Production of safe drinking water: risks, challenges and approaches for evaluating water treatment systems

By Robert S. Donofrio, Ratul Saha, Nabaneeta Saha, Rick Andrew, and Susan T. Bagley

Impact of sanitation and drinking water quality on a global level

Safe drinking water and hygienic sanitation systems are fundamental requirements for achieving and maintaining a healthy human population. Although great strides have been made worldwide over the past decade to increase the accessibility to safe drinking water and basic sanitation, a significant percentage of humans across the globe still do not have access to an improved water supply or basic sanitation facilities. The production and delivery of potable water to the world’s population is of utmost importance in preserving life (Ashbolt 2004). A large portion of the population residing in third-world countries, such as those of Sub-Saharan Africa, is utilizing sub-optimal drinking water sources. These sources may include surface water (lakes, streams and irrigation channels) and unprotected wells or springs. Since 1990, there has been a 5% increase in people worldwide who have access to an improved water supply (78% in 1990 to 83% in 2004) (WHO and UNICEF 2008). Improved water supplies include piped household water connections, rainwater collection, protected wells and springs and public taps.

The United Nations General Assembly declared 2008 the International Year of Sanitation in order to raise awareness of this topic. To understand the magnitude of the situation, one must consider the dichotomy of the world’s population as a whole (urban and rural communities that encompass developed and third-world nations alike), as well as the types of sanitation conditions that currently exist. The types of sanitation available can be broken down into four categories: open defecation, unimproved sanitation (facilities that do not hygienically separate human excrement from human contact), shared sanitation (such as public toilets) and improved sanitation (systems that ensure the separation of human excreta from human contact). A 2006 WHO/UNICEF Joint Monitoring Programme (JMP) for Water and Sanitation analysis claimed that over 2.5 billion people still do not have access to proper/improved sanitation. Almost half of those individuals do not have any access to sanitation facilities and thus must resort to the first demeaning option. The lack of proper sanitation leads directly to adverse health conditions, impoverished communities and reduced personal dignity. However grim the statistics may seem, the situation is improving. Currently 62% of the population is covered by improved sanitation facilities (an 8% increase since 1990). Still, underdeveloped regions, such as Southeast Asia and Sub-Saharan Africa, continue to have less than 33% coverage for improved sanitation (WHO and UNICEF 2008).

The goal of the WHO/UNICEF project is to achieve 90% of the population utilizing improved drinking water sources by 2015. To make this possible, a global effort must be undertaken by individual governments and members of industry to educate the public, implement basic sanitation and water purification technologies, as well as to develop cost-effective methods to treat emerging microbial and chemical threats.

Waterborne pathogens: background and significance to the drinking water treatment industry

Inadequate sanitation practices have a major impact on the production of safe drinking water. Risk assessment studies have identified a number of waterborne pathogens as being threats to our drinking water supply (Ashbolt et al. 2001; FAO/WHO 2003; Guillot and Loret 2009; Medema et al. 2009; Craun et al. 2010). An understanding of the diversity of these biological agents is necessary in order to adequately design a prevention or treatment strategy for potable water.

Waterborne pathogens are diverse and can be divided into four groups: bacteria, viruses, protozoa and helminthes. Their impact on public health is typically
assessed via the consideration of their source, route of transmission, growth characteristics, persistence in water supplies, resistance to disinfection and infectivity (WHO 2006). The majority of the waterborne pathogens are introduced to drinking water via human or animal feces, though some, such as Legionella, can grow in water distribution systems if the proper growth conditions exist. This introduction can be a direct result of poor hygiene and domestic sanitation conditions. Secondary contamination of clothes, food and hands can also play a role in transmitting these pathogens. Transmission and infection can be achieved through ingestion, inhalation and aspiration or direct contact (through bathing) (Moe 2006; WHO 2006; AWWA 2010). A majority of the waterborne agents possess a gastrointestinal route of infection. Legionella, Mycobacterium and Naegleria fowleri, as well as other agents in high concentrations, can cause infection through the respiratory route (Falkinham III et al. 2001; Marciano-Cabral and Cabral 2007). Opportunistic pathogens, such as Burkholderia pseudomallei, Aeromonas and Pseudomonas aeruginosa, may utilize the mucous membranes and skin to enter the host and cause infection (Inglis 2000; Currie 2001). For these opportunistic pathogens, individuals possessing a compromised immune system, as well as the elderly and young children, would be at the highest risk of infection if exposed to contaminated water.

