Environ Monit Assess (2015) 187: 484
DOI 10.1007/s10661-015-4677-1

Evaluation of a field appropriate membrane filtration method for the detection of *Vibrio cholerae* for the measurement of biosand filter performance in the Artibonite Valley, Haiti

Ashley A. Thomson · Claudia K. Gunsch

Received: 22 October 2014 / Accepted: 9 June 2015 / Published online: 3 July 2015
© Springer International Publishing Switzerland 2015

Abstract Biosand filters in the Artibonite Valley of Haiti, the epicenter of the cholera epidemic that began in October 2010, were tested for total coliform and *Vibrio cholerae* removal efficiencies. While coliform are often used as an indicator organism for pathogenic bacteria, a correlation has never been established linking the concentration of coliform and *V. cholerae*, the causative agent for cholera. Hence, a method for field enumeration of *V. cholerae* was developed and tested. To this end, a plate count test utilizing membrane filtration technique was developed to measure viable *V. cholerae* cell concentration in the field. Method accuracy was confirmed by comparing plate count concentrations to microscopic counts. Additionally, biosand filters were sampled and removal efficiencies of *V. cholerae* and coliform bacteria compared. The correlation between removal efficiency and time in operation, biofilm ("schmutzdecke") composition, and idle time was also investigated. The plate count method for *V. cholerae* was found to accurately reflect microscope counts and was shown to be effective in the field. Overall, coliform concentration was not an appropriate indicator of *V. cholerae* concentration. In 90 % of the influent samples from the study, coliform underestimated *V. cholerae* concentration ($n = 26$). Furthermore, coliform removal efficiency was higher than for *V. cholerae* hence providing a conservative measurement. Finally, time in operation and idle time were found to be important parameters controlling performance. Overall, this method shows promise for field applications and should be expanded to additional studies to confirm its efficacy to test for *V. cholerae* in various source waters.

Keywords Biosand filter · Haiti · *Vibrio cholerae* enumeration · Indicator organism · Cholera

A. A. Thomson · C. K. Gunsch (✉)
Department of Civil and Environmental Engineering, Duke University, Box 90287, Durham, NC 27708, USA
e-mail: ckgunsch@duke.edu

Introduction

Vibrio cholerae is now ubiquitous in Haiti (Enserink 2010). The cholera outbreak in Haiti began in late October 2010, just 9 months after a 7.0 earthquake struck the already fragile country (Walton and Ivers 2011). Over 93,000 people have been sickened by the epidemic and over 2100 have died (Chin et al. 2011). This may be, in part, due to the lack of access to improved water sources. Municipal water treatment is not available in most geographical areas in Haiti, and only 51 % of the rural population has access to an improved water source, which could be a household connection, public standpipe, borehole, protected well or spring, or rainwater collection (Water Supply, The World Bank, 2013). Water from these sources is often treated using point-of-use treatment, and while point-of-use treatment is not ideal due to the variability of treatment efficiency between users, user behavior and acceptance, and limiting factors such as cost and availability, point-of-use options do provide a means of water treatment when municipal water treatment is not available. To this end, a large number of biosand filters have been

installed in Haiti over the past decade, in part due to the efforts of the not-for-profit organization, Clean Water for Haiti. Biosand filters provided users with some assurance when the cholera epidemic began in 2010; however, the removal efficiency of *V. cholerae*, the causative agent for cholera, in biosand filters has never been specifically investigated. Although biosand filters have been evaluated for removal efficiency of indicator organisms such as *Escherichia coli* (Elliot et al. 2008), it is unclear if indicator organisms such as coliform bacteria are appropriate as indicators for *V. cholerae*.

A correlation has never been established linking coliform and *V. cholerae* presence, and it has been shown that indicator bacteria do not always accurately represent concentrations of specific pathogens of interest, nor do removal efficiencies necessarily indicate removal of pathogenic organisms as bacteria have different surface proteins and charges which may lead to different attachment efficiencies during biofiltration (Stoodley et al. 2002; Mueller et al. 2007; Gallardo-Moreno et al. 2003). Because indicator organisms such as *E. coli*, total coliform, and fecal coliform may not be appropriate indicators for the pathogens of interest, there is a need for the development of field-based methods to quantify pathogens. In particular, most efforts have focused on coliform with little work on other microbial species. This is problematic as a correlation may not always exist between removal efficiencies of indicator bacteria and the pathogenic target. Thus, there is a clear need for additional field methods.

In the present study, a plate count test utilizing membrane filtration technique was developed which could be applied for the measurement of viable *V. cholerae* cell concentration in the field. The method was compared to traditional coliform plate methods to determine if coliform are an appropriate indicator of *V. cholerae* concentration in source waters and *V. cholerae* removal efficiency in field biosand filters. To this end, 50 biosand filters in the Artibonite Valley of Haiti, the epicenter of the cholera epidemic, were assessed for removal efficiency of *V. cholerae* and total coliform in May of 2011. Schmutzdecke samples were collected from each filter to investigate the correlation between removal efficiency and schmutzdecke composition in terms of extracellular polymeric substance (EPS) amounts and biofilm microbial community. Other parameters including idle time, time in operation, and water source were also analyzed. The specific objectives of the work were to (1) develop a method for field enumeration of *V. cholerae*, (2) perform a field survey in Haiti to determine if indicator bacteria are appropriate surrogates for estimating *V. cholerae* removal in biosand filters, and (3) identify parameters controlling *V. cholerae* removal.

Materials and methods

Bacterial strain and growth conditions

V. cholerae 0395 was obtained from the Kuehn lab at Duke University. Cells from a single colony were grown in Luria-Bertani (LB) broth (10 g NaCl, 5 g yeast extract, 10 g tryptone per liter) to late log phase at 37 °C. Frozen stocks of *V. cholerae* were prepared by mixing 1 mL of late log phase *V. cholerae* in LB broth mixed with 1 mL of glycerol and stored at −80 °C. Each experiment was initiated from a separate −80 °C glycerol stock, streaked onto an LB plate, and incubated overnight at 37 °C. Cells from a single colony were grown in LB broth and were then used in downstream processing. New cultures were used for each experiment. Pure culture stocks were routinely plated to verify community purity.

