Reduction of bacteriophage MS2 by filtration and irradiation determined by culture and quantitative real-time RT–PCR

W. J. Lodder, H. H. J. L. van den Berg, S. A. Rutjes, M. Bouwknegt, J. F. Schijven and A. M. de Roda Husman

ABSTRACT

Molecular methods are increasingly applied for virus detection in environmental samples without rendering data on viral infectivity. Infectivity data are important for assessing public health risks from exposure to human pathogenic viruses in the environment. Here, treatment efficiencies of three (drinking) water treatment processes were estimated by quantification of the indicator virus bacteriophage MS2 with culture and real-time reverse transcription polymerase chain reaction (qRT–PCR). We studied the virus reduction by slow sand filtration at a pilot plant. No decay of MS2 RNA was observed, whereas infectious MS2 particles were inactivated at a rate of 0.1 day^{-1}. Removal of MS2 RNA and infectious MS2 particles was 1.2 and 1.6 log_{10}-units, respectively. Virus reduction by UV and gamma irradiation was determined in laboratory-scale experiments. The reduction of MS2 RNA based on qRT–PCR data was negligible. Reduction of infectious MS2 particles was estimated at 3.0–3.6 log_{10}-units (UV dose up to 400 or 800 J/m^2) and 4.7–7 log_{10}-units (gamma dose up to 200 Gray). As shown in this study, estimations of viral reduction, both inactivation and removal, obtained by molecular methods should be interpreted carefully when considering treatment options to provide virus-safe drinking water. Combining culture-based methods with molecular methods may provide supplementary information on mechanisms of virus reduction.

Key words | bacteriophage, culture, filtration, irradiation, qPCR, quantification

W. J. Lodder (corresponding author)
H. H. J. L. van den Berg
S. A. Rutjes
M. Bouwknegt
A. M. de Roda Husman
Laboratory for Zoonoses and Environmental Microbiology at the Centre for Infectious Disease Control Netherlands,
National Institute for Public Health and the Environment,
P.O. Box 1,
3720 BA Bilthoven,
The Netherlands
E-mail: *willemijn.lodder@rivm.nl*

J. F. Schijven
Expertise Centre for Methodology and Information Services,
National Institute for Public Health and the Environment,
P.O. Box 1,
3720 BA Bilthoven,
The Netherlands

INTRODUCTION

Contamination of water resources by fecal waste poses a serious risk to human health upon exposure. Especially in situations where fecally contaminated source water for drinking-water production is insufficiently treated, consumption may cause (severe) illness and, in certain situations, even death (Swerdlow *et al.* 1992; Hoxie *et al.* 1997). Therefore, protecting source water quality for drinking-water production, maintaining the efficiency of the applied treatment processes and ensuring safe distribution as prescribed in the WHO Water Safety Plans are important tools for the production of safe drinking water (WHO 2009). Several treatment processes can be applied for drinking-water production, such as coagulation, sedimentation, slow sand filtration, membrane filtration and ozone and UV disinfection, through which human pathogens are retained and/or inactivated. Because of the differences in their characteristics, pathogens, including viruses, behave differently in the various water-treatment processes (Allwood *et al.* 2005; Bauer *et al.* 2011; Schijven *et al.* 2013). To date, virus removal by several treatment processes for drinking-water production has mainly been assessed using bacteriophages as indicator viruses for human pathogenic viruses because of their ease of use and their harmlessness to humans. The F-specific RNA phage MS2 is commonly used as a surrogate for human pathogenic RNA viruses, such as entero- and noroviruses due to similarities in

doi: 10.2166/wh.2013.204

characteristics such as size and charge (Bae & Schwab 2008; Mayer *et al.* 2008; Boudaud *et al.* 2012). Waterborne, human pathogenic viruses, such as rotavirus, norovirus and enterovirus, can cause a wide range of symptoms, varying from common cold and gastroenteritis to more severe diseases like meningitis and paralysis (de Roda Husman & Bartram 2007).

Culture-based detection methods are sensitive methods and can be used to quantify infectious viruses in the aquatic environment (Lodder *et al.* 2005, 2010). However, it is still difficult or impossible to reliably culture several common pathogenic viruses, such as norovirus (Duizer *et al.* 2004; Straub *et al.* 2007, 2011, 2013). Furthermore, differentiation of aggregates from single virus cells is not possible with these kinds of infectivity assays, and may therefore lead to underestimation of the virus concentration in the original sample (Teunis *et al.* 2005). Alternatively, molecular detection methods can be used for the detection of noncultivable and cultivable viruses. These methods are sensitive, rapid and are able to quantify aggregated viruses (de Roda Husman *et al.* 2009). One substantial limitation of molecular techniques is that genomes or fragments of genomes are detected and no information is obtained on the infectivity of the virus. Because molecular methods cannot differentiate between infective and defective virus particles, results cannot be directly translated to public health risks.

To obtain more information on the infectivity status of viruses detected by molecular methods, additional procedures, such as treatment of the sample with enzymes such as proteinase K or RNase, prior to molecular detection have been described (Nuanualsuwan & Cliver 2002; Baert *et al.* 2008). Although a decrease in detection of viral genomes could be obtained, these treatments did not lead to the sole detection of infective virus particles (Pecson *et al.* 2009). Other researchers used propidium monoazide (PMA) in a pre-treatment step to detect only infectious RNA viruses (Fittipaldi 2010; Parshionikar *et al.* 2010; Kim & Ko 2012). Although these studies concluded that using PMA treatment prior to the reverse transcription polymerase chain reaction (RT–PCR) succeeded for several of the tested viruses under laboratory conditions, this method was not applicable for all RNA viruses in environmental samples. Also, fragment length has been evaluated as a molecular tool to assess viral infectivity (Wolf *et al.* 2009). In addition, culture and molecular methods have been combined with so-called integrated cell culture–PCR methods to gain information on infectivity of viruses that are difficult to culture (Reynolds *et al.* 2001; Rutjes *et al.* 2009).

Replication of viruses is restricted to susceptible living host cells and therefore infective viruses will only reduce in numbers once released into the environment. The inactivation rate of viruses is dependent on variables such as the pH and temperature of the water, the amount of sunlight (UV) and the virus type. Due to the loss of infectivity in the environment, viruses may be undetectable by culture-based methods, whereas the genomic material still can be detected over a prolonged period of time (de Roda Husman *et al.* 2009).