In regards to the waterborne pathogens, the following organisms are considered the most significant to public health:

**Bacteria** - *Campylobacter jejuni*, pathogenic *Escherichia coli*, enterohemorrhagic *E. coli*, *Legionella* spp., *Salmonella* spp. (including *typhi*), *Shigella* spp., *Vibrio cholerae* and *Yersinia enterocolitica* (Angulo 1997; Thoerner et al. 2003; WHO 2006). *E. coli*, *C. jejuni*, *Legionella* and *Shigella* have a moderate to high relative infectivity (low infectious doses). Other than *Shigella* and *Vibrio*, these bacterial pathogens may persist in the water supply for prolonged periods of time (Kaper et al. 1995; Koenraad et al. 1997; Nataro and Kaper 1998; Alamanos 2000). However, most are relatively susceptible to chlorine disinfection (WHO 2006).

**Viruses** – Adenoviruses, Enteroviruses, Hepatitis A and E, Noroviruses, and Rotaviruses. Each of these viral pathogens possesses prolonged persistence in the water supply, moderate to high chlorine resistance and has low infectious doses (Cuthbert 2001; Grabow et al. 2001).

Protozoa and Helminthes – *Acanthamoeba* spp., *Cryptosporidium parvum*, *Cyclospora cayetanensis*, *Entamoeba histolytica*, *Giardia intestinalis*, *Naegleria fowleri*, *Toxoplasma gondii*, *Dracunculus medinensis*, *Schistosoma* spp. All of these organisms possess a high relative infectivity and most are resistant to disinfection via chlorine or other related halogens (WHO 2006).

### Water treatment systems: importance and limitation of membrane filtration

In the United States, the quality and safety of potable water has been a focus of state and federal regulations since the inception of the Safe Drinking Water Act of 1974 (Montgomery 1985). Multiple amendments to the Act have been made over the past three decades to address chemical contaminants and leachates (i.e., lead), emerging pathogens, and alternative disinfection technologies (AWWA 2010). The area of microbial disinfection has received heightened awareness since the terrorist attacks of September 11, 2001. In 2004, the US Environmental Protection Agency (USEPA) published EPA/600/R-04/063: Water Security Research and Technical Support Action Plan. This document was drafted with the intent to address the need for safer water by encouraging research efforts for the development of water treatment technologies for the inactivation or removal of these specific pathogens (USEPA 2004b).

The types of treatment systems are varied and include ultraviolet (UV) irradiation disinfection systems, ozonation systems, halogens and halogenated resins and membrane based filtration systems (AWWA 2010). The microbial disinfection system that one may select depends on a number of factors, including organism size, charge (isoelectric point) and resistivity to the disinfectant in question. Halogen-based systems, including chlorine and chlorine dioxide, are widely used and are appealing for their residual anti-microbial efficacy exhibited by the active chemical agent. However, the key waterborne pathogens vary greatly in their susceptibility to certain chemical disinfectants. Furthermore, harmful disinfection by-products (DBP) may form via interaction with indigenous compounds, such as humic acids, and thus need to be monitored and controlled. Ozone and UV irradiation have proven to be effective against a wide variety of pathogens (Bolton 1999; Eischedel et al. 2009; Ochiai et al. 2011). However, water supplies with increased turbidity or elevated levels of dissolved organics could reduce the effectiveness of these systems. UV irradiation also lacks any residual effect within the water system. An additional drawback to ozone is the potential to interact with bromide ions naturally present in drinking water to form the DBP of bromate ion (Montgomery 1985; Angulo 1997; Kumar et al. 2011; Genuino and Espino 2012). Because of these issues, many water treatment facilities have found increased value in the incorporation of a size exclusion-based technology.

