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ORIGINAL PAPER

Selective enumeration strategies for *Brevundimonas diminuta* from drinking water

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Abstract *Brevundimonas diminuta* is used as a control organism for validating the efficiency of water filtration systems. Since these protocols use nonselective growth media, heterotrophic plate count bacteria (HPCs) indigenous to the water distribution system may interfere with *B. diminuta* enumeration, thus leading to inaccurate assessment of the filter's microbial reduction capability. This could negatively impact public health as unsafe drinking water may be produced. This study was conducted to evaluate different potential routes for selective enumeration of *B. diminuta* in drinking water. *B. diminuta*'s biochemical and molecular relationships to HPCs recovered from a laboratory drinking-water system were investigated. Of the 24 HPC morphotypes recovered, members of the *Alpha-* and *Betaproteobacteria* were most commonly identified. Based on comparisons of catabolic profiles (generated by the Biolog system) using principal component analysis, *B. diminuta* possessed similar metabolic patterns to several of the *Alphaproteobacteria* (*Sphingomonas* and *Caulobacter*), indicating that development of a selective medium based solely on carbon source was not feasible. Antibiotic susceptibility profiles revealed that the HPCs were least resistant to kanamycin, making it a candidate for future selective applications. Sequence comparisons of partial 16S rRNA sequences did not reveal any distinct similarities. However, basic local alignment search tool (BLAST) alignments of the *gyrB* and *rpoD* sequences for *B. diminuta* did show uniqueness, with the next closest match being to *Caulobacter* (88% and 79% similarity, respectively). Future investigation will focus on applying molecular assays, such as fluorescent in situ hybridization and quantitative real-time polymerase chain reaction (PCR), and incorporating an antibiotic marker or expressed fluorescent protein into the wild-type strain of *B. diminuta* for selective enumeration of *B. diminuta*.

Keywords *Brevundimonas diminuta* · Principal component analysis · 16S rRNA · *gyrB* · *rpoD*

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Introduction

The safety of drinking water has been a focus of state and federal regulations since the inception of the Safe Drinking Water Act of 1974 [20]. Awareness in this area has been heightened due to the terrorist attacks of September 11, 2001 and the several natural disasters that have occurred in the past 5 years. In response to these disasters, the US Environmental Protection Agency (USEPA) published the Water Security Research and Technical Support Action Plan in 2004 that specified the necessity to evaluate the microbial contaminant removal capabilities of point-of-use (POU) and point-of-entry (POE) drinking-water treatment systems [33]. Numerous protocols have been drafted to validate the efficacy of drinking-water filtration systems to remove waterborne pathogens such as enteric viruses, bacteria, and protozoa [32, 34]. Validation of microbial reduction claims, whether performed by the manufacturer or an independent, third-party company, is of utmost importance in instilling consumer confidence as well as ensuring public health. Utilization of a nonvalidated

(or improperly validated) device for remediation of pathogen-contaminated water could result in severe illness or death to the consumer due to product failure, and thus the manufacturer would be liable. Most of these protocols utilize surrogate organisms to assess the removal efficiency of devices. Surrogates are typically selected based on size, charge, hazard level, and ease of cultivation and enumeration [13].

Reverse osmosis (RO) is one POU treatment device technology that has attracted increased attention for use in emergency response situations. Though traditionally utilized in desalination applications and in the consumer sector for water purification, RO devices can be effective in removing pathogens from contaminated water. RO systems incorporate a pressure-driven process which forces contaminated water through a membrane with small pore size ($\sim$0.001 nm). Studies have demonstrated that RO may provide a significant advantage over traditional filtration technologies, such as coagulation and sedimentation and filtration, and that greater than 4-log pathogen removal can be achieved [5, 6].

The USEPA published a validation protocol for POU RO devices that incorporates *Brevundimonas diminuta* as the test surrogate [32]. Organism size was a key determinant for selection of this bacterium, since the smallest identified bacterium of concern, *Francisella tularensis*, is 0.2 μm in diameter [7]. When grown in minimal media such as saline lactose broth, *B. diminuta* has a similar cell diameter (0.3 μm). Titers exceeding 1×10^9 colony-forming units (CFU) per ml at this reduced size can be achieved, making it ideal for use in assessing how "gross" contamination events can impact a filtration system's removal efficiency [15]. *B. diminuta* ATCC 19146 also has been widely used as a test strain for quality control of membrane filters utilized in microbiological water assays (such as those specified in standard methods for enumeration of total coliforms) and as a challenge organism for water treatment devices such as mechanical filters [4, 11, 29].

Brevundimonas diminuta is a Gram-negative, nonfermenting, rod-shaped bacterium belonging to the *Alphaproteobacteria* class and *Caulobacteraceae* family. It has close genetic, metabolic, and morphological similarities to a variety of heterotrophic plate count bacteria (HPCs) found in water distribution systems. The occurrence and prevalence of a diverse array of HPCs, including *B. diminuta*, in water distribution systems is well documented. Previous studies investigating recovery of HPCs from similar nutrient-limited water distribution systems revealed the presence of additional relatives to *B. diminuta* such as species of *Sphingomonas*, *Pseudomonas*, *Alcaligenes*, *Acinetobacter*, and *Ralstonia* [1, 38].

A nonselective medium is required for growth of *B. diminuta* in the RO validation protocols. As a result, microorganisms indigenous to the test system may outcompete, overgrow or be misidentified as *B. diminuta*. Thus, HPCs could potentially interfere with *B. diminuta* enumeration and negatively affect the performance assessment of RO devices. As RO devices have been employed for the production of potable water following environmental catastrophes, any inaccurate assessment of their microbiological removal capability may have profound negative health effects on the individuals consuming this "treated" water [6].

From a public health standpoint, it is important that a more accurate method for quantifying *B. diminuta* from RO test systems be developed. The purpose of this research is to determine the metabolic and molecular relationships of *B. diminuta* to HPCs indigenous to a laboratory deionized-water distribution system. These relationships will be used as a basis for the development of a selective enumeration approach for *B. diminuta* in drinking water. The various approaches applied to the HPCs and *B. diminuta* were comparison of: (a) metabolic fingerprinting (using the Biolog Microbial Identification System and principle component analysis) for possible development of nutrient-based selective medium, (b) antibiotic sensitivities for possible incorporation of a resistance marker, and (c) 16S rRNA, *gyrB* and *rpoD* sequencing, for possible development of species-specific probes with applications in quantitative real-time PCR (qPCR) and fluorescent in situ hybridization (FISH).

