Molecular cloning of *Brevundimonas diminuta* for efficacy assessment of reverse osmosis devices

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ABSTRACT

Brevundimonas diminuta is the test organism specified in the United States Environmental Protection Agency's (USEPA) reverse osmosis (RO) treatment device verification protocol. As non-selective growth medium is employed, enumeration of *B. diminuta* may be impaired due to interference by indigenous heterotrophic bacteria. Thus the microbial removal capability of the filtration system may be incorrectly assessed. As these treatment devices are used in emergency situations, the health of the public could be compromised. The objective of this study was to develop selective approaches for enumerating viable *B. diminuta* in test water. Two molecular approaches were investigated: expression of a kanamycin resistance gene and expression of a fluorescent protein gene. The USEPA protocol specifies a 0.3 μm cell size, so the expression of the selective markers were assessed following growth on media designed to induce this small cell diameter. The kan^R strain was demonstrated to be equivalent to the wild type in cell dimension and survival following exposure to the test water. The kan^R strain showed equivalent performance to the wild type in the RO protocol indicating that it is a viable alternative surrogate. By utilizing this strain, a more accurate validation of the RO system can be achieved.

Key words | *Brevundimonas diminuta*, point-of-use treatment system, reverse osmosis, validation

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INTRODUCTION

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). 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 a pore size 10- to 100-fold less than those used in microfiltration. 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. In addition, bypass of the membrane could occur due to microscopic seal leaks (Sourirajan & Matsuura 1985; NSF/ANSI 2002).

The United States Environmental Protection Agency's (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 2006b). As organism size is a key factor for validation, *Brevundimonas diminuta* ATCC 19146 has been specified as the test organism. *B. diminuta* can achieve cell sizes similar to *Francisella tularensis*, which has a cell diameter of 0.2 μm and is a worst case scenario in terms of bacterial pathogen size (Málková *et al.* 1986; Burrows & Renner 1999). The USEPA method and additional microfiltration evaluation protocols employ R2A agar, a non-selective medium, for the recovery and enumeration of *B. diminuta* (ASTM 2001; USEPA 2006b). Heterotrophic plate count bacteria (HPCs) indigenous to the water distribution system supplying the test device may be recovered since R2A agar is designed to detect injured oligotrophic bacteria (Governal *et al.* 1991;

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Jeppsen & Jeppsen 2003; Reasoner 2004). If present in sufficient concentration, the HPCs may impact the determination of the removal efficiencies for the filtration systems. 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 effects on the individuals consuming this 'treated' water.

To overcome the potential interference the HPCs may pose to enumeration of *B. diminuta*, a selective and/or differential method for this test organism is desirable. Previous research has investigated the applicability of recombinant molecular techniques to confer differential properties to *B. diminuta*. Griffiths *et al.* (2000) utilized an *Escherichia coli* filter-mating transformation procedure for the transfer of plasmids conferring expression of bacterial luciferase (pBSLLUX2) and green fluorescent protein (pBSLGFP1). Tryptic soy agar (TSA) was utilized for the growth of the recombinant strains and allowed for expression of the inserts. However there are several drawbacks to this approach: high cost for the luminescence detection equipment; increased technician time, and fatigue associated with microscopic enumeration. Qualitative and quantitative molecular methods for *B. diminuta* have also been developed using the housekeeping gene *gyrB* and *rpoD* (Han & Andrade 2005; Donofrio *et al.* 2010b). Donofrio *et al.* (2010b) developed a quantitative real time PCR assay and a fluorescent *in situ* hybridization assay for *B. diminuta* enumeration. Though effective in capturing the entire population of *B. diminuta* in a given test water, the assays could not discern between viable and non-viable cells.

The objective of this study was to develop selective methodology for the enumeration of viable *B. diminuta* ATCC 19146 while using R2A as the growth medium. As some treatment devices incorporate antimicrobials within their systems in conjunction with RO, it is desirable for these applications to know the surviving fraction of the test organisms. A direct electroporation approach was investigated to confer kanamycin resistance and expression of two novel fluorescent protein vectors to *B. diminuta* (pAcGFP1 and pDsRed-Monomer). The recombinant strains were compared to the wild type (wt) *B. diminuta* strain in respect to cell size, growth, and expression on R2A, and survival in the test water specified by the USEPA RO protocol. Ultimately the kanamycin resistant strain was subjected to a side-by-side analysis with the wt *B. diminuta* in the USEPA RO protocol using commercially available devices.

