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ORIGINAL ARTICLE

Automated concentration and recovery of micro-organisms from drinking water using dead-end ultrafiltration

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Bacillus spores, concentration, detection, distribution system, micro-organisms, ultrafiltration, water quality.

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Abstract

Aims: Concentration of pathogens diluted in large volumes of water is necessary for their detection. An automated concentration system placed online in drinking water distribution systems would facilitate detection and mitigate the risk to public health.

Methods and Results: A prototype concentrator based on dead-end hollow fibre ultrafiltration was used to concentrate *Bacillus atrophaeus* spores directly from tap water. Backflush was used to recover accumulated particulates for analysis. In field tests conducted on a water utility distribution system, $3{\cdot}2 \times 10^4$–$1{\cdot}4 \times 10^6$ CFU ml^{-1} ($6{\cdot}1 \times 10^6$–$3{\cdot}0 \times 10^8$ CFU) were recovered from the filter when $2{\cdot}9 \times 10^7$–$1{\cdot}0 \times 10^9$ CFU were spiked into the system. Per cent recovery ranged from 21% to 68% for flow volumes of 15–21 l. Tests using spore influent levels <10 CFU l^{-1} (spike < 1000 CFU) yielded 23–40% recovery for volumes >100 l.

Conclusions: *B. atrophaeus* spores at levels <10 CFU l^{-1} were concentrated directly from tap water using an automated dead-end hollow-fibre ultrafiltration system.

Significance and Impact of the Study: The prototype concentrator represents a critical step towards an autonomous system that could be installed in drinking water distribution lines or other critical water lines to facilitate monitoring. Recovered samples can be analysed using standard or rapid biosensor methods.

Introduction

Water supplies are critical resources that are vulnerable to accidental or intentional contamination by hazardous substances, including potential human pathogens (NRC 2002; WHO 2004). Any pathogen present in the water supply system will be diluted by the large volume of water surrounding it; but it might continue to pose a health risk for the end-user. Therefore, there is a global interest in monitoring drinking water supplies for the presence of hazardous microbial agents.

The primary means of securing the safety of drinking water supplies to date has been to ensure adequate treatment of source water that leaves a sufficient disinfection residual in distributed finished water, coupled with discrete targeted sampling and analysis of the finished water. There are problems with this approach in that initial disinfection may be inadequate under certain circumstances, residual disinfectants can be depleted, distribution systems can be breached and current sampling methods only sample a very small portion of the total distribution system at discrete time points and may not be sufficiently representative of the water supply (Medema *et al.* 2003; Robertson *et al.* 2003; Straub and Chandler 2003; Roberson and Morley 2005). Furthermore, standard monitoring methods normally require several days to produce results and detect primarily indicator organisms and/or only a small subset of pathogens that might threaten a water supply, presenting additional problems (Köster *et al.* 2003; Medema *et al.* 2003; Straub and Chandler 2003; APHA *et al.* 2005). Improved water analysis methods that are both rapid and sensitive and that can more reliably indicate potential threats would improve monitoring programmes and be especially useful when

employed in strategic locations throughout a water supply system (Medema *et al.* 2003; Reynolds 2003; Robertson *et al.* 2003; Straub and Chandler 2003).

Because any contaminant introduced into a water resource will likely be rapidly diluted to below the detection limit of most available methods, concentration of micro-organisms from relatively large volumes of water is needed to increase the probability of detection (Köster *et al.* 2003; Medema *et al.* 2003; Reynolds 2003; Straub and Chandler 2003). Protocols to concentrate target micro-organisms from water have been incorporated into several standard methods (Köster *et al.* 2003; APHA *et al.* 2005) and new procedures that can filter larger volumes of water are under investigation (Juliano and Sobsey 1997; Borchardt and Spencer 2002; Morales-Morales *et al.* 2003; Hill *et al.* 2005, 2007; Olszewski *et al.* 2005; Zuckerman and Tzipori 2006; Polaczyk *et al.* 2007). One approach being studied is hollow-fibre ultrafiltration, a versatile and promising technique with the potential to filter large quantities of water and recover a variety of biological contaminants, including viruses, bacteria and protozoa (Juliano and Sobsey 1997; Kuhn and Oshima 2001, 2002; Simmons *et al.* 2001; Morales-Morales *et al.* 2003; Hill *et al.* 2005, 2007). Most hollow-fibre ultrafiltration methods developed to concentrate particulates from water utilize tangential flow, in which a portion of the water pumped through the filter is re-circulated. After filtration, a buffer is re-circulated through the filter to recover particulates and the concentrated material (retentate) is collected. An alternative method is dead-end ultrafiltration in which water is pushed through the filter with no re-circulation. Particulates collected on the filter are backflushed with water or a buffer and the retentate is collected.

Automation of the collection and concentration of micro-organisms from water would facilitate monitoring of water supplies for potentially adverse conditions that indicate a risk to public health. Automated collection/concentration devices could be placed at selected critical points along a water treatment and distribution system and programmed to sample close to continuously. These samples would be more representative of time and location than those that are obtained by existing grab sample approaches. Collected samples would be ready for analysis of index and indicator organisms, as well as specific microbial contaminants, using standard analytical methods. Alternatively, automated sample collection/concentration could be used as part of an online monitoring system that incorporated on-site automated detection. Placement of such near real-time monitoring systems has been stated as a desired long-term goal of water monitoring technology development efforts (Reynolds 2003; Straub and Chandler 2003). Several biosensors exist that could fill the need for autonomous detection (Lim *et al.* 2005; USEPA 2005) but there is currently no fully automated concentration unit that could provide the needed rapid concentration of micro-organisms for these biosensors.

