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ORIGINAL ARTICLE

Detection and enumeration of haloacetic acid-degrading bacteria in drinking water distribution systems using dehalogenase genes

L.H. Leach[1], P. Zhang[2], T.M. LaPara[3], R.M. Hozalski[3] and A.K. Camper[1,4]

1 Montana State University, Center for Biofilm Engineering, Bozeman, MT, USA
2 Microbiology and Molecular Genetics, 6120 Biomedical and Physical Sciences, Michigan State University, East Lansing, MI, USA
3 Department of Civil Engineering, University of Minnesota, Minneapolis, MN, USA
4 Department of Civil Engineering, Montana State University, Bozeman, MT, USA

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Correspondence
Anne K. Camper, 366 EPS Building, Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717, USA.
E-mail: anne_c@biofilm.montana.edu

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Abstract

Aims: To develop a PCR-based tracking method for the detection of a subset of bacteria in drinking water distribution systems capable of degrading haloacetic acids (HAAs).
Methods and Results: Published degenerate PCR primers were used to determine that 54% of tap water samples (7/13) were positive for a *deh* gene, indicating that drinking water distribution systems may harbour bacteria capable of HAA degradation. As the published primer sets were not sufficiently specific for quantitative PCR, new primers were designed to amplify *deh*II genes from selected indicator strains. The developed primer sets were effective in directly amplifying *deh*II genes from enriched consortia samples, and the DNA extracted from tap water provided that an additional nested PCR step for detection of the *deh*II gene was used.
Conclusions: This study demonstrates that drinking water distribution systems harbour microbes capable of degrading HAAs. In addition, a quantitative PCR method was developed to detect and quantify *deh*II genes in drinking water systems.
Significance and Impact of the Study: The development of a technique to rapidly screen for the presence of dehalogenase genes in drinking water distribution systems could help water utilities determine if HAA biodegradation is occurring in the distribution system.

Background

The chlorination of drinking water has been critical in the inactivation of pathogens and the subsequent decrease in water-borne disease (Berry *et al.* 2006). Unfortunately, the addition of chlorine is also known to produce a variety of disinfectant by-products (DBPs), including haloacetic acids (HAAs), which are known rat hepatocarcinogens (Herrenfreund *et al.* 1987; Singer *et al.* 1995; DeAngelo *et al.* 1996; Lebel *et al.* 1997; Williams *et al.* 1997; Moser *et al.* 2004; Tao *et al.* 2004). The United States Environmental Protection Agency (USEPA) has classified dichloroacetic and trichloroacetic acids as probable and possible human carcinogens, respectively (USEPA 2003a,b). Because of the concern over these and other possible health effects, the USEPA promulgated the Disinfectants and Disinfection Byproducts Rule, which imposes a maximum contaminant level of 60 μg l^{-1} for the combined concentration of five HAA species: mono-, di-, and trichloroacetic acids (MCAA, DCAA, TCAA), and mono- and dibromoacetic acids (MBAA and DBAA) (USEPA 2006).

In drinking water distribution systems, HAA levels can vary due to numerous factors including the source water quality, disinfectant type and concentration, temperature, and water age (Lebel *et al.* 1997; Williams *et al.* 1997).

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Although not a consistent phenomenon, some water utilities have observed decreases in HAA concentrations with increasing residence time in the distribution system (Lebel *et al.* 1997; Sung *et al.* 2000; Speight and Singer 2005). The observed loss of HAAs could be due to either chemical or microbial degradation, but is usually attributed to microbial degradation (Hashimoto *et al.* 1998; Ellis *et al.* 2001). Aerobic microbial biodegradation is advantageous as it results in mineralization whereas abiotic degradation results in the formation of other DBPs such as trihalomethanes (hydrolysis) (Zhang and Minear 2002) and lesser halogenated HAAs (reductive dechlorination) (Hozalski *et al.* 2001; Zhang *et al.* 2004). For exposure assessments and regulatory compliance issues, it is useful to know the potential for microbial HAA degradation.

Due to the oligotrophic nature of drinking water distribution systems, it is difficult to track and culture microbes from this environment. To expedite the detection of microbes capable of degrading HAAs, it is important to have a rapid molecular screening method. To design an effective molecular screen, it is necessary to have a functional target gene that is relevant to HAA degradation in drinking water. In bacteria that have the ability to utilize and dehalogenate HAAs, the haloacid dehalogenase enzyme is critical (Hill *et al.* 1999; Janssen *et al.* 2001; van Pee and Unversucht 2003). Dehalogenase genes are abundant in microbial genomes, and microbes with catabolic pathways capable of specifically degrading HAAs have been isolated and characterized (Vanderploeg *et al.* 1991; Weightman *et al.* 1992; Hill *et al.* 1999; Swanson 1999; McRae *et al.* 2004). Hill *et al.* (1999) used a systematic molecular approach to group dehalogenase genes as either *deh*I or *deh*II according to gene similarity. Either the *deh*I or *deh*II genes could provide a good functional target for non-cultivation based detection of HAA degraders (Fetzner and Lingens 1994; Hill *et al.* 1999; Janssen *et al.* 2001).