Materials and methods

Cultures, media, and reagents

Brevundimonas diminuta ATCC 19146 and all reference strains used for phylogenetic comparisons were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The organisms were grown according to the ATCC's instructions. All media used for bacterial isolation and growth were from Difco (Becton–Dickinson, Franklin Lakes, NJ, USA). Unless otherwise noted, incubation temperature of 30°C was used for all enrichment and growth plates and all reagents and chemicals were of American Chemical Society (ACS) reagent grade or higher (Sigma–Aldrich Co., St. Louis, MO, USA).

Water treatment device validation system

A laboratory drinking-water distribution system was used to evaluate POU RO devices. The water characteristics were based on the specifications provided by the USEPA in its environmental technology verification (ETV) report for validating POE RO water treatments systems [32]

and were as follows: pH = 6.0 ± 0.5 or 7.5 ± 0.5, temperature = 20 ± 2.5°C, conductivity ≤2 μS/cm at 25°C. The source was Ann Arbor, MI, USA drinking water that had been dechlorinated and pretreated with a POE RO device to reduce organics. The water system had a flow rate of 1.9 l/min.

HPC recovery and characterization

A comparative nonselective media recovery study was performed on the laboratory drinking-water treatment system. Effluent (2 l) was aseptically collected from the effluent port of the treatment system. Effluent aliquots ranging from 1 l to 0.01 ml were filtered on a 0.45-μm-porosity filter (GN6; Pall Corporation, East Hills, NY, USA) according to the membrane filtration protocol specified in standard methods [3]. The filters were aseptically transferred to tryptic soy agar (TSA), R2A agar, and standard plate count agar (SPCA) and incubated at 30°C. The plates were enumerated after 2 days, as specified by the USEPA RO validation protocol. The plates were observed again at 5 days to see whether additional organisms were present that could impact the molecular assays investigated in this study. Isolated colonies on each medium were separated initially by colony appearance and morphology. All observed bacterial colonies exhibiting unique colonial characteristics were subjected to further biochemical and genotypic characterization.

The isolates were characterized microscopically and were further differentiated via Gram and endospore staining and oxidase and catalase activity. The Biolog Microbial Identification System (Biolog Inc., Haywood, CA, USA) was utilized to generate a carbon source utilization fingerprint and possible identification. The isolates were prepared and inoculated according to Biolog specifications using GN2 (for Gram-negative isolates) and GP2 (for Gram-positive isolates) plates and a 24-h incubation at 30°C.

Statistical analysis of Biolog metabolic profiles

In order to determine whether *B. diminuta* possessed unique metabolic capabilities compared with the HPC isolates, Biolog metabolic profiles were assessed using principle component analysis (PCA) and multivariate analysis. The methods described by Weber et al. [36] were used for performing the data transformation, with a few modifications. The dual absorbance value obtained for all wells was used for PCA. The individual carbon source wells were normalized by average well color development (AWCD). For the carbon source wells common to both Gram-negative and Gram-positive isolates, $n = 57$. The n values for the Gram-negative comparisons of all carbon source wells, carbohydrates only, carboxylic acids only, and amino acids only were 95, 28, 24, and 20, respectively.

The proportion variation and eigenvalue index were calculated for each PCA. The top three principle components for the Gram-negative isolates were compared using cluster analysis. The data pertaining solely to the wells containing carbohydrates, carboxylic acids, and amino acids were analyzed in the same manner. These three groups represent ~75% of the carbon source wells utilized in the Biolog GP and GN identification plates. The PCA results of the wells common to the Gram-negative and Gram-positive isolates, as well as selected reference bacterial strains, were analyzed via the unweighted pair group method using arithmetic averages (UPGMA) method. The squared Euclidean distance (sum of squared deviations between the components) was used as the distance metric. A dendogram was generated to express the relationship of the organisms based on their similarity in Euclidean distance [10, 16].

Antibiotic resistance screening

Susceptibility of the HPC isolates to ampicillin (10 μg), tetracycline (10 μg), kanamycin (50 μg), cefepime (30 μg), levofloxacin (5 μg), and ceftazidime (30 μg) was evaluated using Mueller–Hinton agar and the National Committee for Clinical Laboratory Standards antibiotic disk diffusion method [21]. The plates were incubated at 30°C for 24 h. Sensitivity to the antibiotics was based on the observed zones of inhibition, classified as resistant, intermediate or susceptible.

Genomic DNA isolation and sequencing

All isolates were grown aerobically in 50 ml Luria–Bertani (LB) medium at 30°C on a gyratory shaker (150 rpm) and incubated for 1–5 days, depending on the isolate. Genomic DNA was extracted from the HPCs and *B. diminuta* using the GenElute system (Sigma–Aldrich Co., St. Louis, MO, USA) and Qiagen DNeasy kit (Qiagen, Valencia, CA, USA). Quantification and purity assessments of the isolated genomic DNA were determined by running 10 μl aliquots of the sample DNA on a ultraviolet (UV) spectrophotometer. Purified DNA samples were stored at 4°C until further use.

The 16S rRNA genes were amplified using Microseq 500 kit (Perkin-Elmer, Foster City, CA, USA). ExoSap-It (USB Corporation, Cleveland, OH, USA) was used for PCR product clean-up. Sequencing was performed on an ABI 3100 Genetic Analyzer (Perkin-Elmer, Foster City, CA, USA), and identification was accomplished with Microseq v1.0 software. The 500-bp sequences were further analyzed for genus and species assignment using the Ribosomal Database Project II [9].

The *gyrB* and *rpoD* genes were amplified and sequenced from the isolated genomic DNA using the primers and procedure described by Yamamoto and Harayama [39] with the following modifications. PCR was performed on a Bio-Rad iCycler (Bio-Rad, Hercules, CA, USA). Amplification of the *gyrB* and *rpoD* genes was performed in a total volume of 50 µl with 50 ng chromosomal DNA, 1.25 U Hotstart *Taq* DNA polymerase (Qiagen, Valencia, CA, USA), 1.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.3), 10 pM of each primer, and 200 mM of each individual deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP). Sequencing of the gene products was accomplished via the dideoxy chain termination method using a *Taq* Dye Terminator sequencing kit (Perkin-Elmer, Foster City, CA, USA). Sequencing reactions were carried out in 20 µl volumes: 4 µl of BDv3.1 Reaction Mix, 4 µl halfSEQ BD3.1, 50 ng DNA template, 3.2 pM primer, and nuclease-free water to total volume of 20 µl. The vector pGEM (Promega, Madison, WI, USA) was used as a sequencing reaction control.

