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ORIGINAL PAPER

Quantitative real-time PCR and fluorescence in situ hybridization approaches for enumerating *Brevundimonas diminuta* in drinking water

Robert S. Donofrio · Lorelle L. Bestervelt · Ratul Saha · Susan T. Bagley

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Abstract *Brevundimonas diminuta* is a small Gram-negative bacterium used for validation of membranes and filters used in the pharmaceutical and drinking water treatment industries. Current assays are time consuming, nonselective, and may be subject to interference by competing indigenous microorganisms. The focus of this study is to develop rapid and specific enumeration methodologies for *B. diminuta*. Quantitative real-time polymerase chain reaction (qPCR) and fluorescence in situ hybridization (FISH) assays were developed based on the *gyrB* (1,166 bp) and *rpoD* (829 bp) gene sequences of *B. diminuta* ATCC 19146. Species-specific primers and probes were designed, and a 100–200 bp segment of each gene was targeted in the qPCR studies. For both the qPCR and FISH assays, an internal 25 bp sequence was selected for use as a TaqMan probe (labeled with 6-FAM and a Black Hole Quencher). Probe specificity studies, conducted against Gram-negative and Gram-positive reference strains as well as environmental strains, revealed high specificity of the primer/probe pairs to *B. diminuta*. Sensitivities of the qPCR reactions using purified genomic DNA from *B. diminuta* were determined to be 0.89 pg for *rpoD* and 8.9 pg for *gyrB*. The feasibility of using whole-cell *B. diminuta* suspensions directly with the *rpoD* qPCR protocol was also evaluated. The greatest sensitivity observed for *B. diminuta* was 1×10^3 colony forming units (CFU) per mL when tryptic soy broth was used as the growth medium. When compared with direct microscopic enumeration using a 5′ 6-FAM FISH probe, traditional plating methods showed significant underestimation of *B. diminuta* concentration ($P = 0.01$) when this organism was cultivated in saline lactose broth. The results of this investigation demonstrate that qPCR and FISH are effective methods for rapid (<4 h) enumeration of *B. diminuta* and may be viable alternatives to plating when validating drinking water filtration systems.

Keywords *Brevundimonas diminuta* · *RpoD* · *GyrB* · Real-time PCR · Fluorescence in situ hybridization

Introduction

Multiple protocols exist for determining microbiological removal efficiencies of potable water filtration devices [20, 25, 32]. In these assays, filters are rated by the percentage of microorganisms that they remove. For bacterial studies, enumeration currently involves quantifying viable cells pre and post filtration. The protocols utilize organisms to serve as surrogates for groups of bacteria possessing similar biochemical traits, virulence factors or morphological characteristics. *Brevundimonas diminuta*, a member of the *Alphaproteobacteria* lineage, is used in validation of reverse-osmosis (RO) drinking water filtration devices and submicrometer-porosity pharmaceutical-grade filters due to its small diameter when grown in minimal media [6, 31, 32]. The enumeration protocols for *B. diminuta* use nonselective media and require incubation periods of 48 h. Studies have found that bacteria indigenous to the test system possessing faster growth rates or similar colonial morphologies may

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R. S. Donofrio (✉) · L. L. Bestervelt · R. Saha
NSF International, 789 Dixboro Road,
Ann Arbor, MI 48105, USA
e-mail: Donofrio@nsf.org

R. S. Donofrio · R. Saha · S. T. Bagley
Michigan Technological University,
1400 Townsend Drive, Houghton, MI, USA

interfere with quantification of *B. diminuta* [4, 8, 32]. 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 illness or even death of the consumer.

A limited number of investigations into rapid detection and enumeration techniques for *B. diminuta* have been performed. Griffiths et al. [12] developed a Tn5 transposon insert which conferred a gene expressing luciferase to *B. diminuta*; a recombinant *B. diminuta* strain expressing a green fluorescent protein (GFP) was also developed. Both procedures resulted in decreased processing times for *B. diminuta* enumeration compared with traditional plating methods. However the methods still required at least a 12-h growth period as well as an expensive camera system to detect the fluorescence. Han et al. [14] developed a qualitative polymerase chain reaction (PCR)-based methodology for identification of *B. diminuta* for clinical applications using primers targeting the *gyrB* gene. Though specific for its target, a quantitative approach would need to be developed before this technology could be applied for evaluating filter efficiency.

Although not having been previously utilized for *B. diminuta*, quantitative real-time PCR (qPCR) and fluorescence in situ hybridization (FISH) are molecular technologies that have been used successfully for selective enumeration of numerous bacterial species [7, 13, 26, 37]. The 16S rRNA gene has been the target of many of these methods. Recently *gyrB* and *rpoD*, two housekeeping genes, have gained in popularity for use in molecular quantification assays [16, 23]. *gyrB* and *rpoD* are single-copy genes and are less susceptible to horizontal gene transfer than are rRNAs [18, 28, 36]. These protein-coding genes have been demonstrated to have evolved at a faster rate than rRNAs, thus enabling differentiation of closely related bacterial strains [33]. Studies have also shown that phylogenetic comparisons using *gyrB* and *rpoD* sequences may provide higher resolution than 16S rRNA [17]. As a result, the design of primers and probes based on these genes may be more easily accomplished.

