Efficacy of Hollow–Fiber Ultrafiltration for Microbial Sampling in Groundwater

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Abstract

The goal of this study was to test hollow-fiber ultrafiltration as a method for concentrating in situ bacteria and viruses in groundwater samples. Water samples from nine wells tapping a shallow sandy aquifer in a densely populated village in Bangladesh were reduced in volume approximately 400-fold using ultrafiltration. Culture-based assays for total coliforms and *Escherichia coli,* as well as molecular-based assays for *E. coli*, *Bacteroides*, and adenovirus, were used as microbial markers before and after ultrafiltration to evaluate performance. Ultrafiltration increased the concentration of the microbial markers in 99% of cases. However, concentration factors (CF = post-filtration concentration/pre-filtration concentration) for each marker calculated from geometric means ranged from 52 to 1018 compared to the expected value of 400. The efficiency was difficult to quantify because concentrations of some of the markers, especially *E. coli* and total coliforms, in the well water (WW) collected before ultrafiltration varied by several orders of magnitude during the period of sampling. The potential influence of colloidal iron oxide precipitates in the groundwater was tested by adding EDTA to the pre-filtration water in half of the samples to prevent the formation of precipitates. The use of EDTA had no significant effect on the measurement of culturable or molecular markers across the 0.5 to 10 mg/L range of dissolved Fe^{2+} concentrations observed in the groundwater, indicating that colloidal iron did not hinder or enhance recovery or detection of the microbial markers. Ultrafiltration appears to be effective for concentrating microorganisms in environmental water samples, but additional research is needed to quantify losses during filtration.

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Introduction

In the developing world, diarrheal disease remains one of the leading causes of death among children under age 5, with estimates ranging from 1 to 5 million deaths per year (Parashar et al. 2003). In the United States, Craun (1988) reported that 49% of the 502 reported cases of waterborne disease outbreaks between the years 1971 and 1985 were attributable to contaminated groundwater. In spite of the importance of water in the transmission of diarrheal disease, most groundwater monitoring programs do not measure pathogens directly. This is partly due to the low concentration and intermittent occurrence of pathogens in aquifers. Instead, fecal indicator bacteria such as cultured *Escherichia coli* (Yates 2007) are used as surrogates for pathogen contamination, with a value of <1 colony-forming units per 100 mL typically considered as the acceptable limit for drinking water (Havelaar

et al. 2001). Cultured *E. coli*, however, often correlate weakly with viral and protozoan pathogens (Wilkes et al. 2009), yielding a high percentage of false positives and some false negatives. The weak correlation is due to the intermittent nature of pathogen sources and differences in survival, regrowth, and transport in the environment between fecal indicators and various types of pathogens (Schijven et al. 2000; Woessner et al. 2001; Payment 2009).

Many infectious protozoa, bacteria, and viruses may cause diseases at levels of only 1 to 10 viable particles per liter, which typically requires that water samples undergo a filtration or concentration procedure to improve the detection limit for the pathogen assays (Rendtorff 1954; Willshaw et al. 1994; Gale 2001). In recent years there has been increasing interest in molecular detection methods which can be used for both pathogens and fecal indicators, but these tests use extremely small samples (a few microliters), which further highlights the need for efficient and reliable methods to concentrate the pathogens prior to measurement.

Over the past several decades, a variety of filtration methods have been developed to concentrate viruses and protozoa from large volumes of water (Noble and Fuhrman 2001; Morales-Morales et al. 2003; Lambertini et al. 2008; Hill et al. 2009). These include the U.S. Environmental Protection Agency's method 1623 for concentrating *Cryptosporidium* and *Giardia* using glass wool (Noble and Fuhrman 2001) and the Mark D. Sobsey (MDS) charged filters (American Public Health Association 1995) for concentrating viruses. Generally, these filtration methods are time consuming, cumbersome, and yield low recovery efficiencies. An alternative method recently described by Hill et al. (2005) for the filtration of large volumes of water is hollow-fiber ultrafiltration. This is a form of tangential flow filtration where water is cycled through thousands of fibers with sidewalls that are permeable to water, but not to particles greater than approximately 20 nm in diameter. Larger colloids such as viruses and bacteria remain suspended in the retentate water (RW) during ultrafiltration (i.e., the water not removed by leakage through the fiber walls). This method can be used to concentrate initial volumes of hundreds of liters of water to a few hundred milliliters in several hours. Most importantly, the microorganisms remain in suspension, rather than attached to the filter material, which eliminates the need for steps to resuspend them prior to the measurement with methods such as tissue culture or polymerase chain reaction (PCR).

In laboratory experiments with concentrations of spiked microorganisms ranging from 10^4 to 10^6/mL, Hill et al. (2005, 2007) observed ultrafiltration recovery efficiencies typically ranging between 50% and 100% using a variety of bacterial and viral markers. Recovery efficiency was calculated by dividing the number of microorganisms enumerated in the RW by the known concentration of microorganisms in the initial spiked water sample. Spiked recovery experiments with protozoa, bacteria, and viruses on eight water sources from different regions in the United States, with a minimum of two replicates per source, suggested that recovery efficiency is sensitive to a variety of water chemistry parameters including pH, turbidity, conductance, alkalinity, total Fe, total organic carbon, dissolved organic carbon, and heterotrophic plate count (Hill et al. 2007). In spite of differences in recovery efficiency in water taken from different regions, no statistically significant correlation between recovery efficiency of the markers with the levels of any single water chemistry parameter was observed (Hill et al. 2007). Because most of the water sources used in previous recovery efficiency studies were tap water, it is uncertain how effective ultrafiltration will prove to be across the broader range of physical and chemical conditions found in wells used for water supply. Furthermore, recovery of preexisting microorganisms in samples of WW may differ from recovery of spiked microbial markers added to the water sample after collection.

The primary objective of this study was to evaluate the effectiveness of ultrafiltration as a method for concentrating bacteria and viruses from large (typically 100 L) groundwater samples in the field by measuring in situ concentrations of fecal indicators before and after a 400-fold reduction in volume. The study was carried out at a field site in Bangladesh because the high levels of fecal contamination common at the site increased the likelihood of the presence of a wide range of fecal microorganisms. The ability of ultrafiltration to increase the concentration of microorganisms was tested using a suite of in situ microbial indicators that included total coliforms, *E. coli*, *Bacteroides* and adenovirus. Measurements of microbial indicator concentrations were carried out with culture-based and DNA molecular-based methods (in this case quantitative polymerase chain reaction [qPCR]). The *E. coli* concentrations were measured with both culture-based and molecular methods to determine whether ultrafiltration effectiveness differs with the type of assay.

A secondary objective was to quantify the effect of high concentrations of dissolved reduced [Fe^{2+}], prevalent in aquifers in Bangladesh, on measurements of bacterial and viral markers in the RW. This was performed to address the concern that colloidal FeOOH particles formed by the oxidation of iron due to exposure to atmospheric oxygen during sampling might interfere with the filtration and recovery of bacteria and viruses. These particles could clog the filter or form mineral-microbial aggregates, which would reduce the number of colony-forming units in culture-based assays. In addition, the presence of FeOOH particles in the RW could interfere with the DNA extraction and PCR amplification in the laboratory. To investigate this potential factor, ethylenediaminetetraacetic acid (EDTA) was added to one of the paired groundwater samples from each of nine wells spanning a range of natural [Fe^{2+}] before ultrafiltration to prevent formation of FeOOH with the expectation that EDTA would have the greatest effect in wells with high [Fe^{2+}].