In this study, the efficiencies of virus reduction of three (drinking-) water treatment processes, slow sand filtration, UV and gamma irradiation, were assessed by quantification of the indicator virus bacteriophage MS2 before and after treatment. Quantification was performed by culture and the molecular method quantitative real-time RT–PCR. Furthermore, the feasibility of PCR for the estimation of virus removal and inactivation by such water treatment processes was evaluated.

METHODS

MS2 culture

F-specific bacteriophage MS2 (ATCC 15597-B1) was cultured according to the standard procedure, ISO 10705-1 (1995). The initial concentrations of the viral stocks used in the different experiments were between 2.8×10^6 and 5.8×10^6 plaque forming units (PFU)/mL and these were stored at 4 °C until use.

The detection of F-specific bacteriophages was done using the host strain *Salmonella typhimurium* WG49. The bacteriophage concentration in the original water sample was calculated from the tested volume and the phage counts.

RNA standard

To be able to quantify the PCR-products obtained with a one-step real-time RT–PCR (qRT–PCR) a RNA standard

was developed. For this purpose, a 662 bp PCR product, located at the 5′ end of the MS2 genome, was cloned into a pCRII-Topo vector (Invitrogen, Leek, the Netherlands) according to the manufacturer's instructions. The orientation of the insert was checked with conventional PCR and sequencing using M13 primers supplied by the manufacturer. After several purification steps and restriction enzyme treatments, the RNA was transcribed using T7-polymerase. The resulting RNA (800 nt) was treated with DNase to remove the plasmid DNA and subsequently a final purification step was done. To determine the concentration of the synthesized RNA the optical density (OD) was measured to calculate the RNA copies present in the standard. Serial 10-fold dilutions of the standard RNA, at concentrations ranging from 1.6×10^1 to 1.6×10^6 RNA copies per 5 μL, were aliquoted and stored at −80 °C until use. In each qRT–PCR assay, the serial dilutions of the RNA standard were tested in duplicate to create a standard curve, which was subsequently used to quantify the RNA in the original samples.

MS2 qRT–PCR

An EXPRESS One-Step SuperScript qRT-PCR Kit (Invitrogen) was used according to the manufacturer's instructions. Primers, forward (TGCCATTTTTAATGTCTTTAG) and reverse (TGGAATTCCGGCTACCTAC), and a probe (AGACGCTACCATGGCTATCGC) targeting the 5′ end of the MS2 genome, were used as described earlier to detect the viral RNA (Hill *et al.* 2007). In brief, 50 μL of the original samples were heated to 95 °C for 5 min, to denature the virus capsid and release the viral RNA. Samples were subsequently cooled on ice for 5 min. An aliquot of 5 μL of the heat-released RNA (and 10-fold and 100-fold dilutions), or 5 μL of the aliquoted RNA standard, was added to 15 μL of the qRT–PCR reaction mixture containing EXPRESS Superscript® qPCR SuperMix, 15 μM of each primer, 4 μM of the probe and EXPRESS Superscript® Mix for One-step qPCR. The qRT–PCR protocol was as follows: a reverse transcription at 50 °C for 15 min, an activation step for the Platinum® *Taq* DNA polymerase at 95 °C for 5 min, and 45 cycles of 95 °C for 15 s, and 60 °C for 60 s. The qRT–PCR assays were performed in a LightCycler 480 System (Roche) and the LightCycler software automatically determined the cycle threshold (Ct) point of each qRT–PCR reaction. Both the Ct-data corresponding to the standard RNA and to the viral RNA concentrations were obtained and were subsequently used to calculate the genome concentrations in the samples. The number of phage particles in the samples, either subjected to the treatment processes or not, was determined by the use of a standard curve, assuming that the detection of one copy of the viral genome corresponded with one phage particle. All samples were tested in duplicate as well as the RNA standard and the negative controls. In this study, we interpreted a relative difference of 3 Ct values or less in 10-fold serial dilutions as qRT–PCR inhibition. Theoretically, in a completely efficient qRT–PCR reaction, a difference of 3.3 Ct values relates to every 10-fold dilution of the target nucleic acid in a given sample. The Ct point of each sample was defined as the average of the Ct data of the duplicates.

Treatment processes

Virus reduction by slow sand filtration was studied at pilot-plant scale. A suspension of MS2 was seeded onto a sand filter for a period of 24 hours (temperature 20 °C), as described more extensively elsewhere (Schijven *et al.* 2013). Briefly, $3\,l \times 10^8$ PFU MS2 per liter were added directly to the layer of water on top of the sand filters and were gently mixed with a rotor fixed onto an electric drill in order to immediately achieve the desired C_0 level of approximately 10^6 PFU MS2 per liter. This level was kept constant for 24 hours by proportionally adding seeding suspension. Samples of 100 mL were taken from the influent on top of the filter bed as well as from the effluent every hour during working hours. About 30 effluent samples were collected for 5–7 days. For the inactivation of MS2 during the slow sand filtration, parallel with the seeding experiment, a sample of the seeding suspension was stored at the same temperature as the slow sand filtration and samples were taken on different days. The detection of MS2 by plaque counting was done within 48 hours and by qRT–PCR after subsequent storage at −80 °C (qRT–PCR either with or without the addition of 4.5% Triton X-100 (Merck, Amsterdam, The Netherlands) to the sample).

UV experiments (laboratory scale) were performed using MS2 suspensions in tap water that were exposed to

UV light (wavelength = 253.7 nm) at different fluences. In the first experiment, fluences of 0, 50, 100, 200, 300, 400 and 800 J/m^2 were used and 0, 50, 100, 150, 200, 300 and 400 J/m^2 in the second experiment. Gamma irradiation experiments were conducted with different gamma irradiation doses: 0, 50, 75, 100, 150 and 200 Gray (Gy). The MS2 suspension used in the first experiment had a higher protein content than the MS2 suspension used in the second experiment. Details of the experimental set up are described elsewhere (de Roda Husman *et al.* 2004). Concentrations of MS2 determined by plaque counting were performed directly, detection of MS2 by qRT–PCR was performed after storage at −80 °C.

Statistical methods

During slow sand filtration viruses are removed from the water by inactivation and by attachment to the sand grains. Breakthrough curves were constructed and fitted for the concentration data of the effluent samples using the two-site kinetic model in Hydrus-1D (version 4.14; www.pc-progress.com) in order to obtain values for attachment, detachment and inactivation rate coefficients as described previously by Sadeghi *et al.* (2011). Inactivation rates in the water were determined by fitting the data to the monophasic and biphasic exponential model analysis of the concentration data obtained for samples taken from the water on top of the sand filter at different times at the same temperature as the slow sand filtration experiment (see below).