Phylogenetic alignments and identifications

The Clustal W program was used for sequence alignment [31]. The nucleotide sequence data from *Halobacteria salinarum* DSM 671 AM774415, obtained from the National Center for Biotechnology Information (NCBI) GenBank database, was used to root each tree. Phylogenetic trees were generated using Mega 3.1 neighbor-joining bootstrap analysis with 1,000 iterations.

Isolate identification was attempted via BLAST search of the NCBI GenBank database with the partial 16S rRNA sequences of the individual HPCs [2]. BLAST searches of the GenBank database were also conducted on the Gram-negative HPC *gyrB* and *rpoD* sequences. The sequence data for *B. diminuta* 19146 were deposited to the NCBI GenBank nucleotide sequence database with the following accession numbers: DQ650706 (16S rRNA gene), EF052679 (*gyrB* gene), and DQ979874 (*rpoD* gene). The accession numbers for the HPCs are as follows: GQ891690–GQ891727 (16S rRNA gene), GQ980291–GQ980308 (*gyrB* gene), and GQ980309–GQ980327 (*rpoD* gene).

Results and discussion

HPC recovery and characterization

A thorough understanding of the similarity of *B. diminuta* to the HPCs indigenous to the laboratory drinking-water distribution system used for RO device testing was essential for designing a selective recovery approach. To assess the diversity of HPCs present in the laboratory water distribution system, multiple nonselective media were utilized for isolating these organisms from the laboratory water distribution system. A total of 24 individual HPC morphotypes were identified from the nonselective growth media: 21 after a 2-day incubation period and 3 additional isolates following a 5-day incubation period. Ten organisms were also isolated but could not be recultured. Martiny et al. [18] also documented viability loss of similar HPCs following their isolation from a drinking-water source. As has been reported in other studies with drinking water [17, 28], the highest overall bacterial recovery was found with R2A agar (2.1×10^4 CFU/ml). Compared with TSA and SPCA, total bacterial counts on R2A were higher by 1.01 log (90.2% greater) and 0.08 log (13% greater), respectively.

Four isolates accounted for 75% of the total population observed on TSA agar. For the SPCA and R2A agars, two isolates accounted for 86.6% and 90.6%, respectively, of the total recovered bacterial population. Additional isolates were recovered from each growth medium, albeit in lesser concentrations (<8% of the total population). Of the 24 isolates, all but 2 displayed circular whole-colony appearances with entire edges and 68% possessed colony sizes of $\leq$1.0 mm. Nineteen isolates were Gram-negative bacteria and five were Gram positive. Over 80% of the isolates were single rods. Thus, a majority of the HPCs possessed macroscopic and microscopic appearances similar to those of *B. diminuta*. The recovery of predominantly Gram-negative rods from this sample type with this nonselective media was expected, and similar results have been documented [18, 25, 38].

Population diversity of HPCs

The Biolog data and 16S rRNA, *gyrB* and *rpoD* sequence analyses provided the identification (genus and species) of a number of the HPCs. As identification of the isolates was only a minor objective of the study, only 500 bp of the 16S rRNA was amplified and sequenced. For the 24 HPCs analyzed by both methods, Biolog and Microseq results were in agreement at genus and species level for 38% and 16%, respectively, of the isolates. Differences in identification may be due to numerous factors, including failure of some of the "nutrient-starved" isolates to adapt to the nutrient-rich medium used by Biolog, or absence of the organism in the Biolog database. An additional 20% of the isolates had genus identifiers belonging to the same class (i.e., *Caulobacter* and *Sphingomonas*, both belonging to the class *Alphaproteobacteria*). The percentage of similar genus identifications observed in this study is consistent with previously published research involving environmental isolates [19].

The sequence data obtained from the 16S rRNA, *gyrB* and *rpoD* analyses were used to assign genus and species designations to the isolated HPCs. The results of the sequence BLAST searches against the GenBank database are presented in Table 1. The percentage homology of the HPCs to bacterial strains deposited in GenBank is much lower for *gyrB* and *rpoD*; the GenBank database contains far fewer sequences for *gyrB* and *rpoD* as compared with 16S rRNA. The differences may also be related to the sequence length analyzed for strain alignment (500-bp 16S rRNA partial sequence versus complete sequences of 800 and 1,100 bp for *rpoD* and *gyrB*, respectively). In many instances, the genus matching the HPCs' *gyrB* and *rpoD* genes was in agreement with the bacterial identifier assigned to the 16S rRNA sequences. About half of the HPCs were identified as members of the *Alpha-*, *Beta-*, and *Gammaproteobacteria* (≥99% homology). For the 16S rRNA sequences, 18 of the 24 isolates possessed >97% sequence homology to strains deposited in GenBank. The greatest sequence similarity was to the genera *Caulobacter*, *Sphingomonas*, *Delftia*, *Acidovorax*, *Rhodopseudomonas*, and *Acinetobacter*. A small percentage of the remaining Gram-negative HPCs were identified as belonging to the *Proteobacteria*. Some of the Gram-positive isolates were identified as *Micrococcus* and *Bacillus*. Previous studies analyzing the culturable organisms present in drinking water suggest that the HPCs identified in this study are typical for this type of environment [17, 23, 37]. It should be noted that viable but nonculturable organisms were not investigated in this study, since only culturable organisms could directly interfere with the *B. diminuta* recovery plate assays and thus affect the determination of log removal by RO treatment devices. Additional studies investigating the distribution of HPCs in drinking-water treatment systems and bottled water have recovered similar Gram-negative bacteria in addition to lower levels of *Alcaligenes*, *Brevundimonas*, *Stenotrophomonas*, and *Acidovorax*. Gram-positive organisms from the genera *Bacillus*, *Corynebacterium*, *Micrococcus*, and *Staphylococcus* have all been documented from similar water sources when using minimal-nutrient media for recovery [38].