Membrane technologies are among the most versatile water treatment processes with regard to their ability to effectively remove a wide variety of contaminants (Madaeni 1999; Zhao et al. 2011). Membrane devices, most notably reverse osmosis systems, have often been deployed as emergency water treatment solutions following natural disasters, such as hurricanes and tsunamis (Atkinson 2005). The classifications of the filtration systems are based on the nominal pore size of the filter membrane. Traditional microfiltration utilizes membranes possessing pore sizes between 0.1 and 1.0 μm. Reverse osmosis (RO) treatment devices possess pore sizes 10 to 100-fold less than those used in microfiltration. RO membranes incorporate the principle of cross-flow filtration, that
is, incoming water flows over and parallel to the filter medium. When pressure is applied to the membrane, a majority of the water leaves the system as reject water and contains the concentrated particulate unable to pass through the membrane. A lesser volume of water diffuses through the membrane and is free of contaminants (permeate). The small pore size of the membrane allows RO systems to remove bacteria, viruses and dissolved solids present in the water via size exclusion. Breakthrough of the organisms and particles may still occur if imperfections in the membrane are present. Bypass of the membrane could occur due to microscopic seal leaks (Sourirajan and Matsumura 1985; NSF International 2009). Membrane technologies may also be subject to bacterial fouling due to the presence of heterotrophic plate count bacteria (HPCs) indigenous to the water distribution system (Reasoner 2004; Ivnitsky et al. 2007; Zodrow et al. 2009; Kwon et al. 2011).

**Diversity of HPCs in drinking water**

The occurrence and prevalence of a diverse array of HPCs in water distribution systems is well documented. Example genera of gram-negative bacteria that are commonly found naturally occurring within the distribution system, associated with biofilms or in planktonic form, are *Sphingomonas, Pseudomonas, Alcaligenes, Acinetobacter, Caulobacter* and *Ralstonia*. Gram-positive organisms from the genera of *Bacillus, Corynebacterium, Micrococcus* and *Staphylococcus* have all been documented from similar water sources when using minimal nutrient media for recovery (Mergaert et al. 2001; WHO 2003; Allen et al. 2004; Pavlov et al. 2004; Williams et al. 2004; Martiny et al. 2005; Donofrio et al. 2010a). A significant percentage of the HPCs isolated from drinking water distribution systems has been found to possess resistance to many natural and synthetic antibiotics (Mary et al. 2000; Jeena et al. 2006; Donofrio et al. 2010a). Additional studies show that some HPCs may contain other virulence factors usually associated with pathogenic bacteria, thus indicating that they may potentially act as opportunistic pathogens (Edberg et al. 1996; Papandreu et al. 2000).

**Test surrogates for membrane filtration efficacy**

To achieve the stringent microbial contaminant limits specified by the USEPA’s Water Security Research and Technical Support Action Plan, much effort has been expended in the development of point of use and point of entry water treatment systems (AWWA 2010). Multiple protocols have been drafted to assess the efficacy of drinking water treatment systems using membrane filtration or other disinfectant technologies to remove/inactivate waterborne pathogens (USEPA 2004a; USEPA 2006a; USEPA 2006b; NSF International 2008; NSF International 2011). These protocols utilize select microorganisms to “challenge” the test system. These organisms serve as surrogates for groups of organisms possessing similar biochemical traits, virulence factors or morphological characteristics. Surrogates are typically selected based on size, charge, hazard level and ease of cultivation and enumeration (James 1999). For example, NSF International’s protocol P231 for water purification validation, based on the USEPA guide standard, incorporates the *Raoultella terrigena* and Poliovirus as surrogates for pathogenic bacteria and viruses, respectively (NSF International 2008). Bacteriophages, such as MS2, phiX174 and fr, have also been utilized as non-pathogenic viral surrogates due to their small size and diverse surface charges (USEPA 2006a; USEPA 2006b). In most cases, a non-pathogenic surrogate is preferred to limit exposure risk for the personnel performing the validation assays.

The USEPA, through its Environmental Technology Verification (ETV) program, has developed a protocol to evaluate RO treatment system performance under a simulated intentional or non-intentional microbiological contamination event (USEPA 2004a; USEPA 2006b). This protocol involves validating the removal efficiency by incorporating a series of “challenge” assays. In these assays, water that has been spiked with a known concentration of a bacterial surrogate is delivered to a reverse osmosis unit. Treated permeate is evaluated for the presence the surrogate organism and its concentration is verified. The percent removal of the surrogate is then calculated.

In regard to bacterial surrogate selection, organism size is a key determinant. The USEPA RO protocol is designed for validating the performance of these units under accidental or intentional release of bacterial agents into the water supply (USEPA 2004a). The smallest identified bacterium of concern, *Francisella tularenia*, can possess a minimum size of 0.2 μm in diameter (Burrows and Renner 1999). *Brevundimonas diminuta* was selected as the test surrogate for sub-micrometer porosity filters due to its small diameter (0.2 μm) when grown in minimal media (ASTM 2001; ASTM 2005; USEPA 2006a; USEPA 2006b). Other gram-negative organisms capable of achieving the small cell diameter, such as *Hydrogenophaga pseudoflava* and *Ralstonia pickettii*, have also been considered as well for use as surrogates in these applications.