Total coliform were isolated by filtering activated sludge from the North Durham Wastewater Treatment Plant with a 0.45-μm mixed cellulose filter (Whatman, Piscataway, NJ) and placing the filter on M-Endo agar (Hi media, San Francisco, CA) according to Standard Method 9222B (*Standard Methods for the Examination of Water and Wastewater*, 18th Edition). Briefly, M-Endo agar was prepared by suspending 51 g of M-Endo agar in 1 L of deionized water with 20 mL of non-denatured ethanol. The mixture was heated to boiling and allowed to cool to 60 °C before growth medium was added into each Petri dish. Wastewater samples were serially diluted in PBS buffer and filtered through 0.45-μm mixed cellulose ester filters (Whatman, Piscataway, NJ) using a vacuum pump. Filters were then placed on the prepared plates and incubated at 35 °C for 24 h. A single colony with metallic phenotype typical of total coliform was enriched and cultured in LB broth overnight. We did not sequence this strain for identification purposes. The culture was maintained in glycerol at −80 °C as described above.

Total coliform and *V. cholerae* quantification

Membrane filtration was selected as the basis for method development for the field quantification of bacteria

because of the ease of implementation in the field and its relatively low cost. To this end, bacteria-specific media were selected for total coliform (M-Endo medium; Thermo Fisher Scientific, Waltham, MA) and *V. cholerae* (thiosulfate-citrate-bile salts-sucrose (TBCS) medium; Thermo Fisher Scientific, Waltham, MA). To minimize field equipment requirements, the standard method for membrane filtration was modified and tested in the lab.

Briefly, sufficient powdered growth medium to make 40 mL of M-Endo broth was initially aliquoted into sterile 50-mL plastic tubes. Deionized water was filtered through 0.2-μm mixed cellulose ester filters (Millipore, Billerica, MA) and added to the tubes to a final volume of 40 mL. Plates were prepared by pipetting 2 mL of broth onto a pad. For enumeration, water samples were filtered through 0.45-μm mixed cellulose ester filters (Whatman, Piscataway, NJ) using a vacuum pump. Filters were then placed on the medium-soaked pad and plates were incubated at 35 °C for 24 h. As shown in Fig. 1a, total coliform can easily be differentiated from other bacteria based on their unique phenotype (i.e., they develop a metallic sheen when grown on M-Endo medium). For the *V. cholerae* quantification, the same method was used except that TCBS (Hi media, San Francisco, CA) was substituted for the M-Endo medium. Using this method, *V. cholerae* can easily be differentiated from other bacteria as it expresses a yellow phenotype (Fig. 1b). Similar to the total coliform test which requires follow-up tests to identify fecal coliform, this *V. cholerae* test is a conservative measurement of multiple disease-associated *Vibrio* species. Follow-up tests could be conducted to confirm the species.

To confirm method accuracy, results from the plate count method were compared to hemocytometer direct bacterial cell counts. Hemocytometer quantification allows for the direct microscope count of bacteria suspended in water or growth medium. On the other hand, plate counts make the assumption that a single colony originates from a single cell which may in turn lead to a smaller cell count compared to hemocytometer counts if one colony actually originates from multiple cells. Hemocytometer and plate count quantifications were both performed from frozen bacterial stocks subsequently transferred to LB plates. LB liquid medium was inoculated with cells. The pure cultures were grown with continuous agitation at 250 rpm at 37 °C overnight. Bacterial pellets were obtained by centrifugation, washed, and resuspended in PBS buffer. Serial dilutions were performed in PBS buffer. All dilutions were plated in triplicate for plate counts (10^0 to 10^{-11}). Hemocytometer counts were performed using the following dilutions: 10^0, 10^{-1}, 10^{-2}, and 10^{-3}. For the plate counts, we used the 10^{-8} dilution as this dilution gave colony counts ranging from 100 to 300, the recommended colony count for the coliform count (*Standard Methods for the Examination of Water and Wastewater*, 18th Edition). Both the hemocytometer counts and the plate counts were extrapolated to obtain a concentration estimate. For each dilution, an optical density (OD) reading was taken at 600 nm on a Hach DR/2500 spectrophotometer (Loveland, CO) and hemocytometer counts were performed to generate a standard curve which relates optical density reading to concentration of cells. Hemocytometer counts and membrane filtration technique plate counts were compared to determine if there

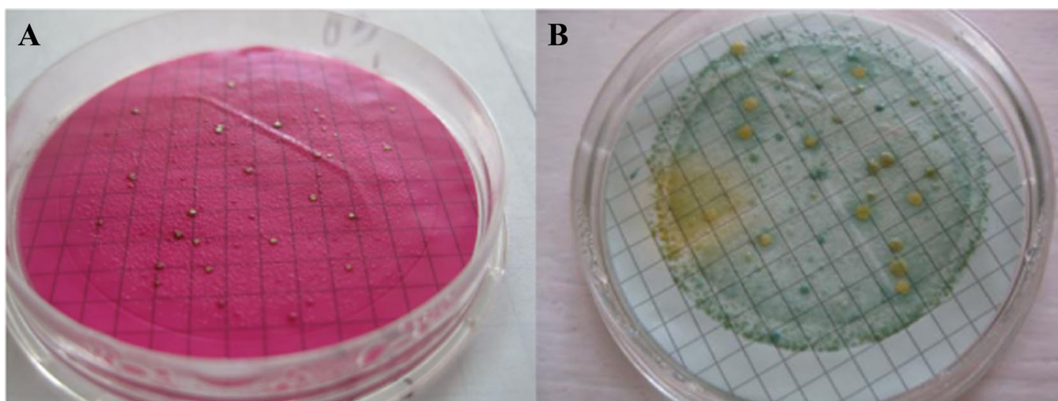

Fig. 1 Mixed culture from field study using described methods plated on **a** M-Endo broth (total coliform shown as *metallic colony*) and **b** TCBS broth (*V. cholerae* shown as *yellow colony*)

was a linear relationship between resulting cell quantifications of the two methods.

