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Validation of hollow fiber ultrafiltration and real-time PCR using bacteriophage PP7 as surrogate for the quantification of viruses from water samples

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ABSTRACT

A quantitative real-time TaqMan® PCR system for *Pseudomonas aeruginosa* bacteriophage PP7 was designed to detect PP7 as surrogate in performance tests of 2 hollow fiber ultrafiltration systems in series. Fifty-six storm water samples from 21 sites representing agricultural, urban and highway locations in California were collected. The optimized procedure gave recoveries of spiked PP7 of 64±4.8% (mean±SEM). The PP7 assay was validated over 5 orders of magnitude with an assay limit of detection of 5 gene copies per reaction volume. Sample-dependent variables like enzymatic inhibition during PCR analysis, filtration recovery and extraction efficiency were quantified and incorporated to calculate a specific sample limit of detection (S_{LOD}) for the spiked surrogate PP7. S_{LOD} values were highly variable among samples; they were independent of physicochemical parameters including conductivity, turbidity, total suspended solids and pH but strongly correlated with the dilution factor required to relieve enzymatic inhibition during PCR analysis. To determine actual gene copies of PP7, a dilution approach was developed that involves assaying several dilutions within a range where inhibitors do not affect the efficiency of amplification and linear regression to determine the theoretical C_t value when there is no inhibition. For the detection of viral pathogens, an internal standard like PP7 can be used to calculate filtration recoveries when quantifying pathogens and to determine whether filtration or inhibitor concentration affect nucleic acid extraction efficiency. Additionally, by defining S_{LOD} values per sample and pathogenic organism analyzed, it should be possible to critically investigate the absence of detects for a particular pathogen and determine probabilities of risk associated with a specific sample limit of detection.

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Nomenclature

A_{LOD}	assay limit of detection
A_{LOQ}	assay limit of quantification
LS	large filtration system
C_t	threshold cycle
E_{ex}	nucleic acid extraction efficiency
F_{LS}	feed in large filtration system
F_{SP}	original PP7 amount spiked to the feed
F_{SS}	feed in small filtration system after removal of solids from RE_{LS} and M_{LS}
HFF	hollow fiber ultrafiltration
I	dilution required to relieve inhibition in TaqMan analysis
M_{LS}	liquid recovered after membrane elution in large filtration system
M_{SS}	liquid recovered after membrane elution in small filtration system
PA	plaque assay analysis
P_{LS}	permeate in large filtration system
P_{SS}	permeate in small filtration system
$R_{filtration}$	overall filtration recovery
R_{LS}	retentate in large filtration system
RE_{LS}	recirculated solution in large filtration system after addition of glycine/NaOH/Tween 80 solution to R_{LS}
R_F	final retentate after 2 consecutive filtration steps, $R_F = R_{SS}+M_{SS}$
R_{PA}	overall filtration recovery determined by plaque assay analysis
R_{SS}	retentate in small filtration system
RT	reverse transcription
R_{TQ}	overall filtration recovery determined by TaqMan analysis
S	solids collected from the large filtration system recirculate
S_{LOD}	sample limit of detection
SS	small filtration system
TQ	TaqMan analysis
TSS	total suspended solids

1. Introduction

Efforts to evaluate the microbiological quality of water have progressed from monitoring indicator organisms, such as coliforms, *Escherichia coli* and streptococci, to include the detection of specific human pathogens in natural and drinking waters (Osborn et al., 2004). Increasingly, molecular methods utilizing PCR are applied to take advantage of low detection limits, as determined in clean buffer systems, and more rapid analysis compared to culture-based enumeration (Thompson et al., 2006). Since pathogens may be present at low concentrations, efficient filtration coupled with sensitive detection should ideally form the cornerstone of a PCR-based pathogen detection protocol applied to aqueous solutions. However, due to the complex physical and chemical properties of water, filtration and concentration techniques may be highly variable or ineffective at recovering pathogens (Farrah, 1982; Guttman-Bass and Catalano-Sherman, 1986; Lukasik et al., 2000). Available methodology to extract and purify nucleic acid from these sample types is limited to small starting volumes and can co-purify PCR inhibitors, a fact that may increase detection limits above acceptable levels for some pathogens (Loge et al., 2002). Further, an explicit measurement of pathogen recovery efficiency is crucial to any investigation of a water body where an assessment of human health risk is the goal. Achieving low detection limits with an environmental pathogen assay is of paramount importance, especially in water samples where the presence of a single organism may result in human illness (Straub and Chandler, 2003).

The inclusion of a spiked surrogate may help to clarify the role of important variables such as sample nucleic acid extraction and purification efficiency, recovery of pathogens from filtration processes, and the effects of PCR inhibition. For example, the benign *Pseudomonas aeruginosa* bacteriophage PP7 has been successfully used as a surrogate for the human enterovirus family in culture-based studies to determine filtration efficiencies of natural waters using hollow fiber ultrafiltration (HFF) (Morales-Morales et al., 2003; Oshima 2001; Winona et al., 2001). We chose PP7 for our studies because of its similarity in size (25 nm) and physicochemical properties to poliovirus, the smallest member of the enterovirus family. Consequently, PP7 simulates a worst case scenario for the filtration of viruses. PP7 viruses are phylogenetically related to small RNA coliphages such as MS2 (Bollback and Helsenbeck, 2001). There are no reports of its occurrence in natural waters.

Currently, most diagnostic analysis of water samples for viral pathogens is performed using PCR rather than culture-based techniques, yet an assessment of viral recovery using a quantitative molecular-based method is usually lacking. Real-time TaqMan® PCR technology allows for quantitative determinations of target numbers when using purified nucleic acid extracts, but PCR inhibitors in natural water extracts can lead to erroneous estimations by TaqMan PCR unless such effects are identified and mitigated (Chandler, 1998; Heid et al., 1996).

Selection of a proper filtration method is also key to successful virus detection. In terms of viral recovery, ultrafiltration has proven to be more reliable and consistent when applied to natural waters than electrostatic microfiltration, which is based on adsorption of virus to filter media (Paul et al., 1991; Winona et al., 2001). HFF is a separation method based on size; selection of the proper pore size allows for the simultaneous removal and concentration of viruses, bacteria, and parasites from large volumes of sample (Morales-Morales et al., 2003). Unlike other filtration systems, recovery of viruses using HFF is largely unaffected by complex chemical constituents found in natural water, as shown in other studies where viral recovery has been determined by conventional plaque assay methods and

qualitative PCR (Morales-Morales et al., 2003; Oshima, 2001; Winona et al., 2001).