All decay rates, in the UV and gamma irradiation experiments, were estimated by maximum likelihood, aiming for the most parsimonious of the monophasic and biphasic exponential models (de Roda Husman *et al.* 2009) and the Weibull model (van Boekel 2002). The likelihood ratio test was used to assess improved fits for the biphasic and Weibull models compared to the monophasic model. The corrected Aikaike Information Criterion (Hurvich & Tsai 1989) was used to select between the biphasic and Weibull models. Decay rate parameters for the infectivity assays were based on virus counts. Decay rate parameters for qRT–PCR were based on genome counts, which were estimated using a likelihood function that estimated jointly the decay rate parameters and the intercept and slope of the RNA standard curve. The 95% intervals for all parameters were obtained by Markov Chain Monte Carlo adaptive rejection sampling using the likelihood functions (Gilks *et al.* 1996). The burn-in period was set to 1,000 iterations and the chain was stopped when 10,000 samples were accepted. The stability of the Markov chains was explored graphically. Virus counts were assumed to be Poisson distributed, genome counts were assumed to be log-normally distributed.

RESULTS

Slow sand filtration

Removal by slow sand filtration is determined by virus inactivation and virus removal by attachment of virus to the sand grains (Schijven & Hassanizadeh 2000). To measure the inactivation rate of MS2 over time, an aliquot of the MS2 spike suspension used in the slow sand filtration experiment was stored under the same experimental conditions as the sand filter for 11 days. Regression analysis revealed that no significant change in MS2 genome concentration was observed over time (Figure 1). After 11 days, a reduction of approximately 5 $\log_{10}$ units of infectious MS2 was observed.

Figure 2 shows the breakthrough curves with normalized concentrations of MS2 as determined by molecular and culture methods. The MS2 RNA standard curve gave reproducible results in different experiments (data not shown). Although PCR inhibition was apparent for undiluted samples of the slow sand filtration experiment, the 10-fold and 100-fold dilutions showed no inhibition. A constant maximum breakthrough concentration was reached; therefore, it may be assumed that a steady state condition applied. This implied that log removal could simply be determined by the differences in the logarithm of the maximum breakthrough concentrations. The difference in seeding concentration amounted to 0.5 $\log_{10}$ units, i.e. the seeding suspension comprised one-third of infectious virus particles and for two-thirds of defective virus particles. Infective MS2 particles were removed at 1.6 $\log_{10}$ units, whereas 1.2 $\log_{10}$ units of defective MS2 particles were removed. Hence, during maximum breakthrough, the

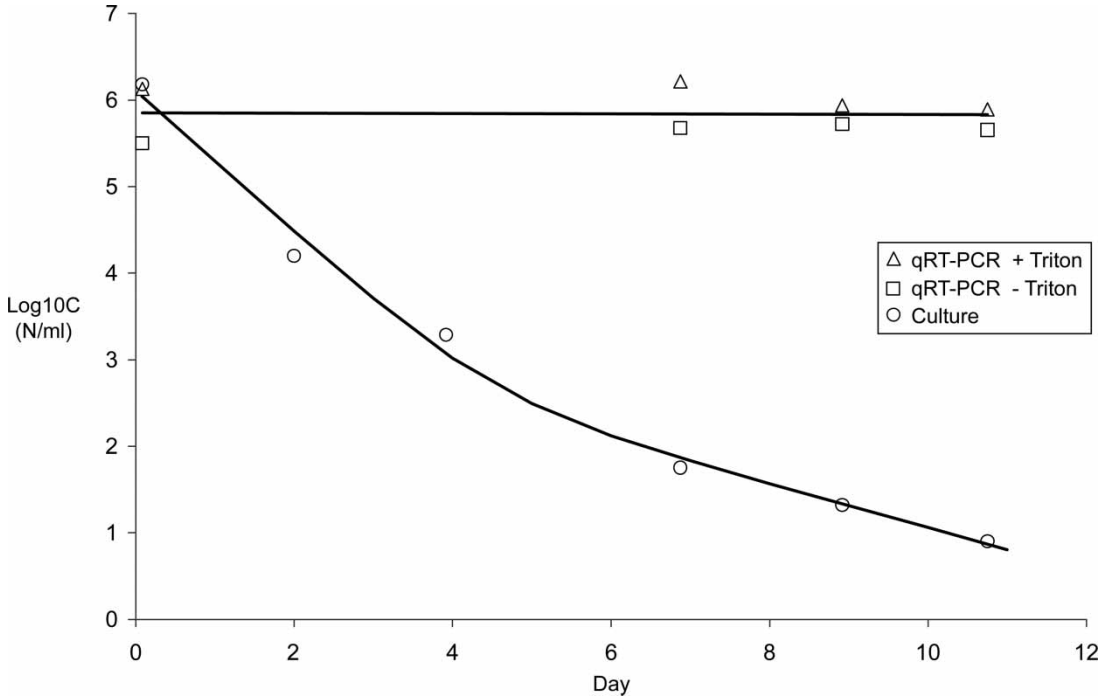

Figure 1 | Inactivation of the MS2 phages in the spike suspension used in the slow sand filtration experiments under experimental conditions (20 °C). Circles represent the MS2 concentration in PFU per mL obtained with the culture method (N/mL) and squares (with Triton) and triangles (without Triton) represent the MS2 concentration in genomes per mL obtained with the qRT–PCR (N/mL). Lines represent the fitted model (parameter values can be obtained on request).

