

Quantitative characterization of the inhibitory effects of salt, humic acid, and heavy metals on the recovery of waterborne norovirus by electropositive filters

MinJung Kim and GwangPyo Ko

ABSTRACT

The virus adsorption-elution technique (VIRADEL) using electropositively charged filters is used frequently for recovering enteric viruses from water. The filter-absorbed virus is typically eluted, concentrated, and subsequently detected by culture or molecular methods. Human norovirus (HuNoV), one of the most important waterborne pathogens, cannot be cultivated by conventional culture methods and is typically detected using a reverse transcriptase-polymerase chain reaction (RT-PCR) assay. However, it is plausible that various inhibitors could be concentrated simultaneously during the VIRADEL process and affect RT-PCR assays. In this study, we evaluated the effect of typical inhibitors, including humic acid, heavy metals, and salt, on the recovery of norovirus by two different electropositive filters: 1MDS and Nanoceram. Known amounts of HuNoV and murine norovirus were inoculated in 1 L of surface water containing various concentrations of humic acid, heavy metals (cadmium and lead), or NaCl. Our results indicate that the presence of heavy metals or salt significantly reduced the recovery of virus from the electropositive filters. Thus, care should be taken when analyzing waterborne norovirus using electropositive filters in environments with high concentrations of heavy metal inhibitors or salts.

Key words | electropositive filter, heavy metal, humic acid, murine norovirus, norovirus, salt

MinJung Kim
GwangPyo Ko (corresponding author)
Department of Environmental Health,
and Institute of Health and Environment,
School of Public Health, Seoul National University,
599 Gwanak-ro Gwanak-gu,
Seoul 151-747,
Republic of Korea
E-mail: gko@snu.ac.kr

GwangPyo Ko
Institute of Microbiology, School of Biological
Sciences, Seoul National University,
Seoul,
Republic of Korea

INTRODUCTION

Human norovirus (HuNoV) is one of the most important waterborne pathogens, causing more than 90% of cases of non-bacterial epidemic gastroenteritis worldwide (Berg *et al.* 2000; Fankhauser *et al.* 2002; Blanton *et al.* 2006). Norovirus (NoV) is a positive single-stranded sense RNA virus belonging to the family *Caliciviridae*. Based on the nucleic acid sequences of its capsid genes, NoV can be classified into five genogroups (GI–GV). Of these, GI, GII, and GIV are known to infect humans (Jean *et al.* 2004; Haramoto *et al.* 2009). HuNoVs are ubiquitous in various types of water, including surface water, groundwater, wastewater, and seawater (Haramoto *et al.* 2009; Karim *et al.* 2009). Due to its high impact on environmental health, HuNoV was

included on the US Environmental Protection Agency's Contaminant Candidate List 3 (US EPA 2009). Because HuNoV cannot be cultivated by conventional cell culture, molecular assays such as reverse transcriptase-polymerase chain reaction (RT-PCR) are generally accepted as the gold standard for viral detection in both clinical and environmental samples (Ando *et al.* 1995; Atmar & Estes 2001; Dreier *et al.* 2006). However, murine norovirus (MNV), which belongs to the GV genogroup of NoV, was recently identified and can be cultivated in conventional cell culture (Doultree *et al.* 1999; Wobus *et al.* 2004). MNV is widely considered to be the most appropriate surrogate for HuNoV because of its biological similarity and high

resistance to various environmental stresses (Karst *et al.* 2003; Lee *et al.* 2008).

Viruses in water are typically present at low concentrations. Thus, it is necessary to concentrate viruses from a large volume of water to detect them. The virus adsorption-elution technique (VIRADEL) using an electropositively charged filter is commonly used to recover waterborne viruses (Haramoto *et al.* 2004; Cashdollar & Dahling 2006; Polaczyk *et al.* 2007; Karim *et al.* 2009; Victoria *et al.* 2009; Gibbons *et al.* 2010). The US EPA recommends use of the Virosorb®1MDS filter (Cuno, Meriden, CT, USA) as a standard method for collecting enteric viruses from large volumes of drinking water (Berg *et al.* 1984). This filter has been widely used and its recovery rates evaluated extensively (Ma *et al.* 1994; Polaczyk *et al.* 2007; Lee *et al.* 2011). Recently, a new electropositive Nanoceram filter composed of a nano-alumina filter has been developed and proven effective for sampling waterborne viruses (Karim *et al.* 2009; Gibbons *et al.* 2010). Most studies have focused on evaluating the recovery of waterborne viruses from tap or less-contaminated surface waters (Berg *et al.* 1984; Lambertini *et al.* 2008; Haramoto *et al.* 2009; Hill *et al.* 2009; Lee *et al.* 2011). However, environmental water conditions can affect the recovery of waterborne viruses (Shields & Farrah 1983; Sobsey & Hickey 1985; Guttman-Bass & Catalano-Sherman 1986; Lukasik *et al.* 2000). For example, a previous study showed very low recovery of a waterborne virus by 1MDS filters from seawater (Polaczyk *et al.* 2007). Like high salt concentrations, humic acid and heavy metals may interfere with the virus adsorption-elution process and reduce recovery. Environmental water samples can contain various levels of salt, humic acid, and heavy metals (Guttman-Bass & Catalano-Sherman 1986), and the effect of salt concentration on viral recovery is important for evaluating the viral contamination of salt water. Humic acid and heavy metals are commonly identified in water samples and may affect adsorption, elution, and RT-PCR assays. Thus, it is critical to fully evaluate how these chemicals affect the recovery of waterborne viruses by electropositive filters. Therefore, the objectives of this study were: (1) to evaluate the effect of salt, humic acid, and heavy metals on NoV recovery efficiency from

electropositive filters; and (2) to compare and characterize the recovery efficiencies from the 1MDS and Nanoceram electropositive filters.