The goal of this research was to develop a prototype automated concentration system for concentrating pathogens from water using dead-end ultrafiltration. The system developed through this effort features water pressure-driven flow that filters drinking water directly from water lines through a hollow-fibre ultrafilter. Sample recovery is accomplished using syringe pump-driven backflush. The retentate recovered from the filter can be analysed using standard microbiological methods or coupled to one of the available rapid biosensor technologies.

Materials and methods

Bacterial spores

Dried viable *Bacillus atrophaeus* spores (received as *Bacillus globigii* or *Bacillus subtilis* variation Niger) (Burke *et al.* 2004) were obtained from the Critical Reagents Program antigen repository (US Army Dugway Proving Ground, Dugway, UT, USA). For initial development of the concentrator, spores were suspended in deionized water (DW) just prior to use to make the desired stock concentration. *Bacillus atrophaeus* spore concentrations in stock suspensions and samples were determined using viable counts. The viable counts tend to underestimate spore concentration and are also subject to large variability; however, microscopic counts of spores in retentates were determined to be less accurate than viable because of the presence of interfering particulates that were also collected and concentrated by the filter. Aliquots of stock suspensions were serially diluted and replicates plated on tryptic soy agar (TSA).

At higher spike levels, retentates were diluted 100- to 1000-fold, depending on the concentration of spores expected in the recovered material, and the two highest dilutions were plated in duplicate on TSA. Additionally, retentate samples were collected before each run and after each cleaning cycle to ensure that no residual contamination with *B. atrophaeus* was present. *Bacillus atrophaeus* colonies were easily distinguished from other microbial growth by colony morphology and their distinctive orange colour. The plates were incubated for 18–24 h at 37°C. All concentration data are reported as CFU ml^{-1}.

For low-level concentration experiments (<10 CFU l^{-1}), 2·5% mineral oil in DW was used to make a stock spore suspension. The suspension was diluted 1 : 10 into DW to a final dilution of 10^{-3} and 100-μl aliquots were distributed into sterile 0·5-μl microfuge tubes and

frozen at −20°C until use. Two 100-μl aliquots were removed and thawed just prior to use. The aliquots were combined and mixed; then, 40–100 μl of the solution was transferred to a 1·5-ml sterile microfuge tube and diluted 1 : 10. When 100-μl aliquots were diluted 1 : 10, 500 μl from each 1 ml dilution volume was transferred to a sterile 1·5-ml tube and heat-shocked at 75°C for 15 min. Replicate 100-μl aliquots ($n = 3$ or 5) from each untreated or heat-shocked dilution tube were plated onto TSA to determine what effect the heat shock procedure might have on viable counts.

Concentration system

The automated concentrator (patent pending), shown in Fig. 1, employs a polysulfone hollow-fibre ultrafilter with an exclusion size of 25–30 nm and a surface area of 0·07 m^2 (Südmo North America, Rockford, IL, USA). Water is pushed through the hollow fibres of the filter using either water line pressure or a pump. The filter cartridge is capped at the opposing end, forcing water through the pores of the fibres in a dead-end filtration path. A permeate line carries water and particles smaller than the pore size of the filter to a drain. Particulates trapped within the fibre cores are removed by backflushing in the reverse direction (from the outside to the inside of the fibres) with buffer pushed by a 50-ml syringe pump (Kloehn Company, Las Vegas, NV, USA) operating at the maximum pump speed setting and the retentate is collected. The retentate can be taken to a laboratory for analysis or the concentrator can be linked to a biosensor for rapid, on-site analysis. A cleaning cycle is incorporated into the procedure to prepare the filter for the next concentration cycle. All parts of the concentration–recovery–cleaning process can be programmed to allow stand-alone operation (fully automated) or each function can be operated via a computer interface (semi-automated). An optional electronic signal is used to trigger automated analysis if a biosensor is linked to the concentrator. For research purposes, a spiking port is located immediately upstream from the filter that enables syringe injection of test micro-organisms.

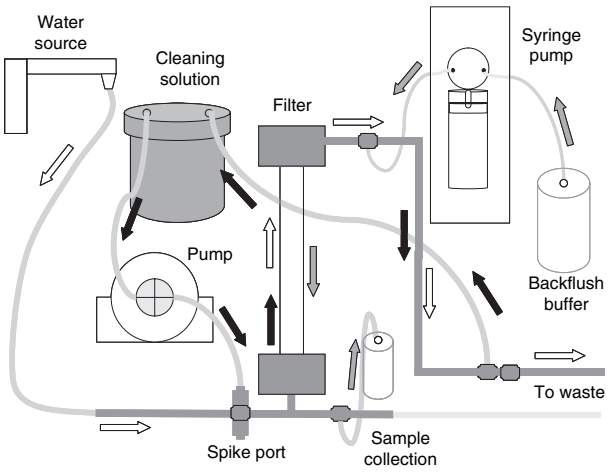

Figure 1 Schematic of dead-end concentration system. Forward flow direction (⇨), backflush flow direction (⇨) and cleaning flow direction (➡).