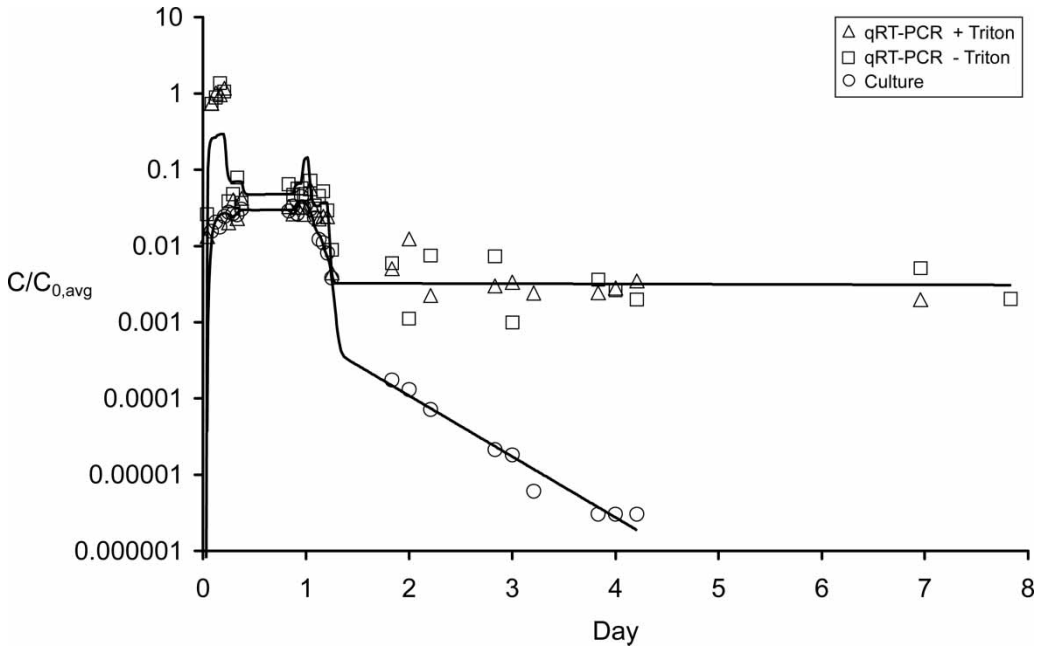

Figure 2 | MS2 breakthrough curves (normalized concentrations $C/C_{0,avg}$) of the slow sand filtration experiment. Squares (without Triton) and triangles (with Triton) are observations obtained with qRT–PCR. Circles are observations obtained with the culture method. The lines represent the fitted two site-kinetic virus transport model using Hydrus-1D.

samples contained about 2.5% infectious and 97.5% defective particles.

The tails of the breakthrough curves represent slow detachment of bacteriophages from the sand after seeding had stopped (Figure 2). The tail of the breakthrough curve for the infectious MS2 particles decreased linearly with time at a rate of about 0.7 $\log_{10}$ units/day, which represents the value of the inactivation rate coefficient of attached infectious MS2 particles.

Inactivation by UV and gamma irradiation

In addition to virus removal by slow sand filtration, disinfection by the mechanistically different treatment processes, irradiation by UV and gamma, was studied. Therefore, reduction of MS2 bacteriophages measured by qPCR was compared with results obtained by culture. The MS2 RNA standard curve gave reproducible results in different experiments (data not shown). No PCR inhibition was observed for the UV and gamma (ir)radiated samples when 5 μL of the original sample was analyzed directly or after the addition of 4.5% Triton X-100 (data not shown). MS2 concentrations of the stocks used in the irradiation experiments determined by plaque assay using the bacteriophage suspensions that were not radiated varied between 3×10^6 and 6×10^6 PFU/mL. MS2 concentrations determined by qRT–PCR were approximately 2 $\log_{10}$ units higher (Figures 3 and 4).

Detection of MS2 genomes by qRT–PCR showed a negligible reduction in MS2 RNA load after UV treatment (Figure 3). UV-irradiation doses up to 800 and 400 J/m^2 showed a 3.6 $\log_{10}$ and 3.0 $\log_{10}$ unit reduction of the infectious MS2 particles, respectively.

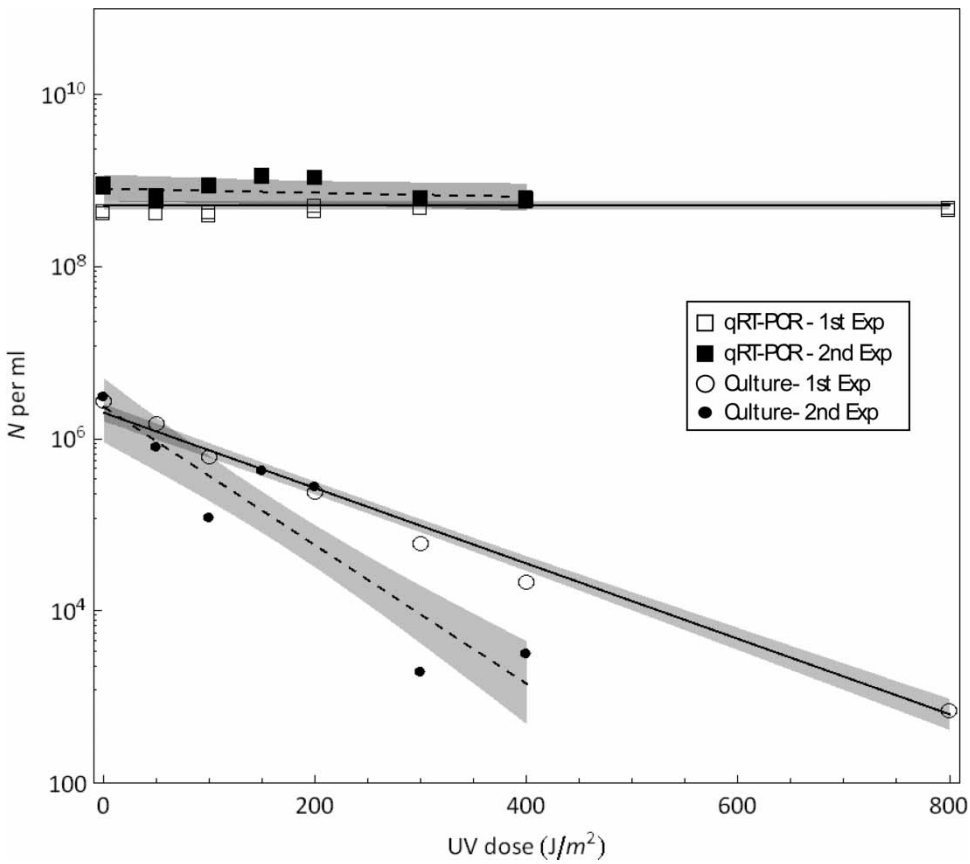

Figure 3 | Concentrations of MS2 bacteriophages (N/mL) after UV radiation with different fluences in the first experiment (open symbols) and the second experiment (closed symbols). Squares represent the MS2 concentration in genomes per mL obtained with the qRT–PCR. Circles represent the MS2 concentration in PFU per mL obtained with the culture method. The shades represent the 95% confidence interval.

