets us obtain any α quantile $\hat{t}^{(\alpha)}$ of T^*, and, in our context, to obtain the following limits of the PI for r_U (further details in Mojirsheibani, 1998):

$$prob(r_U \le r_{U,\,min}) = \frac{\alpha}{2} \quad prob(r_U \le r_{U,\,max}) = 1-\frac{\alpha}{2} \tag{3}$$

The above bootstrap-t PI of r_U lets us derive from Eqs. (1) and (2) two new expressions: the PI of the unknown concentration and its relative error

$$prob\left(\frac{m_U}{r_{U,\,max}} \le Y_U \le \frac{m_U}{r_{U,\,min}}\right) = 1-\alpha \tag{4}$$

$$prob\left(\frac{r_{U,\,min}}{\overline{r_K}} \le e_R \le \frac{r_{U,\,max}}{\overline{r_K}}\right) = 1-\alpha \tag{5}$$

2.8. Statistical evaluation

All of the data were statistically analysed with the 3.1.1 version of the R software (R Core Team, 2016). First, descriptive statistics of all of the recoveries and quantifications were performed. We plotted the actual data together with the normal density, the beta density (estimated using the maximum likelihood criteria) and a non-parametric kernel estimation of the density in order to assess their goodness of fit. The PI (3), (4) and (5) for every organism were computed implementing the equations for T^* and $\hat{t}^{(\alpha)}$ combined with the methods of the *boot* package in R. An R script with our implementation of these PIs can be found in the supplementary material of this paper. Additionally, Wilcoxon signed-rank tests were used to evaluate the difference between the methods of quantification (q(RT)PCR and the infectivity assay) with the recoveries and quantification of RoV. Spearman's rank correlation coefficient was used to evaluate the q(RT)PCR recovery between all of the microorganisms evaluated.

3. Results

3.1. Recovery efficacy

Each water bucket was inoculated with the concentration of microorganisms indicated in Table 1. Bacteria and protozoa were quantified using one method (q(RT)PCR or the infectivity/immunofluorescence assays), whereas viruses were quantified with both methods. The recovery percentage for each microorganism represents the efficacy of recovery using the combination of SMF plus the efficacy of the method of quantification, either q(RT)PCR or the infectivity assay (Table 2). All of the negative control buckets were negative.

3.2. Correlation between the recoveries for the different microorganisms

The recovery results of each of the samples were correlated to determine if there are relationships between the microorganisms for the q(RT)PCR results. Table 3 shows the results of the Spearman's correlation analysis of the values obtained, specifying the number of samples and the *p*-values. It also includes a graphical representation of the data in Cartesian planes inside a correlation matrix between each of the pairs compared. A positive correlation was found between the tested bacteria *H. pylori* and *E. coli* (Table 3). A positive but non-statistically significant correlation also occurred between all the viruses evaluated. *Acanthamoeba* recovery was not correlated with any microorganism evaluated by q(RT)PCR.

3.3. Comparison of the quantification between q(RT)PCR and the infectivity assays

Viruses were enumerated with both q(RT)PCR and infectivity assays; the results for the recoveries are shown in Table 2 and the quantifications in Table 4. The descriptive results of both tables must be carefully interpreted because the small sample size of the infectivity assays does not allow an inferential assessment. For RoV, where 10 replicates are available, the Wilcoxon signed-rank test was computed.

The recoveries with q(RT)PCR and the infectivity assays for RoV show non-significant differences (p-value = 0.37). The descriptive results of HAdV in Table 2 may suggest a similar conclusion. In contrast, also in RoV, we detected significant differences (p-value = 0.002) between the quantities obtained by q(RT)PCR and by infectivity. Table 4 may suggest similar results for the rest of the organisms, but further experiments are required to confirm these preliminary results.

A ratio between the logarithm of the quantification between q(RT)PCR and infectivity was calculated to indicate how many times the quantifications varied relative to one another. The ratios for HAdV, RoV and MS2 were 1.13, 1.35 and 1.07, respectively.

Table 2
Skimmed milk flocculation recoveries for each of the microorganisms evaluated.

Microorganisms	Method	Percent recovery					
		Mean %	CI 95% of mean	*n*	sd	min	max
HAdV	qPCR	66	53.5–78.5	10	17.4	32.2	86.7
	IFA	58.7	4.5–100	3	1.8	8.1	49.8
MS2	q(RT)PCR	23.9	19.6–28.1	13	7	13.8	36.8
	PFU	11.9	9–14.7	4	1.8	9.5	13.9
RoV	q(RT)PCR	28.2	25.6–30.7	19	5.3	16	37.1
	TCID50	26.1	17.1–35.1	10	12.6	43.5	83.7
BVDV	q(RT)PCR	14.7	10.8–18.7	3	1.6	12.9	15.8
	TCID50	0.7	0.4–1.1	3	0.13	0.67	0.89
E. coli	qPCR	59.6	40.3–79	10	27.1	15.6	98.7
H. pylori	qPCR	30.2	24.4–36.1	9	7.6	20.8	41.5
A. castellanii	qPCR	20.5	14.9–26.1	9	7.2	13	32.1
G. lamblia	IFA	17.8	15–20.7	8	3.4	12.8	21.5
C. parvum	IFA	12.8	12.5–15.2	8	2.9	9.6	17.4

q(RT)PCR: quantitative (reverse transcriptase) PCR; IFA: immunofluorescence assay; PFU: plaque-forming units; TCID50: 50% tissue culture infective dose.