To develop a PCR-based detection method, it is also critical to target bacterial strains from water systems that serve as indicator strains representing the larger microbial community capable of HAA degradation. Bacterial cultures with the ability to utilize MCAA or DCAA as the sole carbon and energy source were enriched from distribution system biofilms, filtered tap water biomass, and wastewater activated sludge. The isolation of indicator strains (as identified by 16S rRNA gene fragment sequencing: *Xanthobacter* sp., *Ultramicrobacterium* sp., *Pseudomonas* spp., *Delftia* sp., and *Afipia* sp.) from the enrichment cultures, and the subsequent sequencing of the *deh*II genes, allowed for the development of indicator specific primers targeting the *deh*II gene. A quantitative-PCR protocol was designed to detect *deh*II genes from these bacterial strains. Quantitative PCR evaluation for the presence of *deh* genes in distribution system samples could provide a rapid indication of the potential for HAA-degrading capacity. Indication of a HAA-degrading bacterial community could influence how a utility chooses to deal with HAA concentrations exceeding compliance levels.

Materials and methods

Enrichment of HAA-degrading consortia

To aerobically enrich for MCAA, DCAA, and TCAA-utilizing bacteria, a mineral salts medium supplemented with 1 mmol l^{-1} MCAA, DCAA, or TCAA as sole carbon and energy source was used. The medium contained (per litrr of deionized H_2O): 0·03 g $MgSO_4$, 1·96 g $Na_2HPO_4{\cdot}7H_2O$, 0·37 g KH_2PO_4, 0·50 g NH_4Cl, 0·0006 g $CaCl_2$, and 0·1 ml of SL7 trace mineral solution (0·75 g $FeCl_2{\cdot}4H_2O$, 0·03 g H_3BO_3, 0·05 g $MnSO_4$, 0·06 g $Co(NO_3)_2{\cdot}6H_2O$, 0·066 g $ZnSO_4{\cdot}7H_2O$, 0·0125 g $NiCl_2{\cdot}6H_2O$, 0·007 g $CuCl_2$, 0·0125 g $Na_2MoO_4{\cdot}2H_2O$, and 4·4 ml of 37% HCl per 500 ml DI H_2O). After autoclaving and cooling, the mineral medium was aseptically amended with 1 mmol l^{-1} MCAA, DCAA, or TCAA (i.e. 94·5 mg l^{-1} MCAA, 129 mg l^{-1} DCAA, or 163·5 mg l^{-1} TCAA) by spiking with the respective HAA stocks. The HAA stock solutions were prepared in autoclaved MilliQ water, sterilized by filtration through a 0·2 μm filter, and stored in the dark at 4°C. Samples from water systems and wastewater systems were successfully enriched for bacteria capable of growth using MCAA, DCAA, or TCAA as sole carbon and energy source (Table 1). The wastewater activated sludge sample was collected from the aeration tanks at the Metropolitan Wastewater Treatment Facility (St. Paul, MN, USA) and preserved in 15%

Table 1 Source of inoculum and the HAA that served as both the carbon and energy source for growth of bacteria. Consortia will be referred to as numbered throughout the remainder of the text

Consortia	Source of inoculum	Substrate
1	MN pipe wall biofilm	MCAA
2	Wastewater treatment plant	DCAA
3	Wastewater treatment plant	TCAA
4	Wastewater treatment plant	MCAA
5	PA tap water	DCAA
6	Granular activated carbon used to filter pre-chlorinated water	DCAA
7	Granular activated carbon used to filter pre-chlorinated water	MCAA
8	Granular activated carbon used to filter pre-chlorinated water	TCAA
9	UK tap water	DCAA

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glycerol at −70°C. The water systems sampled included a granular activated carbon (GAC) filter, a drinking water pipe wall biofilm, and two potable tap waters. The GAC sample was collected at the Hershey Water Treatment Plant in Hershey, PA. The filter was fed prechlorinated water containing halogenated disinfection byproducts and was effective at removing HAAs. The chlorine residual was 1·4 mg l^{-1} in the influent and non-detectable in the effluent. A pipe section was collected in St. Paul, in June 2006 from a water distribution main that had been in use for approx. 120 years. The road surface and soil above the water main were excavated. After removing all the adherent soil, the exterior of the pipe was disinfected using a 10% bleach solution. A foot long section of 6 inch (inner-diameter) main was cut, capped at both ends, and transported back to the laboratory in a cooler and processed immediately. The water in the system contained a high chlorine residual of 3 mg l^{-1}. Two tap water samples were collected aseptically from water systems in Lewisberry, Pennsylvania, USA (PA tap water) and Cranfield, Bedforshire, United Kingdom (UK tap water) by filling autoclaved plastic bottles from flame sterilized taps in May 2006. The water samples contained relatively low chlorine residuals (as free chlorine) of <0·5 mg l^{-1} but relatively high DCAA concentrations (44 and 58 μg l^{-1}, respectively). HAA concentrations in the water samples were analysed using gas chromatography with electron capture detection following EPA method 552·3 (USEPA 2003c).

The heterotrophic plate count (HPC) for the PA tap water sample was 6·2 colony forming units [CFU ml^{-1} (the UK water sample was not tested for HPC)]. All samples for inoculation of enrichment cultures were shipped to the University of Minnesota on ice in a cooler and processed within 24 h of collection. The enrichment cultures were incubated at room temperature on a shaker table for up to 102 days. DNA samples from successful enrichments (i.e. those exhibiting HAA loss) were then extracted via the FastDNA® Spin for Soil Kit (QBiogene, Inc., Vista, CA, USA) and shipped on ice to Montana State University.