Whole-cell PCR (i.e., colony PCR), coupled to end-point gel electrophoresis, has been utilized as a quick qualitative screen for multiple Gram-positive and Gram-negative bacteria [22, 30]. This approach involves direct amplification of target genes from a nonextracted DNA template and may be a more rapid alternative to traditional assays which involve DNA extraction and product clean-up prior to analysis [11, 21, 27]. Whole-cell PCR has proven effective in detecting genes of interest from suspensions containing cell concentrations greater than 1×10^5 colony forming units (CFU) per mL [30].

The focus of this study is to develop specific and rapid methods to quantify total *B. diminuta* concentrations in water pre and post filtration treatment. Two methods were investigated: quantitative real-time PCR (qPCR) and FISH methods. These molecular approaches would overcome the interference and specificity issues associated with the time-consuming viable culture plating methodologies currently specified for validating the performance of drinking water filtration systems. Primers and probes based on the *gyrB* and *rpoD* sequences of *B. diminuta* were designed and validated, and the sensitivity and limitations of the methods were assessed. The feasibility of using a whole-cell qPCR approach was also evaluated for its ability to detect *B. diminuta* over the 7-log range required by the water filter validation protocols. The techniques developed in this study could be applied to multiple areas that rely on submicrometer-based filters, including the water treatment, pharmaceutical, and food and beverage industries.

Materials and methods

Media and reagents

All media used for the isolation and growth of the organisms detailed in this study were obtained from Difco (Becton Dickenson, Franklin Lakes, NJ, USA). All reagents and chemicals used in this set of experiments were ACS reagent grade or higher (Sigma–Aldrich Co., St. Louis, MO, USA).

Bacterial strains and growth conditions

The following reference strains included in this study were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA): *Brevundimonas diminuta* ATCC 19146, 17600, and 17599; *Escherichia coli* ATCC 11229 and 19835; *Ralstonia pickettii* ATCC 700590; *Pseudomonas fluorescens* ATCC 13525; *P. aeruginosa* ATCC 15442; and *Staphylococcus aureus* ATCC 6538. Table 1 displays the environmental bacterial isolates that were also included in the study; these were previously isolated from a laboratory RO-treated drinking water distribution system [8]. Multiple isolates were included for a numbers of species, since the isolates each possessed slight differences in metabolic profiles.

For the qPCR and FISH studies, the bacterial strains were grown in tryptic soy broth (TSB) and incubated at 30°C for 24 h. To achieve the necessary cell density, *B. diminuta* was grown in either TSB or saline lactose broth (SLB) at 30°C for 48 h.

Table 1 Specificity results for the *Brevundimonas diminuta gyrB* and *rpoD* qPCR primers and *B. diminuta* fluorescence in situ hybridization (FISH) probes

Organism	qPCR		FISH	
	gyrB	*rpoD*	*gyrB*	*rpoD*
Brevundimonas diminuta ATCC 19146	+	+	+	+
B. diminuta ATCC 17599	+	+	+	+
B. diminuta ATCC 17600	+	+	+	+
Escherichia coli ATCC 11229	–	–	–	–
E. coli ATCC 19835	–	–	NT[c]	NT
Ralstonia pickettii ATCC 700590	–	–	–	–
Pseudomonas fluorescens ATCC 13525	–	–	–	–
P. aeruginosa ATCC 15442	–	–	NT	NT
Staphylococcus aureus ATCC 6538	–	–	–	–
Rhodopseudomonas palustris[a] (4)[b]	–	–	–	–
Variovorax paradoxus[a] (2)	–	–	–	–
Micrococcus luteus[a] (2)	–	–	NT	NT
Acinetobacter radioresistens[a] (2)	–	–	–	–
Hydrogenophaga taeniospiralis[a]	–	–	NT	NT
Acidovorax delafieldii[a] (3)	–	–	–	–
Sphingomonas capsulata[a] (4)	–	–	–	–
Caulobacter leidyi[a] (5)	–	–	–	–
Sphingomonas chlorophenolica[a] (3)	–	–	–	–
Sphingomonas terrae[a]	–	–	–	–
Rubrivivax gelatinosus[a]	–	–	–	–
Delftia acidovorans[a]	–	–	–	–
Ralstonia eutropha[a]	–	–	–	–
Stenotrophomonas maltophilia[a]	–	–	NT	NT
Staphylococcus warneri[a] (2)	–	–	NT	NT
Bacillus mojavenesis[a]	–	–	NT	NT
Bacillus amyloliquefaciens[a]	–	–	NT	NT
Rhodococcus globerulus[a]	–	–	NT	NT

The primer/probes were based on sequences obtained from *B. diminuta* ATCC 19146 [8]

[a] Environmental isolates obtained from a laboratory deionized water distribution system and identified using 500 bp 16s rRNA sequences [8]

[b] The value in parenthesis indicates the number of isolates tested, each having differences in metabolic profiles [8]

[c] NT indicates that this strain was not evaluated against the corresponding primer or probe

Genomic DNA isolation and purity assessment

Genomic DNA was extracted from the reference strains and environmental isolates using the GenElute system (Sigma–Aldrich Co., St. Louis, MO, USA) and Qiagen DNEasy kit (Qiagen, Valencia, CA, USA). The purity and the yield of the genomic DNA were determined using a Genespec I ultraviolet (UV) spectrophotometer (MiraBio, Alameda, CA, USA) and a NanoDrop 1000 spectrophotometer. Purified DNA samples were stored at 4°C until further use.

