BIODEGRADATION OF HALOACETIC ACIDS IN BIOFILTERS AND WATER DISTRIBUTION SYSTEMS: MICROBIOLOGY AND MODELING

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ABSTRACT

There is concern over the potential deleterious health effects of haloacetic acids (HAAs) in drinking water and their removal is of great interest for water utilities. The overall goal of this research was to obtain more detailed information about the diversity of HAA-degrading bacteria and their corresponding dehalogenase (*deh*) genes in drinking water systems and other environments. This research further aimed to model the biodegradation of HAAs in drinking water systems, thus demonstrating that biodegradation could be exploited as a means to effectively remove HAAs during water treatment. A direct plating technique was used to isolate HAA degraders from different environments (river water, tap water and agricultural soil). The obtained isolates included several species that were not isolated in previous studies: *Mycobacterium* sp., *Streptomyces* sp., *Stenotrophomonas* sp. and *Pantoea* sp. Furthermore, a *deh* gene was detected for the first time in a Gram positive bacterium. This gene had 89.4% nucleotide sequence similarity with the *dehII* sequence from an α-*Proteobacterium,* suggesting that lateral gene transfer of *deh* genes across unrelated bacterial species occurred. A culture-independent technique, the terminal restriction fragment length polymorphism (tRFLP) of two classes of *deh* genes (*dehI* and *dehII*), was then used to fingerprint the HAA degraders in drinking water systems. The tRFLP profiles of both *deh* genes showed similar patterns for all analyzed drinking water samples (from Minneapolis, MN; St. Paul, MN and Bucharest, Romania) and for one biologically-active granular activated carbon filter (Hershey, PA). The tRFLP profiles of *dehI* genes from the drinking water samples matched the pattern from *Afipia* spp. that were previously isolated from drinking water. The tRFLP profiles of *dehII* genes did not match any previously characterized *dehII* genes. Finally, a biodegradation kinetic model was developed to predict the fate of HAAs in biologically-active filters and water distribution systems. The model calculations indicated that biodegradation is likely to lead to significant HAA removals in biologically-active filters but not in most distribution systems. A sensitivity analysis showed that while the controlling parameter was the HAA-degrader biomass, physical parameters, such as water flow velocity and pipe length, also had an influence on the HAA removal.

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Chapter 1 : INTRODUCTION

1.1 INTRODUCTION

Providing safe drinking water is vital for all communities and societies. The water treatment process must include a disinfection step to kill any pathogens that might be present in the source water; treated water that goes into distribution systems also must carry a disinfectant residual to prevent re-growth of pathogens. Chlorination is the most common disinfection strategy applied by water treatment facilities, especially in the United States. This disinfection strategy, however, generates hundreds of chlorinated disinfection byproducts (Krasner et al., 2006), among which, haloacetic acids (HAAs) are the second major category. The removal of disinfection byproducts from drinking water is thus a necessity, especially for those compounds that are regulated (such as HAAs).

HAA removal from drinking water was investigated under the use of granular active carbon (GAC) and bacteriological active carbon (BAC) (Tung et al., 2006; Xie & Zhou, 2002; Zhou & Xie, 2002). BAC proved to be more efficient at removing HAAs during prolonged time periods. The microorganisms that develop on BAC and are responsible for HAA removal, however, have not been studied until recently. Zhang et al. (2009a & b) isolated for the first time HAA-degrading bacteria from different locations along drinking water systems, including BAC, and found that *Afipia* spp. appeared to be important HAA degraders in these systems. Of more interest, however, is to estimate the HAA-degrading biomass in drinking water systems and evaluate the HAA loss that can be attributed to biodegradation. Leach et al. (2009) attempted to develop a quantitative method to determine the amount of HAA-degrading biomass in tap water samples, but

their method failed to produce precise quantitative results because further optimization was needed. The authors, however, demonstrated that the use of indicator strains was needed for quantifying the HAA-degrading biomass, thus pointing out the necessity of determining the major HAA-degrading species in drinking water systems.