The study also provided an opportunity to test the utility of *Bacteroides* as a fecal indicator in groundwater and to compare it with other more commonly used

fecal indicators. To the author's knowledge, this study, along with a study by Johnson et al. (this issue), are the first tests of *Bacteroides* as a quantitative fecal indicator in groundwater. *Bacteroides* sp. have the potential to be useful indicators of fecal contamination in water (Bell et al. 2009; Layton et al. 2006; Lee et al. 2008; Yampara-Iquise et al. 2008) because they are present in the intestines of all warm blooded animals and are one of the dominant (10% by mass) bacterial species in human feces (Matsuki et al. 2002; Bernhard and Field 2000). In addition, *Bacteroides* are obligate anaerobes and therefore, unlike *E. coli*, unlikely to grow in subsurface environments. However, *Bacteroides* are difficult to enumerate in the laboratory using culture-based tests. This is why *Bacteroides* had not been quantified prior to the development of qPCR assays (Bernhard and Field 2000; Layton et al. 2006).

Methods

Site Description

The field site selected for this study is a sandy floodplain aquifer underlying the village of Char Para, 23.79 °N 90.63°E, in Araihazar, Bangladesh, herein referred to as Site K (Radloff et al. 2007; Weinman et al. 2008;). The village is located on sand bar deposits which act as an unconfined aquifer and is tapped by dozens of shallow (10- to 20-m deep) tubewells. The shallow aquifer at this location is low in arsenic, relative to many other wells in the region, possibly because rapid vertical recharge has flushed out the mobilizable arsenic over time (van Geen et al. 2008; Aziz et al. 2008). The village is densely populated, with approximately 1500 people living in an area of 30 ha. Hundreds of latrines and approximately fifty ponds, many of which receive discharge from latrines, are scattered throughout the village and serve as point sources of fecal pollution to the aquifer. This site is therefore well suited for the study of microbial sampling methods because of rapid local recharge and abundant sources of fecal contamination.

Well Installation

Two types of wells, 7.6- to 16.8-m deep, were sampled at Site K: (1) private tubewells (five) and (2) wells installed for groundwater monitoring (four). For all wells drilling was done by the traditional hand-flapper method, which is essentially a manual mud circulation method that readily penetrates the loose, wet unconsolidated floodplain deposits throughout the Bengal Basin (e.g., Horneman et al. 2004). The monitoring wells were installed to reduce the likelihood of sample contamination due to poor well seals. The annulus of private wells in Bangladesh is typically filled with material removed from the borehole during drilling, whereas the purposely installed monitoring wells were sealed with cement grout from the top of the sand pack, which itself extends 0.7-m above the 1.5-m screened interval, to the surface. Both types of wells are constructed of 5.1 cm diameter PVC pipes, but private wells are equipped with hand pumps,

whereas the monitoring wells were sampled with an electric-powered submersible pump (Typhoon, Groundwater Essentials, LLC, Sarasota, Florida).

Well Sampling and Ultrafiltration

All wells were purged for at least three standing wellbore volumes before sampling. One wellbore volume ranged from 11 to 30 L, depending on the well depth and the water level. In monitoring well KW-24, high turbidity was initially observed and 10 wellbore volumes were purged until electrical conductivity, temperature, and dissolved oxygen concentrations measured with a multiprobe (556 Multiprobe System, YSI Inc., Yellow Springs, Ohio) stabilized and the water was clear. Steady-state values for the nine wells ranged from 25 °C to 27 °C for temperature; 0.22 to 0.96 μs/cm for electrical conductivity; 6.37 to 7.17 for pH; and 0.2 to 1.1 ppm for dissolved oxygen. Groundwater is typically anoxic in Bangladesh and dissolved oxygen sensors are difficult to calibrate at these very low levels. However, in the particular setting, we cannot rule out that rapid vertical recharge occasionally supplies detectable levels of oxygen to the shallowest aquifer. Monitoring wells were pumped continuously at 7 to 10 L/min with an electric submersible pump and the excess water pumped, when not filling the 20-L sample reservoirs, flowed into a ditch. In contrast, private wells were pumped intermittently with the existing hand pump at an approximate flow rate of 20 to 30 L/min while filling the 20-L sample reservoir. Consequently, monitoring wells were sampled at a constant flow rate, as opposed to intermittent flow, and likely with higher daily pumped volumes than private wells, since the submersible pumps ran continuously. The private wells were also used for domestic purposes between filling the retentate reservoirs but this additional volume pumped was not measured.

The apparatus for performing ultrafiltration (Figure 1) was based on a system described by Hill et al. (2005, 2007). Briefly, groundwater was pumped in a closed loop through a hollow-fiber single-use ultrafiltration cartridge (Rexeed 25S, Dial Medical Supply, Chester Springs, Pennsylvania) under positive pressure (5 to 10 kPa) using a portable peristaltic pump (Solinst Model 410, Pine Environmental Services Inc., Windsor, New Jersey) and Poly Teflon Lined Tubing (TB30120, Pine Environmental Services Inc.). The sidewalls of the capillary tubes in the ultrafiltration cartridge have 20 nm pore sizes.

As a sample cycles through the ultrafiltration cartridge, increasing amounts of water, dissolved constituents and colloids <20 nm are lost through the sidewalls as filtrate water. Colloids >20 nm, which include most bacteria and viruses, remain in the RW which becomes more concentrated during cycling. To concentrate a 100-L groundwater sample, the retentate reservoir was filled five times with 20 L of WW, and then the volume was reduced by ultrafiltration to less than 1 L between each refilling. At the end of the ultrafiltration process, when the retentate reservoir was almost empty, sterile bottled water was used to back flush the tubing and cartridge. The fully saturated volume of the tubing and inner cartridge was calculated
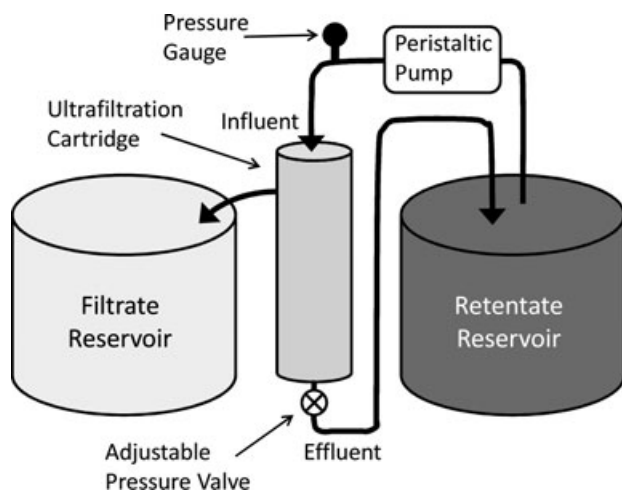
Figure 1. Ultrafiltration apparatus. The retentate reservoir represents the 20-L bucket that was filled with groundwater five times during each 100-L ultrafiltration run. The pressure valve and gauge were used to control the back pressure which influenced the rate of filtrate water exiting the sidewalls of the capillary tubes in the ultrafiltration cartridge.

to be 187 mL. The final retentate sample represented the first 250 mL of RW to exit the back flushed tubing and cartridge, representing approximately 1.3 displaced pore volumes. This method assumes that the microorganisms were in free suspension and not attached to the sidewalls of the capillary fibers in ultrafiltration cartridge. As the original 100-L groundwater sample was reduced in volume 400 times, the concentration of the markers in the retentate was expected to be 400 times higher than in the unfiltered WW sample. Three 10-mL subsamples of this final retentate were diluted with 90 mL of bottled water to measure the cultured *E. coli* and total coliform using the Colilert assay (IDEXX Laboratories Inc., Westbrook, Maine). The remaining retentate (approximately 220 mL) was frozen and transported to the University of Tennessee for molecular DNA analysis.