Quantitative real-time PCR (qPCR)

Primers and probes were designed for the *gyrB* and *rpoD* genes of *B. diminuta* 19146 from the sequence data using the Primer Quest SM program (Integrated DNA Technologies, Coralville, IA, USA). The *B. diminuta* 19146 sequence data used for primer and probe design were obtained from sequences deposited in the GenBank nucleotide sequence database: *gyrB* gene = EF052679; *rpoD* gene = DQ979874 [8]. All primers and probes were prepared and synthesized by Integrated DNA Technologies (Coralville, IA, USA) and are provided in Table 2. The *gyrB* primer set targeted a 184 bp sequence (positions 326–509). The *rpoD* primer set targeted a 101 bp sequence (positions 606–706). The probes, 25 bp in length, were labeled at the 5′ and 3′ ends with 6-FAM and a Black Hole Quencher 1™, respectively. qPCR using the genomic DNA sample of *B. diminuta* and other bacterial strains was performed using a Biorad ICycler (Biorad, Hercules, CA, USA). Individual PCR reactions consisted of a total volume of 25 µl with a maximum of 100 ng chromosomal DNA (10 µl), 12.5 µl ReadyTAQ Mastermix (Roche, Madison, WI, USA), 1 pM of each primer, and 200 nM of probe. For amplification, the following cycling parameters were utilized: conditioning cycle at 50°C for 5 min, initial denaturation cycle at 95°C for 10 min, 40 cycles at 95°C for 1 min and 60°C for 1 min, and hold at 4°C. Specificity studies were performed by screening all *gyrB* and *rpoD* primer/probe sets against the genomic DNA of the environmental isolates as well as selected Gram-negative and Gram-positive reference strains. All reactions were performed with approximately 100 ng genomic DNA from each isolate. To determine the sensitivity of the qPCR reactions, a standard curve for *B. diminuta* was created using a starting genomic DNA concentration of 89 ng. Serial tenfold dilutions in TE buffer were performed to determine sensitivity of the reaction.

Whole-cell qPCR using the *rpoD* primer/probe pair

The density of *B. diminuta* grown separately in TSB and SLB was confirmed via membrane filtration (0.1 µm pore diameter cellulose filters) with R2A agar [3, 4]. The plates were incubated at 30°C for 48 h. The stock concentrations for both cultures were $\sim 1 \times 10^9$ CFU per mL. The cultures were serial diluted tenfold in phosphate-buffered saline. For both the SLB- and TSB-grown cultures, 1 mL stock and each dilution were transferred to separate centrifuge tubes and heated at 94°C for 10 min, then immediately chilled on ice. About 10 µl unheated and heated

Table 2 Primers and probes used in the quantitative real-time PCR (qPCR) and fluorescence in situ hybridization (FISH) experiments

Oligonucleotide name	Oligonucleotide sequence	Label	Reference
EUB338 Universal FISH probe	5′-GCTGCCTCCCGTAGGAGT-3′	5′-TEX 613[a]	[3]
gyrB FISH probe (GYRBSET2)	5′-AAGAAGCACAGCGTCCGCTTCGAGC-3′	5′-6-FAM[b]	NA[c]
rpoD FISH probe (RPODSET2)	5′-TCAAGGCCTATTTCGGCTCGGAGAT-3′	5′-6-FAM	NA[c]
gyrB forward primer	5′-ATCGAGATCATGCTGCACTATGAGGG-3′	None	NA[c]
gyrB reverse primer	5′-TGTTGTTGGTGAAGCACAGCATGG-3′	None	NA[c]
rpoD forward primer	5′-AGTTCCTCAAGGCCTATTTCGGCT-3′	None	NA[c]
rpoD reverse primer	5′-GGCTTCATTCTCGCTGAACTTGGT-3′	None	NA[c]
gyrB real-time probe	5′-ACGTCATCGTCATTCGCGGCCAGAA-3′	5′-6-FAM and 3′-Black Hole Quencher	NA[c]
rpoD real-time probe	5′-AGCGCATCAAGGAGATGGGCGT-3′	5′-6-FAM and 3′-Black Hole Quencher	NA[c]

[a] TEX 613 excitation $X = 598$ nm; TEX 613 emission $X = 617$ nm

[b] FAM excitation $X = 495$ nm; FAM emission $X = 520$ nm

[c] NA, not applicable; primers and probes were designed by the authors

SLB suspensions were run via the qPCR protocol described previously using the *rpoD* primer/probe set. The concentration range examined was 1×10^0 to 1×10^7 CFU *B. diminuta* cells per qPCR reaction.

Fluorescence in situ hybridization (FISH)

FISH probes designed specifically for *B. diminuta* ATCC 19146 were based on the *gyrB* and *rpoD* gene sequences detailed in a previous study [8]. Three oligonucleotides were used: GYRBSET2, RPODSET2, and EUB338. GYRBSET2 corresponds to *gyrB* sequence positions 80–104; RPODSET2 corresponds to *rpoD* sequence positions 614–636. Both GYRBSET2 and RPODSET2 were labeled at the 5′ end with 6-FAM (fluorescein; Integrated DNA Technologies, Coralville, IA, USA). EUB338, a probe specific for all bacteria, was labeled at the 5′ end with TEX 613 (Integrated DNA Technologies, Coralville, IA, USA) [2].