3.4. The impact of recovery on the predicted concentration

The use of q(RT)PCR quantification in QMRA has been previously demonstrated (Rames et al., 2016). To better evaluate the real concentration of microorganisms when the quantification is obtained after SMF, we suggest extrapolation of the q(RT)PCR value incorporating the uncertainty and variability of the method. HAdV, RoV, MS2, *E. coli*, *H. pylori* and *A. castellanii* were used for this purpose. The upper and lower limits, including the real concentration in water samples with a 95% PI, were estimated using the non-parametric bootstrap approach described above. Despite the moderate sample size of our assays (between 9 and 19 replicates), the PIs show a reasonable width of approximately 4–5 units, supporting the applicability of this information for future observations.

As an example of how to use this information in practice, we take here the measurement previously published by Calgua et al. (2008) as a *future* measurement; their reported m_U was 2.73E + 4 genomic copies in 10 L of HAdV. Substituting in Eqs. (4) and (5) the values in Table 5 (bootstrap-t PI method, row HAdV) we obtain:

$$prob(2.73E+4\times 1.027 \le Y_U \le 2.73E+4\times 5.200) =$$

$$prob(2.80E+4 \le Y_U \le 1.42E+5) = 0.95$$

$$prob(29.14\% \le e_R \le 147.57\%) = 0.95$$

In fact, Calgua et al. (2008) state that the real concentration inoculated in the sample was 4.04E + 04 with 68% recovery. Both quantities lie in their respective PIs computed above.

In Table 5, we have additionally computed the PI when a normal distribution of the recoveries is assumed. In some organisms, this PI may show comparable results to the bootstrap-t PI; for instance, in RoV (Table 5) they are almost identical. Fig. 1A can explain this concordance: normal, beta and kernel densities similarly fit the actual data. This is not the general case and, as a consequence, the normal and bootstrap PIs may show different coverages. For instance, in HAdV, the different results in Table 5 can be explained by the different fittings of the densities in Fig. 1B. The main reason to introduce the bootstrap-t predictions was the lack of fit of the normal distribution needed to correctly build prediction intervals.