MATERIALS AND METHODS

Media and reagents

All media used for the isolation and growth of the organisms detailed in this study were 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, St. Louis, MO, USA).

Bacterial strains

The challenge organism, *B. diminuta* ATCC 19146, was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and was resuspended according to the provided instructions. Unless otherwise noted, all incubations were conducted at 30 °C for 48 h while placed on a rotary shaker at 150 rpm. In order to obtain cells possessing a diameter of 0.3 μm *B. diminuta* was grown on saline lactose broth (SLB) (Griffiths *et al.* 2000).

Test system for water treatment devices

A test system was constructed to evaluate the filtration efficiency of RO devices. The system specifications and verification protocol were developed by NSF International in collaboration with the USEPA as part of the ETV program (USEPA 2006a, 2006b). The system was designed to accommodate devices possessing flow rates ranging from 1.9 to 100 L per min. A schematic of the test system is provided in Figure 1. The test system utilized a 2,000 L polyethylene tank to hold the influent test water (challenge water). Table 1 displays the water chemistry parameters used in the test protocol.

Transformation of *B. diminuta*

The EZ-Tn5 <KAN-2> Insertion kit™ (Epicentre, Madison, WI, USA) was selected to confer kanamycin resistance to *B. diminuta*. The target vector pUC19/3.4 was amended with the EZ-Tn5 <KAN-2> transposon via a transposase.

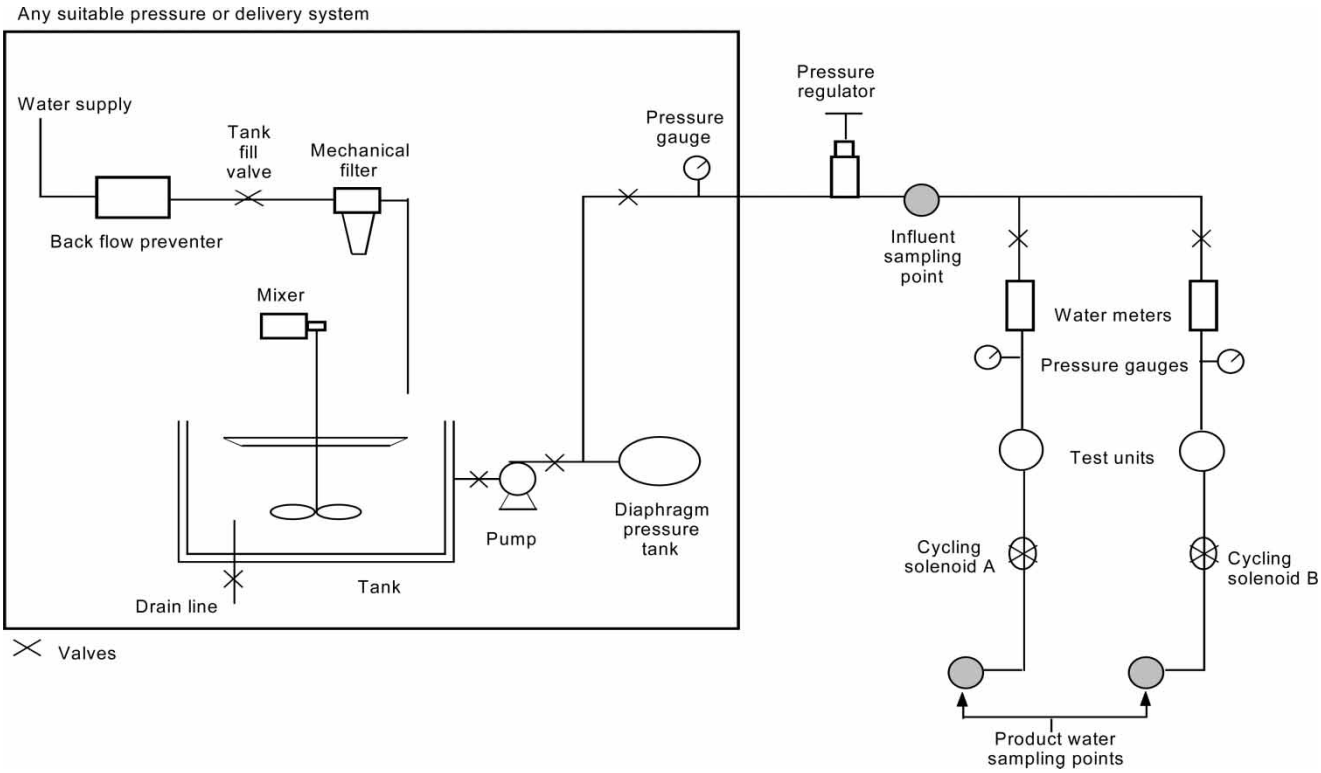

Figure 1 | Schematic of laboratory scale test system for the validation of reverse osmosis devices. Sampling locations are shaded.

