Comparison of enterovirus and adenovirus concentration and enumeration methods in seawater from Southern California, USA and Baja Malibu, Mexico

Lauren M. Sassoubre, David C. Love, Andrea I. Silverman, Kara L. Nelson and Alexandria B. Boehm

ABSTRACT

Despite being important etiological agents of waterborne illness, the sources, transport and decay of human viruses in recreational waters are not well understood. This study examines enterovirus and adenovirus concentrations in coastal water samples collected from four beaches impacted by microbial pollution: (1) Malibu Lagoon, Malibu; (2) Tijuana River, Imperial Beach; (3) Baja Malibu, Baja California; and (4) Punta Bandera, Baja California. Water samples were concentrated using a flocculation-based skim milk method and dead-end membrane filtration (MF). Viruses were enumerated using cell culture infectivity assays and reverse transcription quantitative polymerase chain reaction (RT-QPCR). Across concentration and quantification methods, enteroviruses were detected more often than adenoviruses. For both viruses, MF followed by (RT)QPCR yielded higher concentrations than skim milk flocculation followed by (RT)QPCR or cell culture assays. Samples concentrated by skim milk flocculation and enumerated by (RT)QPCR agreed more closely with concentrations enumerated by cell culture assays than MF followed by (RT)QPCR. The detection of viruses by MF and (RT)QPCR was positively correlated with the presence of infectious viruses. Further research is needed to determine if detection of viruses by rapid methods such as (RT)QPCR can be a useful water quality monitoring tool to assess health risks in recreational waters.

Key words | adenovirus, beach water quality, cell culture, enterovirus, QPCR

Lauren M. Sassoubre
Alexandria B. Boehm (corresponding author)
Environmental and Water Studies,
Department of Civil and Environmental Engineering,
Stanford University,
Stanford,
CA 94305-4020,
USA
E-mail: *aboehm@stanford.edu*

David C. Love
Andrea I. Silverman
Kara L. Nelson (corresponding author)
Department of Civil and Environmental Engineering,
University of California Berkeley,
Berkeley,
CA 94720-1710,
USA
E-mail: *nelson@berkeley.edu*

INTRODUCTION

Globally, exposure to coastal waters polluted with wastewater causes an estimated 120 million gastrointestinal and 50 million severe respiratory illnesses every year (Shuval 2003). Collectively, these illnesses are referred to as recreational waterborne illness. Human viruses are believed to be the main etiologies of recreational waterborne illness (World Health Organization 2003). Human viruses are not routinely monitored in recreational waters, however. Instead, fecal indicator bacteria, *Escherichia coli* and enterococci are routinely measured to assess risk of illness because their concentrations correlate to swimmer illness in epidemiology studies (Kay *et al.* 1994; Wade *et al.* 2003). Despite their link to risk, fecal indicator bacteria are generally not pathogens nor do they universally correlate to concentrations or the occurrence of human viruses in coastal waters (Jiang *et al.* 2001; Noble & Fuhrman 2001; Jiang & Chu 2004; Boehm *et al.* 2009). To better understand health risks to bathers, there is great interest in understanding the sources, transport and decay of human viruses in coastal environments.

Virus concentrations are typically low in coastal waters; samples therefore need to be concentrated before detection. A number of concentration methods have been developed: (1) glass wool filtration (Vilaginès *et al.* 1997); (2) dead-end membrane filtration (MF) with (Katayama *et al.* 2002) and without (Fuhrman *et al.* 2005) additives such as $MgCl_2$ and

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pH adjustors; (3) hollow fiber tangential filtration (Rajal et al. 2007a, b); (4) polyethylene glycol (PEG) precipitation (Jaykus et al. 1996); (5) skim milk concentration (Calgua et al. 2008); and (6) filtration through electropositive filters (Sobsey & Glass 1980). Some of these methods such as hollow fiber tangential filtration are suited for concentrating very large volumes of water (100 L or more), while other methods such as dead-end MF are only capable of concentrating relatively small water volumes (≤1 L). Large-volume concentration can be used to lower detection limits, but it also has the potential to co-concentrate inhibitory compounds for infectivity assays and molecular assays (Rajal et al. 2007a).

After concentration, human viruses have traditionally been enumerated in water samples using cell culture infectivity assays. Permissive cell lines are available for most enteric and respiratory viruses; important exceptions are norovirus (Duizer et al. 2004), for which no reliable infectivity assay is available, and wild-type hepatitis A virus which grows poorly in culture (Konduru & Kaplan 2006). When implemented in the plaque assay format, it is estimated that between one and 10,000 virions give rise to one plaque-forming unit (PFU), depending on the virus (Flint et al. 2004). In culture, human viruses such as adenovirus can take up to 1 week to infect, reproduce and lyse host cells (Lipp et al. 2001); the required time between sample collection and obtaining results is not practical for routine recreational water-quality monitoring.