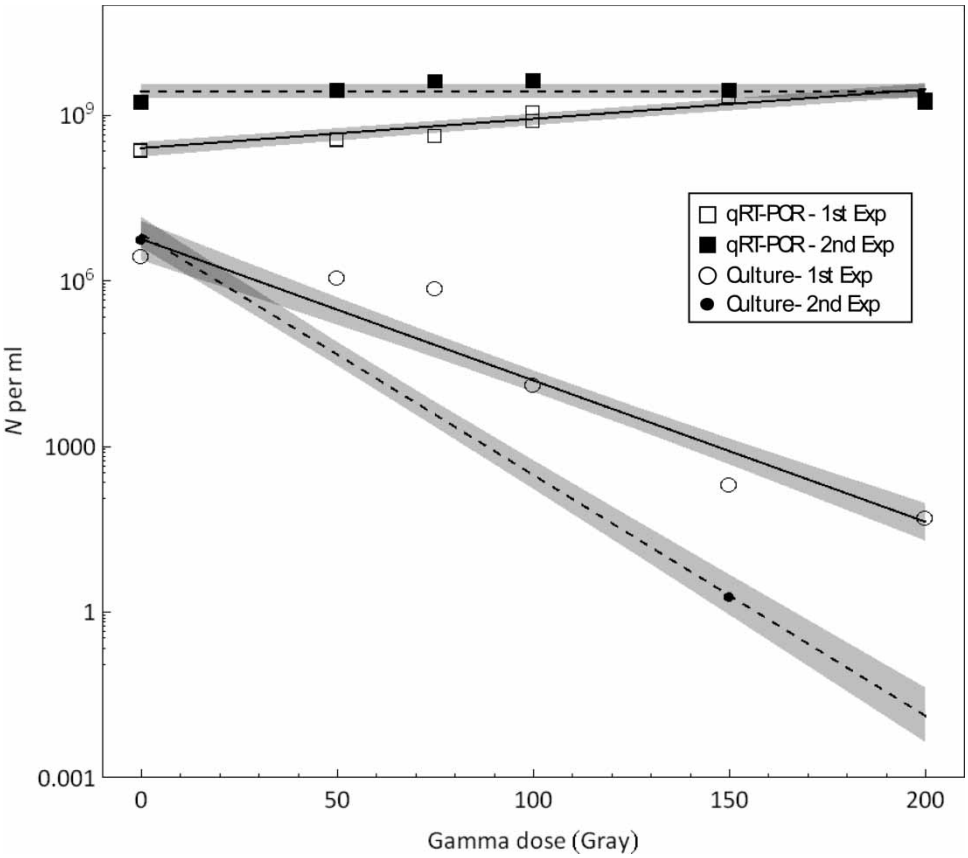

Figure 4 | Concentrations of MS2 bacteriophages (N/mL) after gamma irradiation with different fluences (gamma dose (Gy)) in the first experiment (open symbols) and the second experiment (closed symbols). Squares represent the MS2 concentration in genomes per mL obtained with the qRT–PCR. Circles represent the MS2 concentration in PFU per mL obtained with the culture method. The shades represent the 95% confidence interval.

The qRT–PCR data showed no reduction in the detection of MS2 RNA after irradiation with gamma beams (Figure 4), a 4.7 $\log_{10}$ unit reduction in the first experiment was seen and, because no MS2 could be cultured at the highest irradiation dose of 200 Gy in the second experiment, at least a 7-$\log_{10}$ unit reduction was observed there.

DISCUSSION

Quantitative PCR detection methods are used increasingly for the detection and quantification of (pathogenic) viruses in the environment. Nevertheless, the interpretation of the results on the numbers of viral genomes in the environment for the assessment of potential public health risks upon exposure is challenging. PCR detects both infectious and defective particles with fragment lengths of approximately 300 bp (Gassilloud *et al.* 2003; Al-Hello *et al.* 2008; de Roda Husman *et al.* 2009). Defective particles are unlikely to yield any adverse health effects, although 'defective' has not yet been exactly defined. The defect may interrupt virus binding to the host cell, replication in the host cell or release from the host cell. The application of specific virus detection methods that selectively detect, for example infectious viruses or viral genomes, will indicate whether damage is at the protein or nucleic acid level, or a combination of both (Wigginton & Kohn 2012). In the current study, infectious bacteriophages as well as total bacteriophages (both infectious and defective particles) were detected, and information on at least nucleic acid damage may be obtained.

Slow sand filtration is often used as the last step in the drinking-water treatment process in the Netherlands (Schijven *et al.* 2013). Virus reduction by this process is based on both removal by attachment of virus particles to

the sand grains and inactivation of virus particles. In our study, we compared the breakthrough curves based on either culture data or qRT–PCR data and it was demonstrated that removal occurred by attachment of MS2 RNA-containing particles and by attachment and inactivation of infectious MS2 particles. The tail of the breakthrough curve represents slow detachment of attached infectious MS2 bacteriophages. Apparently, there was 0.4 $\log_{10}$ units (equal to 2.5 times) more reduction by attachment of infectious MS2 particles than of those detected by qRT–PCR. Based on such molecular data, the removal efficiency of slow sand filtration would be underestimated. Our experiment showed that infectious MS2 particles attached more to sand particles than defective particles, or attachment is similar but virus inactivation may occur without detectable genome damage. It would be interesting to clarify the mechanism by carrying out column experiments with, for instance, mutant variants of MS2, or slightly different bacteriophages such as GA. In addition, pathogenic viruses with similar characteristics could be studied to assess their reduction ensuring drinking-water safety.

Our data showed that qRT–PCR was not suitable for studying inactivation by UV and gamma irradiation, because no reduction in MS2 RNA concentrations was seen, not even at the highest fluences, whereas a large reduction of infectious MS2 bacteriophages was observed. The differences in protein content of the MS2 suspensions in the two experiments did not seem to influence results from PCR. Although RNA could be detected and quantified by qRT–PCR after UV or gamma irradiation, no information could be obtained on the possible fragmentation of the MS2 genomes in these samples due to the short target length of 64 nt. Recent research has shown that detection of genome damage in correlation to infectivity is dependent on the length of the amplified nucleic acid (Simonet & Gantzer 2006; Wolf *et al.* 2009). Amplification of long fragments would correlate better with viral infectivity than the amplification of shorter fragments (Pecson *et al.* 2009), but nevertheless it will underestimate virus inactivation. Moreover, it has been shown that also the damage to viral proteins contributes to virus inactivation (Pecson *et al.* 2009; Wigginton *et al.* 2010), which cannot be detected by molecular methods.