Because of its size characteristics, *B. diminuta* has also been used as a test strain for the quality control of membrane filters used for sterilization in the pharmaceutical industry (Carter 1996; Griffiths et al. 2000; ASTM 2001; Sundaram et al. 2001). *B. diminuta*, formerly belonging to the genus *Pseudomonas*, is a gram-negative, non-fermenting, rod shaped bacterium and belongs to the Alphaproteobacteria class (Segers et al. 1994). *B. diminuta*'s closest phylogenetic relatives are members of the Caulobacter family (Segers et al. 1994). In contrast to species of *Caulobacter, B. diminuta* does not possess the ability to form prosthecae (Stahl et al. 1992). Over time, this organism has been observed to produce extracellular polymeric substances (EPS) which could allow for attachment to membrane filters and thus cause fouling and potentially impact filtration efficiency (Badireddy et al. 2008). Clinically, this organism has been documented as causing opportunistic nosocomial infections in cancer patients.
### Table 1. Comparison of microbiological standards and protocols for evaluating the microbial efficacy of drinking water treatment systems

<table>
<thead>
<tr>
<th>Standard / Protocol</th>
<th>Microorganisms/Reduction requirements</th>
<th>Technology covered</th>
<th>Challenge water parameters</th>
<th>General protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proposed NSF Supplemental Std 244-3</td>
<td>R. terrigena - 6 Log MS-2, fr - 4 Log Cysts - 3.3 Log</td>
<td>mechanical filtration, e.g., membrane units; use on safe water only</td>
<td>1 wk each at pH 6, 7.5, 9; at end of test 30 NTU, 10 ppm TOC, 1500 ppm TDS</td>
<td>2 units; 3 wk test; B. diminuta, daily R. terrigena &amp; viruses; 3-48 h stagnations; ends w/ Ceramic purifier challenge; 50 sample pts; cyst-Std 53/ bact as surrogate</td>
</tr>
<tr>
<td>USEPA purifier std/protocol - 1987 (embodied within NSF P231)</td>
<td>R. terrigena - 6 Log Poliovirus 1 - 4 Log Rotovirus SA11 - 4 Log Cysts - 3 Log</td>
<td>UV, halogens, ceramics, adaptable to other technologies; can use on unsafe water</td>
<td>Ceramics/mechanical- 30 NTU, 10 ppm TOC, 1500 ppm TDS, pH 9, 4°C; challenge w/ all organisms</td>
<td>3 units; 10-1/2 day test; 1st 6 days 4 sample pts w/ general test water; 1 stagnation, 4 days w/ challenge water during 4 sample pts; min. 24 sample pts</td>
</tr>
<tr>
<td>Israel std SI 1505 Part 1, Part 2</td>
<td>E. coli - 7 Log E. aerogenes - 7 Log S. faecalis - 7 Log P. aeruginosa - 7 Log no virus/cyst reduction</td>
<td>Part 1- Filter systems, UV Part 2- RO systems; presumed units for use on safe water only</td>
<td>Potable water spiked with bacteria used for challenge water</td>
<td>Initial 5-L challenge w/ 10E+7/100 mL bacteria; 2nd challenge at 90% of capacity; ≤ 1 org/mL allowed; &lt; 5x10E+3 HPC/L allowed</td>
</tr>
<tr>
<td>Japan JIS 3835-1990</td>
<td>P. diminuta - no specific reduction required, report results no virus/cyst reduction</td>
<td>membrane filters; use on safe water only</td>
<td>Sterilized dilute NaCl solution used for challenge water</td>
<td>Challenge must contain ≥ 10E+7 cfu/cm² of filter surface area @ 2-4 mL/min per cm² of filter area w/ max 3P 30 psig; no acceptance criteria, just report results</td>
</tr>
<tr>
<td>Mexico NOM-ISO-SSA-1998</td>
<td>E. coli - 4 Log aerobic bact - 1.3 Log no virus/cyst reduction</td>