Rapid molecular methods are available for most human viruses using quantitative polymerase chain reaction (QPCR) and reverse transcription-QPCR (RT-QPCR), although these methods measure viral DNA or RNA and, alone, cannot distinguish infectious from non-infectious viruses. Hybrid cell culture/molecular detection method assays have been developed to preferentially detect infectious viruses (Ko et al. 2003; Reynolds 2004; Wyn-Jones et al. 2011); these assays typically require a shorter incubation time and do not require the development of cytopathic effects (CPE).

In the present study, we evaluated several virus concentration and quantification methods for enumerating enteroviruses and adenoviruses in recreational water samples from four sites along the Southern Californian and Baja Mexican coast. Two different concentration techniques, flocculation-based skim milk concentration method and dead-end MF method, were compared. Both cell culture infectivity and QPCR or RT-QPCR methods were utilized for virus detection.

MATERIALS AND METHODS

Sample collection

Samples were collected at four sites: (1) Malibu Lagoon, Malibu, CA (ML); (2) the mouth of the Tijuana River, Imperial Beach, CA (TJ); (3) Baja Malibu, Baja, Mexico (BM); and (4) Punta Bandera, Baja, Mexico (MEX) (Figure 1). These four sites have a history of microbial pollution. Malibu Lagoon is potentially impacted by several non-point sources (including wildlife, runoff, beach sand and contaminated groundwater) and Tijuana River is adversely impacted by sewage and urban runoff from Tijuana and Imperial Beach (Reynolds et al. 1998). The Mexican sites are adjacent to Punta Bandera Creek, which discharges a mixture of raw and treated wastewater from the Punta Bandera waste stabilization pond treatment plant.

TJ, BM, MEX and ML were sampled before the sun rose (AM) and after high noon (PM) on a single day between 11 and 13 August 2009, for a total of eight samples. If there was fog at the site, then the PM sample was collected after the fog burned off. At the Tijuana River site, AM and PM samples were collected at the end of the ebb tide. At the time of sampling, the Malibu Lagoon was not open to the coastal ocean; the exit of the lagoon was blocked by a large sand berm.

At all sites except for the Malibu Lagoon site, samples consisted of five 20 L samples collected every 5 min from the exact same location in 10% HCl acid-washed, triple-rinsed cubitainers. At Malibu Lagoon, five 20 L samples were collected at different locations throughout the lagoon. Five samples were collected at each sampling time and location to capture temporal or spatial variation in water quality (Boehm 2007). Water samples were transported in the dark in coolers at 4 °C and processed immediately upon returning to a temporary laboratory at the Tijuana Estuary National Estuarine Research Reserve (Tijuana NERR). Sample processing was completed

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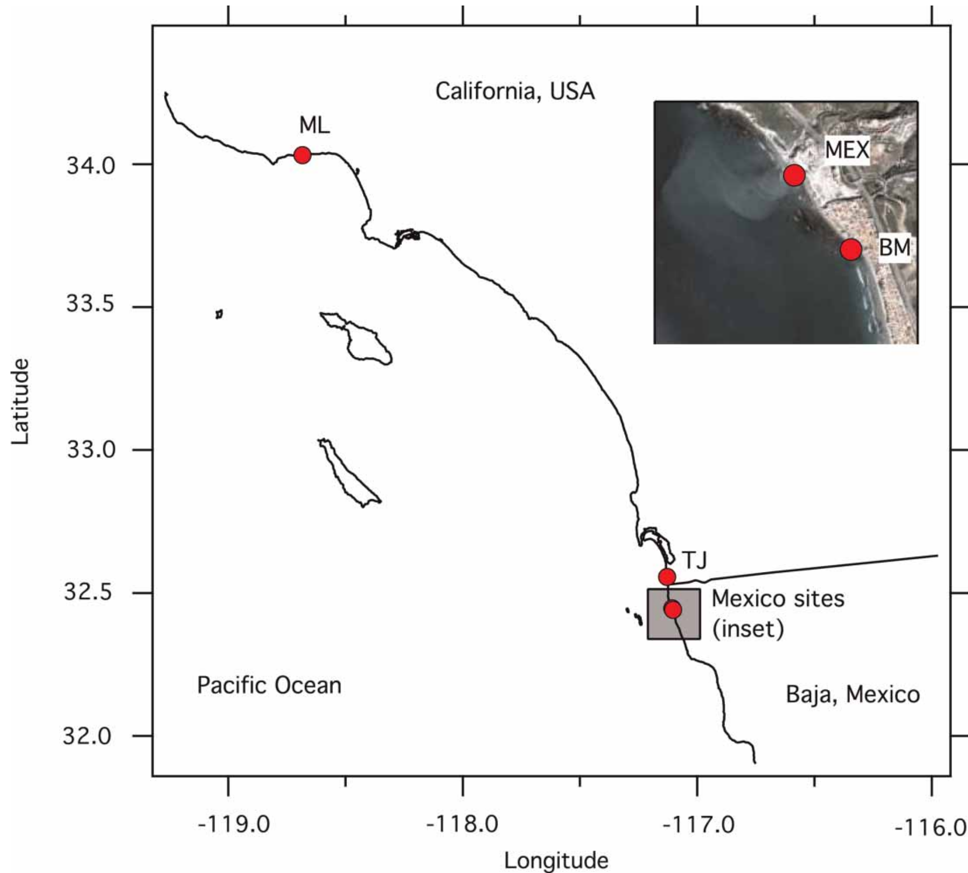