Carbon utilization profiles

As *B. diminuta* could not be distinguished from indigenous HPCs using the current test procedures, the next step was to

Table 1 Sequence homology of the Gram-negative heterotrophic plate count bacteria (HPCs) 16S rRNA, *gyrB* and *rpoD* genes to sequences deposited in GenBank

HPCs[a]	16S rRNA identification[b]	*gyrB* identification	*rpoD* identification
E	*Acinetobacter radioresistens* (99%)	*Acinetobacter radioresistens* (97%)	*Acinetobacter* spp. (79%)
F	*Variovorax paradoxus* (100%)	*Variovorax* spp. (96%)	*Variovorax* spp. (89%)
G	*Rhodopseudomonas palustris* (100%)	*Rhodopseudomonas* spp. (87%)	*Rhodopseudomonas* spp. (85%)
I	*Rhodopseudomonas palustris* (97%)	*Rhodopseudomonas* spp. (90%)	*Rhodopseudomonas* spp. (85%)
K	*Acidovorax delafieldii* (99%)	*Variovorax* spp. (88%)	*Acidovorax* spp. (88%)
M	*Caulobacter leidyi* (99%)	*Stenotrophomonas* spp. (96%)	*Stenotrophomonas* spp. (94%)
N	*Sphingomonas chlorophenolica* (96%)	*Sphingopyxis* spp. (87%)	*Sphingopyxis* spp. (91%)
O	*Sphingomonas terrae* (100%)	*Sphingopyxis* spp. (84%)	*Sphingomonas* spp. (79%)
P	*Sphingomonas chlorophenolica* (96%)	*Sphingomonas* spp. (84%)	*Sphingomonas* spp. (79%)
Q	*Sphingomonas capsulata* (96%)	*Novosphingobium* spp. (86%)	*Novosphingobium* spp. (86%)
R1	*Acidovorax delafieldii* (100%)	*Variovorax* spp. (88%)	*Acidovorax* spp. (87%)
R2	*Rubrivivax* spp. (94%)	Not performed	*Acidovorax* spp. (81%)
T	*Caulobacter leidyi* (99%)	No significant similarity found	*Sphingopyxis* spp. (89%)
W1	*Caulobacter leidyi* (99%)	*Novosphingobium* spp. (85%)	*Novosphingobium* spp. (86%)
X	*Caulobacter leidyi* (99%)	*Caulobacter* spp. (95%)	*Sphingomonas* spp. (81%)
Rig 1	*Sphingomonas capsulata* (98%)	*Novosphingobium* spp. (86%)	*Erythrobacter* spp. (74%)
Rig 2	*Delftia acidovorax* (99%)	*Variovorax* spp. (92%)	*Erythrobacter* spp. (73%)
Rig 6	*Ralstonia eutropha* (96%)	*Ralstonia metallidurans* (98%)	*Acinetobacter* spp. (78%)
2	*Caulobacter leidyi* (97%)	*Verminephrobacter* spp. (93%)	*Verminephrobacter* spp. (89%)

The GenBank maximum identity value is presented in parenthesis. Genus and species are provided for isolates that possess similarity ≥97%, as these sequences are considered operational taxonomic units [37]

[a] Five Gram-positive isolates were also assigned IDs based on their 16S rRNA sequences: isolate 5—*Arthrobacter* spp. (93%), isolate 10—*Bacillus mojavensis* (100%), isolate 11—*Bacillus amyloliquefaciens* (99%), isolate B—*Micrococcus luteus* (99%), and isolate Rig 4—*Bacillus cereus* (100%). Analyses of their *gyrB* and *rpoD* genes were not performed

[b] A 500-bp gene segment was used for the 16S rRNA analysis

evaluate carbon utilization profiles to determine whether a nutrient-based selective medium could be developed. Metabolic fingerprints generated from the Biolog data have been widely used for microbial community analysis studies [36]. Applying this same multivariate analysis strategy to assess the metabolic relationship of *B. diminuta* to the recovered Gram-negative HPCs, no single carbon source was observed as being uniquely metabolized by *B. diminuta*. The Gram-positive isolates were excluded from this particular analysis, as their colony morphologies were distinctly different from that of *B. diminuta*. The results of the PCA cluster analysis with all 95 carbon sources for the Gram-negative HPCs are shown in Fig. 1. Separate analysis of the carbohydrate, amino acid, and carboxylic acid groups was performed, since these are the three most prominent nutrient groups, in terms of number of chemicals, featured on the Biolog GN plate. These analyses also demonstrated that *B. diminuta* clustered with multiple organisms for each carbon source type (data not shown). Figure 2 displays the dendogram of the 21 Gram-negative and Gram-positive HPCs subjected to UPGMA analysis. Only the organisms that were observed after 2 days of growth were included in this analysis, since only these organisms could potentially interfere with *B. diminuta* enumeration due to plate overgrowth or misidentification. When analyzing the cluster plots for the three carbon source groups as well as the UPGMA dendogram, it was evident that *B. diminuta* possessed a metabolic profile that was similar to HPCs determined to be members of the *Sphingomonas* and *Caulobacter* genera (Fig. 2, isolates 2, M, N, O, P, Q, T, W1, and X). This finding is not surprising as *B. diminuta* is a member of the *Caulobacteraceae* family [28]. This indicates that there are multiple, culturable, indigenous HPC isolates that are close relatives to *B. diminuta* in the test system having similar carbon utilization patterns. Because of the similarity in metabolic profiles, designing an isolation strategy for *B. diminuta* based solely on selective carbon source utilization could be difficult.

Antibiotic susceptibility

To further characterize the isolates HPCs and determine their relatedness to *B. diminuta*, antibiotic susceptibility testing was performed. Cefepime, levofloxacin, and ceftazidime were included, since studies indicated that certain strains of *B. diminuta* possess innate resistance to these fluoroquinolones [12]. Additional antibiotics selected for the study are commonly used as antibiotic markers [27]. The results of the antibiotic susceptibility testing are presented in Table 2. When considering the entire set of HPCs analyzed, levofloxacin showed the highest percentage of

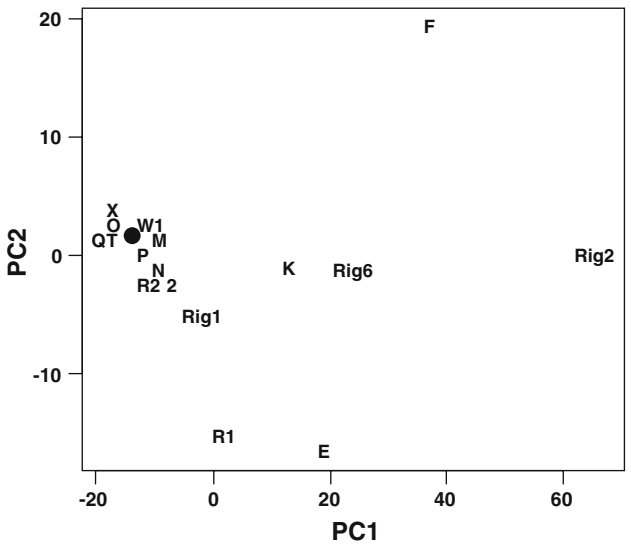

Fig. 1 Cluster analysis of the top two principle component analysis (*PCA*) vectors for all Gram-negative bacteria based on Biolog carbon source utilization data. *Brevundimonas diminuta* ATCC 19146 is denoted by a *bullet*; the designations for the other Gram-negative bacteria are explained in Table 1 ($n = 95$, as all carbon sources were compared). The individual carbon source wells were normalized by average well color development (AWCD) prior to performing the principle component and multivariate analyses