Molecular techniques are particularly useful for microbial source tracking (MST) (Fong *et al.* 2005a) to establish a correlation between viruses isolated from clinical cases and viruses in the possible source responsible for the infection. Bacteriophages have been shown to be useful for MST (Kirs & Smith 2007; Wolf *et al.* 2010), but also specific human pathogenic viruses (Fong & Lipp 2005b). Another useful application of molecular techniques is to monitor the circulation of specific virus types in the human population in a specific area in time (Lodder *et al.* 2010, 2012).

Detection of both infectious and defective virus particles by (RT–)PCR is a well-recognized phenomenon. Multiple PCR-based methods to detect infectious viruses in environmental samples have been suggested (Rodriguez *et al.* 2009; Yeh *et al.* 2009), such as enzymatic pretreatment. Sample treatments prior to the PCR detection, such as PMA treatment, have been studied for the selective detection of genomes derived from infectious particles (Nuanualsuwan & Cliver 2002; Baert *et al.* 2008; Pecson *et al.* 2009). However, these assays also detected a proportion of genomes of inactivated viruses, and the authors concluded that the combination of cell culture and PCR is still the best approach to assess viral infectivity.

There seemed to be more inactivation of the MS2 bacteriophages in the second series of irradiation experiments (both UV and gamma) compared to the first. The spike suspensions used in the irradiation experiments were different in composition with respect to protein content (de Roda Husman *et al.* 2004). High protein content of bacteriophage suspensions did not negatively affect virus reduction by UV irradiation as compared to low protein content. However, virus inactivation by ionizing irradiation was impaired by high protein levels.

Virucidal modes of action have been described for different water-treatment processes including UV disinfection (Sommer *et al.* 2001; Hijnen *et al.* 2006), ozone treatment (Shin & Sobsey 2003) and slow sand filtration (Meschke & Sobsey 2003; Schijven *et al.* 2013). UV irradiation damages the nucleic acids of micro-organisms including viruses (Hijnen *et al.* 2006; Simonet & Gantzer 2006) and the viral capsid (Nuanualsuwan & Cliver 2003). If no repair mechanism is present, as is the case for RNA viruses such as MS2, irradiation will lead to irreversible inactivation of the virus. The degradation rate of the viral genome varies between different RNA viruses, but there does not seem to be a general rule for the UV susceptibility

of viruses with different virus characteristics, i.e. size, genome and weight (de Roda Husman *et al.* 2004). It has been shown in previous studies that viruses with double-stranded genomes, such as adenoviruses, are less prone to damage by UV irradiation as compared with single-stranded RNA viruses, i.e. poliovirus and coxsackievirus (Gerba *et al.* 2002; Duizer *et al.* 2004).

To compare virus reduction as detected by culture and molecular methods, a qRT–PCR was used that detects a fragment of 64 nt of the MS2 phage genome. In this study, the qRT–PCR experiments were performed with heat-released RNA and therefore the hands-on preparation time was low. This rapid approach is applicable when little or no inhibition of the matrix on the detection assay is expected and when test sensitivity is of minor importance as in the case of low protein content spike suspensions with relatively high virus concentrations. Extraction of nucleic acids prior to PCR amplification may increase the sensitivity by lowering the detection limit and removing PCR inhibitors, but also may give rise to loss of virus target, and was not necessary in our application. A one-step qRT–PCR protocol was applied which enabled us to rapidly analyze the presence of MS2 RNA in the different samples taken before and after water-treatment processes.

Detected RNA was quantified by the use of an external single-stranded RNA standard, which was synthesized using *in-vitro* transcription. RNA standards reflect the efficiency of the detection during the entire qRT–PCR assay, including the RNA transcription. RNA standards are susceptible to degradation and should therefore be handled with care to avoid overestimation of the target nucleic acid concentrations. Although DNA standards are less prone to degradation, and therefore more convenient to use, they do not need reverse transcription prior to PCR, possibly leading to mistakes in the estimation of virus concentrations.

The efficiencies of virus reduction by drinking-water treatment processes that cause little to no damage to the viral genome are underestimated when viruses are detected by molecular methods, indicating that loss of infectivity and genome degradation do not coincide. Applying molecular methods to assess virus reduction by water treatment processes would lead to overestimation of possible human health risks from consumption of the treated drinking water. On the other hand, use of molecular data in quantitative microbial risk assessment may aid trend analyses and, if necessary, guide subsequent intervention measures when surges in viral RNA load would be observed. Molecular assays may be utilized in online monitoring systems yielding rapid test results on either indicators or pathogens.

CONCLUSION

As shown in this study, estimations of viral reduction obtained by molecular methods should be carefully interpreted for public health risks. Following disinfection, inactivated and therefore non-infectious viruses may remain present in the sample, and their genomic material will be detected. Combining culture-based methods with molecular methods may provide valuable information on the mechanisms of virus reduction, such as attachment and inactivation of viruses.

ACKNOWLEDGEMENTS

The project was funded by the Strategic Research RIVM (SOR), project number S/330126/01/EP. We gratefully acknowledge the kind cooperation of Regina Sommer (Medical University Vienna, Austria) and her team, who facilitated the irradiation experiments, and Yolanda Dullemont and Michel Colin (Waternet, The Netherlands) with respect to the slow sand filtration experiments.

REFERENCES

Al-Hello, H., Paananen, A., Eskelinen, M., Ylipaasto, P., Hovi, T., Salmela, K., Lukashev, A. N., Bobegamage, S. & Roivainen, M. 2008 An enterovirus strain isolated from diabetic child belongs to a genetic subcluster of echovirus 11, but is also neutralised with monotypic antisera to coxsackievirus A9. *J. Gen. Virol.* **89**, 1949–1959.

Allwood, P. B., Malik, Y. S., Maherchandani, S., Hedberg, C. W. & Goyal, S. M. 2005 Effect of temperature on the survival of F-specific RNA coliphage, feline calicivirus, and *Escherichia coli* in chlorinated water. *Int. J. Environ. Res. Public Health* **2**, 442–446.

Bae, J. & Schwab, K. J. 2008 Evaluation of murine norovirus, feline calicivirus, poliovirus, and MS2 as surrogates for human

norovirus in a model of viral persistence in surface water and groundwater. *Appl. Environ. Microbiol.* **74**, 477–484.