Figure 1 | Map of study sites.

within 2–12 h of sampling, depending on the distance from the site to the laboratory. At the laboratory, 20 L composite samples were created by combining 4 L from each of the five grab samples from each sampling time period. Viral analyses were conducted on these eight composite samples.

Concentration of water samples by dead-end MF

Adenoviruses and enteroviruses were co-concentrated from composite samples by dead-end MF. Between 20 and 1,000 mL of sample (depending on turbidity) were filtered through mixed cellulose HA filters with a pore size of 0.45 μm and a diameter of 47 mm (Millipore, MA) (Fuhrman *et al.* 2005). With every set of samples, a sample processing control (a field blank) was made by filtering sterile water in the same manner as environmental water samples. Filters were stored at −80 °C until analysis.

Concentration of large-volume water samples by skim milk flocculation

Composite water samples (18 L) were concentrated using a skim milk flocculation procedure (Calgua *et al.* 2008). The flocculent was a 1 N HCl acidified solution (pH 3.5) of dissolved skim milk powder (1% wt/vol) in sterile deionized water containing 35 g NaCl, stored at 4 °C until used. Skim milk solution was added to a final concentration of 0.01% (wt/v) to 18 L composite seawater samples in 20 L cubitainers. Samples were mechanically stirred for 8 h then allowed to rest for 8 h for sedimentation of skim milk proteins and viruses; both steps occurred at room temperature. The majority of the supernatant was decanted carefully with a sterile 1 mL pipette tip attached by a rubber hose to a 5 L vacuum flask (i.e. side-arm flask), while the remaining c. 400 mL supernatant and virus containing sediment was collected into a sterile 500 mL

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centrifuge tube. This concentrate was centrifuged at 7,000 × *g* for 30 min at 12 °C and the pellet was resuspended in 11–22.5 mL phosphate buffered saline (pH 7.5) and stored at –20 °C. The method achieved a 800-fold to 1,600-fold concentration.

Fecal indicator bacteria

E. coli and enterococci were measured by MF on mTEC and mEI media, respectively, following US Environmental Protection Agency (USEPA) standard methods (USEPA 2002, 2006). Between 0.1 and 100 mL were filtered, depending on the expected concentration.

Nucleic acid extraction

Adenovirus DNA was extracted from 750 µL of skim milk concentrates using the Ultra Clean Soil DNA kit (MoBio, Carlsbad, CA) following manufacturer instructions. Enterovirus RNA was extracted from 280 µL of skim milk concentrates using the QIAamp Viral RNA extraction kit (Qiagen, Valencia, CA) following the extraction method used by Calgua *et al.* (2008). Both enteroviruses and adenoviruses concentrated by MF were extracted from the filters using the Qiagen All Prep DNA/RNA Micro Kit (Qiagen, Valencia, CA). Qiagen Buffer RLT with β-mercaptoethanol and carrier RNA were added directly to the filter. The lysate was then transferred to the AllPrep DNA spin column and manufacturer instructions were followed. An extraction blank (an extraction carried out without a filter) was carried through to verify that reagents were not contaminated with the target DNA/RNA, and that there was no cross contamination during extractions. Viral nucleic acid extracts were frozen at –80 °C until analysis.

Enterovirus RT-PCR

Enterovirus RT-PCR was performed for confirmation of cell culture positive samples. RT-PCR was performed using a Qiagen RT-PCR buffer (Valencia, CA), dNTPs and enzyme mix and primers from (Jaykus *et al.* 1996; Table 1). Cycling parameters included 50 °C for 30 min, then 94 °C for 15 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and finally 72 °C for 10 min. A positive (RNA with the target sequence) and a negative control (no template control made from RNA-free water) were run in every set of reactions. Amplicons were detected on a 2% agarose gel with ethidium bromide staining.