Concentration protocol

Initial tests to characterize operation of the concentrator used semi-automated operation and either 1-μm carboxylate-modified, yellow-green fluorescent microspheres (FluoSpheres, Molecular Probes, Eugene, OR, USA) or *B. atrophaeus* spores as the test material. Microspheres or spores were injected through the spike port by diluting 0·4–1 ml of stock into a 10-ml syringe with either 0·1 mol l^{-1} phosphate buffer, pH 7·4 (PB) or water and followed by an additional 10 ml injection of water to ensure that the spike material was completely washed into the line leading to the filter. Water (either chloraminated tap or distilled) was run through the filter for 5–10 min after spiking. Forward flow rate was measured once before and once or twice after spiking and was determined by using a stop watch to measure the time taken to collect 1 l of water into a graduated cylinder. Flow rate measurements were used to estimate the volume of water filtered during an experiment. Microspheres were recovered from the filter by backflushing with PB and the spores were recovered using either PB or phosphate-buffered saline with 0·01% Tween 20 (PBST). The filter was cleaned by circulating either hot (55°C) 0·1 N NaOH or 100 ppm buffered bleach (pH < 7·5) through it for 20 min to kill any remaining spores or micro-organisms and then rinsed with DW to remove cleaning solution residue. Filters were stored in 1% bisulfite between experiments to impede microbial growth. Postclean rinse water samples were collected from the filter to monitor the effectiveness of the cleaning process. For some experiments, preconcentration rinse water samples were also collected to monitor the storage process. Aliquots (200 μl) of each were plated in duplicate on TSA.

Procedures for field tests using a fully automated prototype were similar to those using the semi-automated system except that the operation was controlled by a sequencer programme, the total filtration time was 20 min, all tests were done using chloraminated tap water flow and spores were backflushed with sterile DW. The sequencer programme contained all the functions used in semi-automated laboratory experiments and the same programme sequence was used for all experiments. In all field tests, water flow through the filter proceeded for

5 min prior to spiking and for 15 min after. The flow rates were collected once prior to spiking and at least twice after spiking (within first and last 5 min of run) and used to determine the total volume of water filtered. Pressure at the inlet to the concentration system was read at the time of flow measurements using an in-line gauge placed upstream from the spike port. The filter was rinsed manually with DW after the end of the cleaning cycle and stored in 1% bisulfite between experiments. Preconcentration and postclean samples were collected for most runs and plated on TSA as previously described.

To demonstrate that the concentrator could be used to concentrate micro-organisms at levels that might be expected in environmental samples, a final prototype was tested in a simulated application. This prototype differed from the previous in that it included a separate line for backflushing the filter to waste. For these tests, two frozen 100-μl aliquots of *B. atrophaeus* spores were thawed, combined and mixed. After removal of 40–100 μl for viable enumeration, the remainder of the combined aliquots (100–160 μl) was diluted into a 10-ml syringe and used to spike the concentrator. The spiking procedure was similar to that used in prototype development experiments except that the spike port was located just below the tap and transited *c.* 2·7 m of tubing before entering the filter and the spike was injected without stopping forward flow. This ensured greater dilution and mixing of spores in the tap water prior to entering the filter and better mimicked a natural contamination event. To better reflect the large volume of tap water that would need to be filtered in actual applications, the concentration time was programmed for 60 min. The spores were recovered by manually injecting 120 ml of 0·1 mol l^{-1} PB plus 0·01% sodium polyphosphate (NaPP) buffer (PB-NaPP) into the filter through the concentrator spike port and incubating for 5 min. The spores were then backflushed from the filter with the same PB-NaPP buffer using a recovery programme that also included a filter cleaning sequence. Flow rate measurements were recorded as previously described with the first measurement taken within the first 2 min of flow and subsequent measurements taken at 5-min intervals after flow initiation. Thirteen flow rate measurements were obtained during each 60-min experiment. The spore recovery was analysed using a modified culture-based standard method for endospores, method 9218 (APHA *et al.* 2005). The recovered retentate was split into two equal fractions. One fraction was heat-shocked at 75°C for 15 min in a shaking water bath incubator. The temperature was monitored using a thermometer inserted into a container of the same type filled with an equal volume of water. Each fraction was filtered through a 0·45-μm nitrocellulose membrane filter inserted into a 300-ml polyphenylsulfone filter funnel assembly (both from Fisher Scientific, Inc., Pittsburgh, PA, USA). Filters were aseptically removed and transferred to TSA. Prerun (taken after rinsing the filter with tap water) and postclean (taken after dechlorination with sodium thiosulfate) backflush samples were collected, filtered and filters transferred to R2A and/or TSA media. These were done to establish the microbial background on the filter before and after each concentration run and to determine if any *B. atrophaeus* spores could be backflushed from the filter between experiments. Plates were incubated at 35°C for 24–48 h (TSA) or 3–4 days (R2A) before analysis.

Quantification of recovery parameters and statistical analysis of data

The concentration of *B. atrophaeus* spores in each stock suspension was multiplied by the volume injected to determine the total cells spiked into the concentrator. Per cent recovery for experiments at spike concentrations greater than 10 CFU l^{-1} was calculated by first multiplying the concentration of spores in the retentate by the volume collected. This value was divided by the total spores in the spike and multiplied by 100 to determine per cent recovery. In those cases where the retentate was collected in fractions, the total cells recovered in each fraction was calculated and summed. This sum was divided by the total retentate volume collected (sum of fraction volumes) to yield a concentration for the total collected retentate. Per cent recovery for low-level concentration experiments was calculated by counting the number of *B. atrophaeus* colonies on each nitrocellulose filter and multiplying by 2 to get the total recovered for each treatment (heat-shocked and not heat-shocked). The total volume of water filtered was determined by averaging the flow rate measurements in litre per minute and multiplying by the total minutes of flow. For developmental prototypes where flow was stopped for spiking, only the time after spiking was used to calculate volume. In low-level tests which used the final prototype and where the spike was injected into the flow, the entire filtration time was used in the calculation of total volume. Dividing the total cells injected by the total volume filtered provided an estimate of the input concentration.