MATERIALS AND METHODS

Preparation of MNV and HuNoV viral stocks

MNV was obtained from the laboratory of Dr. Virgin at the Washington University School of Medicine (St Louis, MO, USA). MNV was analyzed by a plaque assay as described previously (Lee *et al.* 2008; Lim *et al.* 2010). Briefly, MNV was cultivated using RAW 264.7 cells in Dulbecco's modified Eagle's medium (DMEM; Invitrogen/Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Invitrogen/Gibco, Rockville, MD, USA), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10 mM non-essential amino acids, 10 mM sodium bicarbonate, and 50 µg/mL gentamicin. All cell culture reagents were purchased from Invitrogen (Carlsbad, CA, USA). Cultured RAW264.7 cells were infected by MNV at a multiplicity of infection of 100 and incubated for 3–4 days. The infected cells were then frozen and thawed three times to release the viruses. The resulting cell suspensions were subjected to chloroform extraction using an equal volume of chloroform, followed by centrifugation at 4,000 × g for 20 min at 4 °C. The viral suspension in the upper aqueous phase was recovered and ultra-filtered using an Amicon Ultra-15 filter (Millipore, Billerica, MA, USA). The prepared MNV stock solution was serially diluted and inoculated into six-well plates at a density of $\sim 2 \times 10^6$ cells per plate with RAW 264.7 cells for plaque assays, as described previously (Lee *et al.* 2008; Lim *et al.* 2010). The titer of the viral stock concentrations was estimated to be $\sim 10^8$ plaque forming units (PFU)/mL. The stocks were stored at -80 °C until use.

HuNoV genotype II-4 (GII-4) was provided as a stool sample by the Korean Centers for Disease Control and Prevention. The stool was serially diluted and the titer determined by RT-PCR. Viral RNA was extracted from 150 µL of 10% fecal suspension in phosphate-buffered saline with a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol

and immediately stored at -80°C . The HuNoV GII-4 concentration was estimated to be $\sim 10^6$ RT-PCR viral RNA U/mL.

Quantitative real-time RT-PCR assays for MNV and HuNoV

A real-time RT-PCR assay was performed for the quantitative evaluation of MNV and HuNoV GII-4 recovery from electropositive filters. To prepare the quantitation standard, the capsid regions of MNV and HuNoV GII-4 were amplified and cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) following the manufacturer's protocol. The constructed plasmids were purified using a plasmid DNA purification kit (Labopass; Cosmogenetech Co. Ltd, Seoul, Korea), and confirmed by DNA sequencing. Each MNV and HuNoV GII-4 plasmid was digested with the restriction enzymes *SacI* or *NotI* then verified by agarose gel electrophoresis and purified using a QIAquick Gel Extraction Kit (Qiagen). The linearized DNA plasmid was then subjected to *in vitro* transcription reaction using a Megascript Kit (Ambion, Austin, TX, USA) following the manufacturer's instructions. The concentrations of the RNA transcripts produced were estimated based on the absorbance at 260 nm (ND-1000; NanoDrop Technologies, Wilmington, DE, USA), and used as the standard for quantitative real-time RT-PCR assays as described previously (Lee *et al.* 2011).

For both MNV and HuNoV GII-4, viral nucleic acids were extracted using a QIAamp Viral RNA Mini Kit (Qiagen). Quantitative real-time RT-PCR was performed using an ABI 7300 real-time TaqMan RT-PCR instrument (Applied Biosystems, Foster, CA, USA), as described previously (Lee *et al.* 2008). The reaction mixture was composed of AgPath-ID One-Step RT-PCR reagents (Ambion) with primers specific for HuNoV GII-4 and MNV, as described previously (Lee *et al.* 2008; Lim *et al.* 2010; Park *et al.* 2010). The amplification of HuNoV GII-4 was performed as follows: reverse transcription for 30 min at 48°C , initial denaturation for 10 min at 95°C , and 45 amplification cycles of denaturation for 10 s at 95°C , annealing for 20 s at 50°C , and elongation for 30 s at 72°C . The amplification of MNV was performed as follows: reverse transcription for 30 min at 48°C , initial denaturation

for 10 min 95°C , and 45 amplification cycles of 15 s at 95°C and 60 s at 60°C .

Effect of inhibitors on viral recovery by 1MDS and Nanoceram filters

To measure the recovery of known amounts of virus, VIRADEL using electropositively charged filters was performed following the US EPA Manual of Methods for Virology (Berg *et al.* 1984). A total of either 10^6 RT-PCR units of HuNoV GII-4 or 10^8 PFU of MNV suspended in 1 mL was inoculated into 1 L of distilled water (DW) with or without inhibitors (NaCl, humic acid, or heavy metals). The NaCl concentration was adjusted to 0, 0.5, 0.7, or 1 M. The humic acid concentration (Aldrich Chemical Co., Inc., Milwaukee, WI, USA) was 0, 20, 50, or 100 mg/L, and the lead (Pb) (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and cadmium (Cd) (Kanto Chemical Co., Inc., Tokyo, Japan) concentrations were adjusted to 0, 0.001, 0.1, or 1 ppm and 0, 0.005, 0.5, or 5 ppm, respectively. Either a 142 mm disc 1MDS filter (Cuno) or a 142 mm disc Nanoceram (Argonide, Sanford, FL, USA) filter was placed into the disc filter holder (KS-142-ST; Advantec, Nirasaki, Japan). The virus solution containing inhibitors was connected to the prepared filter holder by a platinum-cured silicon tube. The solution was passed through either the 1MDS or Nanoceram disc filter using a peristaltic pump (Masterflex; Cole-Parmer Instrument Co., Vernon Hills, IL, USA) at a flow rate of 1.7 L/min. The elution and concentration procedures were performed as described previously (Lee *et al.* 2011). Briefly, 500 mL of 1.5% beef extract (Becton Dickinson and Co., Sparks, MD, USA) with 0.05 M glycine (Yakuri Pure Chemicals Co. Ltd, Kyoto, Japan), 0.01% Tween 80 (Honeywell Riedel de Haën, Seelze, Germany), and 0.001% anti-forming agent (Y-30 Emulsion, #A5758; Sigma, St Louis, MO, USA) was prepared as an elution buffer, and its pH was adjusted to 9.0 with NaOH. Two hundred milliliters of elution buffer was poured into the inlet of the filter holder and the elution buffer was allowed to contact the inoculated filter for 30 min at room temperature. The remaining 300 mL of elution buffer was stored in a stainless pressure vessel (Millipore) and forced through the inoculated filters by pressurized nitrogen gas. The resulting

eluate was collected in a sterile beaker containing a stir bar and subjected to acid precipitation. After the pH had been adjusted to 3.5 with 1 M HCl, the eluate was precipitated by centrifugation at $2,500 \times g$ for 30 min at 4°C . The supernatant was then removed. The pellet was resuspended in ~ 15 mL of 0.15 M sodium phosphate solution and the pH adjusted to 9.0; it was stored at -80°C , and then assayed by a plaque assay or real-time RT-PCR. MNV was assayed by both plaque and quantitative real-time RT-PCR assays. HuNoV GII-4 was assayed by only quantitative real-time RT-PCR. Each experiment was repeated three times.