Isolation of HAA-degrading bacterial strains

HAA-degrading bacterial isolates were obtained from the wastewater enrichment cultures using HAA-amended agar plates. To remove organic contaminants, the granular agar was washed 10 times with nanopure water followed by two times with 95% ethanol and then air dried. The mineral medium described previously was used except that the phosphate buffer concentration was increased to 25 mmol l^{-1} to provide improved buffer capacity. A pH indicator (5 mg l^{-1} bromocresol purple) was added, which yields a purple colour at pH ≥ 6·8 and yellow colour at pH ≤ 5·7 (Yu and Welander 1995). The mineral medium, pH indicator, and agar (1·4%) were blended and autoclaved. Then, MCAA, DCAA, or TCAA was added at 10 mmol l^{-1} after the basal medium had cooled to ~60°C. The agar plates were spread with aqueous samples from the enrichment cultures and incubated at room temperature (~23°C). Colonies turning the purple colour to yellow, due to release of HCl during HAA degradation, were putatively identified as HAA-degraders. Such colonies were then streaked onto fresh plates up to three times for further isolation and purification. HAA-degrading ability was confirmed by incubating each purified isolate in liquid mineral medium spiked with 1 mmol l^{-1} of respective HAA and observing a decrease in HAA concentration over time. DNA from the isolated strains was purified using the FastDNA®Spin for Soil Kit and shipped on ice to Montana State University.

Distribution system sampling protocol

To accumulate biomass from tap water samples, cellulose acetate filters (47 mm, 0·2 μm pore size) were placed on a filtration apparatus and autoclaved. The filtration apparatus was then shipped to selected water utilities throughout the USA. (see Table 2). Water was collected from ethanol sterilized taps with as much water as possible (5–10 l), being vacuum filtered through a single filter. The apparatus was then disassembled and the filters were placed in a sterile Petri dish and shipped overnight to Bozeman, MT, USA on ice. Samples were processed within 24 h of being received. The DNA was purified

Table 2 Presence or absence of the *deh*I and *deh*II genes in DNA extracted directly from tap water biomass. (+) indicates a sample positive for the *deh*I or *deh*II gene, (–) indicates a sample negative for the presence of the *deh*I or *deh*II gene

Source of water sample	Volume filtered (l)	*deh*I	*deh*II
Eugene, OR	5	+	+
Mariposa, AZ	6	+	–
Bozeman, MT	5	–	+
Topeka, KN	6	+	–
St. Paul, MN	5	–	–
Minneapolis, MN	5	+	+
Harrisburg, PA	10	–	–
Pinchot State Park, PA	10	–	+
Hershey, PA	5	–	–
Harrisburg, PA	6	+	+
Hummelstown, PA	10	–	–
Helena, MT #1	5	–	–
Helena, MT #2	6	–	–

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using the Power Soil DNA extraction kit (MoBio, Carlsbad, CA, USA) per manufacturer's instructions.

Environmental sample

Samples from either the influent sludge or the aeration tank from the Bozeman, MT, USA wastewater treatment plant was collected for use as environmental DNA samples to determine the specificity of the degenerate PCR primers described below. DNA was extracted from 1 ml of the sample using the FastDNA®Spin for soil.

PCR

PCR protocols using degenerate primers to amplify *deh*I and *deh*II genes in the isolates, consortium, and drinking water distribution system samples were adapted from Hill *et al.* (1999).

*Deh*I *PCR*
Reaction mixture for PCR included GoTaq Green Mastermix (Promega, Madison, WI, USA), 1 μmol l^{-1} of each primer, and 0·2 mmol l^{-1} dNTPs. The touchdown program consisting of 94°C for 2 min; 92°C for 20 s; 70°C (−1° per cycle) for 30 s, cycled 20 times, the adjoining reaction included 92°C for 20 s; 51°C 30 s; 75°C for 30 s; cycled 20 times, with an extension of 75°C for 7 min.

*Deh*II *PCR*
Reaction mixture for PCR included Promega's GoTaq Green Mastermix, and 2 μmol l^{-1} of each primer. Amplification program: 94°C for 10 min; with 36 cycles of 94°C for 45 s; 55°C for 1 min; 75°C for 45 s; finally 75°C with 7 min extension. Primers for both *deh*I and *deh*II were synthesized by Integrated DNA Technologies (Coralville, IA, USA) as published by Hill *et al.* (1999). Reactions were done using a Mastercycler EP Gradient thermocycler (Eppendorf, Westbury, NY, USA).

PCR of 16S rRNA genes for DGGE
Amplification program: 94°C for 5 min; with 30 cycles of 94°C for 45 s; 55°C for 45 s; 72°C for 1·5 min; finally 72°C with 7 min extension. This program was used with either the universal 1070F and 1492R primers or 1070F and 1492R GC clamp primers (Ferris *et al.* 1996; Liu *et al.* 2002).