Statistical analysis of data was performed using SigmaStat 3·1 (Systat Software Inc., Richmond, CA, USA). Differences in per cent recovery between pairs of backflush treatments were evaluated using the Mann–Whitney rank sum test. If the data passed both the normality and variance tests, analysis of significance was evaluated using the *t*-test. To compare retentate concentrations, the data were first log-transformed and then analysed using a *t*-test. For all statistical tests, the significance level was set at 0·05.

Results

Initial characterization of concentration and recovery

Initial studies of concentration and recovery were conducted using a semi-automated prototype so that the performance of the concentrator design could be tested and characterized. Tests were done using both fluorescent microspheres and *B. atrophaeus* spores spiked individually into either DW or tap water flow. The forward flow rate through the filter for these experiments ranged from 0·9 to 1·4 l min^{-1}. An influent pressure gauge was not part of the semi-automated prototype. The fluorescent microspheres permitted rapid assessment because they were easily counted microscopically in the retentate. Microspheres were recovered from the filters using PB. The retentate was collected in fractions for these experiments so that the effectiveness of the backflush sequence could be better assessed. These microsphere tests showed that the concentrator filter collected particulates from a tap water stream and that the collected particulate material could be recovered in a more concentrated suspension using syringe pump-mediated backflush. The average recovery was 98 ± 59%. Fractional collection of the retentate showed that most of the microspheres were recovered in the first 200 ml backflushed from the filter (data not shown).

Table 1 shows results from experiments using *B. atrophaeus* spores. PB or PBST was used to backflush particulates from the filters. The average spore recovery was 81% ± 38% ($n = 7$). Concentrations of spores in the retentates from semi-automated experiments ranged from $3{\cdot}4 \times 10^4$ to $8{\cdot}1 \times 10^5$ CFU ml^{-1} when $3{\cdot}6 \times 10^6$–$1{\cdot}73 \times 10^8$ CFU (equivalent to $3{\cdot}82 \times 10^2$–$3{\cdot}26 \times 10^4$ CFU ml^{-1}) were spiked into the concentrator. Mean per cent recovery was greater when PB was used for backflush (94% ± 48%, $n = 3$) than when PBST was used (71% ± 32%, $n = 4$) but the difference was not significant (*t*-test, $P = 0{\cdot}478$). However, spore recovery tended to be lower in early fractions and higher in later fractions when PBST was used for backflush (data not shown). Higher recovery in earlier backflush fractions minimizes the volume that needs to be collected. In addition, use of PBST resulted in a decrease in the rate of flow through the filter in subsequent experiments. Hill *et al.* (2005) reported a similar decrease in flow rate when water amended with Tween 80 was pumped through a hollow-fibre ultrafilter. Fractional collection of retentates supported the results from microsphere tests in that little additional *B. atrophaeus* spores were recovered after *c.* 200 ml of buffer backflush. Postclean plates showed only low numbers of *B. atrophaeus* colonies indicating concentrations that were several orders of magnitude less than those calculated for retentates and no *B. atrophaeus* colonies were observed on plates from prerun samples.

Per cent recovery is the standard parameter used to evaluate the ability of a sample-processing method to trap and release the target micro-organisms. However, it is important to note that most analysis methods, and especially rapid methods such as immunoassays and polymerase chain reaction (PCR), are dependent on the concentration of target in the recovered material. Hence, the retentate concentration is a more critical parameter than per cent recovery from the perspective of target detection. Characterization experiments on the semi-automated dead-end system showed that more spores could be recovered with increased backflush volume. Although this increased the per cent recovery, it decreased the retentate concentration as relatively few additional spores were recovered after collection of about 200 ml of backflush volume. Consequently, the backflush sequence in subsequent experiments was optimized to provide the highest concentration of target in the retentate.

In order to test the feasibility of direct detection of pathogens in the recovered material, a retentate sample was assayed on the RAPTOR (Research International, Monroe, WA, USA), a fully-automated, evanescent wave-based biosensor platform originally developed at the Naval Research Laboratory in Washington, DC. The

Table 1 Recovery of *Bacillus atrophaeus* spores from water using semi-automated collection/concentration

Back-flush buffer	Total spores spiked (CFU)	Total volume filtered (l)	Influent concentration (CFU ml^{-1})	Retentate volume (ml)	Spore concentration in retentate (CFU ml^{-1})	Total spores in retentate (CFU)	Recovery (%)
PB	$3{\cdot}58 \times 10^6$	9·4	$3{\cdot}82 \times 10^2$	142·5	$3{\cdot}40 \times 10^4$	$4{\cdot}85 \times 10^6$	135·6
PB	$8{\cdot}80 \times 10^6$	9·8	$8{\cdot}96 \times 10^2$	149·5	$6{\cdot}12 \times 10^4$	$9{\cdot}14 \times 10^6$	103·9
PB	$1{\cdot}55 \times 10^8$	6·9	$2{\cdot}24 \times 10^4$	160·5	$4{\cdot}04 \times 10^5$	$6{\cdot}48 \times 10^7$	42·0
PBST	$5{\cdot}93 \times 10^6$	13·9	$4{\cdot}25 \times 10^2$	169	$3{\cdot}64 \times 10^4$	$6{\cdot}15 \times 10^6$	103·78
PBST	$2{\cdot}54 \times 10^7$	17·5	$1{\cdot}45 \times 10^3$	195·5	$4{\cdot}99 \times 10^4$	$9{\cdot}76 \times 10^6$	38·44
PBST	$3{\cdot}82 \times 10^7$	13·9	$2{\cdot}75 \times 10^3$	177·5	$1{\cdot}05 \times 10^5$	$1{\cdot}87 \times 10^7$	48·82
PBST	$1{\cdot}73 \times 10^8$	5·3	$3{\cdot}26 \times 10^4$	198·25	$8{\cdot}10 \times 10^5$	$1{\cdot}61 \times 10^8$	92·82

PB, phosphate buffer, pH 7·4; PBST, phosphate-buffered saline plus 0·1% Tween 20, pH 7·4.

platform uses a standard sandwich immunoassay procedure on a polystyrene optical waveguide to capture and detect specific targets. Waveguides for the experiment were prepared and assays conducted according to published procedures for this biosensor (Kramer and Lim 2004; Simpson and Lim 2005). Polyclonal rabbit anti-*B. atrophaeus* antibodies labelled with biotin or cyanine 5 fluorescent dye (Cy5) were used for capture and detection, respectively. Stock dilutions and sample were incubated on the waveguides, rinsed, incubated with Cy5-labelled antibody and rinsed again before interrogation with a 635-nm laser.