Field water sampling

Fifty biosand filters were tested in five different villages in the Artibonite Valley, the epicenter of the cholera epidemic (Fraser, 2010). The project area is indicated in Fig. 2. Sample villages were chosen based on how long biosand filters were in operation (i.e., either 0 days, 4 days, 1 month, 3 months, or 1 year). Note that because of this, village effects cannot be separated from age of filter. Within each village, there was a range of water sources, including shallow, hand dug wells, deep wells, and water from the Artibonite River. Upon sampling at each home, the users were interviewed and information was collected about the time of sample collection, age of filter, typical use, time the filter was last operated, and water source. Although frequency of use and consistency of use could not be accurately measured during this field study, water sources were annotated. Three samples of the schmutzdecke were collected using a small plastic tube (4″ depth, 0.25″ diameter) and were placed into sterile 1.5-mL microfuge tubes (VWR, Radnor, PA). Schmutzdecke sampling occurred after the influent and effluent water samples were collected as to not have short circuiting in the filter. Also, samples of influent and effluent water were collected in triplicate. Six liters of influent and effluent water were collected gravimetrically, stored in sterile whirlpak bags (Nasco, Fort Atkinson, WI), and kept on ice until they were placed in a 4 °C refrigerator overnight prior to enumeration the

Fig. 2 Map of Haiti with the source of the cholera epidemic (*red star*) and the area sampled (*red rectangle*) (Source—"Haiti (Political) 1987". Perry-Castaneda Library, Map Collection, The University of Texas at Austin. Accessed Oct. 2014 at <http://www.lib.utexas.edu/maps/americas/haiti.gif>)

following day. Effluent water was collected directly from the spout of the biosand filter. Input water was collected from the source water collection bucket with a plastic cup.

Field bacterial removal efficiency measurements

Total coliform and *V. cholerae* concentrations were measured using the membrane filtration technique as described earlier. For each sample, three dilutions were measured to ensure appropriate enumeration. To this end, three different volumes (300, 30, and 10 mL) of each water sample were vacuum filtered through 0.45-μm mixed cellulose ester filters (Whatman, Piscataway, NJ) in triplicate. The filters were then placed on Petri dishes containing absorbent pads (Pall Life Science, Ann Arbor, MI) which were previously treated with either TCBS or m-Endo growth media for *V. cholerae* and total coliform, respectively. The Petri dishes were then incubated at 35 °C for 24 h. Total coliform and *V. cholerae* quantification on both the influent and effluent water samples were performed in triplicate. All equipment was sterilized with ethanol between each filter sample set. Negative controls were performed without water by performing the same procedure as with regular sample tests to ensure no contamination was occurring. The filter was opened, removed with sterilized tweezers, and placed on the filtration device; the pump was turned on and then off after several seconds; and the filter was removed with tweezers, placed onto the respective plate, and incubated at 35 °C. It should be noted that when a routine (or repeat) sample tests positive for total coliforms, the US EPA requires that the sample also be analyzed for fecal coliform or *E. coli*, types of coliform directly associated with fresh feces (US EPA2015). However, this was not done in this study.

Community analysis by T-RFLP

DNA was extracted using a method adapted from Gunsch et al. (2005) and described in Thomson et al. (accepted for publication). PCR amplification of the *16S rRNA* gene, PCR purification, and T-RFLP were performed as described in Thomson et al. (in review) based on methods described in Alito and Gunsch (2014) and Ikuma and Gunsch (2013). Applied Biosystems GeneScan v3.7.1 software (Foster City, CA) was used to inspect terminal restriction fragment length polymorphism (T-RFLP) profiles. T-REX (T-RFLP analysis Expedited) software, available online (Culman et al. 2009), was used. Non-metric multidimensional scaling (nm-MDS) analysis was performed and ordination plots were generated using palaeontological statistics (PAST) (Hammer and Harper 2006).

EPS extraction and analysis

EPS was extracted using a cation exchange resin as previously described (Badireddy and Chellam, 2011). Briefly, 75 mg of the Na^+ form of a polystyrene divinylbenzene microporous ion exchange resin (Dowex 50WX8, 20–50 mesh, Sigma Aldrich)/g volatile suspended solids (VSS) was added to 50 mL of sample and shaken at 900 rpm for 4 h at 4 °C. EPS was harvested by collecting the supernatant in a two-step centrifugation at 4 °C (first at 5000*g* for 15 min and then at 12000*g* for 30 min). The supernatant was then filtered using a 0.45-mm cellulose acetate membrane to remove resin, microorganisms, and residual debris. The filtrate was then analyzed for EPS. After collection from each biosand filter, samples were stored at −20 °C until further analysis. Total suspended solids (TSS) and volatile suspended solids (VSS) of the schmutzdecke were measured according to standard methods as described in Gunsch et al. 2007. Briefly, 0.2-μm glass microfiber filters (VWR, Radnor, PA) were prepared by rinsing with 20 mL of deionized water three times. Then, filters were incubated at 105 and 550 °C to remove any dust or other particles. Filters were first weighed to determine initial mass. Then, 0.1 g of each schmutzdecke sample was filtered using a vacuum pump. The filters were incubated at 105 °C for 1 h and weighed to determine the mass of TSS. Next, filters were incubated at 550 °C for 1 h and then weighed to determine VSS. TSS and VSS for the schmutzdecke samples were measured in duplicate.

Protein was quantified by the Modified Lowry Protein Assay Kit (Pierce Biotechnology, Rockford, IL) with bovine serum albumin standards. Briefly, 40 μL of standard or sample was plated on a microplate (Thermo Fischer Scientific) in duplicate. Two hundred microliters of modified Lowry reagent was added to each well, and the plate was shaken at 1500 rpm for 30 s using a Thermo Electron Corporation Multiskan MCC (Waltham, MA) plate reader. Then, 20 μL of prepared 1X Folin-Ciocalteu reagent was added, and the plate was shaken at 1500 rpm for 30 s. The plate was covered

and incubated at room temperature for 30 min. Then, the absorbance was recorded at 690 nm.

Carbohydrates were measured using the phenol-sulfuric acid method against glucose standards (Badireddy and Chellam, 2011). Briefly, 10 μL of standard or sample was plated on a microplate in duplicate. Five microliters of 80 % phenol solution (*w*/*v*) was added to each well and the plate was briefly shaken at 1500 rpm to mix. Then, 200 μL of sulfuric acid was added to each well. The plate was incubated at room temperature for 10 min and the absorbance was recorded at 490 nm.

Uronic acid was determined as described in Badireddy and Chellam (2011). Briefly, 40 μL of either standard or sample was added to a microplate in duplicate. Two hundred microliters of sulfuric acid (96 % *w*/*w*) containing 120 mM sodium tetraborate was added. The plate was shaken at 1500 rpm to mix and then incubated at 80 °C for 1 h. Then, 100 μL of *m*-hydroxydiphenol was added to each well, the plate was shaken at 1500 rpm, and incubated at room temperature for 15 min. The absorbance was measured at 540 nm.