PCR of 16S rRNA genes for identification of isolated strains
A substantial fragment of the 16S rRNA gene was amplified by PCR (PTC-100 Programmable Thermal Controller; MJ Research, Inc., Watertown, MA, USA) using primers 338F (Lane 1991) and 907 R (Muyzer *et al.* 1995) to identify isolated strains. Each 50 μl PCR reaction mixture included: 1 × Promega PCR Buffer (Promega, Madison, WI, USA), 0·1% bovine serum albumin, 4 nmol l^{-1} of each deoxynucleoside triphosphate (dNTP), 25 pmol l^{-1} of each primer, and 1·25 units of Taq polymerase (Promega). The PCR procedure consisted of a 5 min denaturing at 94°C followed by 30 cycles of 30 s at 92°C, 30 s at 55°C, and 30 s at 72°C and finishing with 7 min at 72°C.

Cloning and sequencing

Sequencing of 16S rRNA gene fragments
To identify strains isolated from the enrichment cultures, PCR products were purified using the GENECLEAN® Gel Isolation and Reaction Cleanup (QBiogene, Inc., Vista, CA USA). The PCR products were then sequenced using primer 338F and 907R, resulting in a single consensus sequence obtained from bi-directional sequence information. The 16S rRNA gene fragments were sequenced at the BioMedical Genomics Centre at the University of Minnesota using an ABI PRISM® 3100 Genetic Analyzer.

Sequencing the deh*II genes*
PCR amplified *deh*II bands from the isolated bacterial strains were excised from agarose gels and purified with the QIAquick Gel Extraction kit (Qiagen, Valencia, CA, USA). Purified DNA was then cloned into the TA vector, pCR2.1 TOPO, and transformed into Mach1-T1 *E. coli* cells (Invitrogen, Chicago, IL, USA). Total DNA was extracted from these solutions with a FastDNA Spin Kit (QBiogene, Inc., Vista, CA, USA) per manufacturer's instructions. Using the M13F primer, the *deh*II gene fragments were sequenced at the Genomic Technology Support Facility at the University of Michigan with an ABI PRISM® 3100 Genetic Analyzer.

Indicator-strain specific quantitative PCR

Primer design
Six strains isolated from wastewater enrichment cultures were used as indicator strains for the design of indicator-specific *deh*II primers. Wastewater-derived HAA degraders were isolated at the beginning of the project and available for quantitative PCR (qPCR) method development. Drinking water enrichments and isolates proved more difficult to generate and hence, were not available at the time the initial method development work was performed. Subsequent studies (data not shown) have demonstrated that there is some overlap between wastewater-derived and drinking water-derived HAA degraders. For example, HAA-degrading *Afipia* sp. isolated from wastewater in this work, are commonly isolated from drinking water-derived enrichments (Zhang *et al.*, unpublished data).

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A DNA alignment of all of the cloned *deh*II genes from the bacterial isolates enriched on MCAA and DCAA was not possible due to lack of significant similarity between the sequences (data not shown). The Sequencer 4.5 GENE-CODES software was used to assemble continuous sequences (contigs) of the *deh*II genes. The contigs were formed automatically by the Sequencer software with the minimum match percentage of 60% and minimum overlap parameter of 10. The *deh*II genes grouped into two contigs with similar gene sequences. The first group of alignments consisted of isolated strains of *Pseudomonas* spp, and *Ultramicrobacterium* sp. (all isolated on MCAA, consortium 4). The second set of alignments included *Xanthobacter* sp. and *Afipia* sp. (isolated on DCAA, consortium 2). These alignments allowed for the rational design of specific primers in non-conserved regions for each strain (Table 3). Primer sets were designed to be specific for each indicator strain. As the *deh*II gene from two *Pseudomonas* spp. were very similar, one primer set was designed to detect both of these pseudomonads. Finally, the *Delftia* sp. (isolated on DCAA, consortium 2) did not align well with either group and, therefore, primers for *Delftia* sp. were designed based on that sequence alone. Primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA).

Quantitative PCR

A Smartcycler II (Cepheid, Sunnyvale, CA, USA) instrument was used for qPCR with *deh*II specific oligo-nucleotide primers for five different strains. To be considered optimized, the reaction had to amplify the *deh*II gene from the target organism without amplification of non-target *deh*II genes.

For *Pseudomonas* spp., the program consisted of an initial melt of 94°C for 570 s, followed 94°C for 45 s, 67°C for 30 s, 72°C for 30 s cycled 45 times. The melting curve was run between 60°C and 95°C in 0·2°C increments. With the exception of the annealing temperature, which is presented in Table 3, this same program was used for all other indicator-strain specific qPCR. For all reactions, the PCR mastermix consisted of SYBR Green Mastermix (Roche, Chicago, IL, USA). The final primer concentration was 0·4 μmol l^{-1}, with the exception of *Xanthobacter* sp., which had a final primer concentration of 0·2 μmol l^{-1}.

Nested PCR

Nested PCR was done using the *deh*II degenerate primers in combination with indicator-specific primers. In this procedure, a second qPCR reaction using indicator-specific primers was performed using the PCR product amplified by the *deh*II degenerate primers as template. The PCR protocols described above were used.