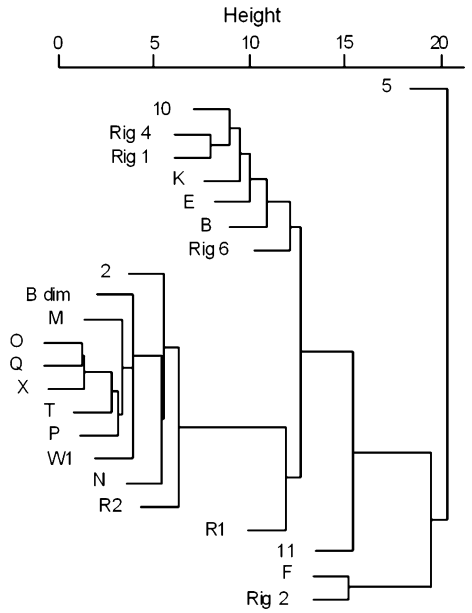

Fig. 2 Comparison of Biolog metabolic profiles for heterotrophic plate count bacteria (HPCs) as well as *Brevundimonas diminuta* ("B dim"). The designations for the other bacteria are explained in Table 1. Only isolates exhibiting visible growth on culture plates within 48 h of incubation at 30°C (i.e., those that could potentially interfere with enumeration of *B. diminuta* due to overgrowth) were included in this analysis. Isolates G and I were excluded. The dendogram is based on UPGMA cluster analysis of metabolic data from all common carbon source wells for Gram-negative and Gram-positive Biolog identification plates. Agglomerative coefficient = 0.61

Table 2 Antibiotic sensitivity of heterotrophic plate count bacteria (HPCs) isolated from a laboratory deionized-water distribution system

Antibiotic	Intermediate (%)	Resistant (%)
All HPCs[a]		
Ampicillin (10 μg)	21	71
Kanamycin (50 μg)	12	32
Tetracycline (10 μg)	29	65
Cefepime (30 μg)	0	29
Levofloxacin (5 μg)	6	18
Ceftazidime (30 μg)	12	53
Alphaproteobacteria[b]		
Ampicillin (10 μg)	17	83
Kanamycin (50 μg)	8	8
Tetracycline (10 μg)	25	75
Cefepime (30 μg)	0	38
Levofloxacin (5 μg)	13	38
Ceftazidime (30 μg)	25	63

Determined by the National Committee for Clinical Laboratory Standards diffusion disc test [21]. *B. diminuta* ATCC 19146 was sensitive to tetracycline, cefepime, and levofloxacin, had intermediate resistance to ampicillin and kanamycin, and was resistant to ceftazidime

[a] $n = 24$ for ampicillin, kanamycin, and tetracycline; $n = 17$ for cefepime, levofloxacin, and ceftazidime (as several isolates lost viability)

[b] $n = 12$ for ampicillin, kanamycin, and tetracycline; $n = 8$ for cefepime, levofloxacin, and ceftazidime (as several isolates lost viability)

susceptible and intermediate resistance responses, followed by cefepime and kanamycin. For the *Alphaproteobacteria* subset, kanamycin showed the highest percentage of susceptible and intermediate resistance responses. *B. diminuta* 19146 was also observed to be susceptible to cefepime and levofloxacin and to have intermediate resistance to kanamycin. The isolates with greatest susceptibility to kanamycin were identified as species of *Caulobacter*, *Sphingomonas*, and *Rhodopseudomonas*. Pavlov et al. [22] described similar observations of HPCs antibiotic reaction when performing disk diffusion studies on populations recovered from drinking water. That study documented resistances of HPCs to ampicillin and kanamycin of 54.3% and 6.9%, respectively, although the concentration of kanamycin tested was lower than that used in the current study (30 μg per disk compared with 50 μg). Jeena et al. [14] also observed elevated resistance of isolated HPCs to ampicillin (70%) and tetracycline (48%). The increased percentage of HPCs susceptible to kanamycin, compared with tetracycline and ampicillin, make this antibiotic ideal for inclusion in a selective media for recovery of *B. diminuta* from drinking water.

Molecular comparisons of HPCs with *B. diminuta*

The genetic similarity of *B. diminuta* to the HPC isolates was examined to assess whether molecular approaches for selectively enumerating *B. diminuta* could be employed. Individual phylogenetic comparisons were generated using the 16S rRNA, *gyrB* and *rpoD* sequences of the organisms (Figs. 3, 4, 5, respectively). Sequences of two housekeeping genes, for the σ^{70} transcription factor (*rpoD*) and the DNA gyrase protein (*gyrB*), were included in this study. Several recent studies have used these protein coding genes for establishing microbial phylogeny [8, 26], as the sequence variation within these genes is greater compared with 16S rRNA. This disparity can permit two or more closely related strains to be distinguished. Additionally, sequence alignment of distantly related strains may be improved due to the protein coding facet of these genes [35, 39].

Phylogenetic comparison of the 16S rRNA partial (500 bp) sequences obtained from the isolates revealed that there were strong similarities between *B. diminuta* and two Gram-negative isolates (G and I) belonging to the class *Alphaproteobacteria*. Analysis of the *gyrB* and *rpoD* bootstrap consensus trees showed that *B. diminuta* displayed the highest degree of homology to isolate G (identified through 16S rRNA sequence analysis as *Rhodopseudomonas palustris*) as well as a close relation to other members of the *Caulobacteraceae*.

A BLAST search of the NCBI GenBank database for *gyrB* and *rpoD* genes of *B. diminuta* showed the closest homology to a species of *Caulobacter* (88% and 79%, respectively). BLAST alignments of the *B. diminuta gyrB* and *rpoD* sequences to their closest relatives revealed that there were 100-bp regions of these genes that showed little (<70%) homology to the deposited strain. This suggests that there are multiple locations within the *B. diminuta gyrB* and *rpoD* genes that could be utilized for species-specific primer/probe design. Until now, the only approach for quantifying the extent of removal by filtration devices has been culture based. By employing a species-specific probe approach for *B. diminuta*, filtration efficiencies could potentially be assessed through more rapid techniques such as real-time quantitative PCR or fluorescent in situ hybridization [24].