Table 1 | Water chemistry specifications for the RO system verification protocol. Municipal drinking water (Ann Arbor, Michigan, USA) was treated by carbon filtration, reverse osmosis, and deionization to make the base water for the tests

Water characteristics	Specification
Alkalinity (as $CaCO_3$)	100 ± 10 mg/L
pH	7.5 ± 0.5 pH
Total dissolved solids	>5 and <500 mg/L
Total chlorine	≤0.05 mg/L
Conductivity	≤2 μS/cm at 25 °C
Total organic carbon	<100 mg/L
Temperature	4 ± 1 °C or 20 ± 2.5 °C
Total organic carbon	<100 CFU per mL

B. diminuta was washed three times using a 10% glycerol electroporation buffer at 4 °C (Miller 1994). Forty microliters of ~10^{10} CFU suspension of electrocompetent *B. diminuta* was electroporated with 1 μg of vector at 2,400 V with a time constant of 5 ms. The total volume post electroporation was brought to 1 mL with ice-cold super-optimal broth with catabolite repression (SOC) medium (Hanahan 1983). The suspension was allowed to equilibrate at 35 °C for 1 h, followed by spread plating 10-fold dilutions of the transformed suspension on Luria Bertani (LB) agar amended with 50 μg/mL kanamycin. The plates were incubated for 48 h at 30 °C. Isolates that were resistant to kanamycin were designated as the strain kan^R. The ability of kan^R strain to grow in SLB medium and R2A agar amended with 50 μg/mL of kanamycin was also assessed.

Fluorescent protein expression by *B. diminuta*

Electrocompetent *B. diminuta* cells were transformed with the pAcGFP1 (green fluorescent protein, GFP) and pDsRed-Monomer (red fluorescent protein, RFP) vectors (Clonetech, Mountain View, CA, USA). Each of the plasmids was approximately 3.3 kb in length and possessed a coding sequence for an ampicillin resistance marker gene. One microgram of each vector was introduced to the cells via electroporation. The electroporation event and cell recovery were performed as described in the transformation section with the exception that the transformants were plated on LB agar amended with

50 μg/mL ampicillin. Individual colonies were screened for expression of the fluorescent proteins using a fluorescent microscope equipped with FITC (excitation 475 nm, emission = 505 nm) and rhodamine (excitation 557 nm, emission = 585 nm) filter sets.

As the EPA protocol calls for R2A to be utilized as the growth media for all influent and effluent test samples, expression of the GFP and RFP on R2A agar was also investigated. The recombinant strains, grown previously in SLB for 48 h at 30 °C, were plated on R2A agar with and without ampicillin amendment.

Confirmation of gene expression

To establish that the vectors had been incorporated into *B. diminuta*, expression of the antibiotic resistance marker genes were evaluated. The kan^R strain was grown in lactose broth amended with 50 μg/mL kanamycin. The GFP and RFP strains of *B. diminuta* were grown in lactose broth amended with 50 μg/mL ampicillin. The suspensions were placed on a rotary shaker (150 rpm) for 24 h at 30 °C. Cellular protein was extracted from 25 mL aliquots of the cell suspensions using the CelLytic™ Express system and protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA). Fifteen microliters of 0.75 mg/mL protein extract for each group was loaded per well of a 15% Tris-HCl Criterion gel (Biorad, Hercules, CA, USA). The gel was run for 50 min at 200 V.