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was done to evaluate the presence of specific strains in the HAA-enriched consortia samples. The DGGE had an acrylamide gradient from 8–12% with a gradient of denaturant from 40–70% (100% denaturant was composed of 7 mol l^{-1} urea and 40% formamide, reagents were from Sigma-Aldrich, St Louis, MO, USA). The DGGE was run on a DCode system (Bio-Rad, Hercules, CA, USA) for 16 h at 60V. The gel was visualized by staining with SYBR Gold (Molecular Probes, Inc., Eugene, OR, USA), and subsequently examined with a FluorChem 8800 fluorescence imager (Alpha Innotech, Inc., San Leandro, CA, USA). 16S rRNA was amplified for the DGGE by PCR as described in the PCR section.

Results

Detection of *deh* genes with degenerate primers

The goal of this project was to determine if HAA-degrading bacteria were present in drinking water distribution

Table 3 Primers designed to amplify the *deh*II genes from selected indicator strains

Primer Set	Forward (5′–3′)	Reverse (5′–3′)	Annealing temperature (°C)
Xantho*	TTGTCTCGATCAGGTCGC	ACGACTACCGCGACTTCTG	62
Delftia†	TGCGAAGCGTACCTAAACCT	GGCTTGAACACTTCGACCTC	61
Ultra‡	TGAAATGGTCGCAATCGAA	CGCTCATTTCGAACTCAAACTC	62
Pseudo§	AGCCTGTCCCTGGGGATG	GAGCAGGTCGTCAGCCA	67
Afipia¶	CTCAAATACGATCCCGCGG	TACGCCGAGCTTCTGTTCGAT	61

*Xantho, primers based on the sequence of *Xanthobacter* sp.

†Delftia, primers based on *Delftia* sp.

‡Ultra, primers based on *Ultramicrobacterium* sp.

§Pseudo, primers based on the alignment of both *Pseudomonas* spp.

¶Afipia, primers based on *Afipia* sp.

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systems. The development of a PCR-based method to screen for HAA-degrading bacteria would allow drinking water utilities to predict if the bacterial community in a drinking water distribution system had the potential to biodegrade HAAs. Initially, presence-absence PCR, using the degenerate PCR primers published by Hill *et al.* (1999), was used to screen DNA extracted directly from samples taken from several drinking water distribution systems. The source of the sample, the amount of water filtered, and the presence/absence of *deh*I and *deh*II genes are summarized in Table 2. Approximately 54% of tap water samples (7/13) were positive for a *deh* gene (*deh*I: 5/13, *deh*II: 5/13, both: 3/13).

In the next step, nine enrichment cultures were screened for the presence of *deh* genes using the degenerate primers in combination with end-point PCR. In the enrichment cultures, the *deh*I gene was detected in five out of nine consortia samples and the *deh*II gene was detected in all samples (Table 4). The high detection prevalence of the *deh*II gene indicated that it would be a promising target for qPCR.

Bacterial strains isolated from the aerobic HAA-degrading consortium cultures were subsequently screened for the presence of dehalogenase genes. By end-point PCR, using the degenerate primer sets, all 15 of the bacterial isolates from HAA-enriched consortia contained *deh*II genes. PCR amplification of the *deh*I was not successful from any of the isolated strains. The *deh*II genes from the isolates were cloned, sequenced, and aligned with the goal of designing non-degenerate primer sets for qPCR targeting *deh*II genes specific to drinking water distribution systems. As only *deh*II genes were detected in the isolates, the *deh*II gene would become the focus for developing a qPCR method. In addition, the detection of a *deh* gene from all of the samples known to degrade HAAs indicates that hydrolysis by way of the *deh* gene is a dominant mechanism for the biodegradation of HAA.

Table 4 Presence or absence of *deh*I and *deh*II amplification products in consortium samples

Consortia	*deh*I	*deh*II
1	–	+
2	–	+
3	+	+
4	+	+
5	+	+
6	–	+
7	+	+
8	–	+
9	+	+

(+) indicates a sample positive for the *deh*I or *deh*II gene, (–) indicates a sample negative for the presence of the *deh*I or *deh*II gene.

Specificity of degenerate primers

To ensure that the developed qPCR assay was not prone to false positive results, the degenerate primers were tested on wastewater samples (from the Bozeman, MT, USA wastewater treatment plant) to determine the specificity of the primers for dehalogenase genes. During this process, it was noted that the degenerate primers would frequently mis-prime environmental samples. This is a significant concern with SYBR Green qPCR, as the non-specific nature of SYBR Green qPCR chemistry could lead to numerous false positive results. Figure 1, lanes 2 and 3 show the amplification of a band at the correct size (422 bp), which correlated with the band obtained after amplifying genomic DNA from *Xanthobacter* sp. (lane 4). In addition to the 422 bp band in lanes 2 and 3, there are numerous other bands that would interfere with quantification during qPCR.