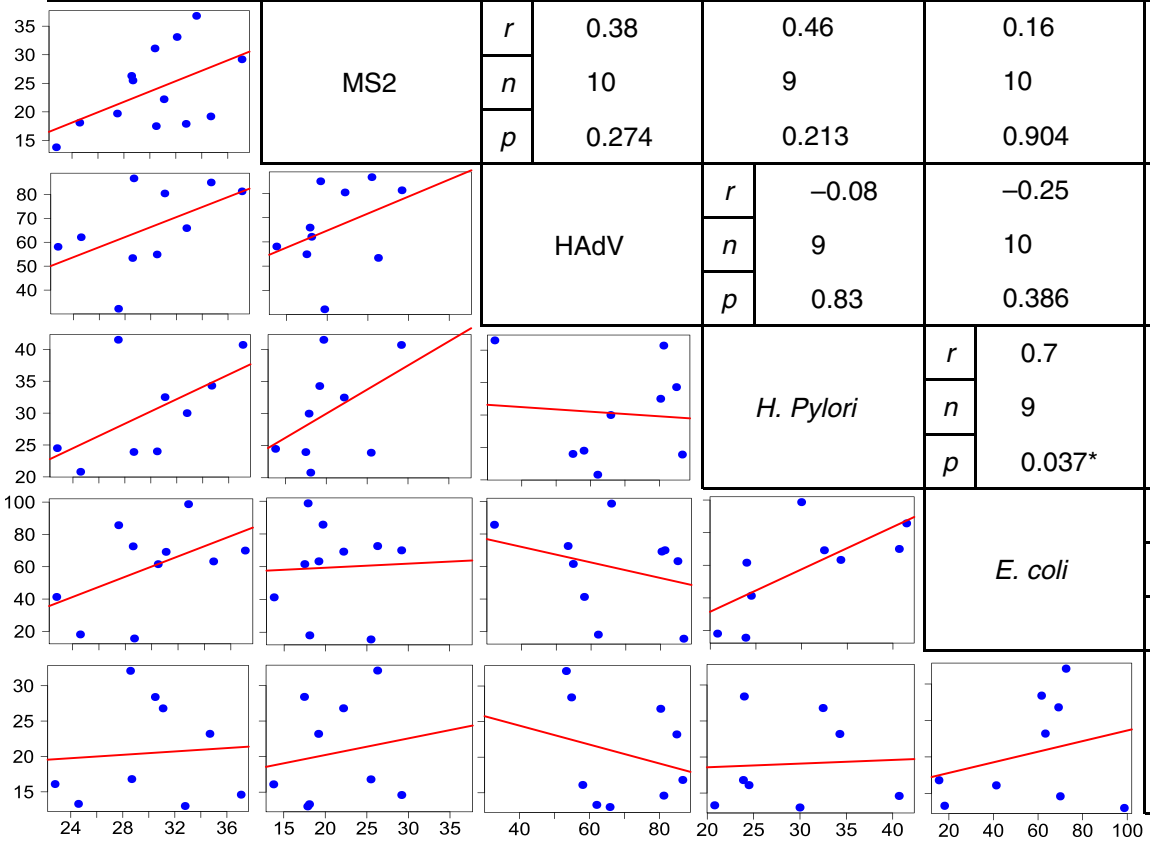
4. Discussion

This is the first study to characterize the simultaneous concentration of viruses (including an enveloped virus), bacteria and protozoa with SMF. Moreover, this is the first study to correlate recoveries and evaluate the uncertainty of the results when using SMF for simultaneous concentration. Additionally, the evaluation of infectious viral particles has been included, since there was no previous information of recoveries with SMF using infectivity assays. The highest recoveries obtained in this experiment were with HAdV and *E. coli*. This method has been previously evaluated by spiking HAdV in seawater, river water and wastewater with recoveries by qPCR of 52%, between 41% and 50%, and between 30% and 95%, respectively (Calgua et al., 2008, 2013a,b). Moreover, SMF has been used to concentrate norovirus (NoV) with recoveries between 34 and 74% (Calgua et al., 2013a). The present study describes the recoveries of HAdV with confidence intervals that include these previous results, with higher sample size and suggesting that SMF with qPCR quantification may be used indistinctly in different water matrices without affecting the efficacy of the method.

SMF has been used in environmental samples to detect DNA viruses used as microbial source tracking tools, such as JC polyomavirus (JCPyV), porcine adenovirus and bovine polyomavirus, in superficial and ground water samples (Bofill-Mas et al., 2011); it has also been used in studies analysing a wide diversity of viruses, such as HAdV, NoV, JCPyV, RoV, Klassevirus, Asfarvirus-like virus and Merckel cell

Table 3
Correlation of the recoveries obtained by q(RT)PCR between microorganisms with the skimmed milk flocculation method.

RoV	*r*	0.51	0.53	0.59	0.51	0.08
	n	13	10	9	10	9
	p	0.073	0.118	0.096	0.134	0.848
MS2	*r*	0.38	0.46	0.16	0.16	
	n	10	9	10	9	
	p	0.274	0.213	0.904	0.676	
HAdV	*r*	−0.08	−0.25	−0.25		
	n	9	10	9		
	p	0.83	0.386	0.52		
H. Pylori	*r*	0.7	0.06			
	n	9	8			
	p	0.037*	0.896			
E. coli	*r*	0.27				
	n	9				
	p	0.48				
A. castellanii						



r = Spearman's rank correlation coefficient, *n* = paired sample size, *p* = *p*-value, *p-value < 0.05.

polyomavirus in river water (Calgua et al., 2013a; Rusiñol et al., 2014, 2015) and HAdV, RoV, PP7 phage and NoV in seawater (Calgua et al., 2008; Rusiñol et al., 2014; Victoria et al., 2014). The SMF protocol with modifications has also been used to quantify HAdV, JCPyV and NoV in sewage water (Calgua et al., 2013b). Additionally, SMF has been modified to detect HAdV and NoV in strawberries with good results (Melgaço et al., 2016).

Enveloped viruses such as BVDV may be more stable than expected in water. Considering the lack of information available on the concentration protocols of enveloped viruses in water, it was decided to include in this study a representative enveloped virus, BVDV, an important pathogen for cattle. The recoveries of this virus using SMF were analysed in triplicate, and the applicability of available methodologies, specifically qPCR and infectivity assays, was also evaluated. The analysis of BVDV, transmitted through inhalation and ingestion as main horizontal routes in cattle (MacLachlan and Dubovi, 2011), in water will produce useful information on the spread of BVDV through contaminated sources of water and animal drinking troughs.

Table 4
A comparison between q(RT)PCR and infectivity results in the viral concentrates after skimmed milk flocculation.

Virus	*N*	Quantification		
		q(RT)PCR	Infectivity	Log 10 ratio q(RT)PCR/infectivity
RoV	10	6.25E + 06	1.06E + 05	1.35
MS2	4	7.38E + 08	1.83E + 08	1.07
HAdV	3	1.87E + 07	2.70E + 06	1.13
BVDV	3	3.09E + 07	4.67E + 03	2.04

The availability of cost-effective techniques for the simultaneous concentration of viruses, bacteria and parasites from water will be very useful when the monitoring of microbial water quality for diverse microbe types is desired (Hill et al., 2005). In addition, it will also be valuable for the application of next-generation sequencing methods and the characterization of the microbial population of water. Until now, there have been no other methods of concentration that allow for the evaluation of a representative volume (10 L), diverse water matrices with high and low turbidity, a high recovery percentage and the simultaneous evaluation of viruses, bacteria and protozoa.