<td>domestic water treatment equipment; for use on “potable” water</td>
<td>Potable water spiked with bacteria used for challenge water</td>
<td>Challenge w/ &gt;10E+4 E. coli/100 mL for 10 min, take 3 samples; ≤ 1 cfu/100 mL allowed; ≥ 95% reduction of aerobic bacteria (HPC)</td>
</tr>
<tr>
<td>Australia/New Zealand AS/NZS 4348:1995</td>
<td>R. terrigena - 6 Log Poliovirus 1 - 4 Log Rotovirus SA11 - 4 Log Cysts - 3 Log</td>
<td>UV, halogens, ceramics, adaptable to other technologies; can use on unsafe water</td>
<td>Ceramics/mechanical- 30 NTU, 10 ppm TOC, 1500 ppm TDS, pH 9, 4°C; challenge w/ all organisms</td>
<td>Same as EPA purifier protocol</td>
</tr>
<tr>
<td>Brazil ABNT NBR 14908</td>
<td>E. coli - 2 Log no virus/cyst reduction</td>
<td>plumbed-in systems; for use on “potable” water</td>
<td>Potable water spiked with bacteria, ≤ 1 NTU</td>
<td>Challenge w/ ≥ 10E+5 cfu/100 mL initially; run to 95% capacity w/ 0.5 ppm Cl₂, retest w/ E. coli</td>
</tr>
<tr>
<td>Venezuela COVENIN 3377:1998</td>
<td>E. coli &amp; P. aeruginosa - no reduction requirement, only to verify claims; no virus/cyst reduction</td>
<td>filtration systems, ozonation devices; presumed for use on safe water only</td>
<td>unknown</td>
<td>Initially flush units w/o org’s to check for coliform &amp; HPC, then challenge with amount of org’s sufficient to verify log reduction claim; retest w/ org’s at end of capacity</td>
</tr>
<tr>
<td>California Guidelines 2004</td>
<td>technology dependent E. coli - 6 Log MS-2 - 4 or 6 Log Cysts - 3.3 Log</td>
<td>Protocol depends on technology; can include POE; can use on unsafe water</td>
<td>For mechanical reduction, high turbidity challenge not required; reduce FR to 0.05 initial flow rate w/ test dust</td>
<td>Depends on specific mechanical reduction technology; similar to EPA ceramic candle protocol; determine max flow rate to 120 psi; POE must meet SWTR or LT2, obtain permit; 6 Log MS-2 reduction for bact surrogate</td>
</tr>
<tr>
<td>WQA ORD0901</td>
<td>E. coli - 3 Log MS-3 - 3 Log</td>
<td>Pour-through, gravity fed systems</td>
<td>pH 6.5 – 8.5, TOC 0.1 – 5.0 mg/L, Turbidity 0.1 – 5 NTU, TDS 50 – 500 mg/L, Temp 17 – 25°C</td>
<td>Operate according to manufacturer’s usage pattern, samples at start-up, 25%, 50%, 75%, and 100% of rated capacity</td>
</tr>
</tbody>
</table>

**Key parameters and conditions for testing microbiological reduction**

- **Pour-through, gravity fed water**
  - unsafe
  - POE; can use on technology; can include elements; safe water only

- **Plumbed-in systems**
  - presumed for use on safe water only

- **Membrane filters**
  - presumed units for use on UV Part 1- RO systems; Part 2- Filter systems, UV Part 2- mechanical filtration, e.g., membrane units; use on safe water only

- **Ceramic elements**
  - presuming for use on UV Part 1- RO systems; Part 2- Ceramic purifier protocol; determine reduction technology; similar to EPA purifier protocol

- **Operation conditions**
  - Temp 17 – 25 ºC
  - TDS 50 – 500 mg/L
  - Turbidity 0.1 – 5 NTU
  - pH 6.5 – 8.5
  - TOC 0.1 – 5.0
  - Min per cm² of filter area @ 2-4 mL/min per cm² of filter area w/ max 3P 30 psig

- **Challenge water**
  - Potable water spiked with bacteria used for challenge water
  - Potential water spiked with bacteria used for challenge water