Baert, L., Wobus, C. E., van Coillie, E., Thackray, L. B., Debevere, J. & Uyttendaele, M. 2008 Detection of murine norovirus 1 by using plaque assay, transfection assay, and real-time reverse transcription-PCR before and after heat exposure. *Appl. Environ. Microbiol.* **74**, 543–546.

Bauer, R., Dizer, H., Graeber, I., Rosenwinkel, K. H. & Lopez-Pila, J. M. 2011 Removal of bacterial fecal indicators, coliphages and enteric adenoviruses from waters with high fecal pollution by slow sand filtration. *Water Res.* **45**, 439–452.

Boudaud, N., Machinal, C., David, F., Freval-Le Bourdonnec, A., Jossent, J., Bakanga, F., Arnal, C., Jaffrezic, M. P., Oberti, S. & Gantzer, C. 2012 Removal of MS2, Qbeta and GA bacteriophages during drinking water treatment at pilot scale. *Water Res.* **46**, 2651–2664.

de Roda Husman, A. M. & Bartram, J. 2007 Global supply of virus safe drinking water. In*: Human Viruses in Water in the Series Perspectives in Medical Virology* (A. Bosch, ed.). Elsevier Science, Amsterdam, pp. 127–162.

de Roda Husman, A. M., Bijkerk, P., Lodder, W. J., van den Berg, H. H. J. L., Pribil, W., Cabaj, A., Gehringer, P., Sommer, R. & Duizer, E. 2004 Calicivirus inactivation by non-ionising (UV-253.7-nanometer-wavelength) and ionising (gamma) radiation. *Appl. Environ. Microbiol.* **70**, 5089–5093.

de Roda Husman, A. M., Lodder, W. J., Rutjes, S. A., Schijven, J. F. & Teunis, P. F. 2009 Long-term inactivation study of three enteroviruses in artificial surface and groundwaters, using PCR and cell culture. *Appl. Environ. Microbiol.* **75**, 1050–1057.

Duizer, E., Bijkerk, P., Rockx, B., de Groot, A., Twisk, F. & Koopmans, M. 2004 Inactivation of caliciviruses. *Appl. Environ. Microbiol.* **70**, 4538–4543.

Fittipaldi, M., Rodriguez, N. J., Codony, F., Adrados, B., Penuela, G. A. & Morato, J. 2010 Discrimination of infectious bacteriophage T4 virus by propidium monoazide real-time PCR. *J. Virol. Methods* **168**, 228–232.

Fong, T. T., Griffin, D. W. & Lipp, E. K. 2005a Molecular assays for targeting human and bovine enteric viruses in coastal waters and their application for library-independent source tracking. *Appl. Environ. Microbiol.* **71**, 2070–2078.

Fong, T. T. & Lipp, E. K. 2005b 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.

Gassilloud, B., Schwartzbrod, L. & Gantzer, C. 2003 Presence of viral genomes in mineral water: a sufficient condition to assume infectious risk? *Appl. Environ. Microbiol.* **69**, 3965–3969.

Gerba, C. P., Gramos, D. M. & Nwachuku, N. 2002 Comparative inactivation of enteroviruses and adenovirus 2 by UV light. *Appl. Environ. Microbiol.* **68**, 5167–5169.

Gilks, W. R., Richardson, S. & Spiegelhalter, D. J. 1996 *Markov Chain Monte Carlo in Practice*. Chapman & Hall, New York.

Hijnen, W. A., Beerendonk, E. F. & Medema, G. J. 2006 Inactivation credit of UV radiation for viruses, bacteria and protozoan (oo)cysts in water: a review. *Water Res.* **40**, 3–22.

Hill, V. R., Kahler, A. M., Jothikumar, N., Johnson, T. B., Hahn, D. & Cromeans, T. L. 2007 Multistate evaluation of an ultrafiltration-based procedure for simultaneous recovery of enteric microbes in 100-liter tap water samples. *Appl. Environ. Microbiol.* **73**, 4218–4225.

Hoxie, N. J., Davis, J. P., Vergeront, J. M., Nashold, R. D. & Blair, K. A. 1997 Cryptosporidiosis-associated mortality following a massive waterborne outbreak in Milwaukee, Wisconsin. *Am. J. Public Health* **87**, 2032–2035.

Hurvich, C. M. & Tsai, C. L. 1989 Regression and time series model selection in small samples. *Biometrika* **76**, 297–307.

ISO 1995 Water Quality – Detection and Enumeration of Bacteriophages – Part 1: Enumeration of F-specific RNA-bacteriophages (1995) ISO 10705-1. International Organization for Standardization, Geneva.

Kim, S. Y. & Ko, G. 2012 Using propidium monoazide to distinguish between viable and nonviable bacteria, MS2, and murine norovirus. *Lett. Appl. Microbiol.* **55**, 182–188.

Kirs, M. & Smith, D. C. 2007 Multiplex quantitative real-time reverse transcriptase PCR for F+-specific RNA coliphages: a method for use in microbial source tracking. *Appl. Environ. Microbiol.* **73**, 808–814.

Lodder, W. J., Buisman, A. M., Rutjes, S. A., Heijne, J. C., Teunis, P. F. & de Roda Husman, A. M. 2012 Feasibility of quantitative environmental surveillance in poliovirus eradication strategies. *Appl. Environ. Microbiol.* **78**, 3800–3805.

Lodder, W. J. & de Roda Husman, A. M. 2005 Presence of noroviruses and other enteric viruses in sewage and surface waters in the Netherlands. *Appl. Environ. Microbiol.* **71**, 1453–1461.

Lodder, W. J., van den Berg, H. H., Rutjes, S. A. & de Roda Husman, A. M. 2010 Presence of enteric viruses in source waters for drinking water production in The Netherlands. *Appl. Environ. Microbiol.* **76**, 5965–5971.

Mayer, B. K., Ryu, H. & Abbaszadegan, M. 2008 Treatability of U.S. Environmental Protection Agency contaminant candidate list viruses: removal of coxsackievirus and echovirus using enhanced coagulation. *Environ. Sci. Technol.* **42**, 6890–6896.

Meschke, J. S. & Sobsey, M. D. 2003 Comparative reduction of Norwalk virus, poliovirus type 1, F+ RNA coliphage MS2 and *Escherichia coli* in miniature soil columns. *Water Sci. Technol.* **47**, 85–90.

Nuanualsuwan, S. & Cliver, D. O. 2002 Pretreatment to avoid positive RT-PCR results with inactivated viruses. *J. Virol. Methods* **104**, 217–225.