In addition to environmental isolates listed in Table 1, the following reference strains were included in the study to assess the specificity of the probes: *E. coli* ATCC 11229; *B. diminuta* ATCC 17599, 17600, and 19146; *R. pickettii* ATCC 700590; and *P. fluorescens* ATCC 13525. The organisms were grown in TSB and SLB to densities of at least 1×10^9 CFU per mL as determined via spread plating on R2A agar [3]. For comparison, the cell density of *B. diminuta* in the same SLB enrichments was estimated via direct microscopy coupled to FISH. Triplicate replicates were performed for the plating methods and microscopic analysis. For the FISH protocol, the procedure described by Sekiguchi et al. [29] was followed, with the following modifications. An aliquot of 100 µl for each of the cultures was incubated with 300 µl 95% ethanol for 10 min at room temperature. Following centrifugation at 12,000 × *g* for 1 min, the pellet was resuspended in hybridization buffer [0.9 M NaCl, 35% formamide, 20 mM Tris–HCl pH 7.4, 0.01% sodium dodecyl sulfate (SDS)] and incubated at 55°C for 10 min. The *gyrB*, *rpoD*, and EUB338 probes (at concentrations of 50, 50, and 20 pmol/µl, respectively) were then added. The suspension was incubated for an additional 30 min at 55°C. Following centrifugation at 14,000 × *g* for 1 min, the pellet was washed with prewarmed buffer (360 mM NaCl, 20 mM Tris–HCl pH 7.4, 0.01% SDS) and placed in a water bath for 15 min at 37°C. The suspension was centrifuged at maximum speed for 1 min, and the pellet was resuspended in 50 µl 2 µg/mL 4′,6-diamidino-2-phenylindole (DAPI)–Dabco (1,4-diazabicyclo[2.2.2]octane)-glycerol solution. A 30-µl sample was placed on a slide, and a coverslip was fixed to the slide. The slides were stored at 4°C in the dark until viewing with a Zeiss Axioskop II Plus fluorescence microscope at 1,000× magnification [29].

Statistical analysis

A two-tailed Student *t*-test ($\alpha = 0.05$) was used to compare cell densities obtained by FISH and traditional plate count methodology for *B. diminuta* grown on SLB.

Results and discussion

Real-time PCR for *B. diminuta gyrB* and *rpoD* genes

Two housekeeping gene sequences were targeted to use as the basis of the *B. diminuta* qPCR protocol: *rpoD* (1,166 bp) and *gyrB* (829 bp) [8]. A total of three primer and probe sets were evaluated for each gene, but only the data for the *gyrB* and *rpoD* sets with the desired specificity are presented in this study. The environmental consortium used to evaluated primer/probe specificity consisted

predominantly of Gram-negative heterotrophic plate count bacteria (HPCs) and included members of the *Caulobacteraceae* as well as strains of the genera *Rhodopseudomonas*, *Rhodococcus*, *Sphingomonas*, and *Acidovorax* [8]. As shown in Table 1, no amplification or detection of either gene was observed when evaluating the genomic DNA extracts of the 9 non-*B. diminuta* reference strains and 18 environmental isolates. The results demonstrate that the designed primer/probe sets possessed high specificity to *B. diminuta* ATCC 19146. It should be noted that the qPCR primer/probe sets showed affinity towards two additional control strains of *B. diminuta*. This is significant as these strains have been used in other product validation standards, such as NSF/ANSI Standard 18 for certification of manual food and beverage dispensing equipment [24], thus broadening the scope of application of this qPCR protocol.

Various concentrations of purified *B. diminuta* genomic DNA were used to assess the sensitivity of the *gyrB* and *rpoD* qPCR assays. The use of purified genomic DNA would offer the best-case scenario for detection of the target genes, as there would be low probability of any PCR inhibitors (i.e., cellular enzymes) being present in the reaction mixture. The results of the sensitivity study are presented in Figs. 1 and 2. Figure 1 demonstrates that the sensitivity for the *rpoD* primer/probe set (0.89 pg) was tenfold greater than the sensitivity for the *gyrB* primer/probe (8.9 pg). The sensitivities of these assays are consistent with the detection limits established in other published qPCR studies [21, 34]. As shown in Fig. 2, the *rpoD* primer set also enabled detection of the genomic DNA at an average of 2.5 cycles sooner than for *gyrB*. Due to the observed increased sensitivity and shorter detection times, the *rpoD* qPCR primer and probe set was selected for use in subsequent whole-cell qPCR studies.