Conclusions

It can be concluded from the identification study of HPCs that the drinking-water system evaluated in this study is consistent with similar drinking-water sources in respect to the culturable bacterial flora present. Furthermore, certain HPCs present in a drinking-water distribution system may

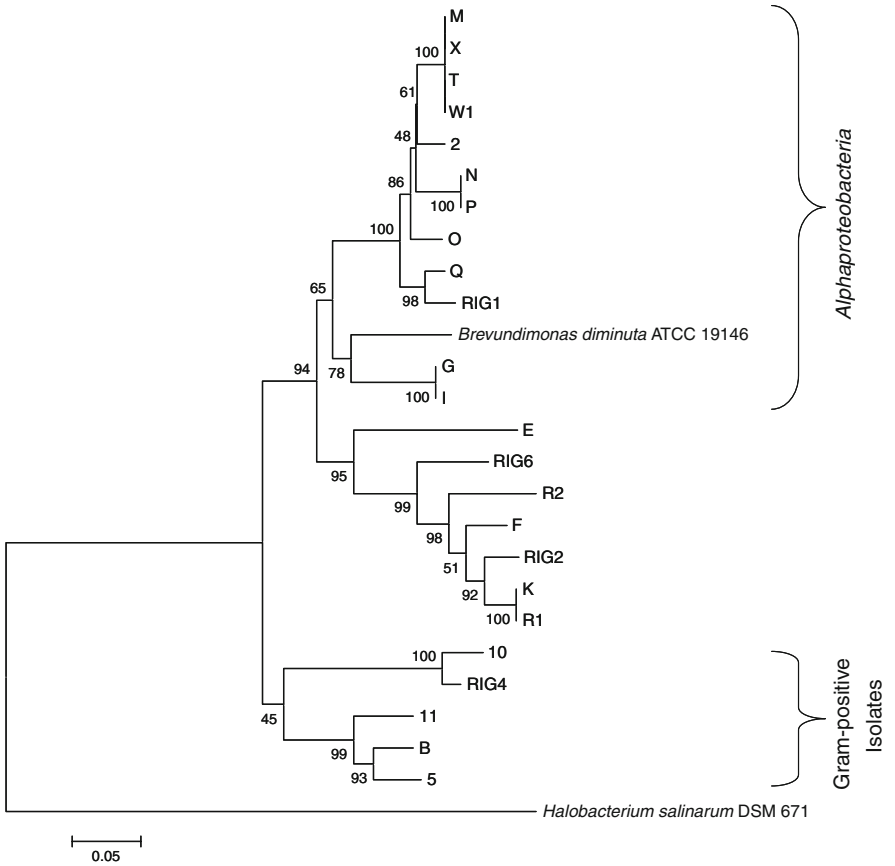

Fig. 3 Neighbor-joining comparison of 16S rRNA sequence alignment data for heterotrophic plate count bacteria (HPCs) isolated from a laboratory drinking-water distribution system. *Brevundimonas diminuta* ATCC 19146 is included in the comparison. *Halobacterium salinarum* DSM671, a halophilic marine Gram-negative obligate aerobic archaeon, was used as the outgroup to root the tree. For each isolate included, a partial gene sequence (500 bp) was used for the analysis. The *lower bracket* indicates which isolates are Gram positive; the remaining isolates are Gram negative, with the *upper bracket* denoting those belonging to the *Alphaproteobacteria* class. The numbers on the branches represent the bootstrap values obtained from 1,000 bootstrap trials, presented as percentage. The *scale bar* corresponds to 0.05 changes per nucleotide. Bootstrap values were obtained from 1,000 bootstrap trials (percentage presented)

display similar colonial morphologies compared with *B. diminuta* when cultivated on R2A agar. This indicates that the observed interference of HPCs with *B. diminuta* enumeration could occur in other laboratories. In relation to the USEPA RO validation protocol, this may impact the validation assay in a negative manner as the HPCs may outcompete and overgrow this target organism on the nonselective media employed in these protocols. Investigation into the metabolic capabilities and profiles of each isolate showed the presence of close relatives to *B. diminuta* in the test system. The absence of obvious catabolic differences between *B. diminuta* and these commonly recovered HPCs indicates that designing a selective enumeration system based on carbon source utilization would be difficult. However, at the molecular level, a feasible approach could be the use of using unique, nonhomologous regions of *B. diminuta gyrB* and *rpoD* genes in techniques such as qPCR and FISH protocols. Since the HPC strains were shown to be susceptible to kanamycin, an approach which could confer resistance of *B. diminuta* to kanamycin could also be the basis for selective medium.

Given the potential of these approaches to serve as selective replacements for current nonselective enumeration plating methods, the authors have also investigated the efficacy of employing the *rpoD* and *gyrB* sequences as a basis for *B. diminuta*-specific probe and primer design. Data has been generated that demonstrate the successful use of qPCR and FISH for quantifying *B. diminuta* concentrations in water. The primers and probes were shown to be specific to *B. diminuta* with no cross-reactivity when screened against the HPC isolates presented in this study as well as a number of Gram-positive and Gram-negative reference strains. 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*. Furthermore, studies have been performed on incorporating a kanamycin-resistance gene into the chromosomal DNA of *B. diminuta* via a Tn*5* transposon/transposase approach. The