Between sampling of each well, all parts of the ultrafiltration apparatus were soaked in dilute bleach and Tween-80 (T164-500, Fischer Scientific) cleaning solution. The ultrafiltration cartridge was discarded after each use. Powdered Chlorox (5 g) and Tween-80 (5 mL) were mixed in 10 L of WW from the next well that was to be sampled. The bleach/Tween solution was cycled through the tubing for 5 min, followed by rinsing with 10 L of WW containing 5 g of sodium thiosulfate (S446-3, Fisher Scientific, Pittsburgh, Pennsylvania) for 2 min. A final rinse with 10 L of WW pumped through the tubing was performed over a period of 2 min. Sterile techniques were employed throughout. The total time for ultrafiltration of 100 L of groundwater including setup, disinfection, and packing up was approximately 3 h, allowing for sampling of two wells per field day.

Each of the markers was measured directly from samples of unfiltered WW immediately before each ultrafiltration run to obtain background concentrations in the WW. Since two ultrafiltration runs were performed on

a well on each field day, unfiltered WW samples were collected twice, once early in the day and once late in the day. For the culture-based assays, triplicate 100-mL Colilert samples for *E. coli* and total coliform were collected from each well at the start of each ultrafiltration run to determine marker concentrations in the well during pumping. The exception to this was KW-12.1 which was only sampled at the start of the second ultrafiltration run, in the middle of the 6-h field day. For the molecular assays, a single 250-mL sample was removed from the first of five mixed 20-L reservoirs of WW at the start of each ultrafiltration run. The final retentate was stored in a sterile 250-mL polypropylene bottle. Each ultrafiltration run included one set of triplicate 100-mL samples of unfiltered WW for culture-based analysis, one 250-mL unfiltered WW sample for molecular analysis and one 250-mL filtered RW sample for both culture-based and molecular analysis.

EDTA Addition and Iron Detection

Concentrations of dissolved iron in WW in the form of Fe^{2+} across Site K were measured using a field Iron Test Kit (Model IR-18B, Hach Company, Loveland, Colorado) and varied widely from <0.1 to 10 ppm (Table 1). Initial lab experiments and field observations demonstrated that FeOOH minerals precipitate out of solution within 20 min when the reduced, high $[Fe^{2+}]$, 5 to 10 ppm, groundwater is exposed to atmospheric oxygen. To test for the influence of this on ultrafiltration, 2.5 g of EDTA disodium salt (02793-500, Fisher Scientific) was added to each 20-L reservoir of unfiltered WW immediately after the bucket was filled to prevent the precipitation of FeOOH particulates. EDTA contains six metal binding sites for each molecule and therefore, theoretically, all dissolved Fe^{2+} should be bound by a concentration of $[EDTA] = 0.17 \times [Fe^{2+}]$ (Essington 2004). However, other divalent metal cations in groundwater such as Mn^{2+} and Ca^{2+} may compete for binding sites with Fe^{2+} (Essington 2004). The concentration of EDTA in each 20-L bucket was 3.36×10^{-4} M, which is twice the maximum

Table 1
Groundwater Wells Sampled and Experimental Design

Well ID	Well Type	Depth (m)	[Fe²⁺] (ppm)	EDTA Added	No EDTA
				Times Sampled	
KW-12.1	Monitoring	7.6	0.5	1	1
UTK-1	Private	9.1	0.6	1	1
KW-24	Monitoring	11.9	3.5	1	1
UTK-8	Private	16.8	3.8	1	1
UTK-7	Private	7.6	6.2	2	1
UTK-31	Private	12.2	7.6	1	1
KW-30	Monitoring	13.7	8.8	1	1
UTK-30	Private	13.7	9.0	1	1
KW-25	Monitoring	15.5	10.0	1	1
Total				10	9

concentration of dissolved Fe^{2+} (10 ppm) measured in the samples. Of the two ultrafiltration runs carried out for each well on a given field day, one involved EDTA addition to each 20-L reservoir and the other was run without. The color and clarity difference between the retentate samples of high [Fe^{2+}] water with EDTA added and without was striking, indicating that EDTA effectively prevented precipitation.

The nine wells that were sampled in the field span a limited depth range (7.6 to 16.8 m) but a wide range of Fe^{2+} concentrations (Table 1). The sequence of sampling at a given well with or without EDTA addition to 100 L of WW was random. The only exception was UTK-7, which was sampled a total of three times (twice with EDTA added) on 2 different days.

Bacterial and Viral Detection Methods

Culture-based and molecular methods based on the analysis of microbial DNA were used to detect fecal indicator bacteria and viruses in all groundwater samples. Samples for *E. coli* and total coliform analysis were stored on ice in the field immediately after collection and processed within 8 h of sampling. Cultured *E. coli* and total coliforms were detected using the Colilert[TM] test kit with the Quanti-tray 2000 (IDEXX Laboratories Inc.). This is a most probable number method (MPN) that splits a 100 mL water sample into 97 testing wells (49 large, 48 small) and the number of wells positive for each bacterial indicator corresponds to MPN/100 mL according to the solution provided by Hurley and Roscoe (1983). Based on the MPN solution for a given 100-mL water sample, the range of possible concentrations ranges from 1 to >2419 MPN/100 mL. Duplicate or triplicate samples taken directly from the well (WW) or diluted (1:10) from the RW were analyzed separately during this study. Because of dilution the detection limit was 10 MPN/100 mL for RW samples, instead of 1 MPN/100 mL for undiluted

WW samples. The MPN solution was used to solve the MPN (Hurley and Roscoe 1983) and associated 95% confidence intervals by combining the numbers of discrete positive wells from all trays of replicate samples. The underlying assumption is that the groundwater from which the 100-mL duplicate or triplicate samples were taken was well mixed, and that the true concentration of bacteria in each 100-mL sample was the same.

Quantitative PCR was used to measure copies of genes for *E. coli*, *Bacteroides,* and Adenovirus in the water samples. To distinguish the cultured *E. coli* values from the molecular *E. coli* values, data collected from qPCR for *E. coli* is denoted as *mE. coli* in this study. For the molecular assays, samples of both unfiltered WW and RW, which is collected after ultrafiltration, were collected in sterile 250-mL polypropylene containers, frozen on dry ice, and brought back to the University of Tennessee for DNA extraction and qPCR analysis. After removal from the −80 °C freezer, samples were thawed in cool water for 3 to 5 h. Two-hundred and fifty milliliter of WW samples and 50 mL of the RW samples were vacuum filtered onto autoclaved 0.45-μm cellulose nitrate filters (47 mm, Whatman filter, Whatman Inc., Piscataway, New Jersey) for DNA extraction. DNA extraction and purification were performed on 1/2 or 1/4 of each filter using a DNA soil extraction kit following the manufacture's protocols (FastDNA®SPIN for Soil Kit, MP Biomedicals, Solon, Ohio). Initial concentrations of gene copies of each marker microorganism per nanogram of DNA extracted were obtained by qPCR following previous published methods (Layton et al. 2006), with primers and probes shown in Table 2.