Statistical analysis

The amount of initially inoculated virus was regarded as 100% to estimate the percentage recovery from the electro-positive filters under each condition. The non-parametric Mann-Whitney U test was used to test the effect of each inhibitor present in the samples compared with DW as a control. In addition, the non-parametric Kruskal-Wallis test was used to evaluate the overall effect of inhibitors on viral recovery in comparison to DW. A statistical analysis was performed using SPSS software v. 15.0 (IBM Corp., Armonk, NY, USA). P -values < 0.05 were considered to be statistically significant.

RESULTS

Effect of NaCl, humic acid, Pb, and Cd on MNV recovery

Figure 1 summarizes the effect of NaCl on MNV recovery as determined by either real-time RT-PCR or a plaque assay. The recovery rates measured by real-time RT-PCR were generally higher than those measured by a plaque assay. The viral recoveries in DW were 57.3 and 45.6% for 1MDS and Nanoceram, respectively, by real-time RT-PCR (Figure 1(a)). However, by a plaque assay, the recovery rates of MNV in DW were 19.5 and 15.2% for 1MDS and Nanoceram, respectively. As the concentrations of NaCl increased, the recovery rates using the 1MDS (Kruskal-Wallis test, $P = 0.025$) and Nanoceram filters (Kruskal-Wallis test, $P = 0.022$) decreased. For example, the mean recovery rates using 1MDS as measured by real-time RT-PCR decreased from 57.3 to 5.9, 1.9, and 1.5% at NaCl concentrations of 0.5, 0.7, and 1 M, respectively. For the Nanoceram filters, the recovery rates at these concentrations also decreased from 45.6 to 10.8, 8.5, and 7.4%. However, the inhibitory effect of NaCl on viral recovery was slightly lower for the 1MDS filter. Consistent results were obtained using plaque assays. As shown in Figure 1(b), the recovery of MNV decreased significantly from 19.5 to 1.5% and from 15.2 to 2.2% using 1MDS and Nanoceram filters, respectively, as the NaCl concentration increased from 0 to 1 M. Based on

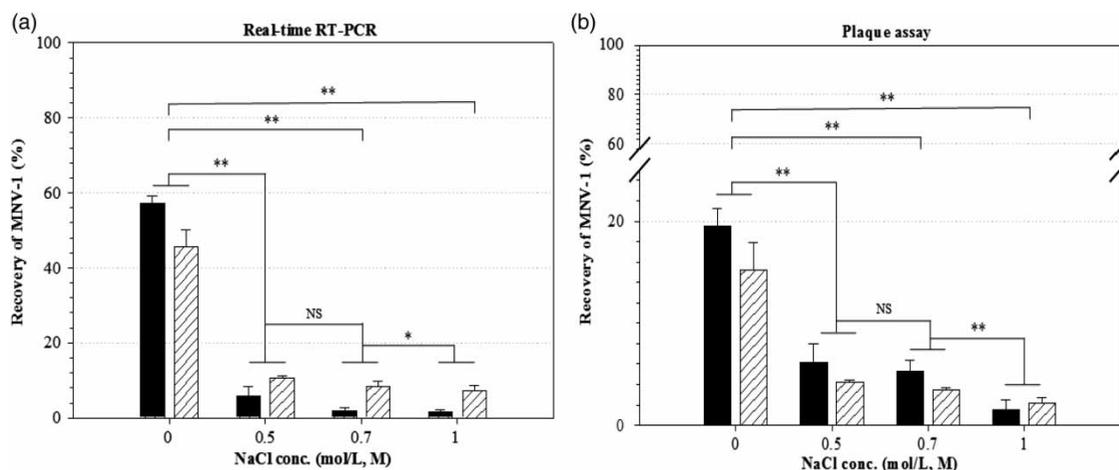


Figure 1 Recoveries of MNV from various concentrations of NaCl using either 1MDS (■) or Nanoceram (▨) filters, measured by (a) real-time RT-PCR and (b) plaque assay. Percentage recovery of MNV was calculated using the initial spiking of MNV (3.8×10^8 PFU/ml) as 100% ($n = 3$). Mann-Whitney U-test was used to compare differences in recovery; NS ($p > 0.05$), * ($p < 0.05$), and ** ($p < 0.01$).

the results of the non-parametric Kruskal–Wallis test, there was a significant difference between the filter types in recovery efficiency at different NaCl concentrations ($P = 0.022$ for the 1MDS filter, and $P = 0.015$ for the Nanoceram filter).

Figure 2 summarizes the effect of humic acid on MNV recovery. Recovery decreased significantly as the concentration of humic acid increased (Kruskal–Wallis test, $P = 0.016$). As the concentration of humic acid increased from 0 to 25 mg/L, viral recovery by the 1MDS filter was reduced by 42.7% based on real-time RT-PCR and 3.5% based on plaque assays. As the concentration of humic acid increased from 0 to 25 mg/L, viral recovery by Nanoceram was reduced by 34 and 8.2% when examined by real-time RT-PCR and plaque assays, respectively. Regardless of filter type, the MNV recovery rate was <10% when the humic acid concentration was >50 mg/L. Overall, the viral recovery rates were reduced to a greater extent by humic acid when the virus was assayed by real-time RT-PCR. Thus, the presence of humic acid significantly reduced the recovery rate for both filters ($P = 0.016$, real-time RT-PCR assay; $P = 0.02$, plaque assay).