Size determination

As size is the key factor for *B. diminuta* ATCC 19146′s inclusion in the USEPA RO test protocol, the ability of the kan^R strain to exhibit the required 0.3 μm diameter was assessed. Scanning electron microscopy was used to confirm cell size for the wild type and kan^R *B. diminuta* strains. An aliquot of 100 μL of bacterial cells were filtered through a 0.1 μm cellulose filter. The filters were transferred to a slide, treated with 3% paraformaldehyde/glutaraldhyde mix for 30 min, and then rinsed with cacodylate buffer twice. The sample was treated with 25, 50, 75, 90, and 95% ethanol for 5 min each, then 100% for 10 min twice. The slides were placed in a desiccator overnight to air dry and then attached to the stub using double-sided carbon tape. Slides were sputter coated with gold (Technics Hummer V Sputter Coater set for 2 min at 90 mTorr, 20–30 mA) and viewed using an AMRAY 1820 I Scanning Electron Microscope at 5.0 kV.

Long-term expression of kanamycin resistance

The stability of the kan^R strain in expressing kanamycin resistance was assessed over a 1-year period. Multiple suspensions of kan^R at a concentration of 1×10^8 CFU per mL were created in sterile phosphate-buffered saline. The suspensions were held at 4 °C for 1 year and the cell density was evaluated for the suspensions twice monthly. The ability of kan^R to express kanamycin resistance post resuscitation from −70 °C storage in a glycerol suspension was also evaluated quarterly over the period of 1 year. For all of the sampling events, the kan^R strain suspensions were diluted in sterile phosphate-buffered saline and the dilutions were spread plated on R2A agar amended with 50 μg/mL of kanamycin.

Affect of test water characteristics on kan^R

The viability of the kan^R strain and the *B. diminuta* wt strain was evaluated after a 6 h exposure to test waters possessing the following abiotic parameters: temperatures of 4 and 20 °C; and total dissolved solids (TDS) of 5 and 500 mg/L. To assess the impact of the test water on low, medium, and high bacterial loads, concentrations ranging from 10^2 to 10^6 CFU per mL were included. These cell concentrations simulated varying magnitudes of bacterial contamination that may be accidentally or intentionally introduced into a water distribution system. Aliquots were removed every 2 h and enumeration of the challenge organisms was accomplished via spread plating on R2A agar (kan^R samples were plated on R2A amended with 50 μg/mL of kanamycin). All plates were incubated for 48 h at 30 °C.

Performance of *B. diminuta* wild type and kan^R strains in USEPA RO treatment device evaluation test

The wt and kan^R strains of *B. diminuta* ATCC 19146 were evaluated against the USEPA RO validation protocol. Three separate RO devices from two different manufacturers were included in the evaluation. These devices possessed a rated service flow of approximately 6 L/min. The systems were

conditioned according to the vendor's instructions using the USEPA base test water at pH 7.5 ± 0.5. At the end of the conditioning procedure, treated water samples were collected from each system as negative controls and analyzed for the challenge organisms. The RO membranes were operated using the base test water for 5 more days prior to challenge testing to ensure that they were performing optimally.

Following the conditioning period, the RO membranes were challenged with the wt and kan^R strains. The pH of the test water was 7.5. Cellular concentrations of 1×10^6 CFU/100 mL were targeted for each bacterial test strains. A 2 L bacterial suspension was prepared for both strains using SLB as the growth medium and the following incubation conditions: temperature of 30 °C; incubation time of 48 h; shaking at 150 rpm. For growth of the kan^R strain, the SLB was amended with 50 μg/mL of kanamycin. After addition of the test organism, the test water was mixed for a minimum of 30 min using a recirculation pump prior to beginning the test. After all systems were shut off, the RO water storage tanks were emptied into separate sterile containers and 1 L samples were collected in sterile polypropylene bottles for challenge organism enumeration. All samples and accompanying dilutions were enumerated in triplicate using the membrane filtration method (0.1 μm pore-size cellulose filter). The membranes were aseptically transferred to the appropriate growth agar. R2A agar was used for growth of the wt and R2A agar amended with 50 μg/mL of kanamycin was used for kan^R. All plates were incubated for 48 h at 30 °C.

Statistical analysis

Two-tailed Student *t*-tests ($\alpha = 0.05$) were used for comparing the survival of the wt and kan^R strain after exposure to the varying test water parameters. Microsoft Office Excel 2000 (Microsoft Corp.) was used to perform the Student *t*-tests.