Specificity of indicator-specific primers

Several methods for improving the specificity for the *deh* genes were considered before the development of a qPCR technique using indicator-specific primers. Among the techniques considered was the use of a Taqman probe, or

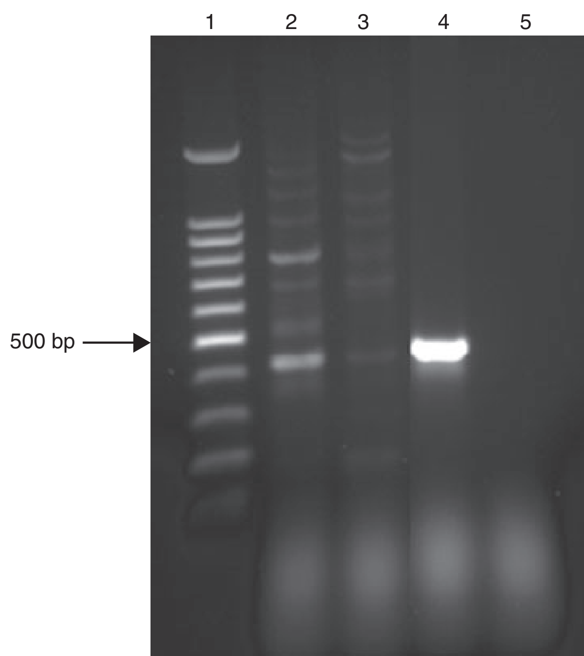

Figure 1 Amplification products obtained from environmental samples using degenerate *deh*II primers. Lane 1: Molecular ladder; lane 2: Wastewater treatment plant sample #1; lane 3: Wastewater treatment plant sample #2; lane 4: *Xanthobacter autotrophicus* genomic DNA; lane 5: DNA free negative control.

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qPCR melt-curve analysis. Although using a Taqman probe in combination with the degenerate primers was considered, the lack of similarity in the *deh*II gene sequences made it difficult to design a Taqman probe that would detect the majority of *deh*II genes. qPCR melt-curve analysis can be used as verification that the qPCR work has amplified the intended target. However, melt-curve analysis does not allow you to determine the actual gene sequence, therefore, relying on melt-curve analysis as the only tool to distinguish between the *deh*II sequence and a false positive is unreliable. For these reasons it was determined that designing indicator-specific primers was the best method for this study. Nucleotide sequence alignments were made in an attempt to design primers to specifically amplify *deh*II genes cloned from the isolates capable of HAA degradation.

The diversity of *deh*II sequences made alignments difficult, and the design of a specific primer set capable of amplifying all sequenced *deh*II genes was not achieved. Therefore, six bacterial strains that degraded either MCAA or DCAA were investigated as possible indicator organisms for tracking the HAA-degrading activity in drinking water distribution systems. The partially sequenced *deh*II genes from the indicator strains were used for the design of specific primer sets targeted to each individual indicator strain. Only one set of primers was designed for two *Pseudomonas* spp. as the sequences had a high degree of similarity. The primers were considered effective when the target species DNA was amplified, and the threshold cycle number (C_t) value of all other available isolates was at or above the C_t value for the DNA-free negative control (data not shown).

Use of indicator-specific primers with consortia samples

The consortium samples were used to test the newly designed indicator-specific qPCR primers. It was important to make sure that the new primers worked successfully on the HAA-degrading consortium samples, especially the two cultures from which the isolates were obtained (consortium 2 and consortium 4) before using them on the drinking water distribution system samples. Hence, DNA extracted from HAA-enriched consortia was subjected to qPCR analysis using the primers designed to detect indicator strains. As expected, qPCR results were positive for *Ultramicrobacterium* sp. and *Pseudomonas* sp. in consortium 4, the consortium from which they were isolated.

In addition, qPCR tested positive for *Xanthobacter* sp. and *Afipia* sp. in the consortium from which they were isolated (consortium 2). This was corroborated by tRFLP results that suggested *Xanthobacter* sp. and *Afipia* sp. were present in significant numbers in consortium 2 (LaPara, unpublished data). Curiously, the *Delftia*-type *deh*II gene was not detected using qPCR despite being isolated from consortium 2. In addition, *Delftia* sp. does not show up in the tRFLP data, which suggests that *Delftia* sp. may not have been a dominant player in consortium 2.

The number of genome copies per ng DNA in each consortium sample tested are shown in Table 5. All primer sets amplified dehalogenase genes from at least one of the consortia samples. Amplification was observed in three different consortia with primers designed to specifically detect *Ultramicrobacterium* (Table 5). Primers designed specifically for *Xanthobacter* amplified the *deh*II gene from two different consortia samples.

Unfortunately, the limited amount of sample prohibited design of more complex internal controls. However, care was taken to protect against both false positive and false negative results. The consortia samples were not PCR inhibited based on successful amplification of one or both *deh* genes using the degenerate primers, ensuring no false-negative results. Melting curve data was analysed to ensure that the primers had amplified the *deh*II gene and

Table 5 qPCR analysis of consortia samples using primer sets targeting indicator-specific *deh*II genes. Results are reported as a range (genome copies/ng DNA) from duplicate samples

	Genome copies ng^{-1} DNA				
	Xantho	Delftia	Ultra	Pseudo	Afipia
Consortium	Range (low–high)				
1					
2	67–76		1708–3112		2364–7544
3					
4			39014–121539	3785–13874	
5					
6	13–622	103–1387	2986–6600		
7					
8					
9					
Positive control	+	+	+	+	+
Negative control	–	–	–	–	–

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that the C_t value was not due to a false-positive reaction. In addition, C_t values above 35 were not included in Table 5 as they were usually non-specific as judged by the melt curve data. All primer sets amplified dehalogenase genes from at least one of the consortia samples. Amplification was observed in three different consortia with primers designed to specifically detect *Ultramicrobacterium* (Table 5). Primers designed specifically for *Xanthobacter* amplified the *deh*II gene from two different consortia samples.