Whole-cell qPCR using *rpoD* primer and probe set

Analysis cost and turnaround time for results are two major issues that testing laboratories are constantly striving to reduce. Current methodologies for assessing *B. diminuta*

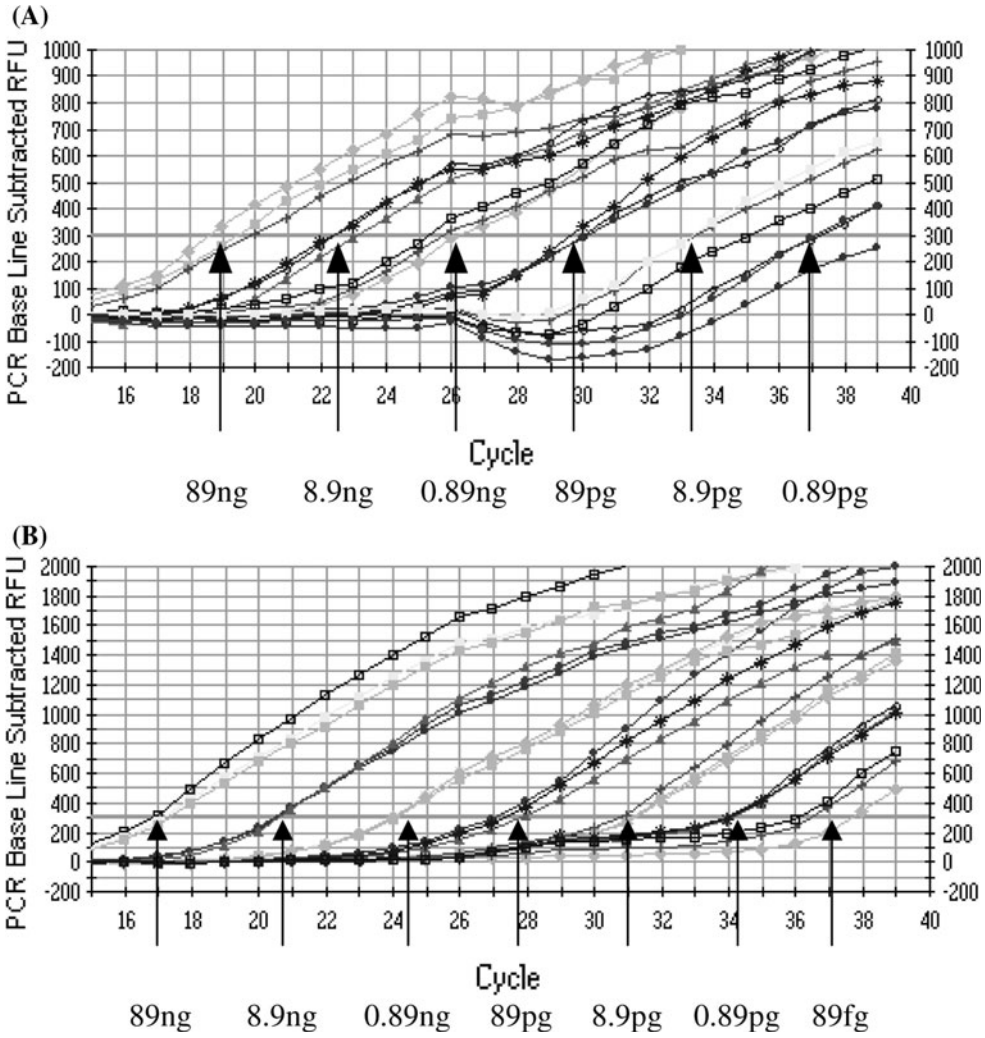

Fig. 1 qPCR sensitivity results using various concentrations of *Brevundimonas diminuta* ATCC 19146 genomic DNA: *gyrB* (**a**) and *rpoD* (**b**). The genomic DNA concentrations present are noted in the figure (per individual 25 µl qPCR reaction). The reactions were performed in triplicate; the threshold was set at 300

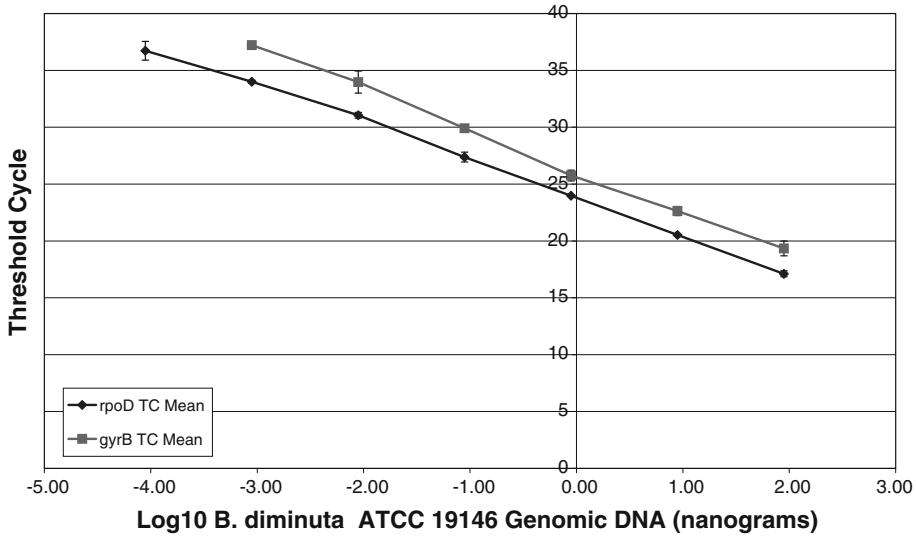

Fig. 2 Comparison of *gyrB* and *rpoD* qPCR sensitivity to varying concentrations of *Brevundimonas diminuta* ATCC 19146 genomic DNA. The following genomic DNA concentrations were used per 25 µl qPCR reaction: 89 ng, 8.9 ng, 0.89 ng, 89 pg, 8.9 pg, 0.89 pg, and 89 fg. All reactions were performed in triplicate. *Error bars* indicate the standard deviation associated with the observed threshold cycle values