Table 1 | Primer and probe sequences used in QPCR and RT-QPCR assays. FAM refers to a 6-carboxyfluorescein dye at the 5′ end. TAMRA refers to a 6-carboxytetra-methyl-rhodamine quencher dye at the 3′ end. BHQ refers to the black hole quencher dye used at the 3′ end. The mixed base N in degenerate primers pairs with any base

Target and method	Primer ID from original article	Sequence (5′ to 3′)	Reference
Enterovirus by RT-QPCR	EVupstream (5′)	CCTCCGGCCCCTGAATG	DeLeon *et al.* (1990); Gregory *et al.* (2006); Walters *et al.* (2009)
	EVdownstream (3′)	ACCGGATGGCCAATCCAA	
	Hydrolysis probe	FAM-ACGGACACCCAAAGTAGTCGGTTC-BHQ	
Enterovirus by RT-PCR	5′ primer	CCTCCGGCCCCTGAATG	Jaykus *et al.* (1996)
	3′ primer	ACCGGATGGCCAATC	
Adenovirus by QPCR	JTVXF (forward)	GGACGCCTCGGAGTACCTGAG	Jothikumar *et al.* (2005)
	JTVXR (reverse)	ACNGTGGGGTTTCTGAACTTGTT	Jothikumar *et al.* (2005); Viau *et al.* (2011)
	Hydrolysis probe	FAM- CTGGTGCAGTTCGCCCGTGCCA -BHQ	Jothikumar *et al.* (2005)
Adenovirus by nested PCR	First round: hexAA1885	GCCGCAGTGGTCTTACATGCACATC	Pina *et al.* (1998)
	First round: hexAA1913	CAGCACGCCGCGGATGTCAAAGT	
	Nested: nehexAA1893	GCCACCGAGACGTACTTCAGCCTG	
	Nested: nehexAA1905	TTGTACGAGTACGCGGTATCCTCGCGGTC	

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Enterovirus RT-QPCR

Enteroviruses were enumerated in skim milk concentrates and MF concentrates by reverse transcription-QPCR (RT-QPCR) on an Applied Biosystems StepOnePlus thermocycler using TaqMan® RNA-to-Ct™ 1-Step Kit (Applied Biosystems, CA) (Walters *et al.* 2009). Twenty microliter reactions consisted of 2 μL of RNA template, 1 × TaqMan RT-PCR Mix, 1 × TaqMan RT Enzyme Mix, 400 nM of each forward and reverse primer and 200 nM probe (Table 1) (Walters *et al.* 2009). Cycling parameters included a 15 min RT step at 48 °C, followed by a 10 min denaturation step at 95 °C and then 45 cycles of 95 °C for 15 s and 60 °C for 60 s. Fluorescence data were analyzed using Applied Biosystems StepOnePlus software v2.0 and the threshold was set at 0.02. Positive (RNA with the target sequence) and negative controls (no template controls made with RNA-free water) were run with every set of samples. RNA standards were made by *in vitro* transcription (MAXIscript *In Vitro* Transcription Kit, Life Technologies, Grand Island, NY) of linearized plasmids extracted from an *E. coli* clone inserted with the enterovirus target (Gregory *et al.* 2006; Walters *et al.* 2009). Transcripts were DNase treated and purified with the MEGAclear Kit (Life Technologies, Grand Island, NY). RNA standards were quantified using a Nanodrop-1000 (Thermo Scientific, Wilmington, DE) and serially diluted to make standard curves. Standard curves were run in triplicate with every set of samples. Standard curves were 'pooled' and used to translate quantification cycles (C_q) to copy numbers based on linear regression (Sivaganesan *et al.* 2010). Copy numbers associated with RNA standard concentrations were based on calculations accounting for the size of the linearized plasmid. The limit of quantification (LOQ) was set by the average C_q of the lowest standard that amplified (approximately three copies per reaction at an average C_q of 37.9 cycles). In units of copies per 100 mL water sample, the LOQ varied with the volume concentrated at each site, as well as with the nucleic acid extraction method used. For enterovirus detection using MF, the LOQ ranged from 20 copies/100 mL to 419 copies/100 mL, depending on the site. For enterovirus quantification using skim milk concentration, the LOQ ranged from 51 copies/100 mL to 105 copies/100 mL. A sample was designated as below the limit of quantification (BLOQ) if the RT-QPCR reaction showed some amplification but was below the LOQ.

Adenovirus nested PCR

Because PCR inhibition can affect QPCR more so than nested PCR (Love *et al.* 2010), a nested PCR method described by Pina *et al.* (1998) (Table 1) was applied to the DNA obtained from the skim milk and MF concentration methods. This method was also used to confirm positives and negatives from the cell culture infectivity assay. A positive (DNA with the target sequence) and a negative control (no template control made from DNA-free water) were run in every set of reactions. Amplicons were detected on a 2% agarose gel with ethidium bromide.