The objectives of this study were (i) to design a quantitative real-time TaqMan reverse transcriptase PCR (RT-PCR) assay for PP7 and (ii) to develop a sample processing and analysis procedure that allows for the reproducible and reliable determination of low microbial target numbers in a large volume of environmental water. Samples were concentrated by HFF and the overall performance of the filtration system was tested by seeding the original sample volume with bacteriophage PP7 (Oshima, 2001). Recovery of PP7 was monitored at each step during the filtration process using a simple culturing method, which was then compared to recoveries obtained by TaqMan RT-PCR analysis. Fifty-six storm water samples from 21 sites representing agricultural, urban and highway locations in California were collected to validate the filtration method in combination with quantitative RT-PCR. Using PP7 as an internal standard, sample-dependent variables were combined to calculate a sample-specific limit of detection for viruses.

2. Materials and methods

2.1. Sample sites and water collection

Grab samples from storm drains and drainage ditches at 21 locations in California were collected in clean, rinsed, 20-L polypropylene carboys over a period of 2 years. A maximum volume of 100 L was collected at each site. The samples were filtered through 3 stainless-steel sieves (75, 53 and 38 μm) to remove gross solids. The turbidity, conductivity, total suspended solids (TSS), and pH of each sample were measured according to Standard Methods (Eaton et al., 1998).

2.2. Filtration and concentration of water samples

To concentrate viruses and to reduce the initial grab sample volume to approximately 100 mL, 2 filtration systems were designed and constructed based on studies reported by Oshima (2001) (Fig. 1). The larger system (LS) with a 50,000 MW cutoff filter (Microza AHP 2013, Pall Life Sciences, East Hills, NY) concentrated the sample from as much as 100 L down to approximately 1.5 L and was designed to be portable and thus useful in the field. The smaller system (SS) was a custom-built bench top unit (Pall Life Sciences) fitted with a smaller filter (Microza AHP 1013, also 50,000 MW cutoff) for the laboratory, and used to further reduce the retentate from LS to a final volume of 50–100 mL.

The raw samples were spiked with 100 μL of the bacteriophage PP7 (ATCC 15692-B2) to a concentration of 10^5–10^6 pfu/mL and mixed by recirculation through the Microza filter or with an electric, mechanical mixer for 10 min. A 10-mL subsample of the feed (F_{LS}) was removed for subsequent analysis. The water was filtered using a peristaltic pump (Watson-Marlow, Inc. Wilmington, MA) through the hollow fiber filter unit at an input pressure of 15–20 psi. Permeate was collected in a plastic carboy and the retentate was recirculated to the steel sample reservoir with a final hold up volume for the system of approximately 1.5 L. Ten-milliliter subsamples of permeate (P_{LS}) and retentate (R_{LS}) were removed for subsequent analysis.

A solution of glycine/NaOH (pH 7.0)/Tween 80 was added to the retentate and the volume was adjusted with the addition

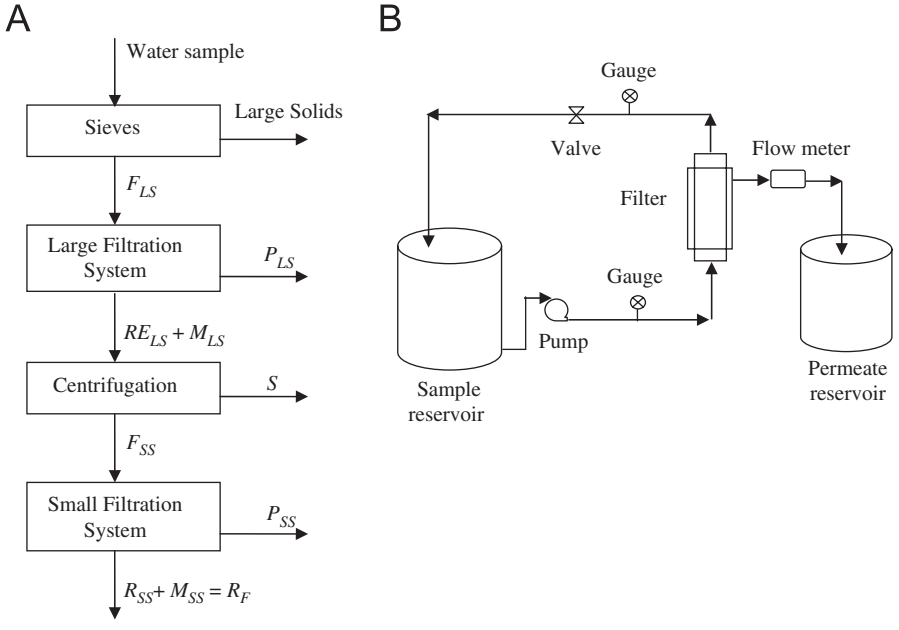

Fig. 1 – (A) Flow chart of the concentration of water samples through the ultrafiltration systems. Subsamples from the feed (F_{LS}, F_{SS}), recirculate (RE_{LS}), membrane (M_{LS}, M_{SS}), and retentate (R_{SS}, R_F) were taken at each step. Solids (S) were also analyzed. (B) A schematic of the filtration systems (large and small), after Oshima (2001).

of double-deionized water to 1.5 L. The final concentrations were 0.05 M for glycine/NaOH and 0.1% for Tween 80. The resulting solution was recirculated through the system (without permeate flow) for 10 min to recover attached virus. A second elution step was performed by the addition of 200 mL of 0.05 M glycine/NaOH (pH 7.0) to the filter, which was shaken for 15–20 min at ambient temperature, and the liquid was recovered (membrane, M_{LS}). Ten-milliliter subsamples from recirculated (RE_{LS}) and membrane (M_{LS}) were also removed for analysis. All subsamples were immediately stored on ice and returned to the laboratory for further processing and analysis.

The RE_{LS} was combined with the M_{LS} and the resultant solution spun at 1000*g* for 10 min at 4 °C to pellet solids. The supernatant was poured into the feed tank of the small filtration system (SS) and subsamples from this feed (F_{SS}) and from the solids (*S*) separated by centrifugation were taken for analysis. The filtration through the small system was performed identically to the large system until the volume had decreased to about 100 mL. Subsamples of retentate (R_{SS}) and permeate (P_{SS}) were removed for analysis.