Statistical analysis

The unpaired, two-tailed student's *t* test was used to identify statistical differences between samples. Results were considered statistically different when the *p* value <0.05. It was not verified that the data was normally distributed, though this method is commonly used for this type of application (Campos et al. 2002; Kennedy et al. 2012; Murphy et al. 2010).

Results and discussion

Plate counts versus hemocytometer counts of *V. cholerae* and total coliform

The plate count method for *V. cholerae* developed in this study was found to accurately reflect microscope counts. No statistical difference was detected ($p > 0.05$) between membrane filtration technique and hemocytometer counts for either total coliform or *V. cholerae* using the described methods (Fig. 3). Linear correlations were observed between *V. cholerae* concentration obtained by membrane filtration and hemocytometer counts (R^2~1) and total coliform concentration obtained by membrane filtration and hemocytometer counts (R^2~1).These data suggest that the described membrane filtration technique methods, both for total coliform and *V. cholerae*, are accurate compared to concentrations obtained via microscope counts and provide an accurate measure of cell concentration.

Total coliform as an indicator of *V. cholerae* in field biosand filters

Source water concentrations of total coliform ranged from approximately 100 to 2000 cfu/100 mL. Source water concentrations of *V. cholerae* ranged from approximately 100 to 3000 cfu/100 mL. Figure 4 shows the measured concentration of *V. cholerae* versus total coliform from source waters tested in the Artibonite Valley, Haiti, using the described membrane filtration technique methods. A 1:1 line is added for comparison purposes. Overall, total coliform concentration was not an appropriate indicator of *V. cholerae* concentration in water. In 90 % of the influent samples from the study, coliform underestimated *V. cholerae* concentration (total $n = 26$). Interestingly, when comparing treatment efficiency, total coliform removal efficiency underpredicted *V. cholerae* removal efficiency ($n = 16$) (Fig. 5). In two cases, a negative total coliform removal efficiency was observed, indicating that higher concentrations of total coliform were measured in the effluent compared to the influent. These data points were associated with biosand filters that had only been operating for 4 days. Thus, one possibility for these results is that the sand within the filter may be contributing to the total coliform load, as during construction, the sand may not have been rinsed thoroughly enough to remove the naturally occurring microorganisms in the sand, which would generally include coliform bacteria. These data suggest that total coliform removal efficiency is not a good predictor of *V. cholerae* removal efficiency; however, in this case, the coliform data provide a conservative measurement as the removal efficiency is higher for *V. cholerae*. Caution should be exercised when interpreting these data as we did not test the full range of bacterial genera known to be included in total coliform counts including *Klebsiella*, Enterobacter, *Serratia*, *Citrobacter*, and *Escherichia* (Edberg et al. 2000). Similar analyses should however be carried out with other pathogens (i.e., *Salmonella* spp., *Shigella* spp., etc.) to determine if this observation remains consistent in other cases. It is likely that schmutzdecke composition and physical/

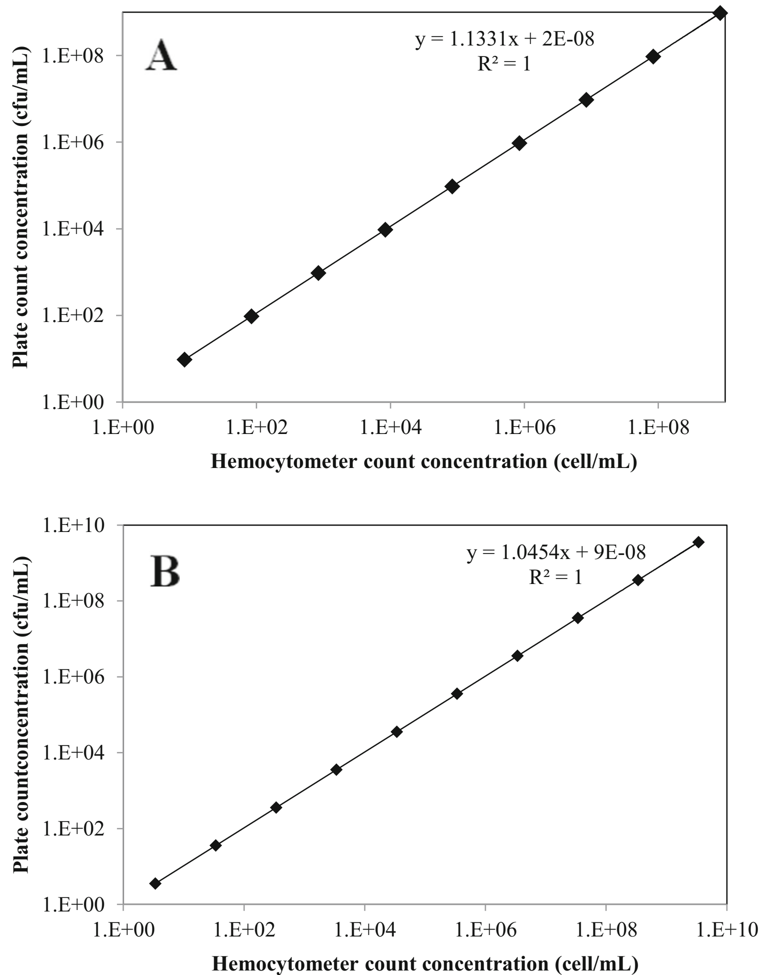

Fig. 3 Experimental concentration (membrane filtration technique) vs. actual concentration (hemocytometer counts) of **a** *V. cholerae* and **b** total coliform. *Error bars* represent the standard deviation of triplicate samples (not visible due to small standard deviations)

chemical attachment play an important role in dictating which bacterial strains will be removed preferentially. Further discussion on this point is presented below.

Time in operation affects removal efficiency of total coliform and *V. cholerae* in field biosand filters

The average removal of *V. cholerae* for all collected samples was 80 ± 31 %. The average removal of total coliform was 63 ± 68 %. However, when samples are separated into groups based on a biosand filter's time in operation, standard deviations are minimized, suggesting that time in operation is an important parameter controlling removal over other variables including water source, idle time, and frequency of use (Fig. 6). This is consistent with previous work by Elliot et al. (2008) who showed that removal efficiency of *E. coli* increased as time in operation increased in a lab study. This improvement was attributed to the formation of the schmutzdecke biofilm.