Figure 2 shows the average change in fluorescent intensity in picoAmps (pA) between PBS blanks and samples for three dilutions of a stock *B. atrophaeus* suspension and a retentate sample from one of the PB backflush experiments that were co-assayed. The results demonstrate positive detection of *B. atrophaeus* in the retentate sample, as determined by a signal above the average channel limit of detection (LOD; mean channel signal change plus three times the SD) for two experiments on the same retentate sample. The average channel signal change for the retentate sample (63·5 pA) was within the expected magnitude based on concentration ($1{\cdot}8 \times 10^6$ CFU ml^{-1} compared with signal changes for stock concentrations (14 and 100 pA at $2{\cdot}1 \times 10^5$ and $2{\cdot}1 \times 10^6$ CFU ml^{-1}, respectively).

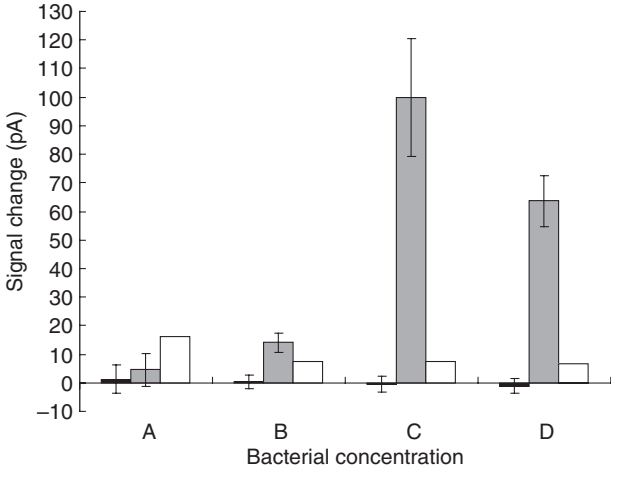

Figure 2 Immunoassay detection of *Bacillus atrophaeus* spores in retentate following collection/concentration using dead-end ultrafiltration. Results were considered positive if they exceeded the limit of detection (LOD) (□), where LOD is the mean signal change of the blanks + (3 × SD of blanks). Samples (▨) consisted of positive controls A, B and C at concentrations of $2{\cdot}1 \times 10^4$, $2{\cdot}1 \times 10^5$ and $2{\cdot}1 \times 10^6$ CFU ml^{-1}, respectively, in phosphate-buffered saline, pH 7·4 (PBS); D is a retentate sample with a concentration of $1{\cdot}8 \times 10^6$ CFU ml^{-1}. Blanks (■) were PBS.

Field test performance using a fully automated prototype

The two goals of the field tests were to demonstrate that collection/concentration of particulates from tap water flow by means of dead-end ultrafiltration could be fully automated and to characterise the automated process. The average per cent recovery for nine field tests was 36% ± 18% (Table 2). Retentate *B. atrophaeus* spore concentrations ranged from $3{\cdot}2 \times 10^4$ to $1{\cdot}4 \times 10^6$ CFU ml^{-1} when $2{\cdot}9 \times 10^7$–$1{\cdot}0 \times 10^9$ CFU of *B. atrophaeus* spores (equivalent to $1{\cdot}5 \times 10^3$–$6{\cdot}0 \times 10^4$ CFU ml^{-1}) were spiked into the concentrator. While these high input concentrations are not representative of amounts that would be expected in contaminated tap water, they were necessary to facilitate trouble-shooting and analysis of prototype performance during this part of the development and testing phase. Flow rates for the nine experiments averaged 1·2 l min^{-1} (range of 1·0–1·4 l min^{-1}) for average influent water pressures of 46·8 ± 0·8 PSI. A decrease in flow rate was observed during each of the experiments with an average decrease of 73 ± 49 ml min^{-1} for all of the tests and also tended to decrease between runs on the same filter despite the cleaning regimen and storage in bisulfite.

Sterile DW was used to backflush particulates from the filter in these experiments because it was thought to be most compatible with an automated system that would operate for long periods without maintenance. It was also considered less likely to react with tap water to produce precipitates that could potentially interfere with rapid analysis methods such as immunoassay or PCR. When compared with the recovery data in Table 1, DW was significantly less effective in removing spores from the filter than PB and PBST (*t*-test, $P = 0{\cdot}007$). This result supports data from the literature (Hill *et al.* 2005; Polaczyk *et al.* 2007), which indicate that buffer solutions containing surfactants or other additives may increase organism recovery from filters.

A sample collection vessel was integrated onto the platform after the first six experiments and resulted in an elevation of the sample collection vessel relative to the retentate output. Prior to this change, sample collection was below the output point. The vessel repositioning required a slightly modified backflush protocol to compensate for differences in the way liquid moved from the filter into the collection vessel and resulted in a slightly reduced retentate volume. The reduction in average volume from 204 ± 4 ml to 190 ± 5 ml was significant (*t*-test, $P = 0{\cdot}003$). The difference in the average *B. atrophaeus* spore recovery before sample vessel repositioning (42 ± 19%) was greater than average recovery after the repositioning (23 ± 6%); however, this difference was not significant (Mann–Whitney rank sum test, $P = 0{\cdot}095$).