filtration efficiency require days to obtain results [2 days for viable culture plating; 1 day for the luciferase and GFP assays described by Griffiths et al.] [12, 31, 32]. Current qPCR assays involve three areas that are labor intensive: extraction and purification of the DNA template (~90 min), performing the PCR assay, and data analysis (~2–3 h) [11, 21, 27]. A whole-cell qPCR approach was included in this study since the absence of a DNA extraction step may reduce the overall time to acquire a result.

The results of the whole-cell study indicate that *rpoD* qPCR is useful for rapid and selective quantification of *B. diminuta*. Results from the whole-cell qPCR studies (Fig. 3) showed that the PCR reaction was inhibited for the suspensions with the highest concentration of cells (1×10^7 CFU per PCR reaction or 1×10^9 CFU per mL). qPCR of *B. diminuta* grown on SLB without heat treatment allowed for a detectable range of 1×10^6 to 1×10^3 CFU per reaction (1×10^8 to 1×10^5 CFU per mL). Thu the whole-cell qPCR protocol detailed in this study will detect *B. diminuta* at the high starting concentrations (1×10^7 CFU per 100 mL) employed in water filtration testing, with or without heat treatment of the sample [31]. When a heating step was included for DNA extraction and protein denaturation, a 5-log cell concentration range of *B. diminuta* was detectable. The sensitivity of the reaction for detecting *B. diminuta* cultivated in SLB increased tenfold, so that a minimum of 1×10^2 CFU per reaction was detected (1×10^4 CFU per mL). In contrast, by growing the organism in TSB and employing the heat treatment, a 6-log range of *B. diminuta* cell concentration could be resolved via qPCR. The detection limit of this qPCR assay was 10 CFU per reaction (1×10^3 CFU per mL).

This qPCR sensitivity reported herein is approximately tenfold greater than what has previously been observed for qPCR using rRNA primers and probes [21, 34]. Sheu et al. [30] reported 100-fold less sensitivity (1×10^5 CFU) when screening environmental water samples for *Ralstonia eutropha*. Degenerate primers based on multiple sequence alignments of polyhydroxyalkanoate-producing bacteria were utilized. They postulated that the low sensitivity observed in their assay was due to impure chromosomal DNA and the degenerate nature of the primers. The high sensitivities observed for *B. diminuta* in this current study may be due to the fact that an automated real-time PCR thermocycler was utilized for PCR product quantification instead of end-point gel electrophoresis used in Sheu et al.'s study. The sensitivity of end-point PCR may be diminished due to inhibition and nonspecific binding of primers and PCR reagents to nontarget DNA [1]. In contrast, qPCR is more sensitive (up to 100-fold) and allows for detection of low volumes of PCR product during the reaction phase. In order to achieve the 7-log sensitivity required by the filter validation protocols, a sample concentration step must be performed prior to performing qPCR. Thus, the whole-cell qPCR approach may be most beneficial as a quality control application, such as for verifying the concentration of the *B. diminuta* stock and influent challenge samples.

rpoD and *gyrB* FISH assays

To assess the feasibility of utilizing a direct microscopic enumeration approach to determine total *B. diminuta* concentration pre and post filtration, FISH assays were investigated using probes selective for *B. diminuta*. The cross-reactivities of the *rpoD* and *gyrB* probes used in the FISH studies were assessed against select bacterial isolates listed in Table 1 [8, 9, 15]. After the optimal hybridization temperature and formamide concentrations were determined,