HAA degraders have been previously isolated from other environments such as soil and activated sludge and their corresponding dehalogenase genes (*deh*) were characterized. The isolates obtained by Zhang et al. (2009a & b) from drinking water systems, however, suggested that these systems select for different HAA-degraders than those found in other environments. Moreover, there was little overlap between the *deh* sequences from drinking water isolates and those previously isolated from other environments and between the *deh* sequences from different drinking water isolates, suggesting that lateral gene transfer is rare among HAA degraders.

This research extended the previous work on HAA degraders isolated from the environment by adding new information on the diversity of HAA isolates and the corresponding *deh* genes. This research also extended the previous work on HAA degraders isolated from drinking water systems by investigating the diversity of *deh* genes and of HAA-degrading bacteria in these systems through a culture-independent technique (terminal restriction fragment length polymorphism). This research further contributed to the applicability of the HAA biodegradation process to drinking water treatment by developing a kinetic model for predicting the loss of HAAs due to biodegradation in biofilters and distribution systems.

1.2 ORGANIZATION OF THE THESIS

This thesis is organized in six chapters. Chapter 2 provides a literature review on the existing information about HAA biodegradation and how this biological process could be applied for the improvement of drinking water treatment. Some of the existing gaps in our knowledge related to HAA degraders and *deh* genes are identified and later addressed in the following chapters.

Chapter 3 focuses on the environmental influence on the phylogenetic diversity of HAA degraders and *deh* genes. Bacterial strains were isolated from different environments that were polluted with HAAs (i.e., agricultural soil, Mississippi River water and tap water) and their phylogenetic diversity and corresponding *deh* genes were analyzed.

In chapter 4, a culture-independent technique, terminal restriction fragment length polymorphism (tRFLP), was developed to fingerprint the HAA-degrading bacterial communities in drinking water systems. Because this tRFLP method targeted relatively short gene fragments (i.e., ~270 bp or ~420 bp *deh* gene fragments), which roughly corresponds to the rate of cutting for frequent-cutting restriction enzymes (i.e., an enzyme recognizing a four-nucleotide DNA pattern should cut, on average, every 256 nucleotides), *in silico* analysis was used to detect optimal restriction enzymes for discerning different *deh* gene fragments.

In chapter 5, a kinetic model was developed to predict the fate of three HAAs (mono-, di- and trichloroacetic acids) along a water distribution system and within a biologically-active filter. For this, a one-dimensional plug flow reactor equation with pseudo-first order biodegradation kinetics was used. Sensitivity analyses were performed

to investigate the effects of physical parameters (e.g., fluid velocity) and biological parameters (e.g., biodegradation kinetics, biomass density) on HAA removal.

Chapter 6 summarizes the main achievements of this research and provides some conclusions that emerged from this study and some ideas for future work.

1.3 SUMMARY

As more information becomes available about the diversity of the organisms capable of degrading HAAs and the *deh* genes they carry, it becomes easier to understand the influence of the environment on the evolution of these organisms and genes. More research, however, is still needed to confirm whether the limited data that exists on these HAA degraders and the corresponding *deh* genes is related to the well known culturing bias or to a relatively little spread of *deh* genes in the environment.

The biodegradation of HAAs has a potentially useful application for drinking water treatment, by contributing to the effective removal of HAAs from drinking water. It is important, however, to have more precise information about HAA-degrading bacteria levels in drinking water systems. Thus, more research is needed concerning the identification and enumeration of major HAA degraders in drinking waters systems.

This thesis aims to expand the existing knowledge on HAA degraders and *deh* genes and to analyze the fate of HAAs in distribution systems. Experimental studies were conducted to isolate novel HAA degraders and fingerprint bacterial communities in tap water and a modeling study was developed to predict the HAA loss due to biodegradation in distribution systems and identify important parameters controlling this process.