The basic PCR protocol used for DNA amplification consisted of 50 °C for 2 min, followed by 95 °C for 10 min, and 40 cycles of 95 °C for 30 s and 55 °C (*E. coli* assay) or 60 °C (AllBac and Adenovirus assays) for 45 s. For each sample and assay, the samples were

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Table 2
Primers and Probes Used for Each Real-Time PCR Assays to Detect *E. coli* and *Bacteroides* rRNA Genes and the Adenovirus Hexon Gene

Assay Name (target organism)	Primer/Probe Name and Sequence (5′–3′)	Size (bp) of Product
EC23S (*E. coli*)[1,2]	EC23Sf 5′ GAG CCT GAA TCA GTG TGT GTG 3′ EC23Sr 5′ ATT TTT GTG TAC GGG GCT GT 3′ EC23Srv1bhq 5′-(FAM)CGC CTT TCC AGA CGC TTC CAC (BHQ-1)-3′	78
AllBac (all *Bacteroides*)[3]	AllBac296f, 5′-GAGAGGAAGGTCCCCCAC-3′ AllBac412r, 5′-CGCTACTTGGCTGGTTCAG-3′ AllBac375Bhqr, 5′-(FAM)CCATTGACCAATATTCCTCACTGCTGCCT(BHQ-1)-3′	106
Adeno (40/41 hexon gene)[4]	AV40/41-117f 5′-CAGCCTGGGGAACAAGTTCAG 3′ AV40/41-258r 5′ -CAGCGTAAAGCGCACTTTGTAA 3′ AV40/41-157BHQ 5′-(Fam)ACCCACGATGTAACCACAGACAGGTC (BHQ-1)-3′	141

[1]Modified from Smith et al. 1999.
[2]Layton et al. 2003.
[3]Latyon et al. 2006.
[4]Rajal et al. 2007.

run in triplicate wells and in a fourth well containing the sample and a plasmid DNA spike to determine PCR inhibition. A standard curve containing a positive plasmid DNA target for each assay ranging from 2.5×10^7 copies to 25 copies was run on each plate along with triplicate blanks. Owing to the potential for cross reactivity of the primers with non-target DNA, when the concentration of the target DNA was <1 copy/ng total extracted DNA, the sample was treated as a non-detect. Because each sample contained a different amount (ng) of total extracted or background DNA, the detection limit varied from sample to sample, resulting in more sensitive detection limits for samples with small amounts of background DNA. The pooled average coefficient of variation based on triplicate qPCR reaction wells was 30% for all assays.

Results

Marker Concentrations in Well Water and Retentate Water

The approach followed in this study was to measure the in situ concentrations of all markers in unfiltered water collected from the wells after purging approximately three well bore volumes or parameter stabilization and then compare these values with measurements from 100-L samples that had been concentrated to a final volume of 250 mL using ultrafiltration (i.e., a 400-fold concentration step). The initial 100-mL samples were referred to as WW samples. The post-ultrafiltration samples are referred to as RW samples. From this final retentate, a subset was initially analyzed for cultured total coliforms and *E. coli* with the Colilert assay and the rest of each sample was frozen and transported to the University of Tennessee for molecular *E. coli*, *Bacteroides*, and Adenovirus assays.

The number of well and RW samples that were positive for each marker, as well as the geometric mean and the range of concentrations are listed in Table 3. The retentate samples contain 9 where EDTA was absent and 10 where EDTA was added. The number of positive samples (i.e., those containing detectable levels of fecal indicators and molecular markers) ranged from 11 to 18 out of the 19 WW samples (Table 3) and a large range of marker concentrations was observed in the samples.

All molecular markers, *mE. coli*, *Bacteroides,* and adenovirus, were more abundant than cultured markers in both unconcentrated WW and RW samples. In WW, the geometric mean concentration of *mE. coli*, *Bacteroides,* and adenovirus were 5100, 2800, and 5000 copies/100 mL, respectively, and the cultured markers, *E. coli,* and total coliforms had geometric means of 5 and 37 MPN/100 mL, respectively (Table 3). In all but 1 out of 83 cases, marker concentrations in the retentate were higher than in the unfiltered WW samples. The addition of EDTA prior to ultrafiltration did not have an obvious impact on the geometric means or ranges of marker concentrations in RW samples. In the retentate samples, the highest marker concentrations were observed for *Bacteroides* which had a geometric mean of 3.4×10^6

Table 3
Summary of Marker Concentrations in 19 Well Water and Retentate Water Samples (9 without and 10 with EDTA added)

Marker	Units	Well Water[1,2] # Samples Positive (%)	Well Water[1,2] Geometric Mean (range)	Retentate Water[3] −EDTA # Samples Positive (%)	Retentate Water[3] −EDTA Geometric Mean (range)	Retentate Water[3] +EDTA # Samples Positive (%)	Retentate Water[3] +EDTA Geometric Mean (range)
E. coli	MPN/100 mL	13 (68)	5.0E+00 (1.0E+00–7.5E+01)	6 (67)	7.6E+01 (1.0E+00–6.5E+03)	9 (90)	1.8E+02 (1.0E+00–7.9E+03)
Total Coliforms	MPN/100 mL	17 (89)	3.7E+01 (1.0E+00–1.1E+03)	9 (100)	1.5E+04 (2.2E+02–6.6E+05)	9 (90)	2.0E+04 (6.6E+03–3.8E+05)
mE. coli	Copies/100 mL	18 (95)	5.1E+03 (6.0E+02–1.4E+05)	9 (100)	8.8E+05 (3.7E+04–1.5E+07)	9 (90)	5.5E+05 (7.2E+04–1.3E+07)
Bacteroides	Copies/100 mL	11 (58)	2.8E+03 (2.9E+02–5.9E+04)	9 (100)	3.4E+06 (1.0E+06–6.8E+06)	8 (80)	9.1E+05 (2.4E+04–1.8E+07)
Adenovirus	Copies/100 mL	15 (79)	5.0E+03 (1.4E+02–7.4E+05)	9 (100)	1.6E+05 (1.1E+04–2.5E+07)	8 (80)	4.5E+05 (1.4E+04–1.1E+07)

[1] A total of 19 well water and 19 retentate water samples were tested.
[2] Well water samples for cultured bacteria were sampled directly from the well while samples for molecular markers were taken from the first of five 20-L well-mixed reservoirs.
[3] Ten milliliter of subsamples of the 250-mL retentate samples were diluted for cultured enumeration and 50 mL was extracted for molecular assays.

and 9.1×10^5 copies/100 mL for samples without and with EDTA respectively. The lowest retentate concentrations were observed for the cultured *E. coli* with geometric means of 76 and 180 MPN/100 mL without and with EDTA respectively. PCR inhibition was detected in only one sample (RW for KW-24, +EDTA), as measured by the lack of PCR amplification of the positive control standard in the DNA sample. This PCR inhibition prevented the detection of any of the molecular marker.