The average percent removal for *V. cholerae* from 4-day old, 1-month, 3-month, and 1-year filters were 65 ± 24 % (n = 10), 92 ± 8 % (n = 10), 96 ± 3 % (n = 9), and 97 ± 3 % (n = 10), respectively. As filter operation time increases, the average removal efficiency of *V. cholerae* increases. This was attributed to the formation of the schmutzdecke (discussed below). Filters having operated for less than 1 month showed the largest overall range of removal efficiencies (ranging from 19 to 94 %). The range became much tighter with extended operation (77 to 99 % for 1-month filters, 90 to 99 % for 3-month filters, and 91 to 99 % for 1-year filters). Beyond 3 months of operation, removal efficiency does not improve significantly ($p > 0.05$), suggesting that the first 3 months of operation play a critical role for biofilm formation. The data also suggest that filters

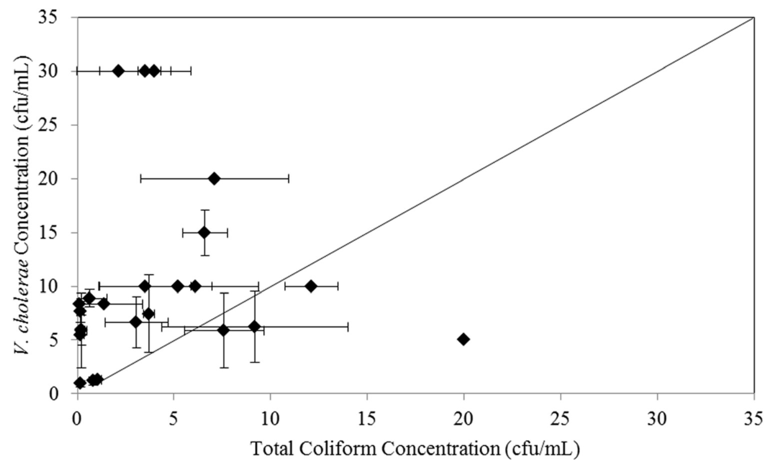

Fig. 4 Total coliform concentration does not correlate well with *V. cholerae* concentration in influent waters. A *1:1 line* is shown for comparison purposes. *Error bars* represent the standard deviation of triplicate samples. ($n = 26$)

reach steady-state removal of coliform and *V. cholerae* within the initial 3 months of operation independent of other variables. It should be noted that users are advised to charge six buckets (~5 gal or 19 L each) of water before consuming the water effluent (personal communication, Clean Water for Haiti, 2011), but this may not be sufficient to provide high level of pathogen removal (i.e., greater than 90 %). Thus, it may be more advisable to design guidelines that take other operational parameters into consideration to better target when the water is safe for consumption. As shown herein, some biosand filters were achieving *V. cholerae* removal in excess of 90 % by the 1-month sampling point. However, it is unclear which parameters contributed to the higher removal rates in some filters over others. A larger study should be carried out to further establish correlations between water characteristics, operational parameters, and water safety in terms of pathogen removal.

Other key factors play a role in removal efficiency

In order to explore factors influencing biosand filter performance besides time in operation, a comparative analysis of the microbial community structure of bacteria in the schmutzdecke was performed using T-RFLP. Using principal component analysis, the data show that time of operation did not explain schmutzdecke microbial community structure suggesting other parameters exist that contribute to biosand filter performance (Fig. 7). Since this is a field study where multiple

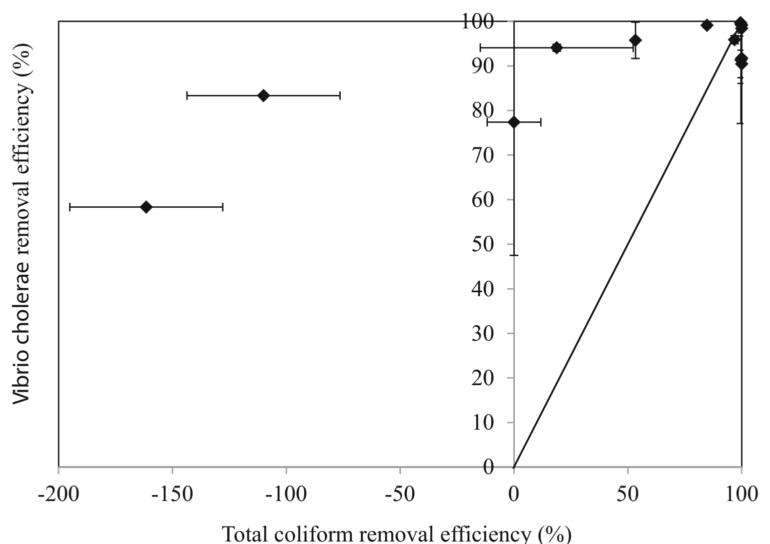

Fig. 5 Total coliform removal efficiency is not a good predictor for *V. cholerae* removal efficiency in biosand filters. A *1:1 line* is shown for comparison purposes. *Error bars* represent the standard deviation of triplicate samples. ($n = 16$)

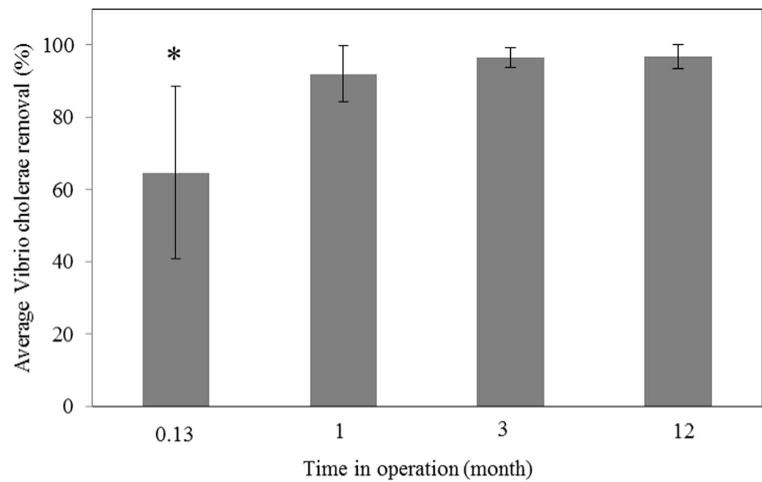

Fig. 6 Average *V. cholerae* removal efficiency (%) based on time in operation. *Error bars* represent the standard deviation within the age group. The *asterisk* indicates statistical difference from filters having operated for 12 months

variable exist and where the sample size is limited (n = 37), this result is not unexpected as microbial profiles will vary greatly based on not only time in operation but also on other factors, such as source water characteristics, idle time, and relative amount of biofilm (indicated by amount of EPS). These factors are explored below.