Adenovirus QPCR

Adenoviruses in skim milk concentrates and MF concentrates were enumerated on an Applied Biosystems StepOnePlus real-time PCR system using TaqMan QPCR (Jothikumar *et al.* 2005) (Table 1). Twenty microliter reactions consisted of 2 μL DNA template, 2 × FastStart universal probe master mix with ROX (Roche Diagnostics, Indianapolis, IN), 0.6 mg mL^{-1} BSA, 1.5% PVP24 (Sigma, St. Louis, MO), 250 nM forward and reverse primers and 125 nM probe. Thermocycling parameters included 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 55 °C for 1 min. Fluorescence data were analyzed using Applied Biosystems StepOnePlus software v2.0 and the threshold was set at 0.05. Positive (DNA with the target sequence) and negative controls (no template controls made with DNA-free water) were run with every set of samples. Standards were generated from *E. coli* plasmid DNA with the adenovirus target insert and standard curves were run in triplicate with every set of samples. All standard curves were 'pooled' and then used to quantify samples (Sivaganesan *et al.* 2010). The LOQ was set by the average C_q of the lowest standard that amplified (approximately 10 copies per reaction at an average C_q of 34.8). In units of copies per 100 mL water sample, the LOQ varied with the volume concentrated at each site as well as with the method of concentration used. For adenovirus detection using MF, the LOQ ranged from 32 copies/100 mL to 922

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copies/100 mL. For adenovirus quantification using skim milk concentration, the LOQ ranged from 33 copies/ 100 mL to 100 copies/100 mL. A sample was designated as BLOQ if the QPCR reaction showed some amplification but was below the LOQ.

PCR inhibition

To assess inhibition, a subset of nucleic acid extracts obtained from the different sites using different concentration and nucleic acid extraction techniques were spiked with a known number of enterovirus RNA or adenovirus DNA plasmid standards. A subset of samples both positive and negative for enteroviruses and adenoviruses were spiked with the corresponding standards and rerun to assess for inhibition using the method of standard addition described by Yamahara *et al.* (2009). This technique requires that the amount of standard added to the sample be of a similar magnitude to the amount of target measured, if any, in the sample (Yamahara *et al.* 2009). While this paper does not quantify and correct for inhibition, inhibition is qualitatively reported. If the measured concentration of the spiked sample was an order of magnitude less than the concentration expected given the amount of spike added to the extract, then the sample was considered inhibited.

Infectivity assays using skim milk concentrate

To detect infectious viruses by cell culture, skim milk concentrates were chloroform extracted (1:1 vol:vol), centrifuged at 7,500 × g for 15 min and the supernatants were recovered for immediate use in tissue culture infectivity. Viruses from MF filters could not be assayed in tissue culture because the DNA or RNA was extracted from the filters.

To detect adenoviruses, 50 μL inoculums of samples were plated on 96-well plates of 95% confluent cell lines (A549 and HEK-293 cells) using a five-dilution, six-replicate most-probable number (MPN) assay. CPE was scored, and positive dilutions were re-plated on fresh cells as a confirmation step. CPE positive wells were assayed by nested PCR to confirm that cell death was caused by adenoviruses (Pina *et al.* 1998). Wells in the same dilution and with the same CPE status (positive or negative) were pooled to create composite samples for nested PCR analysis. Cross reactivity between viruses and cells lines was not tested.

To detect enteroviruses by cell culture, a similar MPN assay was performed using 96-well plates of 95% confluent buffalo green monkey kidney (BGMK) cells (kindly provided by Dr Mark Sobsey, University of North Carolina). CPE was scored, and positive dilutions were re-plated on fresh cells. CPE positive samples were also assayed by RT-PCR (Jaykus *et al.* 1996) as composites from positive dilutions to confirm that cell death was caused by enteroviruses.

RESULTS

Adenovirus

Adenoviruses were concentrated from water using MF and skim milk flocculation (milk) and measured by conventional (nested) PCR, QPCR and infectivity assay using cell lines HEK-293 and A549. Hereafter, concentration and enumeration methods will be abbreviated. For example, a viral concentration measured in skim milk concentrate using cell culture methods will be abbreviated to ‘milk-cell culture’ while a concentration measured by QPCR after MF will be referred to as ‘MF-QPCR’.

All techniques were in agreement that adenovirus was not present above detectable levels at sites TJ and ML (Table 2). At site BM, milk-QPCR and milk-cell culture methods were negative for AM and PM samples; however, milk-nested PCR, MF-nested PCR and MF-QPCR were positive. As measured by MF-QPCR, the concentrations of adenoviruses at BM were 629 and 515 copies/100 mL in the AM and PM samples, respectively. At site MEX, adenovirus was detected using all techniques. The MEX samples concentrated by skim milk flocculation were positive for adenovirus by QPCR (496 and 190 copies per 100 mL in AM and PM, respectively), the HEK cell line (460 and 33 MPN/100 mL in AM and PM, respectively) and the A549 cell line (280 MPN/100 mL in the AM sample, no detection in the PM sample). All cell culture positives were confirmed by nested PCR. The MEX PM sample concentrated by skim milk flocculation was positive by nested PCR, but the MEX AM sample was not. The MEX samples