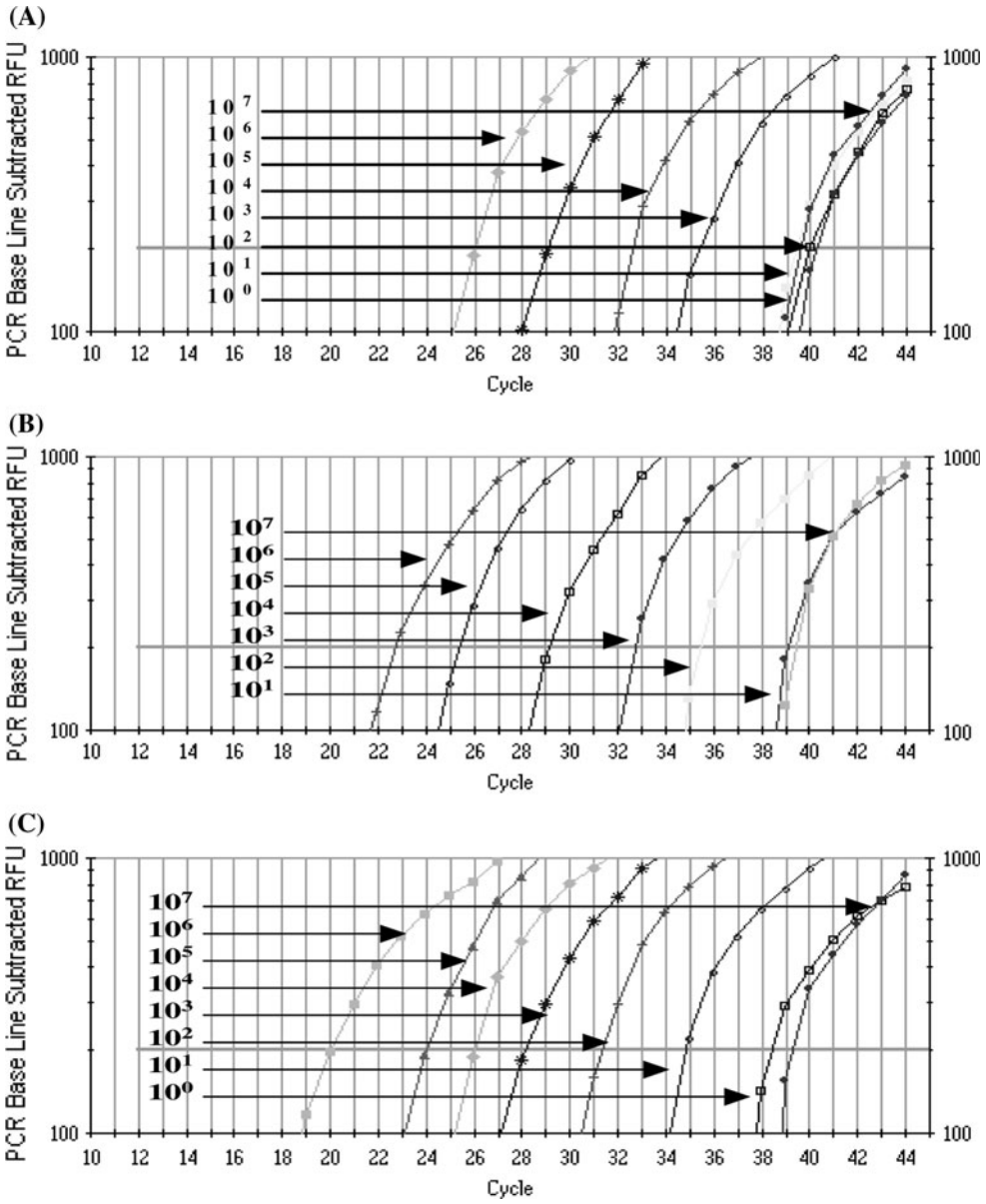

Fig. 3 Results of whole-cell *rpoD* qPCR study assaying varying concentrations of *Brevundimonas diminuta* ATCC 19146. **a** *B. diminuta* grown on SLB for 48 h at 30°C with no heat treatment. **b** *B. diminuta* grown on SLB for 48 h at 30°C then subjected to 10 min heat treatment for DNA extraction. **c** *B. diminuta* grown on TSB for 48 h at 30°C then subjected to 10 min heat treatment for DNA extraction. Ten microliters of each cell suspension (stock and dilutions) were included in the qPCR reactions. The approximate total CFU present in each qPCR reaction are noted on the plots. The threshold was set at 200 for each assay

the probes demonstrated specificity to only the three *B. diminuta* strains included in this evaluation. Representative micrographs of the *rpoD* FISH probes and their reactions with *B. diminuta* and a negative control organism (*E. coli* ATCC 11229) are presented in Fig. 4. Since the *gyrB* FISH probes yielded similar results, the micrographs are not included in this probe set.

A comparison of the ability of FISH and the conventional spread plating technique to enumerate *B. diminuta* was performed. For *B. diminuta* grown in SLB, a Student's *t*-test revealed a significant difference ($P = 0.01$) between the *B. diminuta* concentration estimated by FISH [$(3.4 \pm 0.8) \times 10^8$ CFU per mL] and the bacterial concentration obtained by the standard plating method [$(1.7 \pm 0.4) \times 10^8$ CFU per mL]. This observation is consistent with previous comparative FISH studies that found that plate counts may underestimate the total target bacterial population. Cell clumping is one factor that may be responsible for the disparity between assays [5, 19]. While plate counting only recovers the viable/culturable fraction, the current study supports the fact that FISH will recover all viable and nonculturable cells in suspension. This finding is advantageous when applying this technique to efficacy testing of RO filtration devices and pharmaceutical membranes, where assessing the total microbial removal (viable and nonculturable) is desirable.

Although a suspension-based assay was utilized in this study, in situ hybridization protocols have been modified to include filtration and fixation of the target bacteria to