The effects of Pb and Cd on MNV recovery are summarized in Figure 3. Heavy metals such as Pb and Cd also significantly reduced MNV recovery from the electropositive filters. The recovery rates were more sensitive to the concentration of Cd than to that of Pb. The recovery rates for MNV measured by real-time RT-PCR were reduced by 13.1, 24.8, and 34.2% with the 1MDS filter

(Kruskal–Wallis test, $P = 0.016$) and 21.5, 36.9, and 38.7% with the Nanoceram filter (Kruskal–Wallis test, $P = 0.019$) at lead concentrations of 0.001, 0.1, and 1 ppm. In contrast, the recovery rates measured by real-time RT-PCR were reduced by more than 52.3% even at a Cd concentration of 0.005 ppm. When measured by a plaque assay, the overall recovery rates were lower than by real-time RT-PCR. However, the percentage reduction in viral recovery rose as the heavy metal concentration increased (Figure 3(b) and (d)). MNV recovery by the 1MDS filter as determined by plaque assays dropped significantly from 19.5% to 6.7, 3.9, and 1.5% as the concentration of lead was increased from 0 to 0.001, 0.01, and 1 ppm, respectively ($P = 0.016$). Due to recovery rates <20%, there was no significant difference among the lead concentrations (0.001, 0.1, and 1 ppm) by the Mann–Whitney U test. With the Nanoceram filter, MNV recovery was also significantly reduced from 15.2% to 1.9, 0.9, and 0.4% ($P = 0.019$). As with Pb, the recovery rates were sharply reduced as the Cd concentration increased (Figure 3(d)). The MNV recovery rates were <1% in all Cd-containing solutions for both filters.

Effect of salt and inhibitors on HuNoV GII-4 recovery

Figure 4 summarizes the recovery rates of HuNoV GII-4 in the presence of inhibitors such as NaCl, humic acid, Pb and Cd. In the absence of inhibitors, the recovery rates of HuNoV GII-4 in DW were 82.7 and 43% for the 1MDS

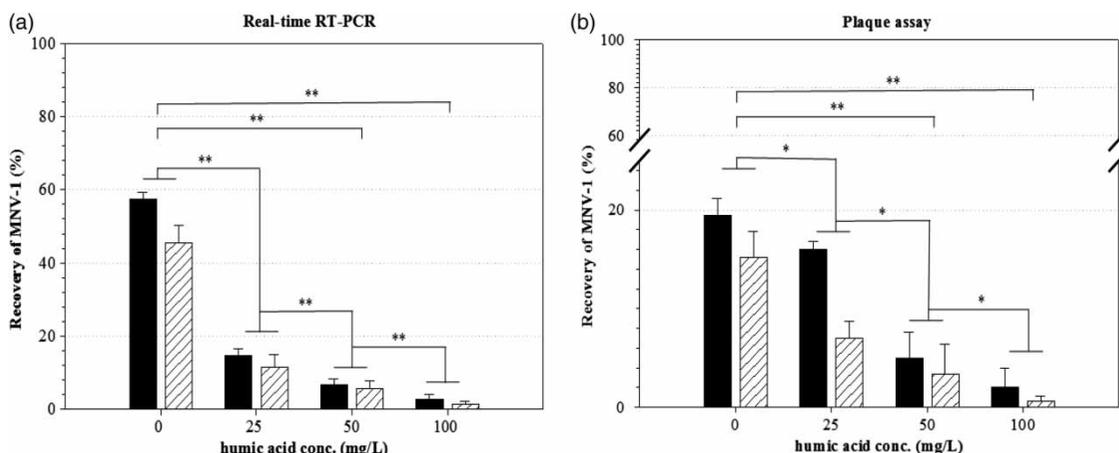


Figure 2 | Recoveries of MNV from various concentrations of humic acid using 1MDS (■) or Nanoceram (▨) filters, measured by (a) real-time RT-PCR and (b) plaque assay. Percentage recovery of MNV was calculated using the initial spiking of MNV (3.8×10^8 PFU/ml) as 100% ($n = 3$). *Mann–Whitney U-test was used to compare differences in recovery; NS ($p > 0.05$), * ($p < 0.05$), and ** ($p < 0.01$).