Nuanualsuwan, S. & Cliver, D. O. 2003 Capsid functions of inactivated human picornaviruses and feline calicivirus. *Appl. Environ. Microbiol.* **69**, 350–357.

Parshionikar, S., Laseke, I. & Fout, G. S. 2010 Use of propidium monoazide in reverse transcriptase PCR to distinguish

between infectious and noninfectious enteric viruses in water samples. *Appl. Environ. Microbiol.* **76**, 4318–4326.

Pecson, B. M., Martin, L. V. & Kohn, T. 2009 Quantitative PCR for determining the infectivity of bacteriophage MS2 upon inactivation by heat, UV-B radiation, and singlet oxygen: advantages and limitations of an enzymatic treatment to reduce false-positive results. *Appl. Environ. Microbiol.* **75**, 5544–5554.

Reynolds, K. A., Gerba, C. P., Abbaszadegan, M. & Pepper, L. L. 2001 ICC/PCR detection of enteroviruses and hepatitis A virus in environmental samples. *Can. J. Microbiol.* **47**, 153–157.

Rodriguez, R. A., Pepper, I. L. & Gerba, C. P. 2009 Application of PCR-based methods to assess the infectivity of enteric viruses in environmental samples. *Appl. Environ. Microbiol.* **75**, 297–307.

Rutjes, S. A., Lodder, W. J., van Leeuwen, A. D. & de Roda Husman, A. M. 2009 Detection of infectious rotavirus in naturally contaminated source waters for drinking water production. *J. Appl. Microbiol.* **107**, 97–105.

Sadeghi, G., Schijven, J. F., Behrends, T., Hassanizadeh, S. M., Gerritse, J. & Kleingeld, P. J. 2011 Systematic study of effects of pH and ionic strength on attachment of phage PRD1. *Ground Water* **49**, 12–19.

Schijven, J. F. & Hassanizadeh, S. M. 2000 Removal of viruses by soil passage: overview of modeling, processes, and parameters. *Crit. Rev. Environ. Sci. Technol.* **30**, 49–127.

Schijven, J. F., Van den Berg, H., Colin, M., Dullemont, Y., Hijnen, W. A. M., Magic-Krezew, A., Oorthuizen, W. & Wubbels, G. 2013 A mathematical model for removal of human pathogenic viruses and bacteria by slow sand filtration under variable operational conditions. *Water Res.* **47**, 2592–2602.

Shin, G. A. & Sobsey, M. D. 2003 Reduction of Norwalk virus, poliovirus 1, and bacteriophage MS2 by ozone disinfection of water. *Appl. Environ. Microbiol.* **69**, 3975–3978.

Simonet, J. & Gantzer, C. 2006 Inactivation of poliovirus 1 and F-specific RNA phages and degradation of their genomes by UV irradiation at 254 nanometers. *Appl. Environ. Microbiol.* **72**, 7671–7677.

Sommer, R., Pribil, W., Appelt, S., Gehringer, P., Eschweiler, H., Leth, H., Cabaj, A. & Haider, T. 2001 Inactivation of bacteriophages in water by means of non-ionizing (UV-253.7 nm) and ionizing (gamma) radiation: a comparative approach. *Water Res.* **35**, 3109–3116.

Straub, T. M., Bartholomew, R. A., Valdez, C. O., Valentine, N. B., Dohnalkova, A., Ozanich, R. M., Bruckner-Lea, C. J. & Call, D. R. 2011 Human norovirus infection of caco-2 cells grown as a three-dimensional tissue structure. *J. Water Health* **9**, 225–240.

Straub, T. M., Honer zu Bentrup, K., Orosz-Coghlan, P., Dohnalkova, A., Mayer, B. K., Bartholomew, R. A., Valdez, C. O., Bruckner-Lea, C. J., Gerba, C. P., Abbaszadegan, M. & Nickerson, C. A. 2007 *In vitro* cell culture infectivity assay for human noroviruses. *Emerg. Infect. Dis.* **13**, 396–403.

Straub, T. M., Hutchison, J. R., Bartholomew, R. A., Valdez, C. O., Valentine, N. B., Dohnalkova, A., Ozanich, R. M. & Bruckner-Lea, C. J. 2013 Defining cell culture conditions to improve human norovirus infectivity assays. *Water Sci. Technol.* **67**, 863–868.

Swerdlow, D. L., Woodruff, B. A., Brady, R. C., Griffin, P. M., Tippen, S., Donnell Jr., H. D., Geldreich, E., Payne, B. J., Meyer Jr., A., Wells, J. G., Greene, K. D., Bright, M., Bean, N. H. & Blake, P. A. 1992 A waterborne outbreak in Missouri of Escherichia coli O157:H7 associated with bloody diarrhea and death. *Ann. Intern. Med.* **117**, 812–819.

Teunis, P. F., Lodder, W. J., Heisterkamp, S. H. & de Roda Husman, A. M. 2005 Mixed plaques: statistical evidence how plaque assays may underestimate virus concentrations. *Water Res.* **39**, 4240–4250.

van Boekel, M. A. 2002 On the use of the Weibull model to describe thermal inactivation of microbial vegetative cells. *Int. J. Food Microbiol.* **74**, 139–159.

WHO 2009 Water safety plan manual (WSP manual): Step-by-step risk management for drinking-water suppliers. Available at: http://www.who.int/water_sanitation_health/publication_9789241562638/en/.

Wigginton, K. R. & Kohn, T. 2012 Virus disinfection mechanisms: the role of virus composition, structure, and function. *Curr. Opin. Virol.* **2**, 84–89.

Wigginton, K. R., Menin, L., Montoya, J. P. & Kohn, T. 2010 Oxidation of virus proteins during UV(254) and singlet oxygen mediated inactivation. *Environ. Sci. Technol.* **44**, 5437–5443.

Wolf, S., Hewitt, J. & Greening, G. E. 2010 Viral multiplex quantitative PCR assays for tracking sources of fecal contamination. *Appl. Environ. Microbiol.* **76**, 1388–1394.

Wolf, S., Rivera-Aban, M. & Greening, G. E. 2009 Long-range reverse transcription as a useful tool to assess the genomic integrity of Norovirus. *Food Environ. Virol.* **1**, 129–136.

Yeh, H. Y., Yates, M. V., Chen, W. & Mulchandani, A. 2009 Real-time molecular methods to detect infectious viruses. *Semin. Cell. Dev. Biol.* **20**, 49–54.

First received 29 October 2012; accepted in revised form 5 March 2013. Available online 18 April 2013