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Table 2 | Adenovirus presence and concentration in composite seawater samples by membrane filtration and skim milk flocculation. The detection limits varied by concentration and enumeration method: 30 MPN/100 mL for the cell culture assays and between 33 and 100 copies/100 mL (skim milk) and 32 and 922 copies/100 mL (membrane filtration) for the QPCR assays

	Membrane filtration		Skim milk flocculation		Cell culture (MPN/100 mL)	
Sample ID	Nester PCR	QPCR (copies/100 mL)	Nested PCR	QPCR (copies/100 mL)	HEK[c]	A549[c]
TJ am	–	BLOQ[a]	–	ND	<30	<30
TJ pm	–	ND[b]	–	ND	<30	<30
ML am	–	ND	–	ND	<30	<30
ML pm	–	ND	–	ND	<30	<30
BM am	+	629	+	ND	<30	<30
BM pm	+	515	+	BLOQ	<30	<30
MEX am	+	352,489	–	496	460	280
MEX pm	+	149,036	+	190	33	<30

[a]Below the limit of quantification (BLOQ) indicates that the RT-QPCR reaction showed some amplification but was not quantifiable.
[b]Non-detect (ND) indicates that the RT-QPCR reaction for that sample showed no amplification.
[c]Cell culture presumptive positives and negatives were confirmed by nested PCR.

concentrated by MF were both positive by nested PCR and QPCR with concentrations of 3.5×10^5 copies/100 mL and 1.5×10^5 copies/100 mL in AM and PM samples, respectively.

The MEX samples were the only samples positive by multiple measurement techniques for adenovirus, allowing for methods to be compared. The milk-QPCR results agreed very well with the cell culture results, particularly those obtained with the HEK cell line; concentrations in copies per 100 mL nearly matched or were within one order of magnitude of concentration in MPN per 100 mL. The MF-QPCR method measured concentrations three orders of magnitude higher than the milk-QPCR method. Milk-HEK293 detected slightly higher concentrations than milk-A549. For example, in MEX AM, 460 MPN/100 mL were measured by milk-HEK293 and 280 MPN/100 mL by milk-A549. In the MEX PM, 33 MPN/100 mL and less than 30 MPN/100 mL were measured by milk-HEK293 and milk-A549, respectively.

Representative DNA extracts were spiked with a known copy number of plasmid adenovirus target to test for inhibition. MEX AM, BM PM and ML PM DNA concentrated by the skim milk method were spiked with 1,000 copies of target and we found no evidence of inhibition. BM AM and ML PM DNA concentrated by MF were spiked with 200 copies of target and we found no evidence for inhibition.

Enterovirus

Enteroviruses were concentrated from water using MF and skim milk flocculation and measured by RT-QPCR and infectivity assay using the BGMK cell line.

All samples that were concentrated by skim milk were positive for enterovirus using cell culture, except for the PM sample collected at ML; concentrations ranged from 0.9 to 102 MPN/100 mL. In contrast, only one skim milk sample was quantifiable for enterovirus by RT-QPCR: the TJ AM sample had 121 copies/100 mL. Both MEX AM and PM samples and the BM PM sample were positive for enterovirus by milk-RT-QPCR but BLOQ (designated BLOQ in Table 3). All samples concentrated using MF were positive for enterovirus by RT-QPCR; concentrations ranged from 60 copies/100 mL at the TJ site to over 10^4 copies/100 mL at the MEX site. Concentrations measured by MF-RT-QPCR and milk-BGMK were significantly positively correlated ($r_p = 0.82$, $p < 0.05$, $n = 8$). However, MF-RT-QPCR measurements were 1.9 log units higher than milk-BGMK concentrations ($p < 0.05$, paired t-test) (Table 3).

Representative RNA extracts obtained from the milk concentrate and MF methods were spiked with known copy numbers of RNA targets to assess inhibition of the RT-PCR. TJ PM, MEX AM, BM AM and ML PM RNA extracts obtained from milk concentrates were spiked with between 33 and 30,000 target copies, depending on the

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Table 3 | Enterovirus presence and concentration in composite seawater samples by membrane filtration and skim milk flocculation. The detection limits varied by concentration and enumeration method: 0.2 MPN/100 mL for the cell culture assay and between 51 and 105 copies/100 mL (skim milk concentration) and 20 and 419 copies/100 mL (membrane filtration) for the RT-QPCR assays. Cell culture results <0.2 MPN/100 mL were confirmed with RT-PCR and were non-detect in all cases

		Skim milk flocculation		
			Cell culture	
Sample ID	Membrane filtration RT–QPCR (copies/100 mL)	RT–QPCR (copies/100 mL)	BGMK (MPN/100 mL)	RT–PCR confirmation
TJ am	60	121.2	1.6	+
TJ pm	61	ND[b]	0.9	+
ML am	129	BLOQ[a]	0.9	+
ML pm	150	ND	<0.2	–
BM am	195	BLOQ	2	+
BM pm	99	BLOQ	12	+
MEX am	10,518	ND	102.2	+
MEX pm	3,217	ND	76.7	+