Table 2 Recovery of *Bacillus atrophaeus* spores from tap water using fully automated collection/concentration

Total spores spiked (CFU)	Total volume filtered (l)	Influent concentration (CFU ml^{-1})	Retentate volume (ml)	Spore concentration in retentate (CFU ml^{-1})	Total spores in retentate (CFU)	Recovery (%)
$2{\cdot}24 \times 10^8$	17·7	$1{\cdot}43 \times 10^4$	200*	$2{\cdot}43 \times 10^5$	$4{\cdot}86 \times 10^7$	21·6
$1{\cdot}30 \times 10^8$	16·7	$7{\cdot}76 \times 10^3$	202*	$4{\cdot}38 \times 10^5$	$8{\cdot}86 \times 10^7$	67·9
$4{\cdot}43 \times 10^8$	16·6	$2{\cdot}67 \times 10^4$	207*	$5{\cdot}40 \times 10^5$	$1{\cdot}12 \times 10^8$	25·2
$2{\cdot}44 \times 10^8$	17·1	$1{\cdot}43 \times 10^4$	205*	$6{\cdot}21 \times 10^5$	$1{\cdot}27 \times 10^8$	52·2
$4{\cdot}80 \times 10^8$	15·4	$3{\cdot}12 \times 10^4$	200*	$7{\cdot}35 \times 10^5$	$1{\cdot}47 \times 10^8$	30·6
$5{\cdot}20 \times 10^8$	15·8	$3{\cdot}28 \times 10^4$	210*	$1{\cdot}42 \times 10^6$	$2{\cdot}97 \times 10^8$	57·1
$1{\cdot}20 \times 10^8$	20·9	$5{\cdot}74 \times 10^3$	185†	$1{\cdot}58 \times 10^5$	$2{\cdot}93 \times 10^7$	24·4
$2{\cdot}92 \times 10^7$	19·4	$1{\cdot}51 \times 10^3$	190†	$3{\cdot}23 \times 10^4$	$6{\cdot}13 \times 10^6$	21·0
$1{\cdot}04 \times 10^9$	17·4	$5{\cdot}97 \times 10^4$	195†	$1{\cdot}19 \times 10^6$	$2{\cdot}32 \times 10^8$	22·4

*Sample collection vessel was positioned below the point of retentate output.
†Sample collection vessel was integrated into the concentrator platform and positioned above the point of retentate output.

The relationship between the number of spores spiked into the filter and retentate spore concentration was explored for the combined data of Tables 1 and 2 (Fig. 3). This graph suggests that the concentration of spores in the retentate increases with increasing total number of spores spiked into the filter although the data were highly variable. A linear regression curve is plotted over the data to demonstrate the general trend and to better display the variability ($R^2 = 0{\cdot}852$). No correlation between spores spiked into the concentrator and per cent recovery could be determined for these experiments.

Concentration of low levels of spores from high-volume tap water samples

The method used for low spike level experiments simulated a contamination situation in which only a few hundred spores were present in over 100 l of unamended tap water that had been disinfected with chloramine and released to the distribution system. The concentration of spores in the contaminated water was <10 spores l^{-1}. Table 3 shows the data from three experiments performed at this level. Only data for stocks and samples that were not heat-shocked are shown, as heat shock was observed to reduce the number of *B. atrophaeus* colonies in both stock and retentate samples and did not appear to offer any advantages in these experiments. Viable counts used to calculate the 100-μl freezer stock concentrations were more consistent than observed in developmental testing of the prototype where stocks were not treated with mineral oil prior to use. Colony counts on the 1 : 10 dilutions of the aliquots used for spiking were 62·3 ± 11·9, 50·2 ± 3·90 and 33·8 ± 4·66. Heat shock reduced calculated concentrations to 42·8 ± 5·67 and 27·8 ± 4·92 for the second and third experiments, respectively. Heat treatment was not done on stock samples from the first experiment.

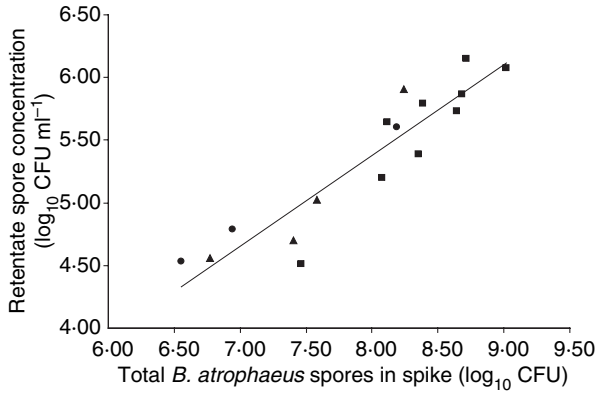

Figure 3 Relationship between *Bacillus atrophaeus* spores spiked into the concentrator and spore concentration in retentate for the combined experiments using three different sterile backflush media. PB (●), phosphate buffer, pH 7·4; PBST (▲), phosphate-buffered saline plus 0·1% Tween 20, pH 7·4; DW (■), deionized water; $R^2 = 0{\cdot}852$.