Due to the importance of finding a suitable indicator of contamination, the correlation between the recoveries of the different pathogens and suggested indicators is relevant information that must be evaluated. In theory, flocs adsorb particles in an acid medium, which increases their weight and facilitates the precipitation of the particles over time (Calgua et al., 2008). These results suggest that the efficacy of the flocs to aggregate the particles in a water suspension may show small changes depending on the type of microorganism. The results also suggest that a single faecal indicator is not feasible; however, the correlations

Table 5
Values used to compute a prediction interval for Y_u with a 95% confidence level (column 2.5% shows $(\frac{1}{r_{U,max}})$, 97.5% $(\frac{1}{r_{U,min}})$).

Virus	n	Bootstrap-t		Normal (unknowns μ and σ)	
		2.5%	97.5%	2.5%	97.5%
RoV	19	2.662	6.431	2.525	5.907
MS2	13	2.544	8.119	2.516	12.499
HAdV	10	1.027	5.200	0.932	4.059
H. pylori	9	2.023	5.734	2.054	8.467
E. coli	10	0.887	3.734	0.886	3.609
A. castellanii	9	2.711	10.990	2.627	34.465

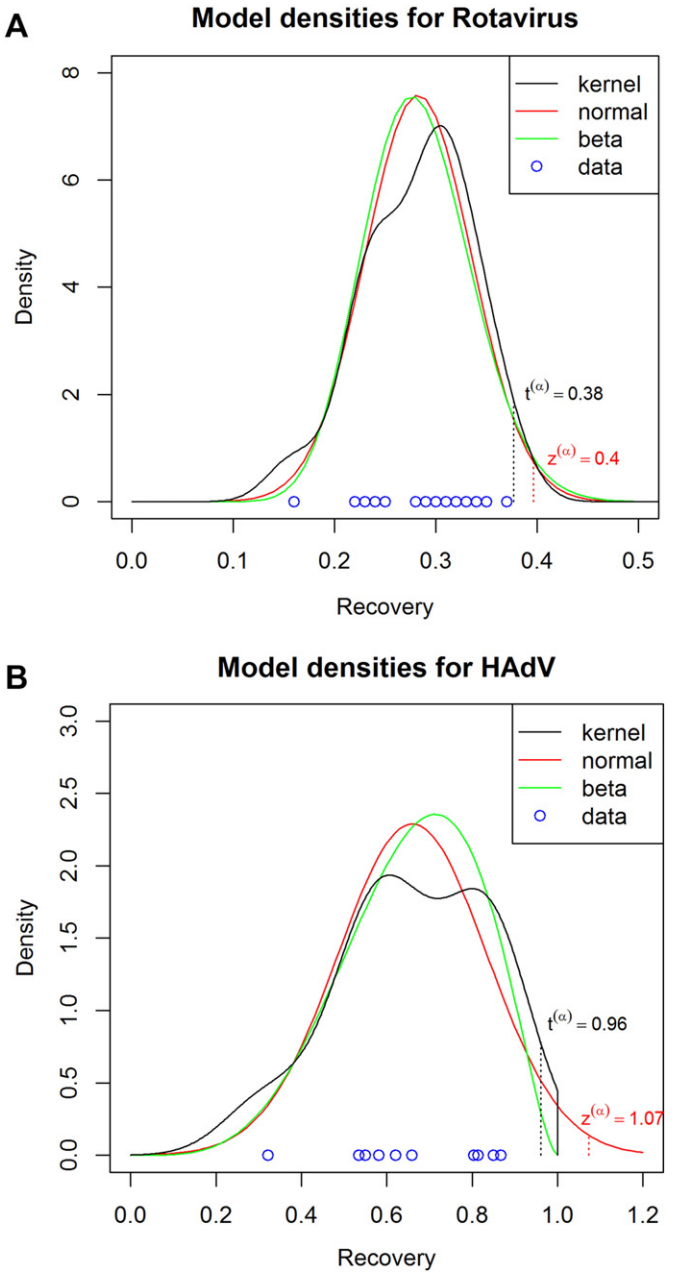


Fig. 1. A: Rotavirus recoveries (dots over the axis) are similarly fitted by the density estimations: normal, beta and kernel. The similar PIs in Table 5 can be explained by the symmetry of the distributions and the similar tails of the 3 models. Notice the value of the quantiles $t^{(\alpha)}$ and $z^{(\alpha)}$ associated to the upper bounds of the bootstrap-t and normal prediction intervals with $1 - \alpha = 95\%$ confidence. B: In contrast, in HAdV, the 3 models show a different fit on the upper right tail of the distribution. The discordance between the intervals for HAdV in Table 5 can be explained by taking into account that $t^{(\alpha)}$ and $z^{(\alpha)}$ are computed precisely on the tails of each distribution.

between bacteria and between viruses support the theory that *E. coli* and HAdV are suitable indicators for bacterial and viral contamination, respectively.