Chapter 2 : LITERATURE REVIEW. BIODEGRADATION OF HALOACETIC ACIDS AND POTENTIAL APPLICABILITY TO DRINKING WATER TREATMENT

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Haloacetic acids (HAAs) are byproducts formed upon the addition of chlorine to water for disinfection purposes. HAAs pose a potential human health risk and their concentrations in drinking water are regulated in many countries. HAAs can be biodegraded under aerobic conditions through a hydrolysis-oxidation pathway. There is experimental and modeling evidence that HAA biodegradation is a potentially effective approach for drinking water treatment in order to reduce HAA concentrations in drinking water and comply with regulations. HAA-degrading bacteria have been isolated from different environments such as soil, activated sludge and drinking water systems. There is, however, very little overlap in the species obtained from water systems and those from other environments, suggesting that the specific conditions in water systems (oligotrophic environment, chlorine residual) select for unique bacteria. As more information becomes available about the diversity of HAA-degrading bacteria and the halocarboxylic acid dehalogenase genes that are involved in the HAA biodegradation pathway, researchers will be able to develop nucleic acid-based quantification methods for determining the HAA-degrading biomass in drinking water systems. This would allow water utilities to predict and possibly control the rate of HAA biodegradation taking place in their systems.

Key words: haloacetic acid, disinfection byproduct, halocarboxylic acid dehalogenase, drinking water systems, maximum contaminant level.

2.1 HALOACETIC ACIDS: PREVALENCE AND CONCERNS

Haloacetic acids (HAAs) are ubiquitous environmental contaminants and their presence in the environment is due both to natural processes and to human activities. HAAs are naturally formed through the biodegradation of organic matter in forest soil (Matucha et al., 2007) and the photodegradation of some herbicides (Wilson and Mabury, 2000). However, much higher amounts of HAAs are released in the environment by human activities. Thus, HAAs were used as food preservatives (Reinmann et al., 1996), trichloroacetic acid (TCAA) was used as herbicide (Ashton & Crafts, 1973) and monochloroacetic acid (MCAA) is still used as precursor for the synthesis of various chemicals (Reinmann et al., 1996). High quantities of HAAs are produced from industrial chlorination processes like pulp bleaching (Juuti et al., 1995) and the chlorination of drinking water and wastewater (Sirivedhin & Gray, 2005; Krasner et al., 1989).

HAAs have been detected in many places such as soil (Peters, 2003; Hoekstra et al., 1999), conifer needles and lichens (Juuti et al., 1996), snow and ice in Antarctica (von Sydow et al., 2000), fog and rainwater (Römpp et al., 2001; Reinmann et al., 1996), sea water (Hashimoto et al., 1998), lake water (Scott et al., 2002), wastewater (Sirivedhin & Gray, 2005) and drinking water (Krasner et al., 1989). Terrestrial concentrations of HAAs range from 1.4 to 120 µg/kg of dry weight in soils (Hoekstra et al., 1999) and from 1 to 180 µg/kg of dry weight in conifer needles (Juuti et al., 1996). HAA concentrations in aquatic environments range from parts per million (ppm) in wastewater (Sirivedhin and Gray, 2005) to parts per billion (ppb) in drinking water (Krasner et al, 1989), and to parts per trillion (ppt) in surface waters (Berg et al., 2000).

HAAs are generally phytotoxic (Hanson & Solomon, 2004a; Norokorpi & Frank, 1995) and toxic to green algae (Kühn and Pattard, 1990), while some HAAs (e.g., dichloroacetic acid (DCAA) and TCAA) have hepatocarcinogenic potential (Herren-Freund et al., 1987). HAAs, however, are not phytotoxic at typical environmental concentrations but their effect on aquatic life is poorly understood as HAAs often occur as mixtures of several HAA species, which could enhance their toxicity (Hanson & Solomon, 2004b).

2.2 HAAs IN DRINKING WATER

Haloacetic acids (HAAs) represent the second most prominent class of halogenated disinfection byproducts (DBPs), after trihalomethanes, in drinking wtaer (Krasner et al., 2006). HAAs are formed in water treatment plants and distribution systems due to reactions between free chlorine, added for disinfection purposes, and the organic matter that is present in natural waters (Rodriguez et al., 2007; Li et al., 2000). HAAs can also form in the distribution systems as a result of the hydrolysis of other DBPs (e.g., haloacetonitriles) (Glezer et al., 1999). Several researchers have reported, however, that HAA concentrations decrease along the distribution systems (Chen and Weisel, 1998; Lebel et al., 1997; Williams et al., 1997). Due to the potential adverse health effects of HAA consumption (Swan et al., 1998; Swan and Waller, 1998; USEPA, 1993), the United States Environmental Protection Agency (USEPA) began regulating HAAs in tap water in 1998 under the Stage 1 Disinfectants/DBPs Rule (D/DBPR) (USEPA, 1998). At present, the maximum contaminant level (MCL) in the United States is 60 µg/L for the sum of five HAAs (HAA_5: MCAA, DCAA, TCAA, monobromoacetic acid (MBAA) and dibromoacetic acid (DBAA)). The European Union, on the other hand,