Initial analyses showed that virus recovery was low for the small filtration system. In contrast to the large system, no additional recirculation step was performed for the small system because the overall length of tubing was shorter and liquid appeared to be trapped mostly in the small filter. Instead, elution of the membrane was modified. After filtration was completed and the retentate (R_{SS}) collected, 50 mL of a solution containing 0.05 M glycine/NaOH (pH 7.0) and 0.1% Tween 80 was added to the Microza filter. The entire volume of liquid was manually pumped through the filter at least 15 times using 60-mL syringes attached to each end. The solution (M_{SS}) was then collected and added to the retentate (R_{SS}), resulting in the final retentate (R_F) after 2 completed filtration procedures. This added elution step greatly improved the overall PP7 recovery (*R*) of the filtration system.

2.3. Growth and storage of PP7

The host *Pseudomonas aeruginosa* (ATCC 15692) was first grown overnight in nutrient broth at 36 °C. The culture was then inoculated with the bacteriophage PP7 (ATCC 15692-B2) and grown overnight at 36 °C. Bacteriophage was harvested according to Morales-Morales et al. (2003). The titer of PP7 was divided into 100-μL aliquots and stored in liquid nitrogen until the day of filtration.

2.4. PP7 plaque assay

Serial 10-fold dilutions of each subsample were assayed for the bacteriophage PP7 using the host *P. aeruginosa* (Oshima, 2001). Each subsample was plated in triplicate. The permeate from each system (P_{LS} and P_{SS}) served as a quality control check to ensure that the filtration system was functioning properly.

2.5. Nucleic acid extraction

During method development, all subsamples (F_{LS}, P_{LS}, R_{LS}, RE_{LS}, M_{LS}, F_{SS}, P_{SS}, and R_{SS}) were extracted using the QIAamp Viral RNA kit (Qiagen, Valencia, CA) according to the manufacturer's directions. For each subsample, 140 μL was extracted and eluted with 80 μL DEPC-treated water. To calculate the overall recovery of PP7, a larger-volume extraction method was used. Ten milliliter of F_{LS} and R_F was added to 200-mL conical plastic centrifuge bottles containing 40 mL of lysis buffer (Boom et al., 1990), and the solution was pulse-vortexed for 15 s. After a 10-min incubation at room temperature, 40 mL of absolute ethanol was added and vortexed again for 15 s. The resultant lysate was spun in a centrifuge for 5 min at 5000*g* to pellet solids. The supernatant was added to a QIAamp Maxi Spin column using a vacuum manifold under a suction of 800 mbar. The column was washed once with 5 mL buffer AW1 (Qiagen), followed by an additional washing step with 5 mL buffer AW2 (Qiagen). The column was placed into a sterile 50-mL collection tube, centrifuged at 4000*g* for 15 min and then incubated at 70 °C for 10 min to remove traces of AW1 and AW2. Nucleic acid was eluted with 2×600 μL of nuclease-free sterile water at 4000*g* for 5 min.

The nucleic acid extraction efficiency (E_{ex}) was measured for 9 samples using a separate spike and TaqMan assay for *Acinetobacter* (McSwain, unpublished data). *Acinetobacter* was used as a surrogate because it gave similar extraction efficiencies to those of PP7 in separate experiments. Ten milliliters of the final concentrated water (R_F) were spiked with aliquots of *Acinetobacter* and extracted immediately. The gene copies present before and after extraction were measured immediately by quantitative PCR. For clean phosphate buffer (PBS), the recovery of nucleic acid was 55%. For 9 concentrated environmental samples, the recovery averaged 7 ± 4%. The recovery was verified on a second day with the extraction of 15 additional concentrated samples.

2.6. PP7 TaqMan PCR system design and validation

The TaqMan RT-PCR system was designed on the replicase gene of PP7 (GenBank accession number NC_001628) using Primer Express (Applied Biosystems, Foster City, CA) software. The sequences are listed in Table 1.

Amplification efficiency and linearity of amplification were tested using 10-fold diluted cDNA obtained from RNA preparations of PP7 phage cultures and known amounts of the plasmid containing the cloned target sequence. A PCR reaction that amplifies the target sequence with 100% efficiency (*E*) would, in theory, double the amount of PCR products with each cycle. The amount of PCR products (C_n) from C_0 input target molecules after *n* cycles could be calculated according to

$$C_n = C_0(1+E)^n. \quad (1)$$

Amplification efficiencies were calculated according to the formula

$$s = -\frac{1}{\log(1+E)}, \quad (2)$$

where *s* is the slope of the standard curve, therefore

$$E = 10^{1/-s} - 1. \quad (3)$$

Table 1 – Oligonucleotides for PP7 TaqMan system

Oligonucleotide	Sequence (5′-3′)
Forward primer	
PP7R-247f	GTTATGAACCAATGTGGCCGTTAT
Reverse primer	
PP7R-320r	CGGGATGCCTCTGAAAAAAG
PP7R-323r	AGGCGGGATGCCTGTGA
PP7R-355r	CGGAAAGCCAACGAGAAATAAG
PP7R-366r	TGGCCAAAAGTCGGAAAGC
Fluorescent probe	
PP7R-274p	6-FAM-TCGGTGGTCAACGAGGAACTGGAAC-TAMRA

2.7. Cloning of target gene sequence of the PP7 replicase gene

In order to generate a standard curve of the PP7 gene copy versus C_t value, plasmids containing the gene sequence were created and quantified. PP7 real-time TaqMan RT-PCR products were purified using Qiagen columns (QIAquick PCR Purification Kit, Qiagen, Valencia, CA) according to the manufacturer's recommendations. The PCR products were cloned into a pCR-TOPO plasmid using the TA cloning strategy (Invitrogen, Carlsbad, CA). The plasmid was transformed into *E. coli* DH5α and recombinant bacteria were selected on ampicillin-containing LB agar. White colonies were screened by real-time TaqMan PCR and 2 positive clones selected for overnight liquid culture propagation. Plasmids were extracted from *E. coli* using Qiagen columns, and inserts were re-sequenced to confirm analytical specificity. Plasmids were quantified by spectrophotometry to generate a standard curve in triplicate by real-time TaqMan PCR.

2.8. One-tube TaqMan RT-PCR

This procedure was used to assay the subsamples for calculation of individual and overall recoveries of PP7 during filtration. Twenty-five microliter of reaction contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, stabilized passive dye ROX (Applied Biosystems), 800 nM each of dATP, dCTP, dGTP and dTTP, 800 nM of each primer, 80 nM of the TaqMan probe, 6 U MMLV-RT (Applied Biosystems), 1.25 U of AmpliTaq Gold DNA polymerase, and 10 μL of the nucleic acid extract. Cycling conditions were 30 min at 48 °C and 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min using an ABI PRISM 7000 sequence detection system (Applied Biosystems).