- **General protocol**
  - Initial 5-L challenge w/ 10E+7/100 mL bacteria; 2nd challenge at 90% of capacity; ≤ 1 org/mL allowed; < 5x10E+3 HPC/L allowed
  - Challenge w/ >10E+4 E. coli/100 mL for 10 min, take 3 samples; ≤ 1 cfu/100 mL allowed; ≥ 95% reduction of aerobic bacteria (HPC)
Under certain growth conditions, *B. diminuta* can reach concentrations exceeding $1 \times 10^9$ CFU per mL, thus making simulation of a gross contamination event possible (Lee et al. 2002; Donofrio et al. 2010a). Current drinking water filtration validation protocols utilize non-selective media for enumeration of *B. diminuta*. In fact, a selective medium for *B. diminuta* has yet to be developed. The reason for this is that traditionally *B. diminuta* has been employed as a test organism for pharmaceutical-grade membrane filter validation. In this application sterile feed water is spiked with *B. diminuta* and the membrane filters are then exposed to the inoculated water (ASTM 2001). In validating RO systems using a drinking water source, interference from HPCs indigenous to the test system may lead to decreased recoveries of the target organism since the colonies of *B. diminuta* require 48 hours until maturity on media such as R2A Agar (Governal et al. 1991; Donofrio et al. 2010a). This may impact the ability to assess the performance of the water treatment devices being evaluated. If the validation of the device is inaccurate, utilization of the unit to remediate water contaminated with waterborne pathogens may result in consumer illness or even death.

**Selective methods for assessing performance of a treatment system with a focus on *B. diminuta***

To overcome the potential interference the HPCs may pose to enumeration of surrogates such as *B. diminuta*, a selective and/or differential method for the test organism is desirable. Key considerations when designing a selective assay are assay time, specificity, ease of use and limit of detection. Rapid turn-around of results is an extremely important factor for the filter manufacturer. The sooner that a manufacturer can assess the performance of a lot of filters, the sooner the lot can be released and freed from the holding process. This ultimately saves cost in storage and allows for a greater volume of the filtration devices to be manufactured and sold. Shorter testing time also allows the manufacturer greater flexibility in the research and development process as a larger number of filtration devices can be assessed in lesser amount of time.

Numerous isolation approaches have been investigated using the surrogate’s metabolic and/or molecular profiles as a basis for selectivity. One approach is to investigate phenotypic traits or utilization of individual carbon sources that might be unique to the surrogate organism. In this scheme, the diversity of the microbial population affecting the test environment is first elucidated (Konopka 1998; Girvan 2003). Girvan (2003) used a polyphasic approach to characterize the effects of different soil types and land management practices had on the microbial ecosystem. The approach incorporated PCR-based techniques (16S rRNA) as well as metabolic fingerprinting using the Biolog System. For the latter, physiological profiling of microbial communities can be conducted (Weber 2007). Biolog Statistical tools such as multivariate or cluster analysis can be employed to discern key metabolic differences between the surrogate and indigenous microbial community (Mardia et al. 1979; Kaufman and Rousseeuw 2005; Gan et al. 2007). Donofrio et al. (2010a) compared the metabolic profiles of organisms indigenous to a laboratory deionized...
water system to which a *B. diminuta* strain had been exposed. Though use patterns did emerge, a sole carbon source to be used as a basis for selective isolation and enumeration was not discerned.

A limited number of investigations into rapid techniques for detection and enumeration of *B. diminuta* have been performed. Previous research has investigated the applicability of using recombinant molecular techniques to confer differential and selective properties to *B. diminuta*. These studies utilized direct electroploration or *E. coli* filter mating transformation procedures for the transfer of plasmids conferring expression of bacterial luciferase (pBSLLUX2) and fluorescent proteins (such as pBSLGFP1, pAcGFP1 and pDsRed-Monomer) allowing for expression using general purpose growth media. Both procedures resulted in decreased procedure times for *B. diminuta* enumeration compared to the traditional plating methods. Drawbacks to this luciferase approach were the high cost for the luminescence detection equipment as well as the increased technician time and fatigue associated with microscopic enumeration (Griffiths et al. 2000; Donofrio et al. 2012). The *Tn5* transposon scheme has been used successfully in other bacterial strains (such as *E. coli* and *Salmonella*) to confer antibiotic resistance or introduce other foreign gene segments (de Lorenzo et al. 1990; Alexeyev et al. 1995; Goryshin et al. 2000). Donofrio et al. (2012) demonstrated that the transposon insertion method generated stable kanamycin-resistant clones of *B. diminuta* which could be isolated effectively from competing HPCs when grown on R2A agar amended with kanamycin. The recombinant *B. diminuta* strain was also found to maintain its ability to be cultivated to 0.3 μm, thus satisfying the size criteria for filter screening protocols.