The increase in marker concentrations between WW and RW samples is substantial for all markers. This was especially notable in the cases (10 out of 83, or 12%) where a marker was not detected in the WW sample (pre-filtration) but was detected in the RW sample (after ultrafiltration). The 1:1 line in Figures 2 and 3, for cultured and molecular markers respectively, indicates the threshold for demonstrating an increase in marker concentration resulting from ultrafiltration. In all but one of the 83 cases for which a marker was detected in the RW sample, the RW vs. WW concentration data point lay above this line (Figure 3c). The 1:400 line on each graph represents the expected concentration factor (CF), assuming that a $400\times$ reduction in sample volume results in a $400\times$ increase in marker concentration. For all markers, the RW vs. WW concentration data points straddled the 1:400 line, but with a high degree of scatter. For total coliforms (Figure 2a) and *Bacteroides* (Figure 3b) markers, about equal numbers of data points lay above and below the 1:400 line. In contrast, for the other markers, more data points lay below the 1:400 line then above. Ultrafiltration resulted in substantial increases in concentration of markers in the retentate relative to the WW samples, but the large amount of scatter in the data indicates that the amount of increase is not consistent between wells or between different samples in the same well.

CF values for each ultrafiltration run were calculated using:

$$CF = \frac{C_{RW}}{C_{WW}} \qquad (1)$$

where C_{RW} is the concentration of the marker in the RW and C_{WW} is the concentration of the same target in the unfiltered groundwater sample. A line is included in Figures 2 and 3 to show the geometric mean CF for each marker based on all the individual ultrafiltration runs. In cases where the marker was detected only in the RW sample, the concentration in the WW sample was set equal to the detection limit for the purpose of calculating the CF. The geometric mean CFs, with associated 95% confidence intervals calculated on the log-transformed data were: 105 (26 to 419) for *E. coli*; 794 (252 to 2503) for total coliforms; 182 (74 to 446) for *mE. coli*; 1023 (491 to 2130) for *Bacteroides*; and 51 (15 to 179) for adenovirus.

Variation in Marker Concentration During Sampling

While planning the study, it was assumed that the concentrations of bacterial and viral markers collected
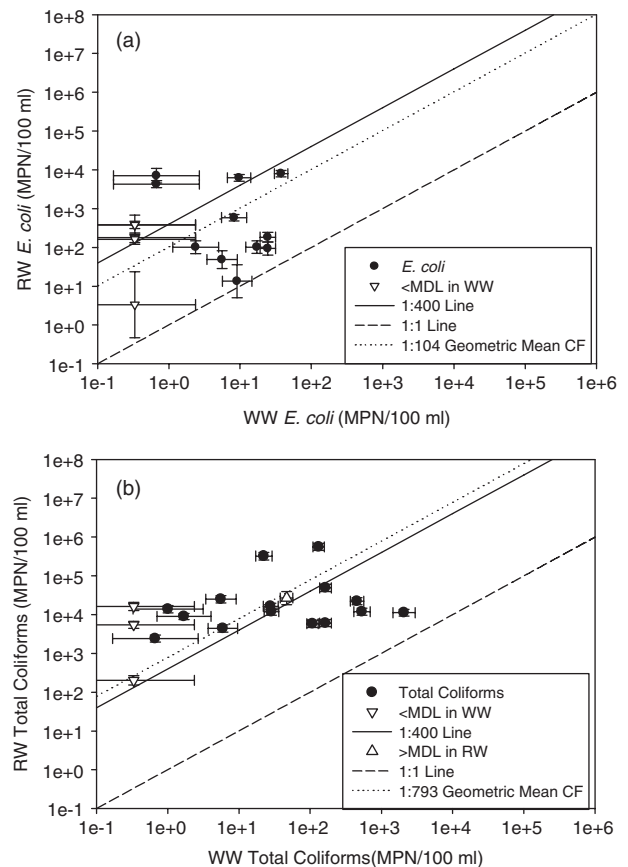

Figure 2. Comparisons of cultured marker concentrations from 250-mL unfiltered well water (WW) samples with 100-L ultrafiltered retentate water (RW) samples. Panels a and b represent *E. coli* and total coliforms respectively. The 1:1 line is where points would lie if there were no increase in marker concentration during ultrafiltration. The 1:400 line is where points would lie if the 250-mL WW sample was representative of the average concentration within the 100-L WW sample, and if no losses occurred during ultrafiltration. The dotted line represents the geometric mean CF, the ratio of the marker concentration in the RW sample over the WW sample. Inverted triangles indicate non-detects in the WW sample only and are plotted at the detection limit on the x-axis of the graph for each marker. Error bars represent 95% confidence intervals.

from the wells would remain relatively constant during the period of sampling and subsequent ultrafiltration for a given RW sample. Contrary to expectations, concentrations varied over the course of the day (while pumping for sampling and/or domestic use continued) by as much as three orders of magnitude. Concentrations of the cultured bacteria, *E. coli* and total coliforms, decreased in every sample collected later in the day in those cases where bacteria were initially detected in early in the day (Figure 4). Early samples were taken from the well at the beginning of the day, whereas late samples were taken after one complete round of ultrafiltration had been completed from the well, before the second round of ultrafiltration had begun. In the case of well KW-30, four WW samples (rather than the usual two) were collected over a 24-h period during which 2000 L of water was
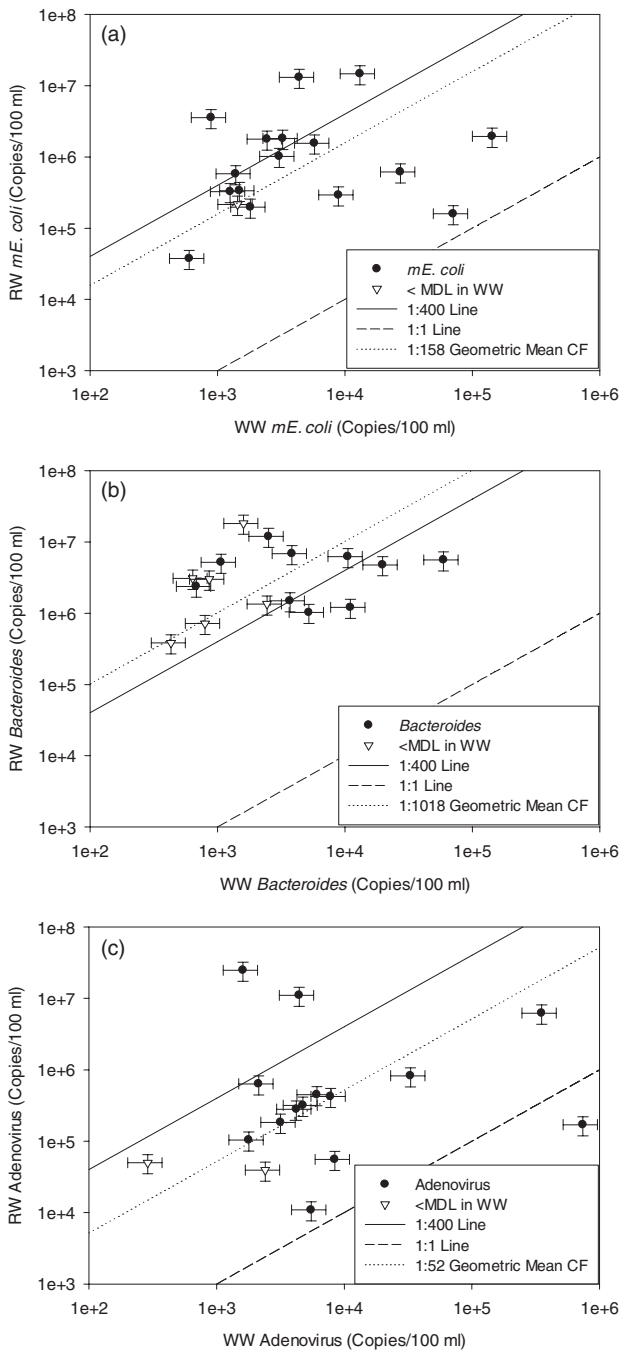
Figure 3. Comparisons of molecular marker concentrations from 250-mL unfiltered well water (WW) samples with 100-L ultrafiltered retentate water (RW) samples. Panels a, b, and c represent *mE. coli*, *Bacteroides*, and Adenovirus. The 1:1 line is where points would lie if there were no increase in marker concentration during ultrafiltration. The 1:400 line is where points would lie if the 250-mL WW sample was representative of the average concentration within the 100-L WW sample, and if no losses occurred during ultrafiltration. The dotted line represents the geometric mean CF, the ratio of the marker concentration in the RW sample over the WW sample. Inverted triangles indicate non-detects in the WW sample only and are plotted at the detection limit on the *x*-axis of the graph for each marker. Error bars represent 95% confidence intervals.
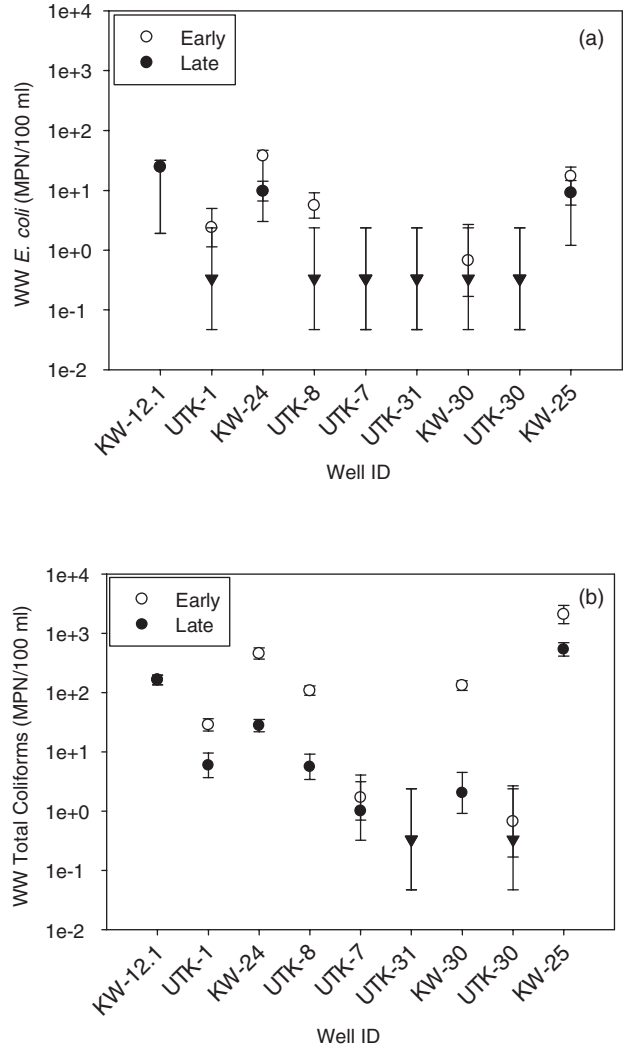