RESULTS AND DISCUSSION

Transformation studies to confer selectivity to *B. diminuta*

In contrast to previous studies that incorporated an *E. coli* filter-mating step for plasmid transfer (Griffiths *et al.* 2000), a direct electroporation of *B. diminuta* with two vector systems was investigated. The first system evaluated was the EZ-Tn5 <KAN-2> Insertion kit. Electroporation of gram-negative bacteria with similar Tn5 systems has been previously described (Goryshin *et al.* 2000). This system allows for the random *in vitro* insertion of a kanamycin resistance selection marker into the target chromosomal DNA. The system incorporates a hyperactive EZ-Tn5 transposition system that displays 1,000-fold greater transposition efficiency compared to traditional Tn5 systems (de Lorenzo *et al.* 1990; Alexeyev *et al.* 1995; Goryshin *et al.* 2000). The recombinant strain displayed robust growth on non-selective nutrient rich medium (such as LB and TSA) as well as on medium amended with 50 μg/mL kanamycin. Figure 2 illustrates the expression of the 32 kD kanamycin resistance protein by the kan^R strain. Repeated growth and recovery studies from cultures held at both 4 and −70 °C performed over the course of 1 year revealed that the recombinant strain retained its selective properties under both storage conditions. It was observed that the wt did not grow on the plates amended with kanamycin. In addition it was observed that none of the HPCs previously isolated from

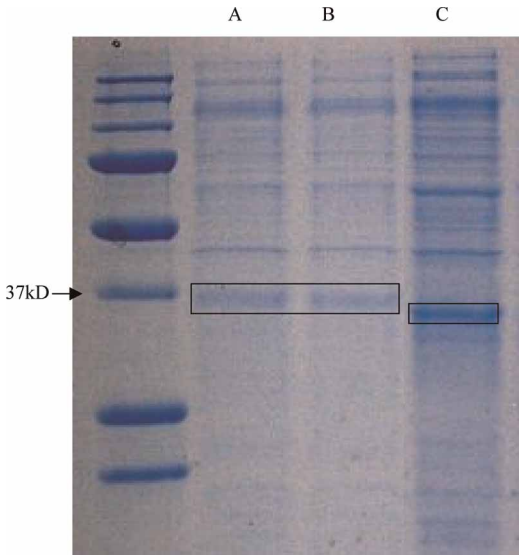

Figure 2 | Expression of kanamycin resistance protein and fluorescent proteins for *B. diminuta* transformants. Lanes A and B: Ampicillin resistance protein (33 kD) expressed by *B. diminuta* transformed with AcGFP (green fluorescent protein) and DsRed (red fluorescent protein). Lane C: Kanamycin resistance protein (32 kD) expressed by the kan^R strain. For culture methods see main text. The full colour version of this figure is available online at: http://www.iwaponline.com/jwh/toc.htm

a laboratory drinking water distribution system at NSF International (Donofrio *et al.* 2010a) grew when plated on this selective medium.

A number of studies have demonstrated that HPCs can effectively colonize water distribution systems. Previous research has also shown that members of the *Alpha, Beta* and *Gammaproteobacteria* were common to drinking water distribution systems (LeChevallier *et al.* 1987; Edberg *et al.* 1996; Kalmbach *et al.* 1997; Martiny *et al.* 2005; Donofrio *et al.* 2010a). A wide range of bacterial genera have been observed in drinking water, including *Pseudomonas, Ralstonia, Flavobacterium,* and *Acinetobacter* (Penna *et al.* 2002). These bacteria have faster growth rates than *B. diminuta* and, if present in sufficient numbers, could potentially outcompete and overgrow *B. diminuta* on non-selective media such as R2A agar and TSA (Bartram *et al.* 2003). Williams *et al.* (2004) observed that the majority of bacterial species isolated from a simulated drinking water distribution system were *Alphaproteobacteria* (i.e., *Caulobacter* and *Sphingomonas*), thus being closely related to *B. diminuta*. Ivnitsky *et al.* (2007) found that many of the same bacteria have been recovered from biofilms that have colonized nanofiltration water treatment systems. The membranes of these devices may be subject to bacterial fouling formation. This could result in the shedding and release of HPCs back in to the test system. In a test application such as the ETV RO device verification protocol, the presence of these HPCs in the effluent samples may result in an underestimation of the filtration efficiency of the treatment device as they may be mistakenly counted as *B. diminuta*. These studies illustrate that multiple strains of HPCs found in similar drinking water environments may possess phenotypic traits (such as size, morphology, and pigmentation) that could potentially interfere with the enumeration of *B. diminuta*.