Figure 8 shows a non-metric multidimensional scaling (nm-MDS) plot of biofilm schmutzdecke microbial communities with vectors for three environmental factors—idle time, time in operation, and amount of EPS. Not surprisingly, based on previous analysis (Fig. 6), time in operation accounted for much of the spread, shown by the vector along axis. Idle time and amount of EPS were inversely related, suggesting that these factors have opposite effects on the microbial community structure. This may be because the biofilms experiencing short idle times do not need as much

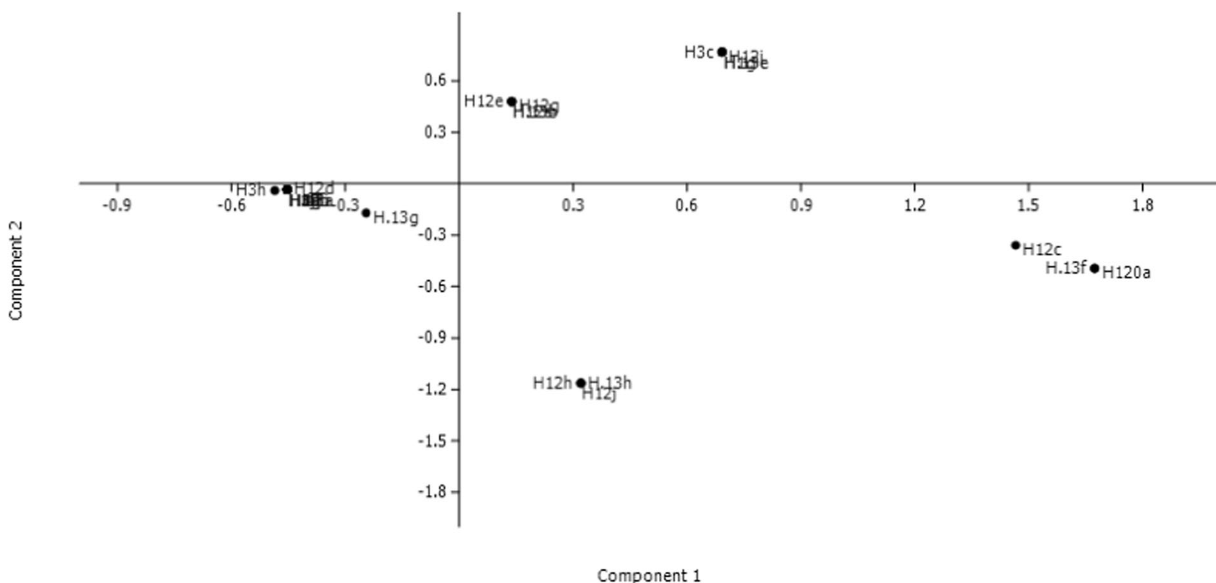

Fig. 7 Principal component analysis (*PCA*) of Haiti schmutzdecke microbial community (*T-RFLP*). Data labeled *H* for Haiti, ## for filter time in operation (months), and then a *letter* designating individual filter number with the age group. Note that several points overlap. Clockwise from the low middle, the following *points* overlap: H.13h, H12j, H12h; H1f, H1m, H1j, H1n, H3e, H3d, H3i, H3g, H3h, H3a, H3b, H3f, H12f, H12b, H12d, H0b, H0a, H0e, H.13a, H.13i, H1q; H.13b, H12a, H12e, H12g; H.13c, H.13e, H1g, H3c, H12i; H.13f, H120a