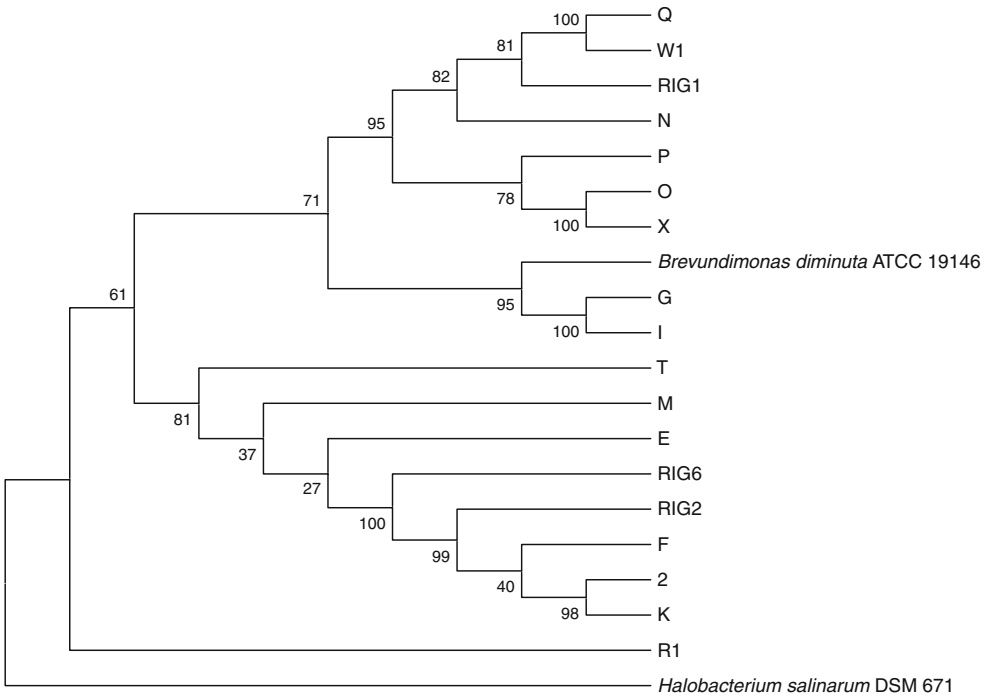

Fig. 4 Bootstrap consensus tree (unscaled) for *gyrB* sequence alignment data of all Gram-negative heterotrophic plate count bacteria (HPCs) isolated from a laboratory drinking-water distribution system. *Brevundimonas diminuta* ATCC 19146 is included in the comparison. *Halobacterium salinarum* DSM671, a halophilic marine Gram-negative obligate aerobic archaeon, was used as the outgroup to root the tree. For each isolate, a full gene sequence (~1,100 bp) was used for the analysis. Isolate R2 was omitted from this analysis due to difficulty in obtaining a complete *gyrB* sequence. The numbers on the branches represent the bootstrap values obtained from 1,000 bootstrap trials, presented as percentage

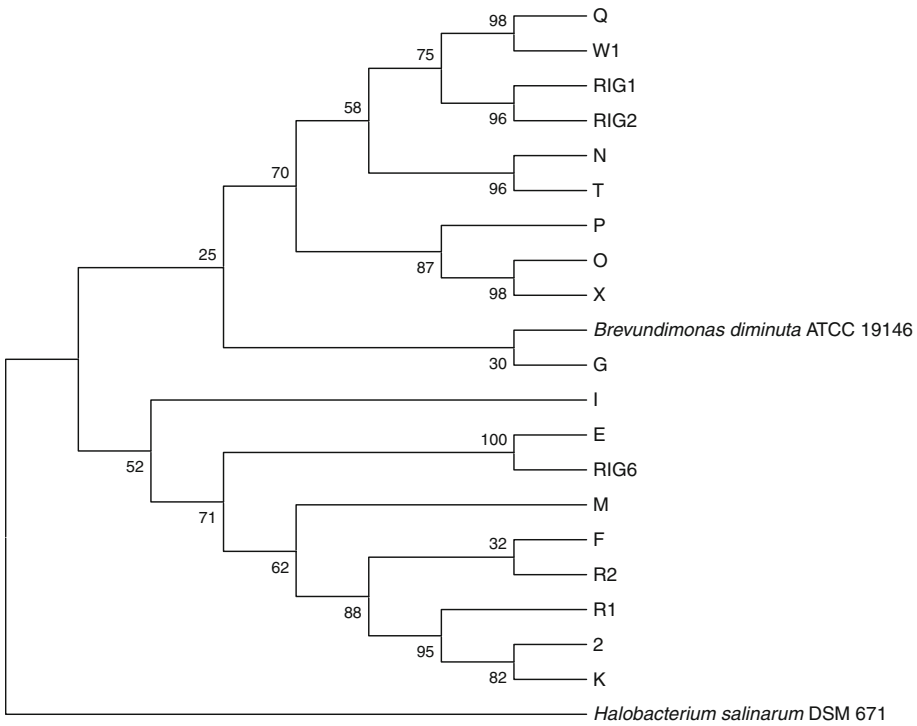

Fig. 5 Bootstrap consensus tree (unscaled) for *rpoD* sequence alignment data of all Gram-negative heterotrophic plate count bacteria (HPCs) isolated from a laboratory drinking-water distribution system. *Brevundimonas diminuta* ATCC 19146 is included in the comparison. *Halobacterium salinarum* DSM671, a halophilic marine Gram-negative obligate aerobic archaeon, was used as the outgroup to root the tree. For each isolate, a full gene sequence (~800 bp) was used for the analysis. The numbers on the branches represent the bootstrap values obtained from 1,000 bootstrap trials, presented as percentage

B. diminuta transformant (kan^R) displayed long-term expression of the gene, and monitoring of bacteria present in the test system showed no transfer of kanamycin resistance from the kan^R strain to indigenous microorganisms. It was concluded that these approaches could be used to aid in the determination of filtration efficiency of

treatment units employed in the production of sterile water or used in the pharmaceutical industry [30]. The complete findings of the qPCR, FISH and Tn*5* transposon studies will be presented in future correspondence. By achieving a more accurate validation approach for RO and other water filtration devices, the production of safe drinking water is assured and public health is preserved.