To overcome HPC interference, selective plating using conferred antibiotic resistance was investigated. Kanamycin was chosen as the target resistance marker based on previous research findings cited in the literature as well as internal screening of HPCs isolated from a laboratory water distribution system at NSF International (Donofrio *et al.* 2010a). When performing disk diffusion studies on populations recovered from drinking water, Pavlov *et al.* (2004) observed HPC resistances to ampicillin and kanamycin of 54.3 and 6.9%, respectively. Jeena *et al.* (2006) noted a large percentage of HPCs recovered from bottled water sources were resistant to ampicillin (70%) and tetracycline (48%). Papandreou *et al.* (2000) found similar ampicillin resistance profiles among Gram negatives isolated from a water distribution system but observed low levels of tetracycline resistance. Clinically isolated strains of *B. diminuta* have been found to possess resistance to quinolones, such as cefepime and ceftazidine (Han & Andrade 2005). Previous work was performed that showed *B. diminuta* ATCC 19146 was sensitive to cefepime and levifloxacin (Donofrio *et al.* 2010a). This study did show that this strain was resistant to ceftazidime. However, a large percentage of HPCs isolated from the test laboratory water distribution system also possessed resistance to this antibiotic and thus kanamycin was chosen as the alternative. The current study illustrated that incorporation of the kan^R strain and its selective medium to water filtration protocols may overcome the HPC interference issue posed when a non-selective medium is used for recovery.

Fluorescent protein transformation studies for *B. diminuta*

As a potential means of differentiating *B. diminuta* from indigenous HPCs, expression of fluorescent proteins by recombinant strains of *B. diminuta* was also investigated. Previous work carried out by Griffiths *et al.* (2000) investigated GFP expression by a recombinant *B. diminuta* strain. In their studies, the transformed strain was enriched in SLB for 24 h, filtered onto 0.2 porosity black membrane placed on TSA plates and incubated for 24 h at 30 °C, and then subjected to enumeration using epifluorescence microscopy. Though sensitive, this technique proved to be time-consuming and physically straining on the technicians. In contrast to the Griffith study, the USEPA RO protocol requires the use of R2A agar as the recovery medium and 48 h incubation in SLB to achieve a mean cell diameter of 0.3 μm. Additionally, incubation conditions have shown to have a major impact on the size of *B. diminuta* (Lee *et al.* 2002). Therefore investigation into the ability of the recombinant strains described in this study to express their differential properties under these growth and recovery constraints were performed.

The expression of the protein conferring resistance to ampicillin (33 kD) by the GFP and RFP recombinant *B. diminuta* strains is illustrated in Figure 3(a) and the

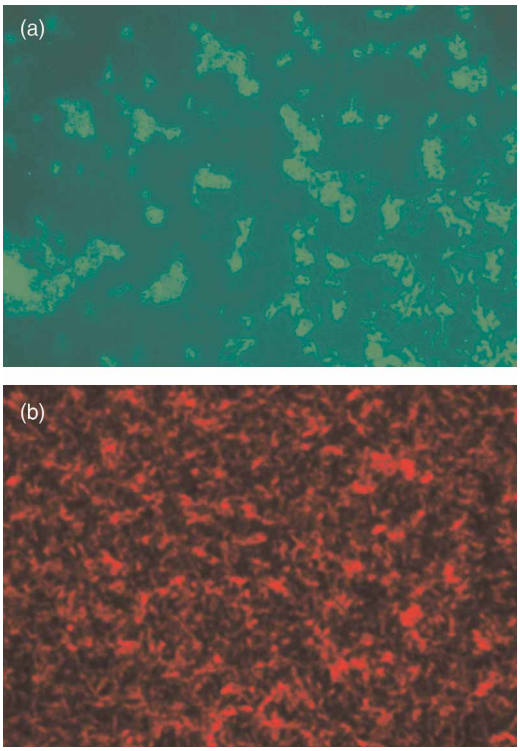

Figure 3 | *B. diminuta* expressing the green fluorescent protein (a) and red fluorescent protein (b). The epifluorescent micrographs are presented at 1,000 × magnification. For culture methods see main text. The full colour version of this figure is available online at: http://www.iwaponline.com/jwh/toc.htm

expression of RFP and GFP in the strains in Figure 3(b) (post-inoculation and enrichment in LB medium). Colonial expression of GFP and RFP was not noticeable when using R2A as the growth medium and microscopic verification was required to confirm expression of the fluorescent proteins. This task proved tedious and time-consuming, especially given the small size of the organism. Given the observed limitations, the recombinant kan^R strain was used for all subsequent studies involving performance verification of RO devices. Future investigation into the coupling of technologies such as flow cytometry for the detection and enumeration of the GFP and RFP recombinant *B. diminuta* may expand the usefulness of these strains (Tombolini *et al.* 1997).