2.9. Calculation of PP7 virus recovery

The partial viral recoveries for both large and small filtration systems, as well as the overall recovery for the overall procedure, were determined using the following general equation:

$$\text{Recovery}_{(\text{Filtration step})}(\%) = \left(\frac{\text{PP7 in sample}_{(\text{After filtration step})}}{\text{PP7 in sample}_{(\text{Before filtration step})}}\right) \times 100. \quad (4)$$

The overall recovery of PP7 (R) from both filtration systems was calculated as follows:

$$\text{Overall recovery for final concentrate (\%)} = \left(\frac{R_F}{F_{LS}}\right) \times 100. \quad (5)$$

2.10. Dilution approach

Extracted nucleic acid was diluted with sterile ddH_2O to determine PP7 recoveries by TaqMan and to assess the effects of PCR inhibition. The dilutions corresponding to C_t values out of the region of inhibition were used to perform a linear regression. The y-intercept of the straight-line equation corresponds to the original target number of PP7 (non-diluted sample).

2.11. Calculation of PP7 concentration in filtration subsamples

Several dilutions of the nucleic acid extracted from each of the subsamples were assayed by one-tube RT-PCR, and the C_t values were determined for replicates. Using the dilution approach as outlined above, the C_t was calculated for a non-diluted template, and this C_t value was substituted in Eq. (6) to calculate the number of PP7 replicase gene copies in the assay, N, based on the standard curve (see Fig. 2).

$$C_t = -3.25 \log(N) + 36.8. \quad (6)$$

Standard curves were reproduced on a regular basis and they did not vary. The total amount of PP7 in a subsample was calculated with Eq. (7) and used to determine the amount of target lost during the filtration process (see PP7 recovery efficiency).

$$\text{Number of PP7 replicase gene copies} = N \times \frac{1}{V_T} \times \frac{V_{el}}{V_{ex-sub}} \times V_{sub}, \quad (7)$$

where V (mL) represents the volumes of nucleic acid template added to the PCR reaction (V_T), of subsample that was extracted (V_{ex-sub}), of eluate from the nucleic acid extraction (V_{el}), and of the total subsample (V_{sub}).

2.12. PP7 assay limit of quantification (A_{LOQ}) and assay limit of detection (A_{LOD})

The A_{LOQ} (gene copies per reaction) was determined by preparing serial 10-fold dilutions of PP7 RNA in ddH_2O and quantifying by TaqMan RT-PCR to determine the lowest concentration of PP7 genomes that remained within the linear range of quantification. This approach is commonly used in real-time TaqMan PCR applications (Seurinck et al., 2005). The A_{LOD} (gene copies per reaction) was obtained for pure water by following the general approach outlined in the US EPA method 40 CFR 136, Appendix B (http://www.dec.state.ny.us/website/dow/bwp/ref750/40cfr136_c.html). First, an estimate was made of the A_{LOD} based on the previously obtained A_{LOQ}. A solution of PP7 that is 1–5 times the A_{LOQ}

was prepared in pure water and quantified 8 times by TaqMan PCR. The standard deviation of 2 similar concentrations was calculated and an *F*-test for 2-sample variance was performed on the 2 datasets to test the hypothesis that the difference between the variances is statistically insignificant. Next the sample variances were pooled and the A_{LOD} was calculated according to the formula: A_{LOD} = Student's $t \times$ pooled standard deviation.

2.13. *Calculation of sample limit of detection (S_{LOD})*

The S_{LOD} was calculated for each original volume of filtered water as

$$S_{LOD} = \frac{A_{LOD}\, I\, V_{el}\, V_{RF}}{V_T\, V_{RF,ex}\, V_S\, R_{filtration}\, E_{ex}}, \quad (8)$$

where V (mL) represents the volumes of final concentrated retentate (V_{RF}), of retentate that was extracted ($V_{RF,ex}$), of eluate from nucleic acid extraction (V_{el}), and of nucleic acid template added to the PCR reaction (V_T). The volume of the original water sample is V_S. Inhibition (*I*) represents the dilution necessary to produce a positive PCR result and is expressed as the inverse of the dilution factor (ranging from 1 to 100). The overall PP7 recovery, as a fraction, is represented by $R_{filtration}$, while E_{ex} (0–1) accounts for the nucleic acid extraction efficiency.

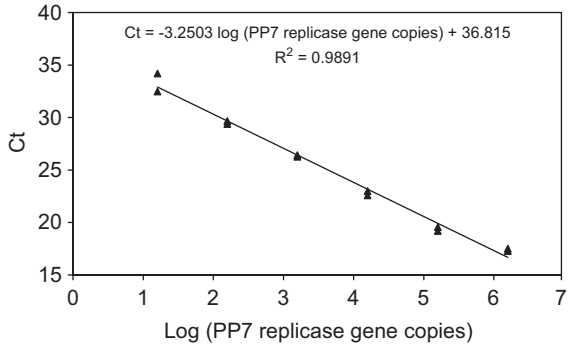

Fig. 2 – Standard curve for the detection of PP7 genomes by TaqMan RT-PCR using 4 reverse primers, 1 forward primer and 1 fluorescent TaqMan probe. C_t, threshold cycle number.

3. Results

3.1. *Analytical sensitivity and specificity of the PP7 TaqMan PCR system*

RT-PCR for PP7 was performed with each of the 4 different sets of reverse primers plus a combination of all primer sets to test the sensitivity and efficiency of amplification (Table 2). While only 3 concentrations are shown, linearity was observed over 5 orders of magnitude for all cases; therefore, the combined efficiency of reverse transcription and amplification remained constant for different target concentrations ranging from 16 to 1.6×10^6 gene copies per reaction volume. There was no significant difference observed in amplification efficiency for the individual primers as the slopes of the straight lines obtained for the different sets of primers were similar. The combination of the 4 reverse primers always produced lower C_t values, resulting in more sensitive detection, whereas the C_t values for PP7-366r or PP7-320r alone had lower standard deviations than did the combination of all 4 primers. A lower standard deviation is generally more preferable than a lower C_t value because broader reactivity by mixing primers may enhance variability of efficiency in amplification. However, the variability in efficiency was found to be minor; hence the 4-primer combination was used to construct a standard curve for the determination of the PP7 replicase gene copy number in an environmental water sample. The A_{LOQ} for PP7 using the combination of all 4 reverse primers (Fig. 2) was 6 gene copies per reaction volume by one-tube TaqMan RT-PCR. The A_{LOD} was 5 gene copies per reaction volume by one-tube RT-PCR. To make the methodology compatible with US EPA terminology (http://www.dec.state.ny.us/website/dow/bwp/ref750/40cfr136_c.html), the A_{LOD} value of 5 was used in all subsequent calculations of S_{LOD}. PP7 was not detected in non-spiked water samples.