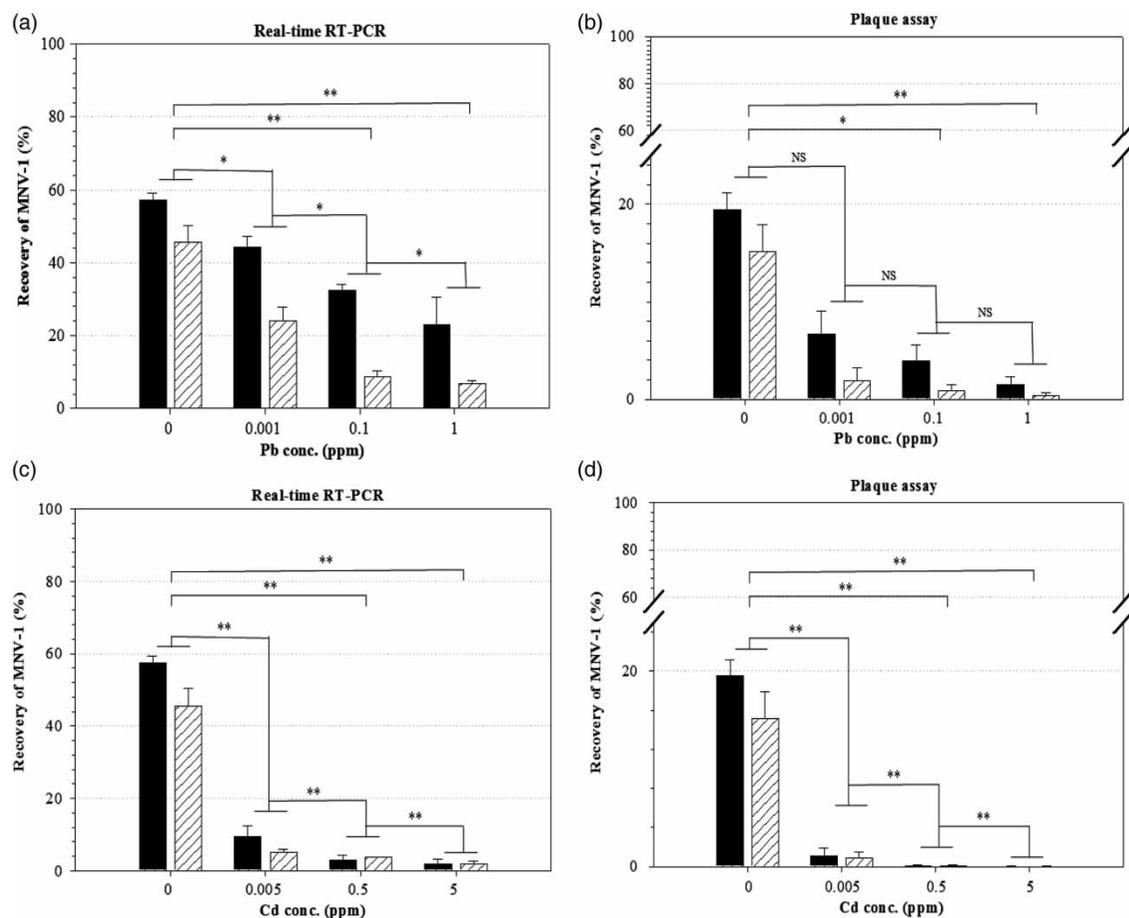


Figure 3 | Recovery of MNV from various heavy metal concentrations (lead, Pb; cadmium, Cd) using 1MDS (■) or Nanoceram (▨) filters, measured by (a), (c) real-time RT-PCR and (b), (d) plaque assay. Percentage recovery of MNV was calculated using the initial spiking of MNV (3.8×10^8 PFU/ml) as 100% ($n = 3$). Mann–Whitney U-test was used to compare differences in recovery; NS ($p > 0.05$), * ($p < 0.05$), and ** ($p < 0.01$).

and Nanoceram filters, respectively. The recovery rates of HuNoV GII-4 decreased significantly for both the 1MDS (Mann–Whitney U test, $P = 0.013$) and Nanoceram (Mann–Whitney U test, $P = 0.021$) filters as the NaCl concentration increased in the water (Figure 4(a)), but there was no significant difference among the NaCl concentrations (0.5, 0.7, and 1 M). The HuNoV GII-4 recovery rates were also greatly affected by the presence of humic acid (Figure 4(b)): recovery was significantly lower regardless of the humic acid concentration for both tested filters (Mann–Whitney U test, $P = 0.013$ for both filters). There was no significant difference among the tested concentrations (25, 50, and 100 mg/L) of humic acid in terms of viral recovery. The HuNoV GII-4 recovery rates were also reduced by both Pb and Cd (Figure 4(c) and (d)). Unlike the other inhibitors, there was a significant difference

among the tested concentrations of Pb (0.001, 0.1, and 1 ppm) for the 1MDS filter (Kruskal–Wallis test, $P = 0.016$) and of Cd (0.005, 0.5, and 5 ppm) for the 1MDS (Kruskal–Wallis test, $P = 0.034$) and Nanoceram (Kruskal–Wallis test, $P = 0.016$) filters, respectively.

Comparison of viral recovery rates between the 1MDS and Nanoceram filters

The recovery rates of the 1MDS and Nanoceram filters were compared. Both filters were sensitive to NaCl, humic acid, Cd, and Pb. Overall, the recovery rates of the 1MDS filter were higher than those of the Nanoceram filter under most conditions for both MNV and HuNoV GII-4. For example, 82.7% of HuNoV GII-4 was recovered from 1MDS filters, whereas only 43% was recovered from

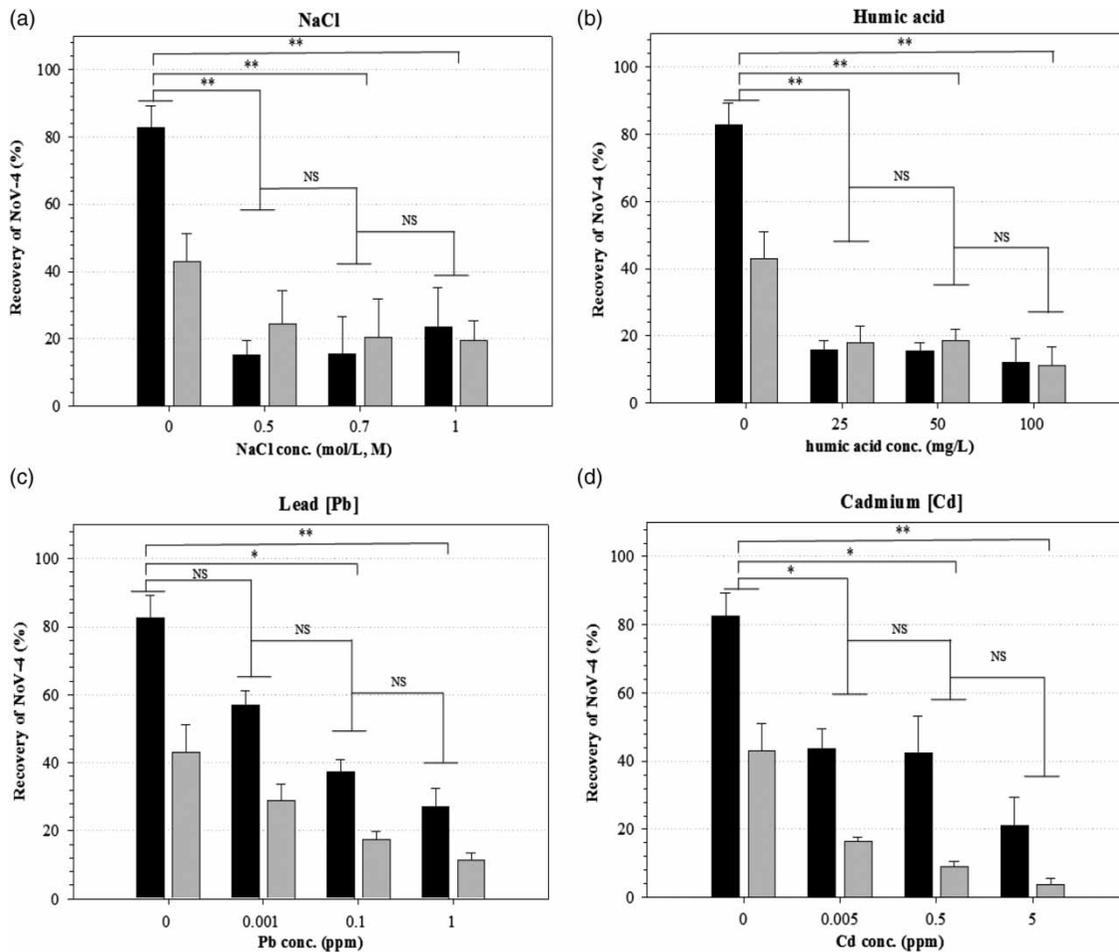


Figure 4 | Percentage recovery of HuNoV GII-4 in various water conditions (NaCl, humic acid and heavy metals [Cd, Pb]) using 1MDS (■) or Nanoceram (□) filters, as analyzed by real-time RT-PCR. Percentage recovery of MNV was calculated using the initial spiking of HuNoV GII-4 (10^6 RT-PCR/unit) as 100% ($n = 3$). Mann-Whitney U-test was used to compare differences in recovery; NS ($p > 0.05$), * ($p < 0.05$) and ** ($p < 0.01$).