does not regulate HAAs (Council Directive 98/83/EC, 1998). Studies done prior to the implementation of the USEPA D/DBPR showed that HAA_5 concentrations in finished waters were sometimes higher than the proposed MCL (Singer et al., 1995).

2.3 DEGRADATION OF HAAs

HAA degradation in aquatic environments and soils is typically attributed to microbial activity (Matucha et al., 2003; Ellis et al., 2001; Thomas et al., 2000). Microbial degradation of HAAs was also detected in water treatment systems such as biologically-active filters (Kim and Kang, 2008; Tung et al., 2006; Wu and Xie, 2005; Zhou and Xie, 2002; Xie and Zhou, 2002) and in water distribution systems (Tung and Xie, 2009; Zhang et al., 2009a & b). HAAs are also capable of undergoing abiotic degradation reactions such as reductive dehalogenation in the presence of zero valent iron (Zhang et al., 2004; Hozalski et al., 2001) or iron minerals (Chun et al., 2007) and hydrolysis (i.e., decarboxylation) (Zhang and Minear, 2002). The abiotic degradation reactions, however, are not likely to be important because either the reactions are slow at environmental pH and temperature values (e.g., the half-life of TCAA at room temperature and neutral pH is 2,190 days according to Zhang and Minear, 2002) or they require a specific combination of conditions (e.g., rapid reductive dehalogenation requires a potent reductant such as zero valent iron and trihalogenated HAAs that contain one or more bromine atoms according to Hozalski et al., 2001). Thus, biodegradation is likely to be a more rapid and efficient degradation process than abiotic processes, which could be applied as a way of reducing HAA concentrations in drinking water.

2.3.1 Aerobic biodegradation of HAAs

HAAs are biodegraded aerobically via a hydrolysis-oxidation pathway (Figure 2.1). This pathway involves an initial substitutive dehalogenation step in which the halogen atom is replaced by a hydroxyl group (Figure 2.1; Ellis et al., 2001). This step is catalyzed by enzymes called halocarboxylic acid dehalogenases.

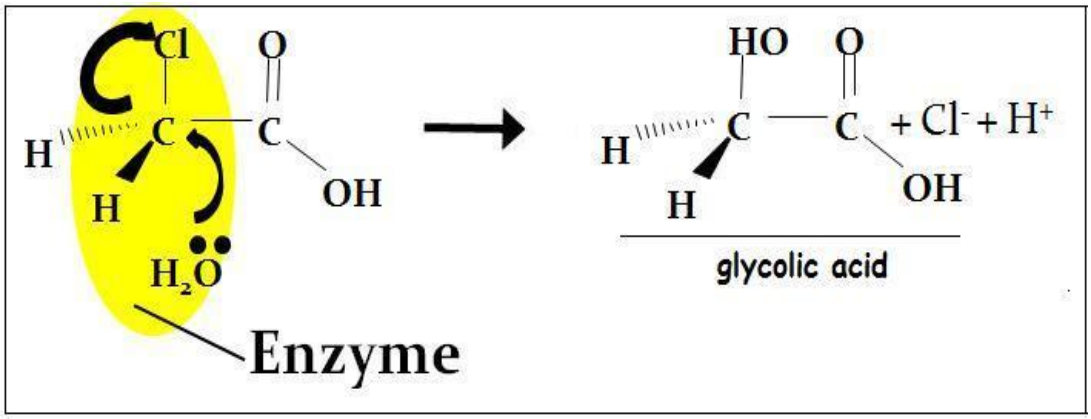

Figure 2.1. Potential biodegradation pathway for MCAA (adapted from Ellis et al., 2001). The resulting biodegradation intermediate, glycolic acid, enters into the general metabolism and gets readily mineralized.