Figure 4. Paired 100-mL pre-filtration well water samples (in triplicate) taken from wells early or late in the day for culturing. Panels a and b represent *E. coli* and total coliforms respectively. All wells were purged for at least three bore volumes, ranging from 33 to 90 L, before sampling. KW-12.1 only had a single sample taken during the day. Total coliforms were not detected in UTK-31 at early or late time. *E. coli* was not detected in UTK-7, UTK-31, and UTK-30 at early or late time. Non-detects are indicated by the Method Detection Limit, with inverted triangles. The error bars describe 95% confidence intervals for combined replicates.

removed from the well. A consistent log-linear decline in concentration of cultured *E. coli* and total coliforms with pumped volume was observed, resulting in decreases of two and three log of *E. coli* and total coliforms respectively (data not shown). Between 7 and 12 mm of daily rainfall occurred on 6 of the 10 consecutive days of sampling at site K during this month in the monsoon season. No systematic relationship was observed between daily precipitation amounts and concentrations of bacteria or viruses in WW during the 10 days of sampling.

Molecular marker concentrations in the unfiltered 100-mL WW samples also showed considerable variability (by up to two orders of magnitude) between paired
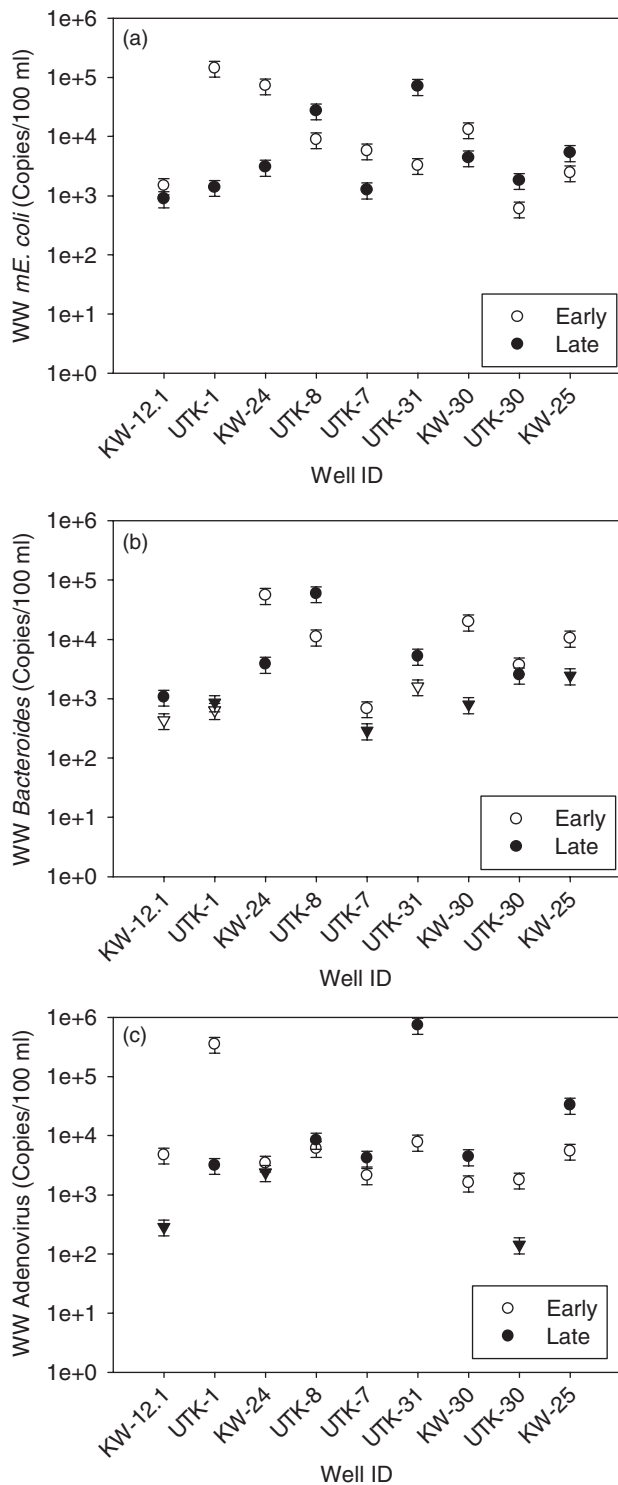
Figure 5. Paired 250-mL well water samples taken from wells early or late in the day for molecular analysis. Panels a, b, and c represent *mE. coli*, *Bacteroides*, and Adenovirus. Non-detects are indicated by the MDL with inverted triangles. The error bars describe 95% confidence intervals for combined replicates.

concentrations of both cultured and molecular markers were not constant in the unfiltered WW for even relatively short time periods (a few hours to a day) or relatively modest volumes pumped (a few hundred to a few thousand liters).