Nanoceram filters. The 1MDS recovery rates appeared to be more sensitive to NaCl, particularly when determined by real-time RT-PCR. However, the 1MDS filter appeared to be more resistant to the effect of Pb regardless of the type of virus (MNV or HuNoV GII-4) and analytical assay (real-time RT-PCR and plaque assays).

DISCUSSION

Our study demonstrates that the presence of salt, humic acid, and heavy metals significantly reduced viral recovery from water using 1MDS or Nanoceram filters and as measured by both plaque and real-time RT-PCR assays. Characterization of the recovery rates under various

environmental conditions is crucial because the limit of detection must be fully evaluated. Previous studies evaluated and optimized VIRADEL recovery rates from tap water by comparing various elution and concentration methods (Ma *et al.* 1994; Katayama *et al.* 2002; Lee *et al.* 2011). However, recovery under various environmental conditions should be evaluated to determine VIRADEL sensitivity in field samples. The recovery rates varied depending on the target microorganism and on the condition of the environmental water samples. For example, previous studies reported relatively higher recoveries of enteric viruses, including NoV, from tap water. However, the recovery rates were much lower when the same or similar methods were applied to environmental field samples such as seawater, groundwater, and surface water (Haramoto *et al.* 2009; Karim *et al.* 2009;

Gibbons *et al.* 2010). In real environmental samples, various chemical compounds coexist with viruses, which likely affect the recovery rates of viruses using 1MDS and Nanoceram filters. Our data are directly applicable to a variety of environmental water samples because the inhibitor concentrations were representative of those in environmental surface water and seawater (Guttman-Bass & Catalano-Sherman 1986; Ullmann *et al.* 2005).

Virus contamination in seawater adversely impacts recreational water activities and shellfish harvests. Seawater contains approximately 3% (0.5 M) NaCl. A previous study reported that the recovery rate of enteric viruses by electropositive filters from seawater was much lower than that from other environmental water sources (Bennett *et al.* 2010). It was also suggested that elevated salt concentrations interfere with viral adsorption to electropositive filters, which may reduce recovery (Lukasik *et al.* 2000). Our data are consistent with these results as the presence of NaCl in water resulted in reduced recovery of NoV. A previous study reported that a variety of chemicals in seawater could affect viral adsorption to the filter depending on the chemical component in question (Lukasik *et al.* 2000). The ability to promote virus adsorption was related to the valence of the cation involved. Generally, trivalent cations such as Al^{3+} were better than divalent cations such as Mg^{2+} , which were better than monovalent cations such as Na^+ (Lukasik *et al.* 2000). Our study attempted to explain only how the presence of NaCl in water could decrease viral absorption and thus the recovery rate.

Humic acid represents the major fraction of humic substances, which are common in streams and surface water at a concentration of ~25–200 mg/L (Kinniburgh *et al.* 1996). Our data indicate that the presence of humic acid also interfered with viral recovery. Humic acid is known to compete with viruses for adsorption onto filter surfaces. As the concentration of humic acid increases, the blocking effect becomes greater (Sobsey & Hickey 1985; Guttman-Bass & Catalano-Sherman 1986). Furthermore, the humic acid used in our study contained 60% ash. This dissolved organic carbon may also prevent viruses from adsorbing to the filter materials (Carter & Suffet 1982). Heavy metals may also be present in surface water. Water contamination with heavy metals such as Pb and Cd has been reported worldwide (Kido *et al.* 1989; Lanciotti *et al.* 1989; Schumacher *et al.*

1990). In many countries, water quality standards require that the concentrations of Pb and Cd be <0.001 and 0.05 ppm, respectively (Ministry of Environment, Republic of Korea, 2005). Like NaCl and humic acid, the presence of heavy metals in water lowered viral recovery. Heavy metals are typically present as divalent cations, which may associate with negatively charged viruses in water. In this way, heavy metals may interfere with virus absorption onto filters.

MNV is considered to be the most appropriate culturable surrogate for HuNoV (Cannon *et al.* 2006). However, the recovery rates of MNV and NoV determined by real-time RT-PCR are somewhat different under most conditions ($P < 0.001$). The recovery of MNV by real-time RT-PCR was higher than by plaque assays. This may be because of (1) the greater sensitivity of real-time RT-PCR, (2) the detection of naked or inactivated viruses by real-time RT-PCR, or (3) a more detrimental effect on cell cultivation through the inhibition of viral infectivity (Lee *et al.* 2008). Regardless of the results, plaque assays remain useful because they provide information on viral infectivity.

In this study, viral recovery rates were determined by both adsorption-elution and analytical assays. Interestingly, our results indicate that different inhibitors had varying effects on each of these steps. For example, NaCl lowered viral recovery as measured by both real-time RT-PCR and plaque assays, whereas humic acid affected viral recovery only when determined by real-time RT-PCR. These results suggest that NaCl affected the adsorption-elution steps, but did not preferentially inhibit any specific analytical assays. However, humic acid showed greater inhibition of the real-time RT-PCR assay. Unlike humic acid, viral recovery in the presence of heavy metals was sharply reduced when it was measured by a plaque assay. These data may also be affected by the fact that heavy metals might have greater inhibitory effects on cell cultures than molecular assays. Among the three inhibitors tested, salt concentration and humic acid were more likely to affect physical viral recovery, while heavy metals such as Cd were more likely to affect the biological recovery of virus during cell culture. Additionally, these inhibitors could co-exist and react with one another in sample water, which could cause different effects on viral recovery. Combinations of inhibitors should be fully evaluated in future studies.