Use of indicator-specific primers with drinking water distribution system samples

The drinking water distribution system samples that yielded a product when screened with the *deh*II degenerate primer set (Table 2) were further screened for the presence of particular *deh*II genes using the indicator-specific primers. As only samples that were positive for *deh*II genes using the degenerate primers were tested with the qPCR technique, PCR inhibition was not a concern. No amplification was observed with the indicator-specific primer sets (data not shown). The qPCR protocol worked well in consortium samples, but failed to amplify any of the drinking water distribution system samples. Therefore, it was suspected that this was due to low DNA concentrations both because of the oligotrophic nature of these systems and the presence of disinfectant. To increase sensitivity, a nested PCR protocol was conducted. In a first amplification round, the degenerate primers were used to amplify the aforementioned 422 bp fragment of DNA. The indicator-specific primers, which target sites within the amplified DNA fragment, were then used in a second round of amplification. The two rounds of PCR amplification increased the sensitivity of the method such that detection was possible from the distribution system samples. After the nested PCR step, all of the drinking water distribution system samples were positive for at least one of the indicator-specific *deh*II genes (Table 6). Primers designed to detect *Delftia*, *Pseudomonas* and *Afipia* were positive for amplification in all the drinking water distribution system samples that had been pre-screened for the presence of *deh*II. After qPCR analysis the melting curves were examined to ensure that the PCR amplicon from the drinking water distribution system samples had the same melting temperature as the *deh*II gene isolated from that specific strain.

Table 6 Nested qPCR analysis was used to determine the presence or absence of indicator-specific *deh*II genes. Samples were deemed positive if the C_t value was less than 35

Sample	Primer set				
	Xantho	Delftia	Ultra	Pseudo	Afipia
Pinchot State Park, PA	−	+	+	+	+
Minneapolis, MN	−	+	+	+	+
Harrisburg, PA	+	+	−	+	+
Eugene, OR	−	+	+	+	+

Justification of the accuracy of indicator-specific *deh*II primers

In support of the previous data, further studies were done to ensure that the *deh*II PCR primers, used in the qPCR analysis, were specific for the strains indicated. Therefore, strains from the consortia samples were identified both by PCR using *deh*II indicator-specific primers and by DGGE of PCR-amplified 16S rRNA gene fragments. Indicator-specific *deh*II primers allowed for the amplification of the *deh*II gene to predict the species of bacteria present in the sample. PCR-DGGE profiles obtained from the consortia samples were compared to the PCR-DGGE bands obtained from pure isolates of the indicator strains (Fig. 2). In this manner, it could be determined if the isolated strain was present in the consortium. In nine different consortia samples, the species profile obtained by PCR-DGGE and the species profile predicted by indicator-specific *deh*II qPCR showed a 75·5% correspondence. This was calculated by tabulating both the positive matches (5/45), negative matches (29/45), and mismatches (11/45) (Table 7).

Discussion

Bacteria capable of degrading HAAs have been isolated, characterized and consistently found to require a haloacid dehalogenase gene for activity (Vanderploeg *et al.* 1991;

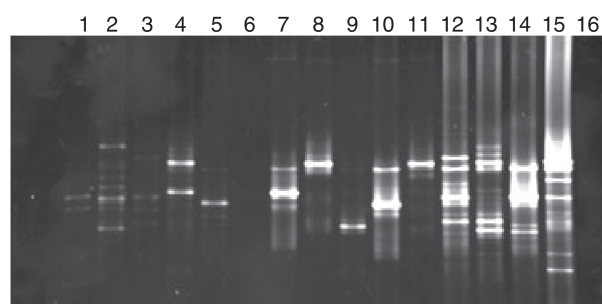

Figure 2 DGGE of 16S rRNA gene fragment using universal primers on isolated bacteria and consortia samples. (1) consortium 1; (2) consortium 2; (3) consortium 3; (4) consortium 4; (5) consortium 5; (6) DNA free control; (7) *Pseudomonas* sp.; (8) *Ultramicrobacterium* sp.; (9) *Xanthobacter* sp.; (10) *Afipia* sp.; (11) *Delftia* sp.; (12) consortium 9; (13) consortium 7; (14) consortium 8; (15) consortium 6; (16) DNA free control.

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Table 7 Comparison of enrichment consortia community profiles based on the 16S DGGE patterns or *deh*II indicator-specific PCR amplification

	1		2		3		4		5		6		7		8		9	
	D	Q	D	Q	D	Q	D	Q	D	Q	D	Q	D	Q	D	Q	D	Q
Pseudo			x				X	X										
Ultra				x			X	X			X	X	x					
Xantho				x								x					x	
Afipia				x		x			X	X					x			
Delftia							x				X	X	x					

D, Dominant strains by PCR-DGGE. Q, Strains predicted by strain-specific *deh*II qPCR amplification. Lower case x indicates strains detected by either PCR-DGGE or qPCR. Capital X indicates samples where both the *deh*II qPCR and the PCR-DGGE profile indicated presence of the same bacterial strain.