Correlations of Markers in Retentate Water

The correlation between the different markers in RW samples was calculated using the Spearmann rank order correlation coefficient (Table 4). The strongest correlations were observed between *E. coli*, *mE. coli*, and total coliforms ($p < 0.01$). The *E. coli* Colilert assay is a subset of the total coliform assay so it would be expected to be correlated. However, the strong correlation between the *mE. coli* assay and total coliforms, which are based on independent assays, suggest that the fecal indicator bacteria are the principal source of coliform bacteria. The other fecal indicator bacteria, *Bacteroides*, did not correlate strongly with either *E. coli* or *mE. coli* in the RW, indicating either different die-off (in the environment or during sampling) or transport rates for this bacterium. Correlations were not calculated for the unfiltered WW samples due to the large number of non-detects resulting in comparatively small data sets.

The relative proportion of cultured *E. coli* to *E. coli* genomes, assessed by the molecular assay and the Colilert method, is shown for each sample in Figure 6. The ratio represents the geometric mean of the number of cultivable *E. coli* to the total number of 23S genes detected. For the RW samples this ratio was 1:6315 (2679 to 14887). Assuming six copies of the ribosomal gene in *E. coli* (Klappenbach et al. 2001) the data indicate that the cultivable proportion represents 0.1% of the *E. coli* genomes. The overall proportion of cultivable *E. coli* did not change greatly for unfiltered WW samples and filtered RW samples, indicating that ultrafiltration does not inactivate a large proportion of cultivable *E. coli* cells.

Table 4
Correlation Matrix of Marker Concentrations in Retentate Water (RW)

	E. coli	Total Coliforms	*mE. coli*	*Bacteroides*	Adenovirus
E. coli	1.00				
Total coliforms	**0.67**	1.00			
mE. coli	**0.68**	**0.80**	1.00		
Bacteroides	0.02	0.30	0.36	1.00	
Adenovirus	0.28	0.39	0.29	−0.37	1.00

Numbers represent the nonparametric Spearmann rank order correlation coefficient (r_S). Numbers in bold indicate statistically significant correlations in paired ranks ($p < 0.01$). Paired data set sample sizes vary between 18 and 19, with non-detects included at their respective detection limits.

samples collected at the beginning and the end of the same day, but with approximately equal numbers of cases where concentrations increased or decreased during the day (Figure 5). Together, these findings indicate that the
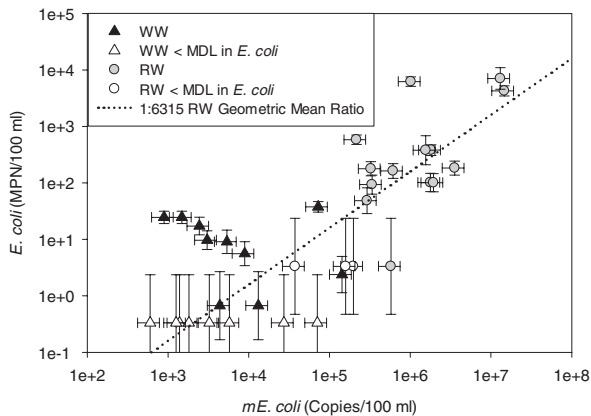
Figure 6. Comparison of cultured and molecular *E. coli* assays in both WW and RW samples. The geometric mean of the cultivable to molecular *E. coli* ratios in all RW samples is represented by the 1:6315 line, representing approximately 0.1% cultivable *E. coli*. Error bars represent 95% confidence intervals.

EDTA and Fe Effect

The addition of EDTA to WW prior to ultrafiltration did not have any systematic effect on concentrations of the five markers in the retentate (Figure 7). There is no evidence that EDTA improved the recovery of any of the five markers, even in a subset of high $[Fe^{2+}]$ waters, as none of markers with EDTA are consistently higher or lower than those without EDTA. One-sided *t*-tests were performed on the differences between log-transformed concentrations (with and without EDTA added) of each marker pooled from all wells using the statistical software NCSS (version 07.1.14, NCSS, LLC, Kaysville, Utah). The null hypothesis that there was no difference in marker concentration in RW samples with and without EDTA (H_o: μ = 0) was not rejected ($p = 0.05$) for any of the five markers. The total coliform marker data set failed normality tests (skewness and kurtosis) due to a single outlier (UTK-31, 7.6 ppm Fe^{2+}) where 2 $\log_{10}$ greater RW concentration was observed for the sample with EDTA added (Figure 7). Although there was no systematic effect of EDTA or $[Fe^{2+}]$ on molecular marker concentrations in RW samples, there was a high degree of variability between subsequent 100-L ultrafiltered samples, which frequently differed by more than an order of magnitude (Figure 7). This is consistent with the high degree of variability observed for all the markers in pre-filtration WW samples (Figures 4 and 5). The differences between measured concentrations of markers in RW samples taken from the same well on the same day were apparently random, and could not be explained by any linear combination of parameters measured from the well, such as pumped volume, electrical conductivity, temperature, or pH, as assessed by multiple regression ($p = 0.05$) using the software NCSS.

Discussion

Ultrafiltration resulted in substantial increases (geometric mean CFs of 52 to 1018, relative to an expected

value of 400) in concentration of in situ bacterial and viral markers from groundwater in 99% of cases where the marker was quantifiable in the RW sample (Figures 2 and 3). For each marker, measured concentrations in the RW sample tended to be higher for wells which started out with higher concentrations in the pre-filtration WW. There was, however, a substantial range (several orders of magnitude) of CFs calculated for each marker for the different ultrafiltration runs. CFs for total coliforms and *Bacteroides* tended to be higher than the predicted value of 400 (based on the 400-fold volume reduction and the measured concentration of each marker in the pre-filtration WW). The other three markers (*E. coli*, *mE. coli*, and Adenovirus) tended to have CFs that were lower than the expected value of 400.

The large variability in calculated CFs was at least partly caused by the variability in marker concentrations in the unfiltered WW samples, as shown in Figures 4 and 5. This variability in WW samples could be due to a heterogeneous distribution of microbial markers in the aquifer, but it is perhaps more likely related to conditions in the well. Kwon et al. (2008) found that 36 wellbore volumes were required to reach quasi-steady state in total bacteria cell concentrations and a stable microbial community, however substantial changes in these continued up to 230 wellbore volumes. A possible explanation for the unstable bacteria and virus concentrations in the present study is that pumping could mobilize microorganisms attached to biofilms in the well, or it could draw in contaminated water through cracks in the well casing. Losses related to the ultrafiltration process (e.g., attachment to the filter or die-off during filtration) would also influence CFs, but such effects cannot be distinguished from that of marker variability in the pre-filtration water on the basis of the available data.

Several previous ultrafiltration studies (Hill et al. 2005, 2007) have involved carefully controlled experiments where the sample is spiked with a known concentration of a marker, prior to ultrafiltration to focus on losses due to ultrafiltration. However, it is often not practical to spike samples in the field (especially in Bangladesh) and there would still be uncertainty as to whether ultrafiltration losses of the spiked marker would be similar to losses of in situ markers from the sampled aquifer. To separate WW variability from potential ultrafiltration artifacts, a 100-L sample could have been homogenized before ultrafiltration.