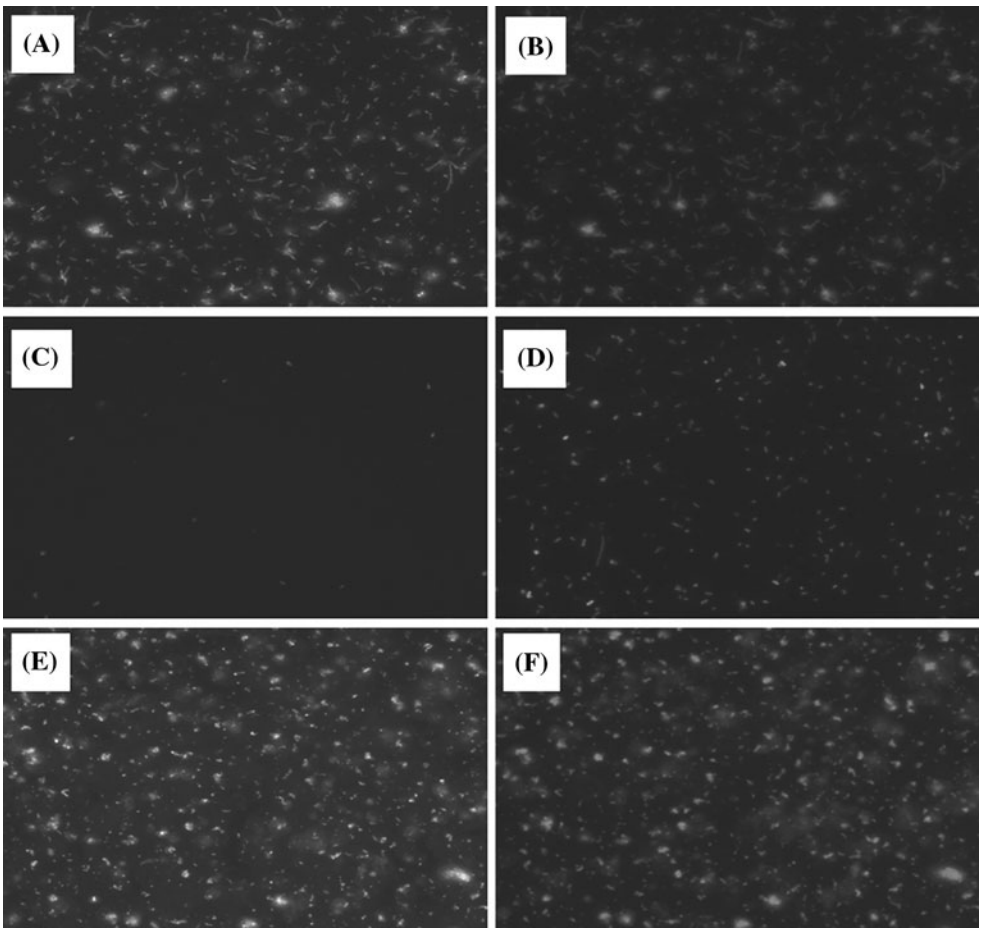

Fig. 4 Epifluorescence micrographs (magnification 1,000×) of *rpoD* FISH study using *Brevundimonas diminuta* ATCC 19146: **a** *B. diminuta* ATCC 19146 grown in TSB for 48 h at 30°C and labeled with RPODSET2 probe; **b** *B. diminuta* ATCC 19146 grown in TSB for 48 h at 30°C and labeled with EUB338 universal probe [3]; **c** *E. coli* ATCC 11229 grown in TSB for 48 h at 30°C and labeled with RPODSET2 probe; **d** *E. coli* ATCC 11229 grown in TSB for 48 h at 30°C and labeled with EUB338 universal probe; **e** *B. diminuta* ATCC 19146 grown in SLB for 48 h at 30°C and labeled with RPODSET2 probe; **f** *B. diminuta* ATCC 19146 grown in SLB for 48 h at 30°C and labeled with EUB338 universal probe

cellulose filters with 0.45 μm pore size [18]. A filtration step could easily be applied to the *rpoD* and *gyrB* FISH detection method, thus further reducing the detection limit. A 0.1 μm pore size filter would be recommended given the small diameter of *B. diminuta* when cultured with SLB medium. Such adaptation would also provide the ability to concentrate samples, thus lowering the potential detection limit of the FISH assay.

It should be noted that the FISH assay is more labor intensive than qPCR. Given the small cell size of *B. diminuta* (0.3 μm), manual enumeration by FISH may prove tedious and be prone to error due to potential interference by any autofluorescent background particulates in the sample. To reduce error, technologies such as flow cytometry may be applied to future studies involving FISH [10, 35]. Positive aspects of the FISH protocol are that it can easily be performed in less than 2 h and, when run under stringent conditions, the assay is extremely specific and selective in its binding to the target cells, thus limiting the occurrence of false positives.

Conclusions

Through the utilization of species-specific primers and probes for the *gyrB* and *rpoD* genes, FISH and qPCR were able to detect viable and nonviable *B. diminuta*. As the goal of filter validation protocols is to ascertain the true efficiency of the filtration system, detection of the entire population (viable and nonculturable) of the challenge organism present in the test system is desirable. The existing methods use nonselective plating methodologies that may not recover injured or stressed challenge organisms, thus leading to overestimation of filtration efficiency. Injured cells may arise from the antimicrobial activity associated with treatment technologies, such as halogenated

resins, that may be coupled to filtration devices in order to achieve a broad spectrum of organism removal and inactivation. Molecular technologies allow for the total concentration of the challenge organism present in a given system to be quantified. These technologies allow for much more rapid determination of *B. diminuta* concentrations compared with the traditional plating methodologies currently specified in water filtration test standards. By employing these technologies in the validation of water filtration treatment systems, more accurate assessment of their microbial removal efficiency can be achieved. As a result, a safer drinking water product will be produced, and the health of the consumer will be preserved.

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