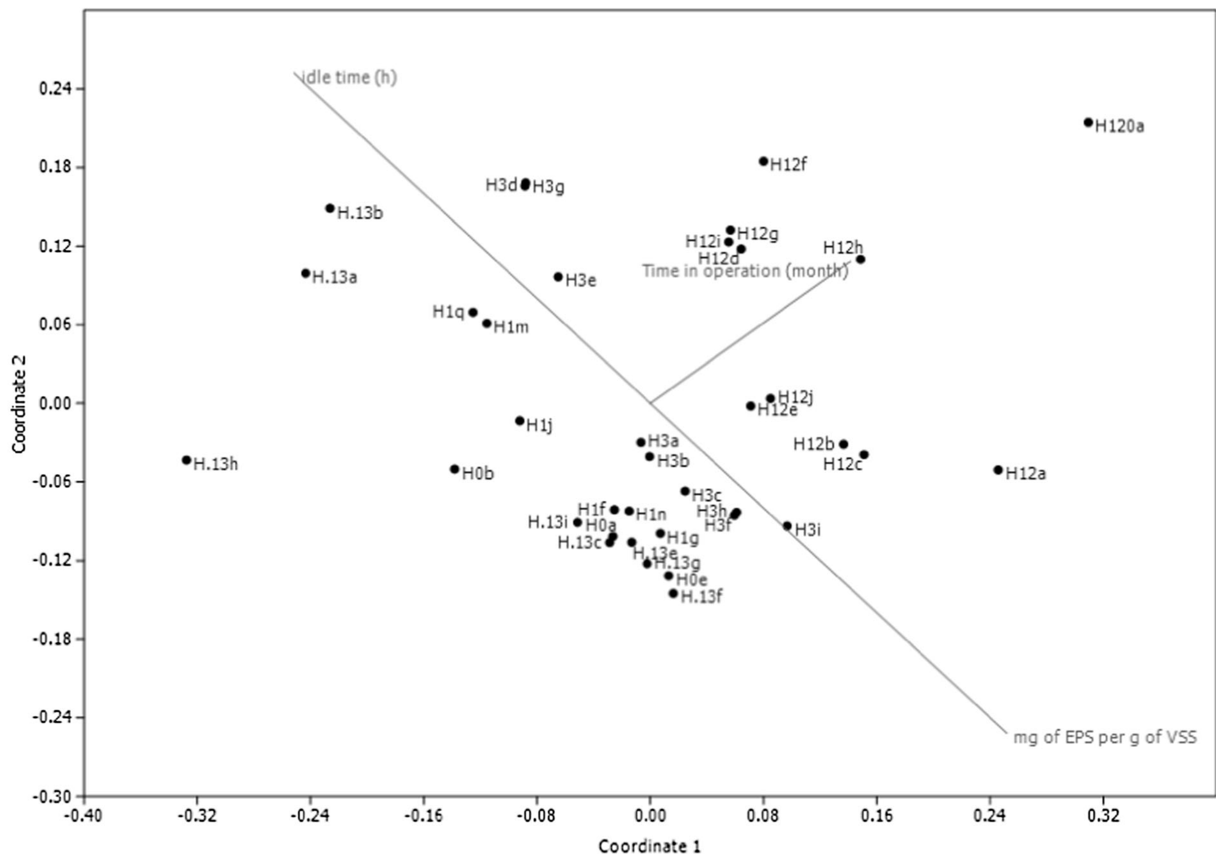

Fig. 8 Non-metric multidimensional scaling (*nm-MDS*) plot of Haiti schmutzdecke microbial community (T-RFLP analysis) with three environmental factors (idle time, filter time in operation, and amount of EPS)

EPS as bacteria are always present. However, the biofilms experiencing longer idle times may need more EPS because bacterial growth may be limited. Analyzing the nm-MDS plot with PAST, time in operation was associated with component 1, which accounts for 47.5 % of the microbial community differences while idle time was associated with component 2, which accounts for 37.1 % of the microbial community differences. These data suggest that time in operation and idle time significantly affect the biofilm and, hence, filter performance. Additionally, there is no clear correlation between time in operation and amount of EPS (Fig. 9). These data suggest that there are other key parameters contributing to removal efficiency besides time in operation. A possible explanation for this finding is that time in operation does not necessarily indicate schmutzdecke age. The standard protocol calls for users to stir the schmutzdecke when the effluent flow rate becomes too slow for the user. When mixing occurs, some schmutzdecke detachment will occur thereby increasing the effluent flow rate. The schmutzdecke will then need to be reconstituted (i.e., grow) prior to the biosand filter reaching an optimal removal efficiency once more. Thus, it is likely that users with water sources containing higher total organic carbon (TOC) and higher turbidity will need to stir the schmutzdecke more frequently, thus resetting the schmutzdecke age, as compared to users with a deep well as a water source (corresponding to water with low TOC and low turbidity).

In addition to water source characteristics, other parameters including idle time, frequency of use, and consistency of use may play a critical role in treatment efficacy. If a filter experiences too long of an idle time (>48 h) or an inconsistent use, others have shown that the biofilm may begin to deteriorate due to starvation or lack of oxygen (Lawrence et al. 2004; Hunt et al. 2004;

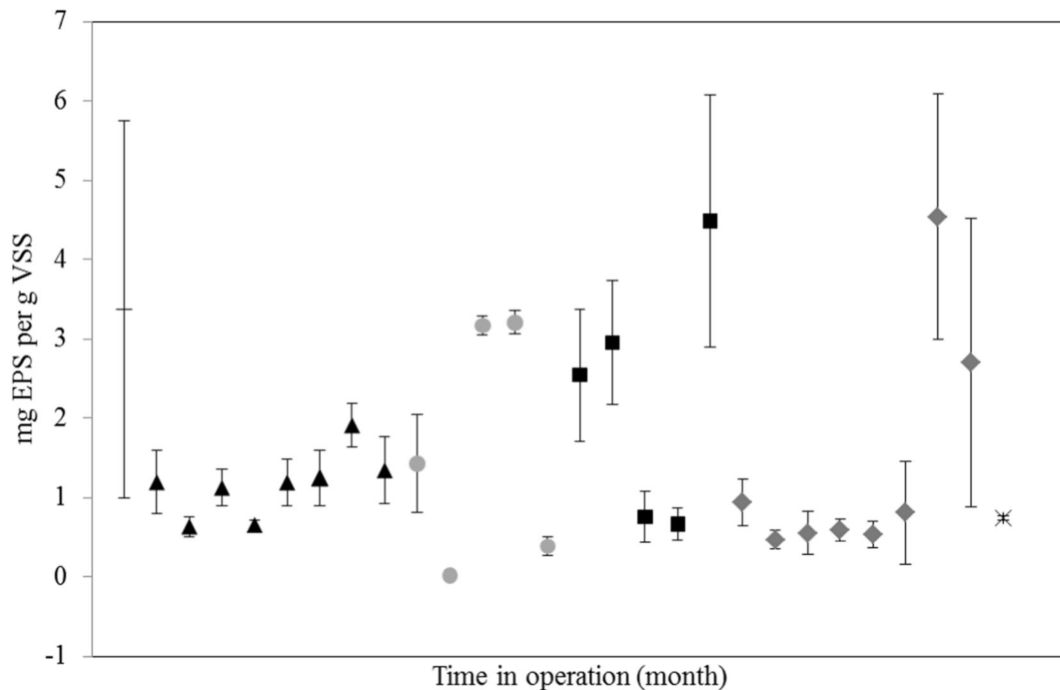

Fig. 9 EPS in Haiti biosand filter schmutzdecke samples, filter time in operation of less than 1 day (*plus sign*), 4 days (*black triangle*), 1 month (*gray oval*), 3 months (*black square*), 12 months (*gray diamond*), and 120 months (*small letter x*). *Error bars* represent the standard deviation of duplicate samples

van der Kooij et al. 2003). On the other hand, if the biosand filter is used too often (i.e., more than twice per day), the residence time may not be long enough for a biofilm to develop or for other removal mechanisms to take place, such as starvation (which occurs in the depths of the filters due to lack of nutrients and/or oxygen) or predation (which occurs in the schmutzdecke) (Elliot, 2008; Hunt, 2004). Such frequent use may also lead to more biofilm shearing events and ultimately decrease the amount of EPS. Although frequency of use and consistency of use could not be accurately measured during this field study, water sources were annotated and there was strong variability of water source within each age group. Follow-up studies should consider the importance of source water TOC on the development of a robust schmutzdecke layer and the resulting treatment performance.