A ratio between the logarithm of the quantification between q(RT)PCR and infectivity suggests that the difference in quantification for these viruses was related to the proportion of non-infectious particles that may be produced in the cell lines where they have been cultured and the different sensitivities of the assays. The number of HAdVs detected in water using qPCR are typically 1 to 2 logs higher than estimates using culture-based methods (Rames et al., 2016). Moreover, HAdV and RoV are recognized to be resistant to pH, and their infectivity is not affected by the acidification of the sample in the SMF protocol (Attoui et al., 2012; Harrach et al., 2012). Although MS2 shows the smallest difference in quantification, the percentage of recoveries differ between q(RT)PCR and plaque-forming units. However, for an enveloped virus such as BVDV, either the recovery or the quantification was higher in q(RT)PCR (ratio of 2.03), which could be due to the acid pH (3.5) treatment (for approximately 16 h) that is used in the SMF protocol. In general, the sensitivity of infectivity assays has been traditionally considered to be lower in comparison with PCR techniques (Amini and Kraatz, 2014).

It is important to note that the model captures the random character of the unknown recoveries, but does not capture the random character of the concentrations in the sampled region. Therefore, the fitted error in the expression above refers to the technical error in the measurements but not the actual distribution of the organisms' concentrations in the water.

We strongly recommend using the extrapolation method with samples previously spiked with a surrogate virus or process control, such as the MS2 used in this study. We recommend verification of the recovery obtained with this surrogate, which might be between 10% and 38% (within 2 standard deviations of the mean of recovery) and is an interval that allows us to describe the variability and uncertainty of the SMF method in our laboratory. Another way to determine the recovery percentage in each laboratory is to estimate the mean and standard deviation of the surrogate under the particular laboratory conditions. Although variability is an intrinsic characteristic of each variable, the uncertainty introduces subjective "variability" into the variable (Vose, 2008). In addition, it may be increased or decreased by the expertise of the operator developing the SMF, the equipment, and the reagents. These may cause differences in the recoveries between operators or laboratories and should be taken into account.

The SMF recoveries are susceptible to improvement, and it is important to minimize the attachment of flocs to the lateral wall of the bucket after the sedimentation step and the loss of small pieces of flocs in the decanting process after the centrifugation step at 8000 rpm for 30 min. Although the use of non-adherent buckets may increase the recovery efficacy, this will make the SMF method more expensive. The fungible materials per sample were estimated to be low cost and can be disinfected or reused for other purposes or recycled.

The parameters that provide variability and uncertainty in HAdV and *E. coli* will be very useful in future studies. Under controlled conditions, the variability between the samples for the percentage of recovery in HAdV (CI 95%: 53.5–78.5%) includes the results of previous studies

with SMF (Calgua et al., 2008; Rusiñol et al., 2014, 2015). Therefore, we suggest modelling the recovery with the purpose of having a better approach for the real risk of the presence of the microorganism in water.

While the risk to a population is dictated by the frequency of contamination and the distribution of the dose, the probability of infection of an individual is ultimately based on the number of pathogens ingested (Ross, 2008). Errors in the precision of the quantification can underestimate the real concentration. Therefore, an extrapolation method that permits estimation of the real concentration of microorganisms in water samples is important in obtaining a better approach for future QMRA.

The distribution of the recovery under the controlled condition does not suggest that the distribution of the microorganisms in the environment occurs in the same way. It is important to consider that sampling methods of water in the environment have always been a limitation and require more work to determine the right way to describe the distribution of microorganisms in the environment (Petterson et al., 2015). Microbial water quality often varies rapidly and over a wide range. Short-term peaks in pathogen concentrations may increase disease risk considerably and may trigger outbreaks of water-borne disease, and furthermore, by the time the microbial contamination is detected, many people may have been exposed (Gorchev and Ozolins, 2011).

In summary, the low cost, repeatability, low variability, and applicability to the methods described for the simultaneous concentration of a diversity of microorganisms support SMF as a useful tool for the control of water safety. In addition, the possibility of obtaining intervals, which allows the prediction of the actual amount of microorganisms in the samples, including the uncertainty of the method, shows that SMF is an efficacious and efficient method for concentration and should be considered a robust procedure for evaluating the microbiological quality of water and the associated public health risk.

5. Conclusions

SMF can be used to efficiently and simultaneously concentrate viruses, bacteria and protozoa with repeatable results.

Statistically significant positive correlations were found between the recoveries of the bacteria evaluated, *E. coli* and *H. pylori*. Although the correlation between the recoveries of the viruses was not statistically significant, a positive correlation between them shows that HAdV is a suitable indicator for viral contamination.