Validation of the kan^R strain as a suitable challenge organism

Studies were performed to assess the stability of the recombinant kan^R strain when exposed to the varying abiotic conditions of the test water specified in the USEPA RO validation protocol. During the RO device verification test, the challenge organism must be able to remain viable and maintain stable cell densities for the duration of the test.

Table 2 | Results of the *B. diminuta* wild type (wt) and kanamycin resistant strain (kan^R) strain stability studies. Challenge organism concentrations are presented as geometric means of three replicates (standard deviations are included)

		***B. diminuta* wt**		***B. diminuta* kan^R**	
Water characteristics	**Exposure time (hours)**	**Log_{10} (SD)**	**Log reduction**	**Log_{10} (SD)**	**Log reduction**
4 °C, 50 mg/L TDS[a]	0	6.03 (0.09)	–	5.80 (0.04)	–
	2	5.93 (0.04)	0.09	5.76 (0.04)	0.04
	4	6.00 (0.03)	0.02	5.75 (0.02)	0.05
	6	6.00 (0.03)	0.03	5.82 (0.04)	0
4 °C, 500 mg/L TDS	0	4.88 (0.06)	–	4.81 (0.02)	–
	2	4.78 (0.02)	0.1	4.76 (0.03)	0.05
	4	4.73 (0.05)	0.15	4.76 (0.03)	0.14
	6	4.51 (0.05)	0.37	4.61 (0.07)	0.20
20 °C, 50 mg/L TDS	0	2.85 (0.04)	–	2.89 (0.05)	–
	2	2.80 (0.06)	0.08	2.88 (0.03)	0.02
	4	2.80 (0.09)	0.09	2.84 (0.06)	0.06
	6	2.82 (0.04)	0.06	2.86 (0.08)	0.04
20 °C, 500 mg/L TDS	0	3.88 (0.03)	–	5.08 (0.06)	–
	2	3.26 (0.24)	0.62	4.85 (0.09)	0.23
	4	3.69 (0.21)	0.19	4.76 (0.07)	0.32
	6	3.39 (0.36)	0.49	4.70 (0.21)	0.38

[a]Total dissolved solids.

Table 2 displays the results for this experiment. The water temperatures and TDS levels were selected to represent the regional variations that may exist in the abiotic parameters of drinking water systems, which may feed commercially available RO systems. A 6 h exposure time of the organism to the test water was selected. This timeframe typically reflects the maximum time a suspension of challenge organism may reside in an influent feed tank prior to the verification test. No significant differences ($p>0.05$) were observed between kan^R and wt *B. diminuta* for survivability over the course of the study for each variable tested. Both strains showed the greatest reduction in viable cells at 500 mg/L (~0.5 log after 6 h exposure). To account for this loss of viability, starting challenge concentrations should be increased by 0.5 logs.

Cellular size is a crucial quality control parameter for the USEPA protocol. The ability of the recombinant kan^R strain to attain the 0.3 μm size requirement was investigated. Figure 4 illustrates the size of the kan^R strain when grown in SLB and TSB for 48 h at 30 °C. The wt strain of *B. diminuta* ATCC 19146 was also grown under the same conditions in order to make size comparisons. No significant difference ($p>0.05$) was observed when comparing the cell diameters of the wt and recombinant strain following growth in SLB medium (mean diameter of 0.37 ± 0.05 μm for *B. diminuta* ATCC 19146 wt versus 0.34 ± 0.05 μm for the *B. diminuta* kan^R strain).