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References

1. Allen MJ, Edberg SC, Reasoner DJ (2004) Heterotrophic plate count bacteria—what is their significance in drinking water. Int J Food Microbiol 92:265–274. doi:10.1016/j.ijfoodmicro.2003.08.017
2. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402
3. American Public Health Association (1998) Standard methods for the examination of water and wastewater, 18th edn. American Public Health Association, Washington, DC
4. American Society of Testing Materials Standard (2001) D 3862-80, Standard test method for retention characteristics of 0.2-µm membrane filters used in routine filtration procedures for the evaluation of microbiological water quality. ASTM International, West Conshohocken, PA. doi:10.1520/D3862-80R01
5. American Water Works Association (1999) In: Letterman RD (ed) Water quality and treatment, 5th edn. McGraw-Hill, New York
6. Atkinson S (2005) Membrane and filtration companies aid Katrina recovery efforts. Membr Technol 2005:7–8. doi:10.1016/S0958-2118(05)70549-8
7. Burrows WD, Renner SE (1999) Biological warfare agents as threats to potable water. Environ Health Perspect 107:975–984
8. Cladera AM, Bennasar A, Barcelo M, Lalucat J, Garcia-Valdes E (2004) Comparative genetic diversity of *Pseudomonas stutzeri* Genomovars, clonal structure, and phylogeny of the species. J Bacteriol 186:5239–5248. doi:10.1128/JB.186.16.5239-5248.2004
9. Cole JR, Wang Q, Cardenas E, Fish J, Chail B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh T, Garrity GM, Tiedje JM (2008) The ribosomal database project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res 37:141–145
10. Gan G, Ma C, Wu J (2007) Data clustering theory, algorithms, and applications. SIAM, Philadelphia
11. Griffiths MH, Andrew PW, Ball PR, Hall GM (2000) Rapid methods for testing the efficacy of sterilization-grade filter membranes. Appl Environ Microbiol 66:3432–3437
12. Han XY, Andrade RA (2005) *Brevundimonas diminuta* infections and its resistance to fluoroquinolones. J Antimicrob Chemother 55:853–859. doi:10.1093/jac/dki139
13. Janas JJ (1999) Verifying membrane filter-based bacterial removal using titer reduction. Med Device Diagn Ind 21:81–87
14. Jeena M, Deepaa P, Mujeeb Rahimana K, Shanthia R, Hatha A (2006) Risk assessment of heterotrophic bacteria from bottled drinking water sold in Indian markets. Int J Hyg Environ Heal 209:191–196. doi:10.1016/j.ijheh.2005.11.003
15. Kaltenbach CM, Moss CW, Weaver RE (1975) Cultural and biochemical characteristics and fatty acid composition of *Pseudomonas diminuta* and *Pseudomonas vesiculare*. J Clin Microbiol 1:339–344
16. Kaufman L, Rousseeuw PJ (2005) Finding groups in data: an introduction to cluster analysis. Wiley, New York
17. Kawai M, Matsutera E, Kanda H, Yamaguchi N, Tani K, Nasu M (2002) 16S ribosomal DNA-based analysis of bacterial diversity in purified water used in pharmaceutical manufacturing processes by PCR and denaturing gradient gel electrophoresis. Appl Environ Microbiol 68:699–704. doi:10.1128/AEM.68.2.699-704.2002
18. Martiny A, Albrechtsen H, Arvin E, Molin S (2005) Identification of bacteria in biofilm and bulk water samples from a nonchlorinated model drinking water distribution system: detection of a large nitrite-oxidizing population associated with *Nitrospira* spp. Appl Environ Microbiol 71:8611–8617. doi:10.1128/AEM.71.12.8611-8617.2005
19. McKinsey PC, Franck MM, Fliermans CB (2000) Biolog ID as compared to 16S ribosomal RNA ID for environmental isolates from the deep subsurface. US Department of Energy. WSRC-MS-2000-00306
20. Montgomery JM (1985) Water treatment principles and design. Wiley, New York
21. National Committee for Clinical Laboratory Standards (2003) Performance standards for antimicrobial disk susceptibility tests, 8th edn. Approved standard M2-A8. National Committee for Clinical Laboratory Standards, Wayne
22. Pavlov D, de Wet C, Grabow W, Ehlers M (2004) Potentially pathogenic features of heterotrophic plate count bacteria isolated from treated and untreated drinking water. Int J Food Microbiol 92:275–287. doi:10.1016/j.ijfoodmicro.2003.08.018
23. Penna V, Alzira S, Martins M, Mazzola P (2002) Identification of bacteria in drinking and purified water during the monitoring of a typical water purification system. BMC Public Health 2:13. doi:10.1186/1471-2458-2-13
24. Qin X, Emerson J, Stapp J, Stapp L, Abe P, Burns JL (2003) Use of real-time PCR with multiple targets to identify *Pseudomonas aeruginosa* and other nonfermenting gram-negative bacilli from patients with cystic fibrosis. J Clin Microbiol 41:4312–4317. doi:10.1128/JCM.41.9.4312-4317.2003
25. Reasoner DJ, Geldreich EE (1985) A new medium for the enumeration and subculture of bacteria from potable water. Appl Environ Microbiol 49:1–7
26. Saavedra MJ, Figueras MJ, Martinez-Murcia AJ (2006) Updated phylogeny of the genus *Aeromonas*. Int J Syst Evol Microbiol 56:2481–2487. doi:10.1099/ijs.0.64351-0
27. Sambrook J, Russell D (2001) Molecular cloning: a laboratory manual, 3rd edn. CSHL Press, New York. ISBN 978-087969577-4
28. Sly LI, Cox TL, Beckenham TB (1999) The phylogenetic relationships of *Caulobacter*, *Asticcacaulis* and *Brevundimonas* species and their taxonomic implications. Int J Syst Bacteriol 49:483–488. doi:10.1099/00207713-49-2-483
29. Sundaram S, Eisenhuth J, Howard G Jr, Brandwein H (2001) Method for qualifying microbial removal performance of 0.1 micron rated filters. Part I: characterization of water isolates for potential use as standard challenge organisms to qualify 0.1 micro rated filters. PDA J Pharm Sci Technol 55:266–267
30. Taylor MA, Alambra EF, Anes J, Behnke J, Enachescu B, Fitzgerald CL, Fortado M, Sizelove ML (2004) Remote site

production of sterile purified water from available surface water. Prehosp Disaster Med 19:266–277
31. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680
32. US Environmental Protection Agency (2004) NSF 04/14/EP-ADWCTR. Environmental Technology Verification report: physical removal of microbial contamination agents in drinking water
33. US Environmental Protection Agency (2004) Water security research and technical support action plan
34. US Environmental Protection Agency (1988) Guide standard for the water purifiers
35. Watanabe K, Nelson J, Harayama S, Kasai H (2001) ICB database: the *gyrB* database for identification and classification of bacteria. Nucleic Acids Res 29:344–345
36. Weber KP, Grove JA, Gehder M, Anderson WA, Legge RL (2007) Data transformations in the analysis of community-level substrate utilization data from microplates. J Microbiol Meth 69:461–469. doi:10.1016/j.mimet.2007.02.013
37. Williams MM, Domingo JWS, Meckes MC, Kelty CA, Rochon HS (2004) Phylogenetic diversity of drinking water bacteria in a distribution system simulator. J Appl Microbiol 96:954–964. doi:10.1111/j.1365-2672.2004.02229.x
38. World Health Organization (2003) In: Bartram J, Cotruvo J, Exner M, Fricker C, Glasmacher A (eds) Heterotrophic plate counts and drinking-water safety: the significance of HPCs for water quality and the human health. IWA, London. ISBN 1843390256
39. Yamamoto S, Harayama S (1998) Phylogenetic relationships of *Pseudomonas putida* strains deduced from the nucleotide sequences of *gyrB*, *rpoD* and 16S rRNA genes. Int J Syst Bacteriol 48:813–819. doi:10.1099/00207713-48-3-813