This study also compared NoV recovery rates using 1MDS and Nanoceram filters. The 1MDS filter has been frequently used worldwide and is commonly applied to environmental samples (Polaczyk *et al.* 2007; Haramoto *et al.* 2009; Karim *et al.* 2009; Bennett *et al.* 2010). Nanoceram is a more recently developed electropositive filter that has become popular due to its low cost and good recovery rate (Karim *et al.* 2009; Gibbons *et al.* 2010; Deboosere *et al.* 2011). In this study, the recovery rates of NoV by 1MDS were slightly higher than those from the Nanoceram filter; however, the difference was not statistically significant (Mann–Whitney U-test, $P > 0.05$). Our study indicates that both filters showed a reasonably good waterborne virus sampling efficiency and could be applied to environmental water samples. A limitation of our study is that only disc-type filters were used to measure the recovery rates of waterborne viruses. However, 1MDS or Nanoceram cartridge-type filters are commonly used in the field because they can sample much larger volumes of water (Borrego *et al.* 1991; Polaczyk *et al.* 2007; Lambertini *et al.* 2008; Deboosere *et al.* 2011; Ikner *et al.* 2011). Although we believe that the effects of the tested chemicals should be the same or similar, we suggest that cartridge-type filters should also be evaluated.

CONCLUSIONS

In conclusion, the presence of various chemicals such as salt, humic acid, and heavy metals resulted in lower waterborne virus recovery rates by the 1MDS and Nanoceram electropositive filters. The level of inhibition depended on the analytical method used and the inhibitors present. Therefore, the chemical composition of field water samples should be well characterized prior to using these electropositive filters.

ACKNOWLEDGEMENTS

This research was supported by a grant from the Korean Food & Drug Administration (10162KFDA995) and a grant from the National Research Foundation of Korea funded by the Korean government (MEST) (2010-0029113).

REFERENCES

- Ando, T., Monroe, S. S., Gentsch, J. R., Jin, Q., Lewis, D. C. & Glass, R. I. 1995 Detection and differentiation of antigenically distinct small round-structured viruses (Norwalk-like viruses) by reverse transcription-PCR and southern hybridization. *J. Clin. Microbiol.* **33**, 64–71.
- Atmar, R. L. & Estes, M. K. 2001 Diagnosis of noncultivable gastroenteritis viruses, the human caliciviruses. *Clin. Microbiol. Rev.* **14**, 15–37.
- Bennett, H. B., O'Dell, H. D., Norton, G., Shin, G., Hsu, F. C. & Meschke, J. S. 2010 Evaluation of a novel electropositive filter for the concentration of viruses from diverse water matrices. *Water Sci. Technol.* **61**, 317–322.
- Berg, D. E., Kohn, M. A., Farley, T. A. & McFarland, L. M. 2000 Multi-state outbreaks of acute gastroenteritis traced to fecal-contaminated oysters harvested in Louisiana. *J. Infect. Dis.* **181**, S381–S386.
- Berg, G., Safferman, R. S., Dahling, D. R., Berman, D. & Hurst, C. J. 1984 USEPA Manual of Methods for Virology, US EPA, Washington, DC.
- Blanton, L. H., Adams, S. M., Beard, R. S., Wei, G., Bulens, S. N., Widdowson, M. A., Glass, R. I. & Monroe, S. S. 2006 Molecular and epidemiologic trends of caliciviruses associated with outbreaks of acute gastroenteritis in the United States, 2000–2004. *J. Infect. Dis.* **193**, 413–421.
- Borrego, J., Cornax, R., Preston, D., Farrah, S., Mcelhaney, B. & Bitton, G. 1991 Development and application of new positively charged filters for recovery of bacteriophages from water. *Appl. Environ. Microbiol.* **57**, 1218–1222.
- Cannon, J. L., Papafragkou, E., Park, G. W., Osborne, J., Jaykus, L. A. & Vinje, J. 2006 Surrogates for the study of norovirus stability and inactivation in the environment: a comparison of murine norovirus and feline calicivirus. *J. Food Prot.* **69**, 2761–2765.
- Carter, C. W. & Suffet, I. H. 1982 Binding of DDT to dissolved humic materials. *Environ. Sci. Technol.* **16**, 735–740.
- Cashdollar, J. L. & Dahling, D. R. 2006 Evaluation of a method to re-use electropositive cartridge filters for concentrating viruses from tap and river water. *J. Virol. Methods* **132**, 13–17.
- Deboosere, N., Horm, S. V., Pinon, A., Gachet, J., Coldefy, C., Buchy, P. & Vialette, M. 2011 Development and validation of a concentration method for the detection of influenza A viruses from large volumes of surface water. *Appl. Environ. Microbiol.* **77**, 3802–3808.
- Doultree, J., Druce, J., Birch, C., Bowden, D. & Marshall, J. 1999 Inactivation of feline calicivirus, a Norwalk virus surrogate. *J. Hosp. Infect.* **41**, 51–57.
- Dreier, J., Stormer, M., Made, D., Burkhardt, S. & Kleesiek, K. 2006 Enhanced reverse transcription-PCR assay for detection of norovirus genogroup I. *J. Clin. Microbiol.* **44**, 2714–2720.
- Fankhauser, R. L., Monroe, S. S., Noel, J. S., Humphrey, C. D., Bresee, J. S., Parashar, U. D., Ando, T. & Glass, R. I. 2002 Epidemiologic and molecular trends of 'Norwalk-like viruses'