3.2. *Dilution approach to account for PCR inhibition*

We designed a dilution approach to adequately account for the inhibition of *Taq* DNA polymerase in PCR reactions. The analysis of multiple dilutions also ensured that reverse transcription and amplification occurred with maximum efficiency. Different levels of inhibition were

Table 2 – C_t values for different amounts of PP7 from one-tube TaqMan RT-PCR[a]

Replicase gene (no. of copies)	Reverse primers				
	PP7-323r	PP7-355r	PP7-366r	PP7-320r	All 4 primers
1.6×10^6	18.72 ± 0.07	19.66 ± 0.13	19.01 ± 0.01	18.33 ± 0.13	17.39 ± 0.15
1.6×10^4	25.16 ± 0.31	24.71 ± 0.32	24.41 ± 0.07	24.16 ± 0.02	22.79 ± 0.29
1.6×10^1	35.94 ± 1.03	34.25 ± 1.01	34.03 ± 2.48	34.34 ± 0.35	33.30 ± 1.21
Efficiency[b]	0.97	1.15	1.11	1.03	1.03

[a] Mean (± SD).
[b] According to Eq. (3).

observed in nearly all of the subsamples, leading to an underestimation of the target RNA unless corrective measures were employed.

Samples ORI and DPA are examples of the effect of inhibition on TaqMan results (Fig. 3). A C_t value of 40 corresponds to a negative result by TaqMan RT-PCR. The first 2 dilution points for sample DPA were negative for PP7. Further dilution decreased the concentration of inhibitors in the sample, and the detection signal was recovered (third point on curve for DPA, Fig. 3). Successive dilutions were assayed to the point where inhibitors did not affect the efficiency of amplification, as indicated by the linear range. Prior to this point, the detection is positive but any calculation based on the given C_t values will underestimate the target number of PP7. Conversely, any calculation using C_t values from dilutions within the linear range will yield similar final target numbers. Using this approach, the C_t from the lowest dilution in the linear range could be used to calculate the number of PP7 gene copies, but such a calculation would only include 1 data point. Alternatively, the y-intercept of the linear regression equation can be used as the theoretical C_t value when there is no dilution ($\log 1 = 0$). The latter approach is more robust since it considers all data points within the linear range. To demonstrate these approaches, the actual PP7 replicase gene copy number for each point of the curve for the sample DPA was calculated. For dilutions within the linear range, the gene copy numbers ranged from 5.4×10^3 to 7.4×10^3. Using the y-intercept of the linear regression equation, a value of 5.3×10^3 gene copies was obtained.

Sample ORI did not contain a high concentration of inhibitors and the undiluted sample was positive for detection of PP7 RNA with $C_t = 30$. However, inhibitors impacted amplification until the third dilution step after which a linear range was obtained.

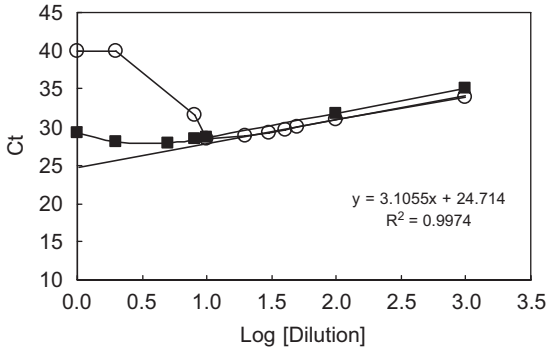

Fig. 3 – Dilution approach for the detection of PP7 by TaqMan RT-PCR for samples ORI (■) and DPA (○) exhibiting different levels of inhibition. Linear regression was performed for the DPA dilutions that were not affected by inhibition. The y-intercept corresponds to the calculated C_t for the undiluted sample in the absence of inhibition. A C_t value of 40 corresponds to a negative result by TaqMan RT-PCR. C_t, threshold cycle number.

3.3. PP7 recovery during ultrafiltration

The PP7 recovery was determined by plaque assay and TaqMan RT-PCR for each step during the concentration of water samples. While plaque assay estimates the number of infectious phages in the subsample that is assayed, all the phage particles (including non-infectious ones) extracted from the same subsample are detected by TaqMan RT-PCR.

Three different types of recovery were determined: individual recoveries from each step, partial recoveries from each filtration system, and the overall recovery from the total system. Analyses of the specific recoveries helped improve individual filtration steps. The recoveries obtained for the feed subsamples (F_{LS}) were always lower than 100% of the known spiked amount, and in some occasions lower than the corresponding recovery for the recirculated (RE_{LS}) subsample. Such behavior can be explained by noting that the spiked feed was sampled after 10 min of recirculation (no permeate flow). During that recirculation period, PP7 may have adsorbed to the lines, plastic feed tank, and filtration membrane, but it could later be recovered in the liquid phase (RE_{LS}) when the elution with glycine and Tween was performed. Analysis of the partial recoveries for the large system (using the known spiked amount as reference) were usually larger than 100%, both by plaque assay and by TaqMan RT-PCR, again reflecting a possibility of phages being attached to the lines, feed tank, and filter during the initial recirculation period, as explained previously.

Attachment of the virus to the plastic feed tank was confirmed by sampling the tanks' internal surfaces with sterile wet cotton swabs (data not shown). Other researchers reported that the use of a blocking agent prior to filtration consisting of a solution of 5% calf serum (Oshima, 2001) reduced binding of virus to the filters. In the present study, addition of this step not only resulted in reduced PP7 recovery, but also was time-intensive and expensive. To reduce loss of PP7 during mixing by recirculation and to avoid recovery results greater than 100%, the 50-L plastic feed container was replaced with a stainless-steel 100-L vessel with motorized mixing impellers, which resulted in overall recoveries (using feed and retentate subsamples) less than or equal to 100%, as would be expected.

Evaluation of the recoveries from each filtration step also revealed the importance of the recirculation step, during which glycine and Tween 80 are passed through the membrane repeatedly. This step was not initially performed on the small filtration system, but an elution step was added after analyzing the first set of samples. Elution on the small membrane was performed with the same glycine and Tween solution and by manual syringe pumping.