Quantification by q(RT)PCR and infectivity methods shows ratios that suggest similar recoveries for HAdV-35 and RoV. They may be used indistinctly to evaluate these microorganisms with an SMF method of concentration.

The quantification of BVDB, which is sensitive to pH in the process of flocculation, is more efficiently conducted using q(RT)PCR than infectivity assays.

The estimation of the inoculums using q(RT)PCR quantification and the 95% bootstrap PI using the sample of the recovery estimates for each microorganism permits the acquisition of intervals that predict the real concentration of pathogens or indicators and may be used as a measure of uncertainty in QMRA studies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2017.01.006.

References

Amini, K., Kraatz, H.-B., 2014. Recent advances and developments in monitoring biological agents in water samples. Rev. Environ. Sci. Biotechnol. 14:23–48. http://dx.doi.org/10.1007/s11157-014-9351-5.

Anonymous, 1995. ISO 10705-1: Water Quality—Detection and Enumeration of Bacteriophages-Part 1: Enumeration of F-specific RNA Bacteriophages. International Organisation for Standardisation, Geneva, Switzerland.

Attoui, H., Mertens, P., Becnel, J., Belaganahali, S., Betgoin, M., Brussaard, C., Chappell, J., Ciarlet, M., de Vas, M., Dermody, T., Dormitzer, P., Duncan, R., Fang, Q., Graham, R., Guglielmi, K., Harding, R., Hilman, B., Makkay, S., Marzachì, C., Matthijnssens, J., Milne, R., Mohd Jaafar, F., Mori, H., Noordeloos, A., Omura, T., Patton, J., Rao, S., Mann, M., Stolts, D., Suzuki, N., Upadhyaya, N., Wei, C., Zhou, H., 2012. Family reoviridae. In: King, A., Adams, M., Carstens, E., Lefkowitz, E. (Eds.), Virus Taxonomy, Classification and Nomenclature of Viruses. Elsevier Academic Press, Davis, pp. 541–637.

Bai, C., Bickel, P., Olshen, R., Marcus, M.B., 1990. Hyperaccuracy of bootstrap based prediction. In: Eberlein, E., Kuelbs, J. (Eds.), Probability in Banach Spaces VII Proceedings of the Seventh International Conference. Birkhauser, Boston, Cambridge, Massachusetts, pp. 31–42.

Bofill-Mas, S., Hundesa, A., Calgua, B., Rusinol, M., Maluquer de Motes, C., Girones, R., 2011. Cost-effective method for microbial source tracking using specific human and animal viruses. J. Vis. Exp. http://dx.doi.org/10.3791/2820.

Bofill-Mas, S., Rusiñol, M., Fernandez-cassi, X., Carratalà, A., Hundesa, A., Girones, R., 2013. Quantification of human and animal viruses to environmental samples. Biomed. Res. Int.:2013 http://dx.doi.org/10.1155/2014/675857.

Calgua, B., Mengewein, A., Grunert, A., Bofill-Mas, S., Clemente-Casares, P., Hundesa, A., Wyn-Jones, A.P., Lopez-Pila, J.M., Girones, R., 2008. Development and application of a one-step low cost procedure to concentrate viruses from seawater samples. J. Virol. Methods 153:79–83. http://dx.doi.org/10.1016/j.jviromet.2008.08.003.

Calgua, B., Barardi, C.R.M., Bofill-Mas, S., Rodriguez-Manzano, J., Girones, R., 2011. Detection and quantitation of infectious human adenoviruses and JC polyomaviruses in water by immunofluorescence assay. J. Virol. Methods 171:1–7. http://dx.doi.org/10.1016/j.jviromet.2010.09.013.

Calgua, B., Fumian, T., Rusinol, M., Rodriguez-Manzano, J., Mbayed, V.A., Bofill-Mas, S., Miagostovich, M., Girones, R., 2013a. Detection and quantification of classic and emerging viruses by skimmed-milk flocculation and PCR in river water from two geographical areas. Water Res. 47:2797–2810. http://dx.doi.org/10.1016/j.watres.2013.02.043.

Calgua, B., Rodriguez-Manzano, J., Hundesa, A., Sunen, E., Calvo, M., Bofill-Mas, S., Girones, R., 2013b. New methods for the concentration of viruses from urban sewage using quantitative PCR. J. Virol. Methods 187:215–221. http://dx.doi.org/10.1016/j.jviromet.2012.10.012.

Calgua, B., Carratala, A., Guerrero-Latorre, L., de Abreu Correa, A., Kohn, T., Sommer, R., Girones, R., 2014. UVC inactivation of dsDNA and ssRNA viruses in water: UV fluences and a qPCR-based approach to evaluate decay on viral infectivity. Food Environ. Virol.:260–268 http://dx.doi.org/10.1007/s12560-014-9157-1.

Estes, M., Greenberg, H., 2013. Rotaviruses. In: Knipe, D., Howley, P. (Eds.), Fields Virology. Lippincott Williams & Wilkins, Philadelphia, pp. 1347–1401.