[a]Below the limit of quantification (BLOQ) indicates that the RT-QPCR reaction showed some amplification but was not quantifiable.
[b]Non-detect (ND) indicates that the RT-QPCR reaction for that sample showed no amplification.

viral concentration measured in the sample (Yamahara *et al.* 2009). MEX AM was the only RNA extract that showed evidence of inhibition; the spiked RNA measured less than one-tenth of the expected copy number given the spike. All RNA extracts obtained using the MF concentration method were spiked with between 33 and 3×10^6 RNA target copies to test for inhibition; no sample showed evidence of inhibition.

Comparison of viral concentrations between sampling times

Time of day (AM versus PM) was not a significant factor affecting enterovirus concentrations measured by MF-RT-QPCR and milk-BGMK across all sites ($p < 0.05$, paired *t*-test). Adenoviruses were detected in two of four sites; at those sites adenoviruses were detected at a higher concentration in the AM versus PM sample, regardless of the quantification method. We recognize that comparisons of virus levels between an AM and a PM sample at a single site are highly speculative and should be interpreted with caution.

Comparison of viral concentrations and fecal indicator bacteria

E. coli and enterococci concentrations were highest at sites MEX and BM, which were also the two sites where both viruses were detected by MF-(RT)QPCR (Table 4). Indicator bacteria and viral concentrations (measured by multiple methods) were positively correlated when data from all four sites were combined. Adenovirus concentrations measured by MF-QPCR positively correlated with both *E. coli* and enterococci (Spearman's rank correlation, $r_s = 0.913$, $p = 0.002$ for *E. coli* and $r_s = 0.723$, $p = 0.043$ for enterococci). Enterovirus measured by MF-RT-QPCR positively correlated with *E. coli* (Spearman's coefficient, $r_s = 0.833$, $p = 0.10$) but not with enterococci ($p = 0.139$). Enterovirus measured by cell culture (BGMK) was positively correlated with *E. coli* ($r_s = 0.814$, $p = 0.014$) and with enterococci at an alpha level of 0.1 ($r_s = 0.671$, $p = 0.069$).

Table 4 | *E. coli* and enterococci concentrations in the composite samples measured by membrane filtration and platting on selective media

Sample ID	*E. coli* (CFU/100 mL)	Enterococci (CFU/100 mL)
TJ am	5	3
TJ pm	10	14
ML am	51	110
ML pm	17	4
BM am	108	361
BM pm	2,760	1,160
MEX am	220,000	2,609
MEX pm	3,427	146

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Correlations between indicator bacteria and virus concentrations measured by other methods were not assessed due to the high number of non-detects (ND) and data points BLOQ.

DISCUSSION

This study compares concentration and enumeration methods for enteroviruses and adenoviruses in seawater from four sites in Southern California and Baja Mexico. The highest concentrations were measured with MF-(RT)QPCR, even though the skim milk methods assayed 18–90-fold larger volumes. A perceived drawback to using rapid molecular detection methods such as MF-(RT)QPCR from a water quality management perspective is that this method detects viral genomic material and cannot indicate whether these viruses are infectious and present risks to bathers. We found that molecular detection of human viruses overestimated but was positively correlated with the presence of infectious viruses; 75% of samples (nine of 12 samples) that were positive by MF-(RT)QPCR were also positive for infectious human viruses by milk-cell culture. Viruses measured by both MF-(RT)QPCR and cell culture were also positively correlated with *E. coli* and enterococci, fecal indicator bacteria that are routinely measured in recreational waters. Additional research is needed to investigate the consistency of the relationship between viral concentrations measured by different detection methods by examining the relationships at other sites.

When comparing enumeration methods using skim milk concentrates, more samples were positive for enteroviruses by milk-cell culture than by milk-RT-QPCR. Previous studies have also found more enteroviruses by cell culture than by RT-PCR (Reynolds *et al.* 1998; Moce-Llivina *et al.* 2005). Reynolds *et al.* (1998) suggested this finding is due to the co-concentration of inhibitory compounds for PCR and/or the low volume of sample added to the RT-PCR compared to cell culture assays (Reynolds *et al.* 1998), yielding higher detection limits for PCR assays in their study. While our inhibition testing for the MEX sample suggests inhibitory compounds may have been co-concentrated with enteroviruses, inhibition results from the other three sites suggest inhibition was not a problem. However, each RT-QPCR and cell culture assay contained the equivalent of between 6–12 and 40–82 mL of sample water, respectively, which is consistent with the suggestion by Reynolds *et al.* (1998) regarding detection limits. Further investigation of inhibition could aid assessment of whether inhibition played a role in the reduced detection of enterovirus in the skim milk extracts (D'Agostino *et al.* 2011; Wyn-Jones *et al.* 2011). Other possible explanations for higher concentrations found by milk-cell culture compared to milk-RT-PCR are preferential concentration of infectious viruses by skim milk and reduced extraction yields from the skim milk concentrate.