The markers, total coliforms, *E. coli,* and *mE. coli* all correlated strongly with one another in the retentate samples (Table 4). This is expected since *E. coli* is a subset of total coliforms. In contrast, *Bacteroides* did not correlate with *E. coli*. Adenovirus, which has been proposed as a possible viral fecal indicator, correlated only weakly with the other fecal indicator bacteria. This could be due to different processes controlling transport through porous media for viruses than bacteria (Schijven et al. 2000; Woessner et al. 2001). *E. coli* represents the cultivable subset of all *E. coli* genomes present in the water sample. Because the *mE. coli* primer targets
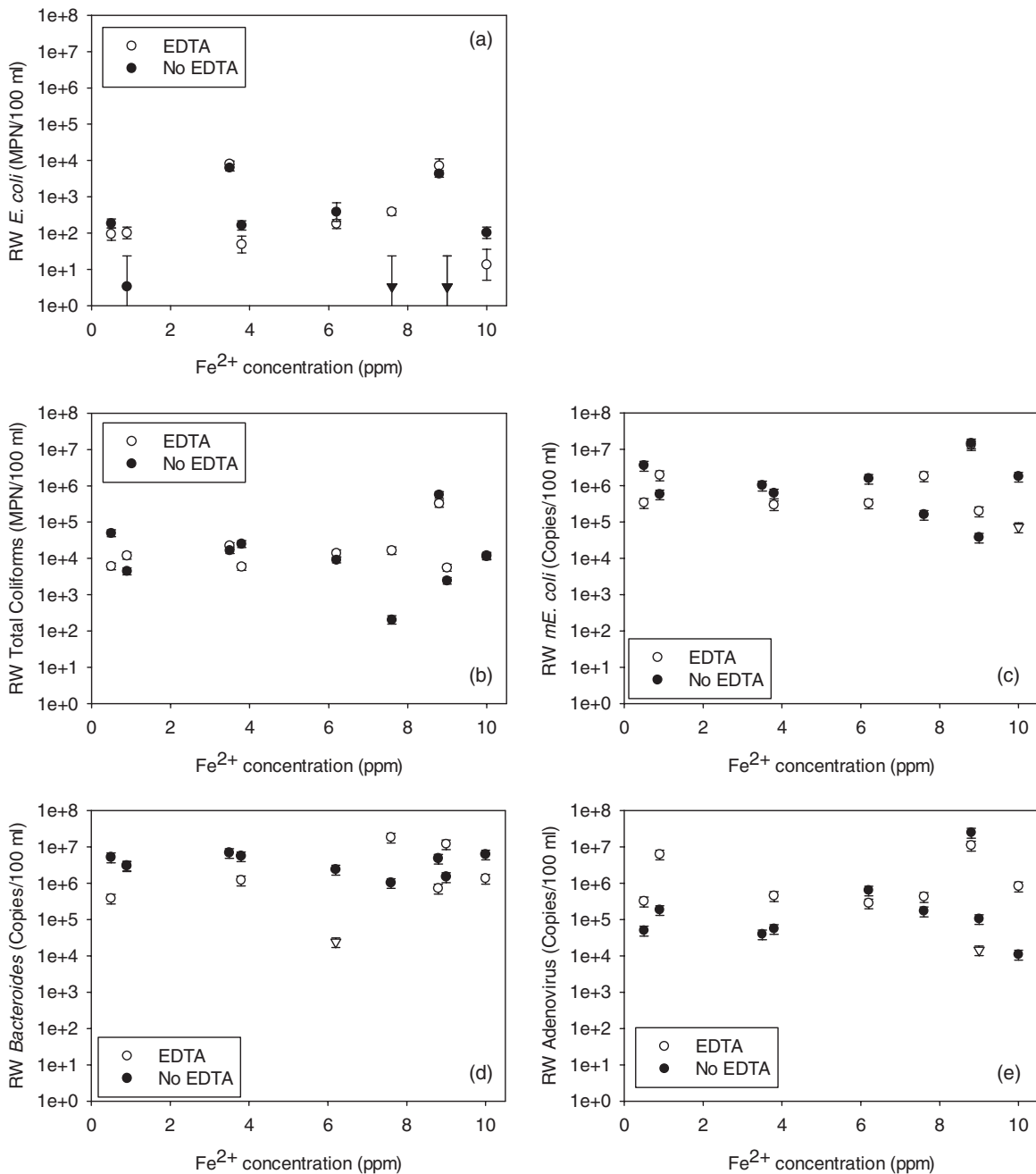
Figure 7. Comparison of retentate water samples from ultrafiltration runs with EDTA and those without EDTA added. Panels a, b, c, d, and e represent *E. coli*, total coliforms, *mE. coli*, *Bacteroides,* and adenovirus. No significant difference was found between the two categories across the range of Fe^{2+} concentrations present in the water. Error bars represent 95% confidence intervals.

the 23S rRNA gene on the *E. coli* genome and this sequence is repeated approximately six times on each genome (Klappenbach et al. 2001), the results of the qPCR assay will give an approximate $6\times$ larger value than the number *E. coli* genomes present in the water sample. Figure 6 shows that cultivable *E. coli* typically consists of 0.1% of the total copies of *E. coli* genomes in RW samples, somewhat less than the 1% in previous reports of percent cultivable *E. coli* in low nutrient waters (Garcia-Armisen and Servais 2004). In the present study, substantial changes in the percent cultivable *E. coli* were

not observed between WW ($n = 13$) and RW ($n = 15$) samples suggesting that ultrafiltration was not inactivating the bacteria in large numbers.

Although there was considerable variability in concentrations of some markers in paired retentate samples taken from the same well, the addition of EDTA did not explain this variability even in high [Fe^{2+}] wells. It was expected that the negatively charged bacteria and viruses would become attached to the positively charged FeOOH particles, resulting in clumping of bacteria and viruses and perhaps denaturation of the viral protein coat as occurs

with viral attachment to metal oxide coated porous media (Abudalo et al. 2005). The lack of a negative correlation between [Fe^{2+}] and cultured bacterial concentration in the retentate in the absence of EDTA suggests that FeOOH particles had no effect on the measured concentration of *E. coli* and total coliforms in the retentate samples. This agrees with other studies which found that larger microorganisms such as bacteria and protozoa do not attach as readily as viruses to FeOOH minerals (Abudalo et al. 2005; Dong et al. 2002). The lack of an observable [Fe^{2+}] effect with the molecular markers indicates that FeOOH colloids did not interfere with recovery of markers during the ultrafiltration process, via clumping and denaturing of viral protein coats, nor did it interfere with DNA extraction and qPCR analysis.

Conclusions

Groundwater from nine wells was concentrated for fecal microorganisms from a contaminated shallow aquifer in Bangladesh. By measuring concentrations of five in situ markers before and after ultrafiltration, it was verified that ultrafiltration resulted in a substantial increase of all the markers in most cases. Measurements on samples collected immediately prior to or during ultrafiltration indicated that both cultured and molecular bacterial and viral concentrations vary greatly with time or pumped volume from both private tubewells and monitoring wells. This suggests that more research is needed to develop better sampling methods for obtaining representative samples of microorganisms from groundwater. The fact that high [Fe^{2+}] in groundwater did not depress the retentate concentrations indicates that FeOOH colloids neither interfered with the persistence of the molecular markers during filtration nor qPCR detection in the laboratory.

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