Eusebi, L.H., Zagari, R.M., Bazzoli, F., 2014. Epidemiology of *Helicobacter pylori* infection. Helicobacter 19:1–5. http://dx.doi.org/10.1111/hel.12165.

Gascón, J., 2006. Epidemiology, etiology and pathophysiology of traveler's diarrhea. Digestion 73:102–108. http://dx.doi.org/10.1159/000089785.

Gorchev, H.G., Ozolins, G., 2011. WHO guidelines for drinking-water quality. WHO Chron. 38:104–108. http://dx.doi.org/10.1016/S1462-0758(00)00006-6.

Harrach, B., Benkö, M., Both, G., Brown, M., Davidson, A., Echavarría, M., Hess, M., Jones, M., Kajon, A., Lehmkuhl, H., Mautner, V., Mittal, S., Wadell, G., 2012. Family Adenoviridae. In: King, A., Adams, M., Carstens, E., Lefkowitz, E. (Eds.), Virus Taxonomy, Clasification and Nomenclature of Viruses. Elsevier Academic Press, San Diego, pp. 125–141.

Hernroth, B.E., Conden-Hansson, A.-C., Rehnstam-Holm, A.-S., Girones, R., Allard, A.K., 2002. Environmental factors influencing human viral pathogens and their potential indicator organisms in the blue mussel, *Mytilus edulis*: the first Scandinavian report. Appl. Environ. Microbiol. 68:4523–4533. http://dx.doi.org/10.1128/AEM.68.9.4523.

Hill, V.R., Polaczyk, A.L., Hahn, D., Narayanan, J., Cromeans, T.L., Roberts, J.M., Amburgey, J.E., 2005. Development of a rapid method for simultaneous recovery of diverse microbes in drinking water by ultrafiltration with sodium polyphosphate and surfactants. Appl. Environ. Microbiol. 71:6878–6884. http://dx.doi.org/10.1128/AEM.71.11.6878-6884.2005.

Hill, V.R., Narayanan, J., Gallen, R.R., Ferdinand, K.L., Cromeans, T., Vinjé, J., 2015. Development of a nucleic acid extraction procedure for simultaneous recovery of DNA and