Weightman *et al.* 1992; Hill *et al.* 1999; Marchesi and Weightman 2003). Therefore, dehalogenase genes provided targets for cultivation-independent detection of HAA-degrading bacteria in drinking water distribution systems. The method presented in this study allows samples from drinking water and wastewater systems to be evaluated for the presence of indicator-specific dehalogenase genes known to encode functional enzymes. This is advantageous, as amplifying a cryptic or silent *deh* gene with a non-specific primer set could lead to false positives (Hill *et al.* 1999).

Cultivation-independent techniques have an important advantage over cultivation-based methods, which can introduce bias and skew the interpretation of environmental samples. In fact, culture-based biases have previously been observed during the enrichment and subsequent isolation of HAA-degrading microbes. McRae *et al.* (2004) reported that species isolated on solid media supplemented with MCAA and TCAA were not the dominant organisms in their respective enrichment cultures. Marchesi and Weightman (2003) reported that isolates containing dehalogenase genes that were most abundant in the enrichment cultures were not the predominant members of the original environmental samples. The solid media isolation technique used in our study also has limitations, as it proved difficult to isolate *deh*I-containing bacteria from consortia that were positive for the presence of a *deh*I gene.

The development of indicator-specific primers in this study relied initially on DNA sequences amplified from bacterial strains isolated from enrichment cultures inoculated with activated sludge. The developed indicator-specific primers amplified *deh*II genes from the drinking water distribution system, suggesting that our isolates were representative of species found in the distribution system. Nevertheless, nested PCR was necessary to increase the detection sensitivity, thereby suggesting very low abundances of the organisms in the drinking water distribution system. This is not surprising as drinking water systems are intentionally harsh environments for bacteria with chlorine added to maintain low bacterial numbers in the bulk water to minimize the risk of waterborne disease. Another possible reason necessitating a nested PCR step was that the HAA-degrading wastewater isolates used for PCR primer development are not prominent in drinking water distribution systems. Future work with primer development could consider *deh*II genes from strains isolated from drinking water systems.

In using a PCR test as an indicator of the ability for systems to remove HAAs it would be important to determine if the DNA being amplified is naked DNA found in the water, or nucleic acids extracted from whole cells. Particularly in the drinking water distribution system, an increase in contact time with the disinfectant may cause the release of naked DNA from ruptured cells. Unfortunately, this study was initiated before the development of a technique that uses propidium monoazide (PMA) to distinguish between DNA from live cells and DNA from the surrounding environment (Nocker *et al.* 2007). In further development of this or similar techniques the inclusion of a PMA treatment step would be strongly recommended.

Hill *et al.* (1999) proposed that 95% of haloacid dehalogenases belong to either the *deh*I or *deh*II subgroups. In this study, a screen for the *deh*II gene was developed because *deh*II genes predominated in the isolated strains. The distribution of *deh*I and *deh*II in the environment is unclear, however a study by Marchesi and Weightman (2003) found a predominance of *deh*II genes. Additionally, Hill *et al.* (1999) found that out of 54 soil and sediment samples, 43 were positive for *deh*II genes and eight were positive for *deh*I. Although *deh*II genes maybe more predominant, the *deh*I gene was also detected in consortium and drinking water samples using the degenerate primers in this study. This suggests that the *deh*I gene should also be considered as an additional target to make the method more robust. With coverage of both *deh*I and *deh*II a majority of the known haloacid dehalogenase genes would be represented.

DGGE of 16S rRNA gene fragments was used to substantiate primer design for the qPCR work. These results also have implications in microbial ecology. Once DNA

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has been extracted from an environmental sample, there is no technique that can provide a direct connection between the 16S rRNA genes and a corresponding functional gene. If the indicator-specific *deh*II gene consistently appeared with the 16S rRNA gene of the same species, one could make assumptions about the species diversity based on a functional gene. It appears that the isolated *deh*II genes are often, but not always, associated with a particular species. Dehalogenase genes of *Pseudomonas putida* Pp3 are located on mobile genetic elements, indicating the possibility of lateral transfer (Slater *et al.* 1985; Thomas *et al.* 1992a,b; Weightman *et al.* 2002). Therefore, any further work to identify systems with the capacity to biodegrade HAAs will need to focus on the functional dehalogenase genes rather than 16S rRNA gene sequences.

The development of a technique to rapidly screen for the presence of dehalogenase genes in a drinking water distribution system could help water utilities determine if HAA biodegradation is occurring in their distribution systems. In this research, *deh*II genes were detected in tap water biomass samples with indicator-specific primers developed from bacterial strains enriched on HAAs. Amplification of specific *deh*II genes from indicator strains was only possible via nested PCR. As PCR is an exponential amplification technique, it appears that the concentration of indicator-specific dehalogenase genes in bulk tap water is low. The typically low nutrient levels and presence of a residual disinfectant in the bulk drinking water present a harsh environment for bacteria. Nevertheless, bacteria in drinking water distribution systems can survive by attaching to biofilms in the pipe walls where they are afforded some protection from residual disinfectant (Camper *et al.* 1999; Berry *et al.* 2006). A better approach would be to sample and test biofilms scraped from pipe walls, but it is difficult and costly to obtain such samples. In the absence of biofilm samples, the results from bulk tap water would provide a conservative estimate of the potential for HAA degradation in the drinking water distribution systems.

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