These improvements increased overall virus recovery and stabilized the feed (F_{LS}) PP7 concentrations. The original filtration procedure had an average recovery of $24 \pm 9\%$ (mean $\pm$ SEM) for 16 samples analyzed. The addition of a stainless steel tank and mechanical mixer increased this to $35 \pm 12\%$ for 14 samples. Finally, the incorporation of the elution step with the small system optimized the overall process, resulting in an average recovery of $64 \pm 4.8\%$ (mean $\pm$ SEM) for 22 samples. A one-way ANOVA confirmed a statistically significant difference ($P < 0.05$) between the

original procedure and the improved method including mechanical mixing and elution.

Permeate subsamples from both systems were negative for PP7 by plaque assay and TaqMan RT-PCR, confirming that the hollow fiber ultrafiltration was functioning properly. There was only one exception, for sample MEN, where 0.4% of the spiked amount was detected by TaqMan RT-PCR in the permeate.

Finally, adsorption of PP7 to solids was identified as a factor in lowering overall PP7 recovery. The final volume of concentrate from the large system was centrifuged to remove solids. Up to 10% of PP7 remained attached to solids in some samples, based on plaque assay and TaqMan RT-PCR mass balances of the subsamples.

3.4. *Effect of ultrafiltration on PP7 infectivity and E_{ex}*

The standard curve used for quantitative PP7 determinations by TaqMan RT-PCR refers to any phage particle, infectious or not. Thus the total phage titer in the plaque assay (PA) can be lower than that in the TaqMan RT-PCR assay (TQ) for the same sample, because not all phage particles in the spiked sample will be infectious and because more than 1 phage particle can infect a single bacterial host cell. To address whether the filtration procedure affected infectivity of the PP7 bacteriophage on the host *P. aeruginosa*, the overall PP7 recovery by plaque assay was calculated according to Eq. (7). The results for PP7 recovery by plaque assay and TaqMan are presented in Table 3 for the 22 samples that were concentrated using the optimized filtration process. The average overall PP7 recovery by plaque assay was slightly lower at 53 ± 3.5% (mean ± SEM) than the average recovery of 64 ± 4.8% by TaqMan. A Student's t-test showed the means to be statistically different ($P < 0.05$), indicating that the infectivity of PP7 was slightly affected by the filtration process. However, the plaque assay and RT-PCR TaqMan methods are inherently different, and each method introduces its own biases, which must be considered when comparing the methods.

During the plaque assay, the actual number of infective phage particles may be underestimated if plagues overlap. The impact of this bias is minimized by performing triplicate plating for multiple dilutions. In contrast, the TaqMan method relies on the efficiency of nucleic acid extraction and the quality of extracts. When analyzing the storm drain samples, F_{LS} and R_F represent 2 extremes in terms of concentrations of suspended solids, chemicals, and biological constituents that contribute to reduced extraction and purification efficiencies (Harry et al., 1999; Miller et al., 1999). In the feed, F_{LS}, there was no additional concentration inhibitory substances due to filtration, and TaqMan PCR amplification can be considered to have proceeded at greater efficiency than in the concentrated retentate, as indicated by

Table 3 – Overall recovery of PP7 and ratio between PP7 determined by TaqMan (TQ) and infectious PP7 determined by plaque assay (PA) for natural water samples, before and after concentration by ultrafiltration

Sample	Overall recovery (R)			Feed (F_{LS})		Final retentate (R_F)	
	TQ (%)	PA (%)	TQ/PA (gc pfu^{-1})[a,b]	I[c]	TQ/PA (gc pfu^{-1})	I	TQ/PA (gc pfu^{-1})
MAL-2	9.7	71.5	0.14	1	0.038	10	0.005
TRA-2	50.0	42.5	1.18	10	0.127	10	0.150
TPN-2	57.7	47.3	1.22	1	0.056	100	0.068
SMO-2	97.9	45.0	2.18	1	0.030	1	0.066
PCH-2	95.3	72.5	1.32	1	0.048	100	0.063
FO 2	75.7	38.9	1.94	1	0.049	50	0.095
MEN 2	82.0	59.6	1.38	1	0.092	10	0.127
MAD 2	53.3	33.0	1.62	1	0.082	10	0.133
SDN-3	65.0	42.8	1.52	5	0.356	50	0.540
SLR-3	68.1	42.2	1.61	5	0.082	50	0.133
SDR-3	37.3	33.7	1.11	5	0.011	100	0.012
CHO-3	77.8	70.3	1.11	5	0.283	50	0.313
ENC-3	44.1	52.2	0.85	1	0.012	50	0.010
EFS-3	87.9	89.7	0.98	2	0.053	10	0.052
TRA-3	87.8	46.9	1.87	1	0.210	50	0.392
MAL-3	76.7	80.4	0.95	1	0.086	50	0.082
TPN-3	74.9	53.5	1.40	2	0.761	50	1.065
SMO-3	45.8	52.9	0.87	2	0.063	50	0.055
CAR-3	53.0	66.4	0.80	2	0.051	10	0.040
ORI-3	82.1	44.5	1.84	1	0.174	10	0.320
CWC-3	44.8	50.6	0.89	2	0.400	10	0.354
COL-3	38.2	25.1	1.52	1	0.970	50	1.473

[a] gc: gene copy.
[b] pfu: plaque forming unit.
[c] Dilution factor required to overcome inhibition for Taqman detection.

the differences in inhibition factor values (I) (Table 3). The I values for F_{LS} ranged from 1 to 10, whereas the values for R_F ranged from 10 to 200. Inhibition of the TaqMan RT-PCR reactions was overcome using the dilution approach discussed previously and hence did not affect calculations.

It was also possible that inhibiting substances, which are elevated in the concentrated retentate compared to the original feed sample, would introduce a bias in the nucleic acid extraction and PP7 recovery calculations. Such an effect can be inferred from the ratio of viruses determined by each method (TQ/PA) for the original sample (F_{LS}) and the final concentrated sample (R_F) (Table 3). For a given sample, the TQ/PA should be constant for F_{LS} and R_F if the nucleic extraction efficiency and plaque infectivity remained constant. An increase in TQ/PA for the concentrated retentate would suggest a proportionate decrease in plaque infectivity, whereas a decrease in TQ/PA would indicate a proportionate decrease in the E_{ex}. For the 22 environmental samples presented in Table 3, all ratios remained constant or increased by up to a factor of 2, except for sample MAL-2. Hence there was no effect of sample filtration and inhibitor concentration on E_{ex}.