The average per cent recovery was 30 ± 9% for spikes of 338–997 CFU in flow volumes of 109–116 l (equivalent to 3·11–8·64 CFU l^{-1}) (Table 3). Per cent recovery appeared to increase with decreasing number of spores in the spike. Retentate concentrations ranged from 0·78 to 1·56 CFU ml^{-1}) and increased with higher spike amounts. Influent flow rate for these experiments averaged 1·88 l min^{-1} (range of 1·81–1·92 l min^{-1}) at average influent pressures of 32·7 ± 0·5 PSI and decreased with subsequent experiments performed on the same filter, despite the cleaning regimen and storage in bisulfite. The flow rate also tended to decrease during the 60 min run time of each experiment, with terminal flow rates being 69–82% of the initial flow rates. As with the average flow rate between runs, the flow rate decrease within runs was least for the first experiment and greatest for the last

Table 3 Recovery of low levels of *Bacillus atrophaeus* spores from tap water using automated collection/concentration

Total spores spiked (CFU)*	Total volume filtered (l)	Concentration of spores in tap water (CFU l^{-1})	Retentate volume (ml)	Total spores in retentate (CFU)*	Spore concentration in retentate (CFU ml^{-1})	Recovery (%)
997	115·5	8·64	146	228	1·56	22·86
502	114·8	4·37	162	142	0·88	28·29
338	108·6	3·11	160	130	0·78	40·24

*Data are from stock and retentate samples that were not heat-shocked.

experiment. Moderate-to-high background levels of organisms other than *B. atrophaeus* were recovered on the R2A and TSA plates from both prerun and postclean samples. Prerun and postclean sample volumes used in the standard method tests ranged from 36 to 45·5 ml. No *B. atrophaeus* colonies were observed on any of the TSA plates from prerun or postclean samples.

Discussion

The feasibility of using an automated concentrator based on dead-end ultrafiltration for collection and concentration of micro-organisms from a water stream was evaluated by analysing recovery of *B. atrophaeus* spores spiked into tap water flow. The results demonstrate that *B. atrophaeus* spores were successfully collected and concentrated directly from drinking water, even at levels of <10 CFU l^{-1}, when filtered through a hollow-fibre ultrafilter integrated into an automated platform that used only existing water pressure to drive filtration. The data from these studies support previous work (Juliano and Sobsey 1997; Simmons *et al.* 2001; Kuhn and Oshima 2002; Morales-Morales *et al.* 2003; Hill *et al.* 2005, 2007; Olszewski *et al.* 2005) demonstrating the effectiveness of ultrafiltration to collect and recover particulates from a liquid stream. However, the previous efforts used hollow-fibre ultrafilters in cross-flow configuration, in which a portion of the water is re-circulated through the filter until a minimum volume is reached. Recovery is generally accomplished by re-circulating a buffer through the filter, although Hill *et al.* (2005) found that filter backflush could be used to increase the recovery of micro-organisms. This study used dead-end ultrafiltration in which water was forced through the filter in one direction and particulates recovered from the filter by backflushing in the reverse direction with buffer or water.

The reported advantages of cross-flow ultrafiltration are that it exerts less stress on micro-organisms collected on the filter, increases recovery of microbes from the filter by decreasing the extent to which they embed and adhere tightly to the filter matrix and reduces filter fouling. It is difficult to compare specific recovery values for cross-flow and dead-end filtration because water input and micro-organism recovery processes were so different among the different studies. However, the data from this study clearly show that low levels (<10 CFU l^{-1} or <1000 total CFU) of viable *B. atrophaeus* spores can be concentrated and recovered from large volumes (>100 l) of tap water using dead-end ultrafiltration. An important advantage of the dead-end ultrafiltration method described in this report is that the micro-organisms were concentrated directly from the unmodified water stream; i.e. no dechlorinating, surfactant or dispersing agents were added. Previous studies used finite sample volumes of water amended with chemical additives (Juliano and Sobsey 1997; Kuhn and Oshima 2001, 2002; Morales-Morales *et al.* 2003; Hill *et al.* 2005, 2007). The minimum treatment used was dechlorination. Direct filtration of water simplifies the filtration procedure and is essential for online monitoring.

This study demonstrates that detection is the limiting factor for methods to be used in microbial monitoring applications. For the higher spike inputs used in the prototype development and testing, concentrations are within the detectable range of most standard and rapid methods. At these concentration levels, sufficient sample volume was produced (140–200 ml) to allow analysis by several different test methods. However, the lower retentate concentrations obtained at low spike concentrations (<10 CFU l^{-1}) were not detectable without further concentration. A standard culture-based method provided the necessary sensitivity to enable detection and quantification of spores but this method required most of the sample be analysed, was not rapid and will not work for organisms that do not remain viable in the unamended tap water. Previous research in which grab samples were obtained and treated with thiosulfate to neutralize disinfectant before spiking (Juliano and Sobsey 1997; Kuhn and Oshima 2001, 2002; Morales-Morales *et al.* 2003; Hill *et al.* 2005, 2007) do not accurately reflect the problems associated with detection of micro-organisms in treated waters. For organisms that do not remain viable in the environmental sample and/or for which rapid detection methods are desired, the lower range of detection is

generally 10^2–10^3 CFU ml^{-1} for nucleic acid methods such as PCR and 10^4–10^5 CFU ml^{-1} for immunoassay-based methods (USEPA 2005) such as that used for detection in this research. These detection limits include losses resulting from the required sample preparation steps. Detection of micro-organisms in retentate produced by any of the published concentration methods for environmental water samples would therefore require a secondary concentration step and/or filtration of a larger volume of water to increase the number of targets collected in the ultrafilter. If some target organisms could reasonably be expected to survive, enrichment might also be used to increase the number of target organisms to detectable levels. Methods to concentrate and detect vegetative bacteria that are more sensitive to the effects of disinfectant (e.g. *Escherichia coli*) are currently under investigation in our laboratory.