Both molecular and culture-based quantification methods employed in this study can detect a range of enteroviruses including polioviruses, echoviruses and coxsackieviruses (Dahling & Wright 1986). The enterovirus concentrations detected by MF-RT-QPCR and milk-cell culture were highly correlated. MF-RT-QPCR yielded concentrations nearly two orders of magnitude higher than the skim milk-cell culture (BGMK) method, with a PCR to MPN infectivity ratio range of 9–143 (Table 3). Previously reported particle-to-PFU ratios for poliovirus, a representative enterovirus, range from 30 to 1,000 (Atmar *et al.* 1993; Flint *et al.* 2004) which is consistent with our findings. Another possible explanation for the high concentrations of enterovirus measured could be that the *in vitro* transcribed RNA standards contained residual DNA leading to an overestimate of their copy number. However, RNA standards were generated following standard techniques and included a DNase incubation, so this is unlikely. Additionally, the milk-RT-QPCR enterovirus concentrations were not similarly high, further suggesting that the RNA standards were not a source of error.

Adenoviruses were detected less frequently than enterovirus; only two sites, BM and MEX, had quantifiable levels of adenoviruses. Results from the various measurement methods were consistent at both sites. Higher adenovirus concentrations were measured at site MEX, with quantifiable results measured by all three concentration-quantification combinations (MF-QPCR, milk-QPCR and milk-cell culture). MF-QPCR concentrations were three orders of magnitude higher than milk-QPCR concentrations, and two to three orders of magnitude higher than the milk-cell culture concentrations. As with enterovirus, differences in adenovirus concentrations measured by MF-QPCR, milk-QPCR and milk-cell culture could be due to: (1) QPCR inhibition associated with the milk concentration method; (2) selective flocculation of

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infectious adenoviruses; or (3) poor extraction efficiency from the milk concentrate. We did not find any evidence of inhibition in the DNA obtained from the milk concentrate method, leaving options (2) and (3) as possibilities.

The virus particle-PFU ratio of adenovirus is estimated to be between 20 and 100 (Flint *et al.* 2004). Our MF-QPCR measurements compared to the milk-cell culture measurements at site MEX suggest a higher ratio of non-infectious to infectious adenovirus (MF-QPCR to cell culture ratio of 4500:1), perhaps due to a loss of virus infectivity in the environment or treatment processes at the source. He & Jiang (2005) reported a 1,000:1 ratio of non-infectious (measured by QPCR) to infectious (measured by cell culture) adenovirus in primary treated wastewater; this ratio is consistent with what we observed in this study between MF-QPCR and milk-cell line concentrations.

Although we had low occurrence of infectious adenovirus, the HEK-293 cell line yielded higher titers and more frequent detects than the A549 cell line. The HEK-293 cell line is susceptible to more species of adenovirus than the A549 line (Choi & Jiang 2005). The former can be infected by adenovirus 2, 5, 40 and 41 while the latter by adenovirus 2 and 5. It is important to note that the A549 cell line can also detect enteroviruses; however, previous research shows that the BGMK line better selects for enteroviruses and the A549 line better selects for adenoviruses (Lee *et al.* 2004; Choo & Kim 2006).

At sites with detectable viruses, AM and PM samples either had similar virus concentrations or the PM concentrations were lower in concentration than the AM samples. Previous work has shown that enteric viruses and bacteriophages are inactivated by sunlight (Boehm *et al.* 2009; Love *et al.* 2010), consistent with lower levels in the afternoon relative to before sunrise. However, viral concentrations in the source waters were not measured and the study was underpowered to investigate the effects of sunlight on viruses at these sites. Further research is needed to assess the effect of sunlight on viral concentrations throughout the day.

CONCLUSION

Enteroviruses and adenoviruses were detected at four beaches in Southern California and Baja Mexico using multiple concentration and enumeration methods. Both viruses were detected at sites BM and MEX, which are impacted by a point source of partially treated wastewater. Viral concentrations measured by skim milk flocculation followed by either cell culture assay or (RT)QPCR agreed, suggesting skim milk flocculation could be used to concentrate infectious viruses in large sample volumes. The highest viral concentrations were measured by MF followed by (RT)QPCR. Despite yielding higher concentrations, results from MF followed by (RT)QPCR were correlated with infectious virus concentrations measured by skim milk flocculation followed by cell culture. Additional research is needed to compare cell infectivity assay results and molecular method results for different viruses and at different sites to determine the variability of this relationship.

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