3.5. S_{LOD}

The S_{LOD} values were highly variable among samples (Table 4). There was no effect of physicochemical properties, which also varied widely for conductivity, turbidity and TSS, on S_{LOD} values as determined by regression analysis (results not shown). In contrast, the S_{LOD} value was strongly related ($r^2 = 0.89$) to the dilution required to relieve inhibition in TaqMan analysis as would be expected based on Eq. (8).

4. Discussion

4.1. A_{LOQ}, A_{LOD}, and S_{LOD}

The A_{LOQ} for PP7 was 6 gene copies per reaction volume by one-tube RT-PCR; the A_{LOD}, was 5 gene copies per reaction. This limit was lower than that reported for bacteriophage MS2 by other authors. For example, the detection limit of MS2 was 10^4 virions/mL or 50 virions per reaction volume using a microchip PCR array system, called Advanced Nucleic Acid Analyzer (ANAA), and verified by a TaqMan PCR assay. The detection limit was determined based on the lowest amount

Table 4 – Physicochemical properties, PP7 recovery by TaqMan, and sample limit of detection based on TaqMan analysis for samples processed through the optimized filtration system

County (in CA)	Sample	Runoff origin	pH	Conductivity (mS)	Turbidity (NTU)	TSS (mg L^{-1})	Recovery (%)	S_{LOD} (gc mL^{-1})[a]
Los Angeles	MAL-2	Urban/tidally influenced	8.52	2.71	4.70	5.47	9.7	305
	TRA-2	Urban	8.46	0.86	4.40	0.20	50.0	9
	TPN-2	Urban	8.34	0.01	16.2	9.23	57.7	145
	SMO-2	Highway	8.56	0.94	14.4	28.60	97.9	1
	PCH-2	Urban	8.5	1.44	18.6	29.13	95.3	154
Fresno	FNO-2	Highway	8.26	171	277	79.25	75.7	47
	MEN 2	Agricultural	8.56	95.8	164	74.75	82.0	10
	MAD-2	Highway	8.28	108	153	46.00	53.3	26
San Diego	SDN-3	Natural	8.33	0.05	0.0	0.40	65.0	80
	SLR-3	Urban	8.06	2.00	35.0	34.00	68.1	52
	SDR-3	Urban	8.27	0.013	44.0	21.00	37.3	200
	CHO-3	Urban	8.93	0.01	15.0	7.50	77.8	84
	ENC-3	Urban/tidally influenced	7.98	3.37	4.0	2.25	44.1	86
Los Angeles	EFS-3	Natural	8.83	365	10.1	3.20	87.9	6
	TRA-3	Urban	8.28	1145	1.0	0.87	87.8	51
	MAL-3	Urban/tidally influenced	8.08	3062	16.0	3.40	76.7	91
	TPN-3	Urban	8.61	1519	2.70	1.68	74.9	44
	SMO-3	Highway	8.85	1207	2.60	1.92	45.8	58
Solano	CAR-3	Urban/tidally influenced	8.17	1736	13.2	4.50	53.0	12
Contra	ORI-3	Urban	8.52	560	3.40	1.75	82.1	8
Alameda	CWC-3	Urban	8.62	630	7.50	3.83	44.8	12
	COL-3	Urban/tidally influenced	8.25	0.01	46.5	12.50	38.2	99

[a] gc, gene copy.

that could be measured (2) and hence corresponds to our definition of A_{LOQ}. When comparing limits of quantification (LOQs) and limits of detection (LODs) obtained in different studies it is important to consider how they are defined. The International Conference for Harmonization (ICH) proposed the term LOQ to determine assay sensitivity and defined the term Limit of Detection (LOD) as a concentration of analyte that is still detectable but not quantifiable because it is outside the linear response range of the instrument or method used (Moffat et al., 2000). We have followed this convention and hence use both terms, LOQ and LOD, for assay sensitivity and only the term LOD for sample limit of detection. Such definitions and their mathematical basis are important to state but are often not reported in the literature. By defining S_{LOD} values per sample and pathogenic organism analyzed, it should be possible to critically investigate the absence of detects for a particular pathogen and determine probabilities of risk associated with an S_{LOD} using risk-based analysis (Olzewski et al., 2005).

4.2. *Dilution approach*

Dilution of nucleic acid extracts to overcome inhibition of PCR amplification is commonly applied in environmental, clinical, and food analysis (Audemard et al., 2004; Drosten et al., 2002; Fode-Vaughan et al., 2003; Harms et al., 2003; Kulesh et al., 2004). In this work, successive dilutions were assayed for each subsample to the point where inhibitors did not affect the efficiency of amplification, as indicated by the linear range for sample DPA in Fig. 2. For all 56 environmental samples analyzed for the internal standard PP7, a linear range for amplification was observed. Using this approach, we were able to overcome inhibition and quantify PP7 for all samples.

Inhibition of reverse transcriptase has been reported for RT-PCR reactions with template concentrations less than 10^5–10^6 copies of starting RNA (Chandler et al., 1998). For our experiments, RT-PCR was performed in 1 tube, and multiple dilutions and starting concentrations were analyzed for each sample. Since reverse transcription is done for each dilution, any inhibition of reverse transcription should be observed as nonlinear amplification as the template concentration decreases. According to our dilution approach, any dilution exhibiting such RT-inhibited reactions would not be included in final calculations. The non-diluted retentate samples had 10^4–10^5 starting copies of RNA, and a linear range of amplification was typically observed through dilutions to 10^{-2} and 10^{-3} as shown in Fig. 3. These results indicate that inhibition of the RT step was not a major concern.

The approach outlined here may be used to obtain amplification of pathogen cDNA and DNA. However, for environmental samples, there may not be amplification for more than 1 dilution if there are very low target numbers in the sample combined with a high degree of inhibition of RT-PCR. In this case, C_t values would be used to calculate pathogen numbers from reactions that are still subject to inhibition, with efficiencies of amplification less than that of the standard curve. Such a PCR result should be qualified and can underestimate the true target number due to inhibition and molecular sampling error (Chandler, 1998). By developing and validating nucleic acid extraction methods, which efficiently remove PCR inhibitors, the underestimation of pathogens at very low numbers may be corrected.