Conclusions

A method for the quantification of *V. cholerae* in the field was developed and implemented. The plate count method for the enumeration of *V. cholerae* in the field was shown to be accurate as compared to hemocytometer counts, yielding differences between the two methods that were not statistically significant. This test offers a relatively inexpensive (comparable to the total coliform membrane filtration test) and simple way to visualize *V. cholerae* colonies in a mixed culture setting by using a selective growth medium. Total coliform removal did not correlate with *V. cholerae* removal suggesting a need for pathogen specific tests when evaluating biosand filter performance especially in an outbreak situation. Finally, time in operation and idle time were found to be key parameters in determining the removal of *V. cholerae* in field biosand filters.

Acknowledgments

This material is based upon work supported by the National Science Foundation Graduate Research Fellowship Program and Dare to Love: Project Haiti. Any opinions, findings, conclusions, or recommendations expressed in this material are those of the author(s)

and do not necessarily reflect the views of the NSF. We would like to thank our collaborators at Clean Water for Haiti for their support in this research.

References

Alito, C., & Gunsch, C. (2014). Assessing the effects of silver nanoparticles on biological nutrient removal in bench-scale activated sludge sequencing batch reactors. *Environmental Science & Technology*, *48*(2), 970–976.

Badireddy, A., & Chellam, S. (2011). Bismuth dimercaptopropanol (BisBAL) inhibits formation of multi-species wastewater flocs. *Journal of Applied Microbiology*, *110*(6), 1426–1437.

Campos, L. C., Su, M. F. J., Graham, N. J. D., & Smith, S. R. (2002). Biomass development in slow sand filters. *Water Research*, *36*, 4543–4551.

Chin, C., Sorenson, J., Harris, J., Robins, W., Charles, R., Jean-Charles, R., Bullard, J., Webster, D., Kasarskis, A., Peluso, P., Paxinos, E., Yamaichi, Y., Calderwood, S., Mekalanos, J., Schadt, E., & Waldor, M. (2011). The origin of the Haitian cholera outbreak strain. *New England Journal of Medicine*, *364*(1), 33–42.

Culman, S. W., Bukowski, R., Gauch, H. G., Cadillo-Quiroz, H., & Buckley, D. H. (2009). T-REX: software for the processing and analysis of T-RFLP data. *BMC Bioinformatics*, *10*, 171.

Edberg, S. C., Rice, E. W., Karlin, R. J., & Allen, M. J. (2000). *Escherichia coli*: the best biological drinking water indicator for public health protection. *Journal of Applied Microbiology*, *88*, 106S–116S.

Elliott, M., Stauber, C., Koksal, F., DiGiano, F., & Sobsey, M. (2008). Reductions of E-coli, echovirus type 12 and bacteriophages in an intermittently operated household-scale slow sand filter. *Water Research*, *42*(10–11), 2662–2670.

Enserink, M. (2010). Infectious diseases: Haiti's outbreak is latest in cholera's new global assault. *Science*, *330*(6005), 738–739.

Fraser, B. (2010). Haiti still gripped by cholera as election looms. *Lancet*, *376*(9755), 1813–1814.

Gallardo-Moreno, A., Gonzalez-Martin, M., Bruque, J., Perez-Giraldo, C., Sanchez-Silos, R., & Gomez-Garcia, A. (2003). Influence of the growth medium, suspending liquid and measurement temperature on the physico-chemical surface properties of two enterococci strains. *Journal of Adhesion Science and Technology*, *17*(14), 1877–1887.

Gunsch, C., Cheng, Q., Kinney, K., Szaniszlo, P., & Whitman, C. (2005). Identification of a homogentisate-1,2-dioxygenase gene in the fungus *Exophiala lecanii-corni*: analysis and implications. *Applied Microbiology and Biotechnology*, *68*(3), 405–411.

Gunsch, C., Kinney, K., Szaniszlo, P., & Whitman, C. (2007). Relative gene expression quantification in a fungal gas-phase biofilter. *Biotechnology and Bioengineering*, *98*(1), 101–111.

Hammer, Ø., & Harper, D. A. T. (2006). *Paleontological data analysis*. Blackwell.

Hunt, S., Werner, E., Huang, B., Hamilton, M., & Stewart, P. (2004). Hypothesis for the role of nutrient starvation in biofilm detachment. *Applied and Environmental Microbiology*, *70*(12), 7418–7425.

Ikuma, K., & Gunsch, C. (2013). Successful genetic bioaugmentation with *Pseudomonas putida* for toluene degradation in soil columns: engineering in situ microbial adaptation: a case study for genetic bioaugmentation in soil columns. *Environmental Chemistry Letters*, *11*(4), 365–370.

Kennedy, T. J., Hernandez, E. A., Morse, A. N., & Anderson, T. A. (2012). Hydraulic loading rate effect on removal rates in a biosand filter: a pilot study of three conditions. *Water Air Soil Pollution*, *223*, 4527–4537.

Lawrence, J., Chenier, M., Roy, R., Beaumier, D., Fortin, N., Swerhone, G., Neu, T., & Greer, C. (2004). Microscale and molecular assessment of impacts of nickel, nutrients, and oxygen level on structure and function of river biofilm communities. *Applied and Environmental Microbiology*, *70*(7), 4326–4339.

Mueller, R., McDougald, D., Cusumano, D., Sodhi, N., Kjelleberg, S., Azam, F., & Bartlett, D. (2007). *Vibrio cholerae* strains possess multiple strategies for abiotic and biotic surface colonization. *Journal of Bacteriology*, *189*(14), 5348–5360.

Murphy, H. M., McBean, E. A., & Farahbakhsh, K. (2010). Nitrification, denitrification and ammonification in point-of-use biosand filters in rural Cambodia. *Journal of Water and Health*, *8*(4), 803–817.

Stoodley, P., Sauer, K., Davies, D., & Costerton, J. (2002). Biofilms as complex differentiated communities. *Annual Review of Microbiology*, *56*, 187–209.

van der Kooij, D., Vrouwenvelder, J., & Veenendaal, H. (2003). Elucidation and control of biofilm formation processes in water treatment and distribution using the unified biofilm approach. *Water Science and Technology*, *47*(5), 83–90.

Walton, D., & Ivers, L. (2011). Responding to cholera in post-earthquake Haiti. *New England Journal of Medicine*, *364*(1), 3–5.