- associated with outbreaks of gastroenteritis in the United States. *J. Infect. Dis.* **186**, 1–7.
- Gibbons, C., Rodriguez, R., Tallon, L. & Sobsey, M. 2010 Evaluation of positively charged alumina nanofibre cartridge filters for the primary concentration of noroviruses, adenoviruses and male specific coliphages from seawater. *J. Appl. Microbiol.* **109**, 635–641.
- Guttman-Bass, N. & Catalano-Sherman, J. 1986 Humic acid interference with virus recovery by electropositive microporous filters. *Appl. Environ. Microbiol.* **52**, 556–561.
- Haramoto, E., Katayama, H. & Ohgaki, S. 2004 Detection of noroviruses in tap water in Japan by means of a new method for concentrating enteric viruses in large volumes of freshwater. *Appl. Environ. Microbiol.* **70**, 2154–2160.
- Haramoto, E., Katayama, H., Utagawa, E. & Ohgaki, S. 2009 Recovery of human norovirus from water by virus concentration methods. *J. Virol. Methods* **160**, 206–209.
- Hill, V. R., Polaczyk, A. L., Kahler, A. M., Cromeans, T. L., Hahn, D. & Amburgey, J. E. 2009 Comparison of hollow-fiber ultrafiltration to the USEPA VIRADEL technique and USEPA method 1623. *J. Environ. Qual.* **38**, 822–825.
- Ikner, L. A., Soto-Beltran, M. & Bright, K. R. 2011 New method using a positively charged microporous filter and ultrafiltration for concentration of viruses from tap water. *Appl. Environ. Microbiol.* **77**, 3500–3506.
- Jean, J., D'Souza, D. H. & Jaykus, L. A. 2004 Multiplex nucleic acid sequence-based amplification for simultaneous detection of several enteric viruses in model ready-to-eat foods. *Appl. Environ. Microbiol.* **70**, 6603–6610.
- Karim, M. R., Rhodes, E. R., Brinkman, N., Wymer, L. & Fout, G. S. 2009 New electropositive filter for concentrating enteroviruses and noroviruses from large volumes of water. *Appl. Environ. Microbiol.* **75**, 2393–2399.
- Karst, S. M., Wobus, C. E., Lay, M., Davidson, J. & Virgin, H. W. 2003 STAT1-dependent innate immunity to a Norwalk-like virus. *Science* **299**, 1575–1578.
- Katayama, H., Shimasaki, A. & Ohgaki, S. 2002 Development of a virus concentration method and its application to detection of enterovirus and norwalk virus from coastal seawater. *Appl. Environ. Microbiol.* **68**, 1033–1039.
- Kido, K., Takehiko, S., Takao, W., Haruo, N., Mariko, O. & Masayuki, I. 1989 Correlation of tetrachloroethylene in blood and in drinking water: a case of well water pollution. *Bull. Environ. Contam. Toxicol.* **43**, 444–453.
- Kinniburgh, G. D., Milne, J. C., Benedetti, F. M., Pinheiro, J. P., Filius, J., Koopal, K. L. & Van Riemsdijk, H. W. 1996 Metal ion binding by humic acid: application of the NICA-Donnan model. *Environ. Sci. Technol.* **30**, 1687–1698.
- Lanciotti, E., Comodo, N., Gambassini, L., Cerbai, E., Vallone, G., Griffini, E. & Mugelli, A. 1989 Survey of barium in Italian drinking water supplies. *Bull. Environ. Contam. Toxicol.* **43**, 833–837.
- Lambertini, E., Spencer, S. K., Bertz, P. D., Loge, F. J., Kieke, B. A. & Borchardt, M. A. 2008 Concentration of enteroviruses, adenoviruses, and noroviruses from drinking water by use of glass wool filters. *Appl. Environ. Microbiol.* **74**, 2990–2996.
- Lee, H. T., Kim, M. S., Paik, S. Y., Lee, C. H., Jheong, W. H., Kim, J. & Ko, G. 2011 Evaluation of electropositive filtration for recovering norovirus in water. *J. Water Health* **9**, 27–36.
- Lee, J. E., Zoh, K. D. & Ko, G. 2008 Inactivation and UV disinfection of murine norovirus with TiO₂ under various environmental conditions. *Appl. Environ. Microbiol.* **74**, 2111–2117.
- Lim, M. Y., Kim, J. M. & Ko, G. 2010 Disinfection kinetics of murine norovirus using chlorine and chlorine dioxide. *Water Res.* **44**, 3243–3251.
- Lukasik, J., Scott, T. M., Andryshak, D. & Farrah, S. R. 2000 Influence of salts on virus adsorption to microporous filters. *Appl. Environ. Microbiol.* **66**, 2914–2920.
- Ma, J. F., Naranjo, J. & Gerba, C. P. 1994 Evaluation of MK filters for recovery of enteroviruses from tap water. *Appl. Environ. Microbiol.* **60**, 1974–1977.
- Ministry of Environment, Republic of Korea 2005 *Drinking Water Quality Standards*. Ministry of Environment, Republic of Korea.
- Park, H. J., Kim, M. J. & Ko, G. P. 2010 Evaluation of various methods for recovering human norovirus and murine norovirus from vegetables and ham. *J. Food Prot.* **73**, 1651–1657.
- Polaczyk, A. L., Roberts, J. M. & Hill, V. R. 2007 Evaluation of 1MDS electropositive microfilters for simultaneous recovery of multiple microbe classes from tap water. *J. Microbiol. Methods* **68**, 260–266.
- Schumacher, M., Bosque, M. A., Domingo, J. L. & Corbella, J. 1990 Lead and cadmium concentrations in marine organisms from the terragona coastal waters, Spain. *Bull. Environ. Contam. Toxicol.* **44**, 784–789.
- Shields, P. A. & Farrah, S. R. 1983 Influence of salts on electrostatic interactions between poliovirus and membrane filters. *Appl. Environ. Microbiol.* **45**, 526–531.
- Sobsey, M. & Hickey, A. 1985 Effects of humic and fulvic acids on poliovirus concentration from water by microporous filtration. *Appl. Environ. Microbiol.* **49**, 259–264.
- Ullmann, F., Gerhartz, W., Yamamoto, Y. S., Campbell, F. T., Pfefferkorn, R. & Rounsaville, J. F. 2005 Ullmann's Encyclopedia of Industrial Chemistry.
- US EPA 2009 Drinking Water Contaminant Candidate List 3, Federal Register (73 FR 9627).
- Victoria, M., Guimaraes, F., Fumian, T., Ferreira, F., Vieira, C., Leite, J. P. & Miagostovich, M. 2009 Evaluation of an adsorption-elution method for detection of astrovirus and norovirus in environmental waters. *J. Virol. Methods* **156**, 73–76.
- Wobus, C. E., Karst, S. M., Thackray, L. B., Chang, K. O., Sosnovtsev, S. V., Belliot, G., Krug, A., Mackenzie, J. M., Green, K. Y. & Virgin, H. W. 2004 Replication of norovirus in cell culture reveals a tropism for dendritic cells and macrophages. *PLoS Biol.* **2**, e432.

First received 21 August 2012; accepted in revised form 28 June 2013. Available online 17 August 2013