Another advantage of the described dead-end filtration method is that no pretreatment of the filter was required. Previously reported cross-flow filtration methods required that filters be blocked before use by incubating them in buffers containing additives such as Laureth-12 (Juliano and Sobsey 1997), calf serum/fetal bovine serum (Kuhn and Oshima 2002; Morales-Morales *et al.* 2003; Hill *et al.* 2005, 2007) and sodium polyphosphate (Hill *et al.* 2005) for periods ranging from 1 h to *c.* 16 h. Lack of a blocking step simplifies filter preparation and maintenance and permits continuous reuse of the filter after cleaning, which facilitates automation for online monitoring.

Furthermore, dead-end ultrafiltration is generally faster than cross-flow ultrafiltration. Hill *et al.* (2005) reported filtration times of 12–14 min (corresponding to flow rates of 0·7–0·83 l min^{-1}) for 10-l dechlorinated water samples and filtrate rates of *c.* 1200 ml min^{-1} for 100-l samples using cross-flow. Juliano and Sobsey (1997) reported times of 200–260 min to process 50 l of tap water (equivalent to flow rates of 0·19–0·25 l min^{-1}). The flow rates were greater than 1 l min^{-1} for all nine field test experiments using the automated dead-end concentrator and approached 2 l min^{-1} for the three low-level concentration experiments. Higher flow rates may be possible with higher input pressures and/or higher capacity filters.

A decrease in flow rate was observed during each of the experiments and between experiments using the same filter. The decrease in flow rate suggests the occurrence of filter fouling, which may be caused by the accumulation of organic and/or inorganic material on the filter. A slight decrease in flow rate was also observed between experiments on the same filter, despite the cleaning regimen and storage in bisulfite. This suggests that the cleaning function was not able to completely restore the filter and that some residual impediment to flow remained. However, there was no correlation between average flow rate for an experiment or flow rate decrease and either per cent recovery or retentate concentration (data not shown). Hill *et al.* (2005) reported filter fouling to be a problem in their tangential flow filtration method only when Tween 80 was added to water samples prior to filtration. Insufficient data were given to determine if flow reduction occurred during filtration of 10-l water samples without Tween 80 addition. The extent of flow reduction in other tangential flow ultrafiltration studies (Juliano and Sobsey 1997; Kuhn and Oshima 2001, 2002; Simmons *et al.* 2001; Morales-Morales *et al.* 2003) could not be determined from the given data. The results presented in this report suggest that filter fouling during dead-end ultrafiltration of volumes of tap water up to *c.* 115 l does not adversely affect either flow rate or recovery and provides a faster means of processing water samples than tangential flow. Filters reused for at least three large-volume (>100 l) concentrations with cleaning and storage in bisulfite between runs showed little degradation in flow rate demonstrating that the filters can be cleaned and reused, which will reduce operating costs.

Experiments during the development and testing of the prototypes showed large variability in calculations of stock and retentate concentrations and per cent recovery for spores. The variability was initially attributed in large part to inconsistency of spore germination (Setlow 2003; Jones *et al.* 2005) in the viable method used for enumeration. Viable enumeration was preferred over direct counts as the presence of large amounts of other particulates in the retentate made it difficult to reliably discriminate spores. For experiments at spike levels of <10 CFU l^{-1}, a more reliable method for determining the number of spores spiked into the concentrator was needed to increase confidence in the number of spores that were being introduced to the filter. During attempts to develop a more reliable method of enumerating the stock suspension, extensive clumping of spores was observed microscopically in both initial stock suspensions and dilutions of this stock. The clumping was observed to be a major contributor to variability in direct counts of the stock and suspected of contributing to the high variability in viable counts. As spore aggregation has been attributed to hydrophobic interactions resulting from the surface properties of spores (Mamane-Gravetz and Linden 2005; Almeida *et al.* 2006), addition of a small amount of mineral oil was tested as a means of more effectively dispersing spores in suspension. When examined microscopically, the mineral oil was observed to break up most spore aggregates, leaving only a few pairs and triplets. Dilution of these stocks into DW with vortexing reduced the per cent of mineral oil but did not result in increased aggregation as long as the suspensions were not allowed to settle. Viable enumeration of diluted suspensions

prepared using this method gave less variable results than suspensions made up in either DW or PBS alone. Therefore, spore aggregation was determined to be a major contributor to variability in viable counts of stock suspensions and likely in retentate samples as well. More experiments at both high and low spike levels are needed to fully evaluate the effect of reduced aggregation in the spike on subsequent variability in retentate concentrations and per cent recovery for spores and to determine the benefits of this procedure on enumeration of other organisms undergoing testing on the concentrator.

This research presents an initial characterization of a completely automated prototype for collecting and concentrating micro-organisms from water based on dead-end ultrafiltration. Average recovery of the test organism, *B. atrophaeus* spores at high spike levels, was 36% ± 18% from unaltered chloraminated tap water with no pretreatment of the filter and using only sterile DW for backflush recovery. Average recovery for low levels (<1000 total) of *B. atrophaeus* spores recovered from high-volume (>100 l) unamended water samples using PB containing sodium polyphosphate was 30·5% ± 9%. The results demonstrate that dead-end ultrafiltration can be used to concentrate target particulates from water even at low levels. Detection of target in retentate samples will depend on the concentration in the retentate and the technology used for detection. Detection at very low contamination levels continues to be problematic because of limitations of available detection technology. To the authors' knowledge, this is the first report of completely automated concentration and recovery of a microbial target from water and is particularly notable because concentration at environmentally relevant levels was demonstrated. It represents a first step towards automated monitoring of critical water resources for potential pathogens and proposes that detection technology is the primary limiting factor in developing a fully automated online monitor for microbial targets in water.

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