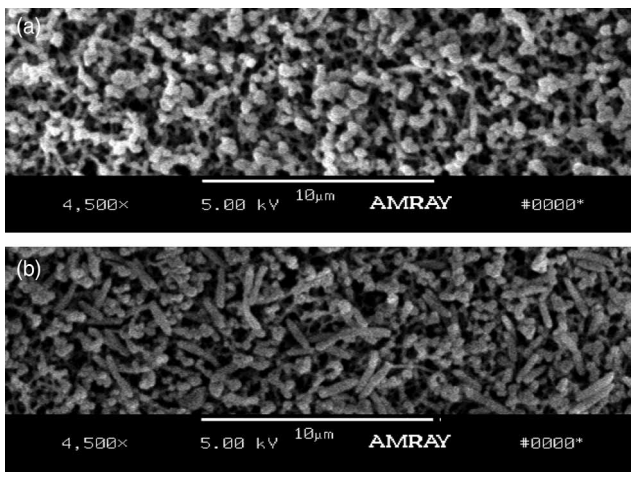

Figure 4 | Size analysis of kan^R *B. diminuta* strain. (a) Scanning electron micrograph of kan^R strain grown on SLB medium amended with 50 μg/mL of kanamycin. (b) kan^R strain grown in TSB without antibiotic supplement. For culture methods see main text.

Performance of kan^R in the USEPA RO protocol

In this study the kan^R strain has been shown to be similar to the wt strain in terms of size expression and its ability to remain viable over an extended period of time when exposed to the test water. Therefore, a side-by-side evaluation of the two strains using the USEPA RO treatment device verification protocol was performed. Different RO models were included in the test evaluation since the protocol was designed to be independent and applicable to all makes or models of RO treatment devices. The results of the evaluation are provided in Table 3. For all test runs, the minimum target influent concentration of 1×10^6 CFU/100 mL was achieved for both the wild type and recombinant *B. diminuta* strain. The treatment devices varied in their ability to remove *B. diminuta* from the challenge water. Device 2 displayed the greatest removals (>7 log). Devices 1 and 3 failed to exceed reduction efficacies greater than 2.7 logs for either strain, while log reduction values observed for Device 1 and 2 were similar. The data illustrate that the kan^R strain could effectively diagnose a failing device. Evaluation of Device 3 revealed that the kan^R strain displayed log reduction that was 0.9 logs greater that what was observed for the wt strain.

Observations as to the number of morphologically different colonies present on the recovery plates were also made. For Devices 1 and 2, only one distinct colony type was observed (which displayed coloration and colonial size typical of *B. diminuta*). For the evaluation of Device 3, one distinct colony type was present on the R2A

Table 3 | Comparative performance of kanamycin resistant strain (kan^R) and wild type (wt) strains of *B. diminuta* against the ETV protocol for verifying RO water treatment devices. Three different RO devices were evaluated. Removal efficiencies were based on enumeration of the test organisms performed at test startup (Time 0), 15 and 30 min. All samples were processed in triplicate. The geometric mean of the log reduction is presented

RO device	Test organism	Average influent conc. (CFU/100 mL)	Log reduction
1	wt	2.0×10^7	2.5
	kan^R	6.9×10^6	2.7
2	wt	8.2×10^7	7.9
	kan^R	5.4×10^7	7.7
3	wt	1.6×10^7	1.5
	kan^R	2.6×10^6	2.4

agar plates amended with kanamycin. For the non-selective R2A plates used for the wt enumeration, multiple HPCs were present at each sample dilution evaluated. As some of the colonies displayed morphological similarity to *B. diminuta*, an over-estimate of the concentration of *B. diminuta* in the effluent/treated water samples may have occurred. This is an example of the HPC interference that could impair the accurate assessment of the RO's microbial removal efficiency.

CONCLUSION

This was the first known study to utilize a recombinant *B. diminuta* strain that was resistant to kanamycin in the verification of the performance of a RO treatment device. The experiments demonstrated that the Tn5 vector was successfully integrated into *B. diminuta* ATCC 19146 and that its expression is stable and robust. The viability of this strain was stable at a wide range of temperatures, pH, and TDS. The strain also achieved the strict size requirements of the protocol. The recombinant strain was found to be equivalent in performance to its wt counterpart when testing against the USEPA RO protocol. The selectivity characteristics of the recombinant *B. diminuta* kan^R strain allowed for a more accurate assessment of the removal efficiency of RO treatment devices. In addition, the use of this selective strain could be applied to other membrane filtration verification protocols which utilize *B. diminuta* as the test organism. As a genetically engineered strain is being proposed for future use in the ETV RO protocol, appropriate monitoring and disinfection procedures should be implemented to ensure that this strain is not released into the environment. Ultimately, the development of a more precise evaluation method for these devices will benefit public health by ensuring that the systems validated for drinking water production can meet their stated microbial removal claims.

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