4.3. *PP7 recoveries*

Recoveries of PP7 in the subsamples were monitored both by plaque assay and TaqMan PCR for all water samples. There were no correlations between those recoveries and other properties measured in the original water sample. However, there was a clear benefit, in terms of recovery as estimated by TaqMan or plaque assay analysis, in performing a glycine elution step after concentration in the large filtration system (LS). The recovery of PP7 for the subsample after the glycine recirculation step (RE_{LS}) was 1.2–54 times greater compared to the recovery before glycine recirculation. In contrast, the elution of the ultrafiltration membrane did not affect the overall PP7 recovery unless the sample contained many solids (up to 15%). Additionally, the information related to the permeate subsamples for both systems was useful as a quality control test of the integrity of the ultrafiltration membrane, since PP7 virus should not pass through the membrane and into the permeate.

Few studies have used large-scale filtration and spiked surrogate viruses to determine recoveries as a prerequisite to quantification of human viruses. For example, spiking with Poliovirus Sabin type 1 was carried out on 2 samples to determine the efficiency of concentration and extraction of water samples (Donaldson et al., 2002), but these values were not incorporated into calculations of human viral pathogen concentrations. Most published procedures make use of only 1 dilution step and do not characterize inhibition as part of the sample limit of detection. In a recent study by Fuhrman et al. (2005), filtration of small volumes of water (0.5–1 L) using cellulose acetate/nitrate filters was coupled with real-time RT-PCR of enteroviruses. Varying amounts of poliovirus were spiked with better recoveries, around 50%, when higher amounts of poliovirus were added. Recovery efficiencies were calculated by including filtration, extraction and PCR inhibition variables and by using an average correction factor, but the authors did not provide a mathematical approach for their procedure (Fuhrman et al., 2005). Recently developed real-time PCR and nucleic acid sequence-based amplification (NASBA) assays that estimate enteroviruses and noroviruses in large-volume water samples did not report recoveries of surrogates or sample limits of detection (Lodder and Husman, 2005; Rutjes et al., 2005) but acknowledged that some form of surrogate spiking would be necessary for quantitative detection (Rutjes et al., 2005).

Our ultrafiltration method was based on the original design of Oshima and colleagues (Morales-Morales et al., 2003; Olzewski et al., 2005; Oshima 2001; Winona et al., 2001). The main process modification was the omission of the pre-blocking stage, which in our hands produced lower recoveries and increased processing time and expenses. Recoveries of PP7 were comparable at $64 \pm 3.8\%$ (mean $\pm$ SEM) in this study and reported values of $\geqslant 70\%$ for a large-scale hollow-fiber ultrafiltration system capable of filtering 100 L (Olzewski et al., 2005). A smaller unit recovered from 62.5% to 76.1% of spiked PP7 bacteriophage (Olzewski et al., 2005). However, there are major differences in how the systems were operated and

recoveries were calculated. First, in the present study a large-scale (100 L) and a small-scale (2 L) filtration step were combined with the intention of reducing the final hold-up volume to about 50 mL. Sequential filtration was deemed necessary to improve the ability to detect very low numbers of target pathogens. The hold-up volume after l00 L has been filtered is about 1.5 L. Second, recoveries were estimated from plaque assays in the studies by Oshima and colleagues but were calculated based on a newly designed real-time TaqMan RT-PCR assay for PP7 in the present study, with the rationale that PCR techniques are increasingly used to detect pathogens in the aqueous environment. Subsequently, these recoveries can be used to calculate sample limits of detection (S_{LOD}) for human pathogenic viruses.

In the past PCR results for water samples have often been reported as positive or negative, without regard to limits of detection (LODs) associated with individual samples. However, LODs are important considering that contact with contaminated water may pose a health risk if concentrations of pathogens reach a critical level (Loge et al., 2002). Factors that determined S_{LOD} in our study included volume of original sample, PP7 recovery efficiency, final volume of retentate, volume of sample extracted, eluted nucleic acid volume, E_{ex}, volume of nucleic acid added to PCR reaction, and inhibition of PCR. Of these, sample volume, recovery efficiency, E_{ex}, and PCR inhibition had the greatest effect on detection limits. All of the aforementioned factors were considered when calculating detection limits for stormwater samples. However, the E_{ex} was not optimized for the recovery of PP7 RNA because the objective was to use only one method of nucleic acid extraction for the detection of a range of human viruses containing either RNA or DNA and other pathogenic organisms. Further, it must be noted that E_{ex} will vary somewhat with target concentration and hence it can be expected to be higher for PP7 compared to viral pathogens. Improvements in extraction procedures of DNA and RNA would further decrease S_{LOD} values for specific pathogens, a feature that may be desirable if the pathogens of interest are more narrowly defined.

5. Conclusions

- A TaqMan PCR-based approach was validated to quantify microbial targets present at low numbers in natural waters. Bacteriophage PP7 was chosen as the internal standard for environmental analysis of viruses, and other surrogates should be used when bacteria or protozoa are the target organisms. The hollow fiber ultrafiltration method for concentration of large volumes of water proved reliable in our hands after several modifications, including sequential filtration steps to reduce the hold-up volume. The procedure facilitates low sample limits of detection and provides a high degree of confidence in the data obtained when human pathogens are determined to be absent or present in low numbers. Additionally, filtration of large volumes of water statistically improves the chances of finding pathogens when a grab or composite sample is taken. The ultrafiltration systems are appropriate for the fast concentration of water samples.
- The methodology is simple, and the portable design offers the advantage of performing the concentration step in the field, thus diminishing the risks and the costs of transportation and handling of large volumes of sample. Virus determinations were obtained within 8 h from sampling to results.
- The recovery efficiencies for the filtration step were variable and sample dependent. TaqMan RT-PCR recovery analysis requires a careful consideration of nucleic acid extraction efficiencies, detection limits, and PCR inhibition. E_{ex} was not optimized in this work but should be the subject of further methodological research because of the difficulties of detecting low levels of viruses and other pathogens in concentrated water samples.
- Sample limit of detection (S_{LOD}) values were highly variable among samples. By defining S_{LOD} values per sample and pathogenic organism analyzed, it should be possible to critically investigate the absence of detects for a particular pathogen and determine probabilities of risk associated with a specific S_{LOD}.
- The advantages of using quantitative real-time TaqMan RT-PCR are speed, sensitivity, and specificity of detection. The method also provides the versatility and potential to extend surrogate recovery to the final calculation of microbial target numbers in the original water samples. In addition, the recovery efficiency analysis was helpful in identifying pitfalls during the filtration steps as well as in introducing modifications to the design.

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