RNA from diverse microbes in water. Pathogens 4:335–354. http://dx.doi.org/10.3390/pathogens4020335.
Johnson, C.H., Rice, E.W., Reasoner, D.J., 1997. Inactivation of *Helicobacter pylori* by chlorination. Appl. Environ. Microbiol. 63, 4969–4970.
Khan, I.U.H., Gannon, V., Kent, R., Koning, W., Lapen, D.R., Miller, J., Neumann, N., Phillips, R., Robertson, W., Topp, E., van Bochove, E., Edge, T.A., 2007. Development of a rapid quantitative PCR assay for direct detection and quantification of culturable and non-culturable *Escherichia coli* from agriculture watersheds. J. Microbiol. Methods 69: 480–488. http://dx.doi.org/10.1016/j.mimet.2007.02.016.
Lambrecht, E., Baré, J., Chavatte, N., Bert, W., Sabbe, K., Houf, K., 2015. Protozoan cysts act as survival niche and protective shelter for foodborne pathogenic bacteria. Appl. Environ. Microbiol. 81. http://dx.doi.org/10.1128/AEM.01031-15 (AEM.01031-15).
Losurdo, M., Mari, V., Lucente, M.S., Colaianni, M.L., Padalino, I., Cavaliere, N., Buonavoglia, C., Decaro, N., 2015. Development of a TaqMan assay for sensitive detection of all pestiviruses infecting cattle, including the emerging HoBi-like strains. J. Virol. Methods 224:77–82. http://dx.doi.org/10.1016/j.jviromet.2015.08.013.
MacLachlan, J., Dubovi, E., 2011. Fenner's Veterinary Virology. fourth ed. Academic Press, San Diego http://dx.doi.org/10.1016/B978-0-12-375158-4.00030-4.
Madigan, M., Martinko, J., Bender, K., Buckley, D., Stahl, D., 2014. Brock Biology of Microorganisms. 14th ed. Pearson, Harlow.
Marciano-Cabral, F., Cabral, G., 2003. *Acanthamoeba* spp. as agents of disease in humans. Clin. Microbiol. Rev. 16:273–307. http://dx.doi.org/10.1128/CMR.16.2.273-307.2003.
Melgaço, F.G., Victoria, M., Correa, A.A., Ganime, A.C., Malta, F.C., Brandao, M.L.L., de Mello Medeiros, V., de Oliveira Rosas, C., Bricio, S.M.L., Miagostovich, M.P., 2016. Virus recovering from strawberries: evaluation of a skimmed milk organic flocculation method for assessment of microbiological contamination. Int. J. Food Microbiol. 217: 14–19. http://dx.doi.org/10.1016/j.ijfoodmicro.2015.10.005.
Mojirsheibani, M., 1998. Iterated bootstrap prediction intervals. Stat. Sin. 8, 489–504.
Moreno, Y., Ferrús, M.A., 2012. Specific detection of cultivable *Helicobacter pylori* cells from wastewater treatment plants. Helicobacter 17:327–332. http://dx.doi.org/10.1111/j.1523-5378.2012.00961.x.
OIE, 2015. Bovine viral diarrhea. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Office International des Epizooties, Paris, France, pp. 1–22.
Otto, P.H., Reetz, J., Eichhorn, W., Herbst, W., Elschner, M.C., 2015. Isolation and propagation of the animal rotaviruses in MA-104 cells—30 years of practical experience. J. Virol. Methods 223:88–95. http://dx.doi.org/10.1016/j.jviromet.2015.07.016.
Petterson, S., Grøndahl-Rosado, R., Nilsen, V., Myrmel, M., Robertson, L.J., 2015. Variability in the recovery of a virus concentration procedure in water: implications for QMRA. Water Res. 87:79–86. http://dx.doi.org/10.1016/j.watres.2015.09.006.
Pina, S., Puig, M., Lucena, F., Jofre, J., Girones, R., 1998. Viral pollution in the environment and in shellfish: human adenovirus detection by PCR as an index of human viruses. Appl. Environ. Microbiol. 64, 3376–3382.
Qvarnstrom, Y., Visvesvara, G.S., Sriram, R., Da Silva, A.J., 2006. Multiplex real-time PCR assay for simultaneous detection of *Acanthamoeba* spp., *Balamuthia mandrillaris*, and *Naegleria fowleri*. J. Clin. Microbiol. 44:3589–3595. http://dx.doi.org/10.1128/JCM.00875-06.
R Core Team, 2016. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria (URL https//www.R-project.org/).
Rames, E., Roiko, A., Stratton, H., Macdonald, J., 2016. Technical aspects of using human adenovirus as a viral water quality indicator. Water Res. 96:308–326. http://dx.doi.org/10.1016/j.watres.2016.03.042.
Ross, T., 2008. Microbial ecology in food safety risk assessment. In: Schaffner, D. (Ed.), Microbiological Risk Assessment of Foods. ASM Press, Washington DC, pp. 51–98.
Rusiñol, M., Fernandez-Cassi, X., Hundesa, A., Vieira, C., Kern, A., Eriksson, I., Ziros, P., Kay, D., Miagostovich, M., Vargha, M., Allard, A., Vantarakis, A., Wyn-Jones, P., Bofill-Mas, S., Girones, R., 2014. Application of human and animal viral microbial source tracking tools in fresh and marine waters from five different geographical areas. Water Res. 59:119–129. http://dx.doi.org/10.1016/j.watres.2014.04.013.
Rusiñol, M., Fernandez-Cassi, X., Timoneda, N., Carratala, A., Abril, J.F., Silvera, C., Figueras, M.J., Gelati, E., Rodó, X., Kay, D., Wyn-Jones, P., Bofill-Mas, S., Girones, R., 2015. Evidence of viral dissemination and seasonality in a Mediterranean river catchment: implications for water pollution management. J. Environ. Manag. 159:58–67. http://dx.doi.org/10.1016/j.jenvman.2015.05.019.
Santiago, P., Moreno, Y., Ferrús, M.A., 2015. Identification of viable *Helicobacter pylori* in drinking water supplies by cultural and molecular techniques. Helicobacter 20: 252–259. http://dx.doi.org/10.1111/hel.12205.
USEPA, 2005. Method 1623: Cryptosporidium and Giardia in Water by Filtration/IMS/FA. EPA 815-R-05-002. Off. Water, Washington, DC.
van Duin, J., Olsthoorn, R.C.L., 2012. Family Leviviridae. In: King, A., Adams, M., Carstens, E., Lefkowitz, E. (Eds.), Virus Taxonomy, Classification and Nomenclature of Viruses. Elsevier Academic Press, Davis, pp. 1035–1043.
Victoria, M., Fumian, T.M., Rocha, M.S., Dalmao, F., Leite, J.P.G., Girones, R., Miagostovich, M.P., 2014. Gastroenteric virus dissemination and influence of rainfall events in urban beaches in Brazil. J. Appl. Microbiol. 117:1210–1218. http://dx.doi.org/10.1111/jam.12592.
Vose, D., 2008. Risk Analysis a Quantitative Guide. third ed. John Wiley and Sons, Wiltshire, England.
Wold, W.S., Horwitz, M., 2013. Adenoviruses. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology. Lippincott Williams & Wilkins, Philadelphia, pp. 1732–1767.
World Health Organization, 2014. Preventing Diarrhoea Through Better Water, Sanitation and Hygiene. pp. 1–32 (Online).
Zeng, S.Q., Halkosalo, A., Salminen, M., Szakal, E.D., Puustinen, L., Vesikari, T., 2008. One-step quantitative RT-PCR for the detection of rotavirus in acute gastroenteritis. J. Virol. Methods 153:238–240. http://dx.doi.org/10.1016/j.jviromet.2008.08.004.