Quantitative real time (q)PCR and fluorescent in situ hybridization (FISH) are two additional molecular technologies that have been used successfully for the selective enumeration of numerous bacterial species including those that could be found in drinking water such as *Ralstonia* spp., *Salmonella* spp., *B. diminuta* and *Pseudomonas* spp. (Sekiguchi et al. 1999; Weller et al. 2000; Penna et al. 2002; Yilmaz and Noguera 2004; Reynisson et al. 2008; Donofrio et al. 2010b; Saha et al. 2010). The qPCR assays typically subject the sample to a DNA extraction and cleanup step (Nadkarni et al. 2002). This step is time consuming, though effective in providing a DNA template free of PCR inhibitors. Whole cell (i.e., colony) PCR involves the direct amplification of target genes from a non-extracted DNA template (Sambrook et al. 2001; Fuchs and Podda 2004). The template DNA is released through cell lysis in the denaturing step of the PCR run. Whole cell PCR, coupled to gel electrophoresis, has been utilized as a quick qualitative screen for multiple gram-positive and gram-negative bacteria (Naofuni et al. 2000; Sheu et al. 2000). This technique has proven effective in detecting the genes of interest from suspensions containing cell concentrations greater than 1 x 10^5 CFU (Sheu et al. 2000). The whole cell approach has also been successfully coupled to qPCR to provide quantitative measurements of cell density for organisms such as *Pseudomonas* sp. and *B. diminuta* (Saha et al. 2010; Donofrio et al. 2010b).

In addition to specific virulence factors, the 16S rRNA gene has been the target of many of these methods. Nadkarni et al. (2002) successfully demonstrated that qPCR can be used for estimating the bacterial load present in a water sample using 16S rRNA universal probes. Recently two housekeeping genes, *gyrB* and *rpoD*, have gained in popularity as the basis for the molecular quantification methods due to a number of similarities and additional advantageous traits they possess compared to the rRNA gene target (Nies 2004). Additionally, Watanabe et al. (2001) have created the ICB database that is searchable for numerous organisms based on their *gyrB* sequences. Both *gyrB* and *rpoD* may have similar horizontal transmission rates to rRNAs (Yamamoto and Harayama 1998; Yamamoto et al. 2000). Both genes possess a single copy number, as opposed to 16S rRNA which possesses a variable copy number (Yamamoto et al. 1998; Yamamoto et al. 2000; Kenzaka et al. 2005). These protein-coding genes have been demonstrated to have evolved at a quicker rate than rRNAs and have even displayed higher resolution than 16S rRNA. Because of these characteristics, the *gyrB*-based methods have demonstrated the ability to differentiate closely related bacterial strains (Venkateswaran et al. 1998; Hiroaki et al. 2000; Wantanabe et al. 2001). Studies have also shown that phylogenetic comparisons using *gyrB* and *rpoD* sequence may provide higher resolution than 16S rRNA. As a result, the design of primers and probes based on these genes may be more easily accomplished (Kenzaka et al. 2005). In addition, Han and Andrade (2005) have developed a PCR based methodology for identification of *B. diminuta* for clinical applications demonstrating the feasibility of this methodology. In this study, primers were designed around the housekeeping gene *gyrB*. The *gyrB* and *rpoD* genes were targeted by Donofrio et al. (2010b) for a qPCR and FISH approach to evaluate the concentration of *B. diminuta* in laboratory deionized water systems. These approaches could be used to validate the removal efficiency of filtration-based treatment systems for drinking water systems.

Summary

The availability of safe water for human consumption will always be an area of great importance for maintaining and preserving public health. Manufacturers of water treatment technologies must address the risk posed by established as well as emerging microbial pathogens. Thus the validation of such systems must include organisms that represent a broad spectrum of pathogens that are expected to threaten the water supply. Furthermore the methods employed for system validation must be of adequate specificity and sensitivity to account for the infectious dose of the target pathogens. By integrating molecular assays such as qPCR and FISH into the validation scheme, manufacturers will have a more accurate and time efficient means of evaluating the ef-
ficacy and performance of the water treatment technologies. Ultimately a stronger validation and certification protocol will result in heightened consumer confidence in this industry and an overall improvement in the quality and safety of the potable water supply.

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