Information concerning the aerobic biodegradation of HAAs (i.e., pathway, kinetics and the genes involved) largely comes from work with enrichment cultures and isolates obtained from soil and wastewater environments and from studies with other α-halocarboxylic species like monochloro- and dichloropropionic acids (MCPA and DCPA). HAA-degrading bacterial strains such as *Burkholderia* sp., *Xanthobacter* sp., *Sphingomonas* sp. and *Chrysobacterium* sp. were isolated from soil and activated sludge (Hill et al., 1999; McRae et al., 2004). A recent study by Zhang et al. (2009a & b) revealed some of the HAA degraders from drinking water systems including previously unknown HAA degraders such as *Afipia* spp. Marchesi and Weightman (2003)

demonstrated that the α-halocarboxylic degraders isolated by enrichment cultures are not necessarily the environmentally-relevant organisms due to the culturing bias (Dunbar et al., 1997). Kerr and Marchesi (2006) further showed that the culturing conditions (i.e. temperature, pH, oxygen supply and choice of batch cultures or direct plating) are the key elements in isolating novel bacteria, which could lead to the future isolation of a broader range of HAA degraders.

McRae et al. (2004) was the first group to perform batch experiments to evaluate the HAA biodegradation kinetics at the low HAA concentrations similar to those found in surface waters and drinking water systems (<< 1 mg/L). MCAA and TCAA degradation followed pseudo-first order reaction kinetic models with MCAA being degraded faster than TCAA. The bacteria used in these experiments were enriched from a wastewater activated sludge inoculum where HAA concentrations are generally higher than in drinking water distribution systems. Recently, Zhang et al. (2009a & b) showed that isolates from drinking water systems have a wide range of HAA-degrading abilities at drinking water HAA concentrations, with the faster DCAA degrader, an *Afipia* species, having a 4-fold faster DCAA biodegradation rate than the slowest DCAA degrader, a *Methylobacterium* species. The drinking water isolates were able to degrade DCAA faster than MCAA and MCAA faster than TCAA. There was little overlap in the bacterial species isolated from wastewater and drinking water enrichment cultures, suggesting that drinking water distribution systems select for unique HAA-degrading bacteria, perhaps because of the oligotrophic conditions present therein (Zhang et al., 2009a & b; McRae et al., 2004).

2.4 HALOCARBOXYLIC ACID DEHALOGENASES AND CORRESPONDING GENES

Halocarboxylic acid dehalogenases catalyze the initial step in the biodegradation pathway of HAAs. Several genes encoding those enzymes have been sequenced and grouped into two phylogenetically unrelated groups, called *dehI* and *dehII* (Hill et al., 1999). These two groups of *deh* genes are characterized by high intra-group genetic diversity and are divided into several phylogenetic subgroups (Figures 2.2 & 2.3; Hill et al., 1999). Studies on the genetic localization of the *deh* genes from different bacterial strains showed that many of these genes are found on the chromosome (Ohkouchi et al., 2000; Cairns et al., 1996; Barth et al., 1992; Murdiyatmo et al., 1992), which explains their high genetic variability. Some of the *deh* genes, however, are carried by plasmids (Sota et al., 2002; Brokamp et al., 1997; Kawasaki et al., 1994; Kawasaki et al., 1992) and can be found on transposable genetic elements (Sota et al., 2002; Weightman et al., 2002).

The genetic diversity between the two *deh* groups is also reflected in the mode of action of the corresponding enzymes on the substrate. The dehalogenases from group II proceed via a nucleophilic attack resulting in a covalent ester-enzyme link between an aspartate residue and the dechlorinated substrate (Ridder et al., 1999), while the dehalogenases from group I do not form any ester bond with the substrate (Nardi-Dei et al., 1999). Additionally, the two groups of halocarboxylic acid dehalogenases have stereospecificity towards optically active substrates such as 2-monochloropropionic acid (2MCPA) (Hill et al., 1999). Thus, the group I of halocarboxylic acid dehalogenases is active with both L- and D-isomers of 2MCPA, while the other group is only active with

the L-isomer. Moreover, the dehalogenases from group II are members of the haloacid dehalogenase (HAD) superfamily and are structurally more closely related to other enzymes like phosphatases and epoxidases (Hill et al., 1999).

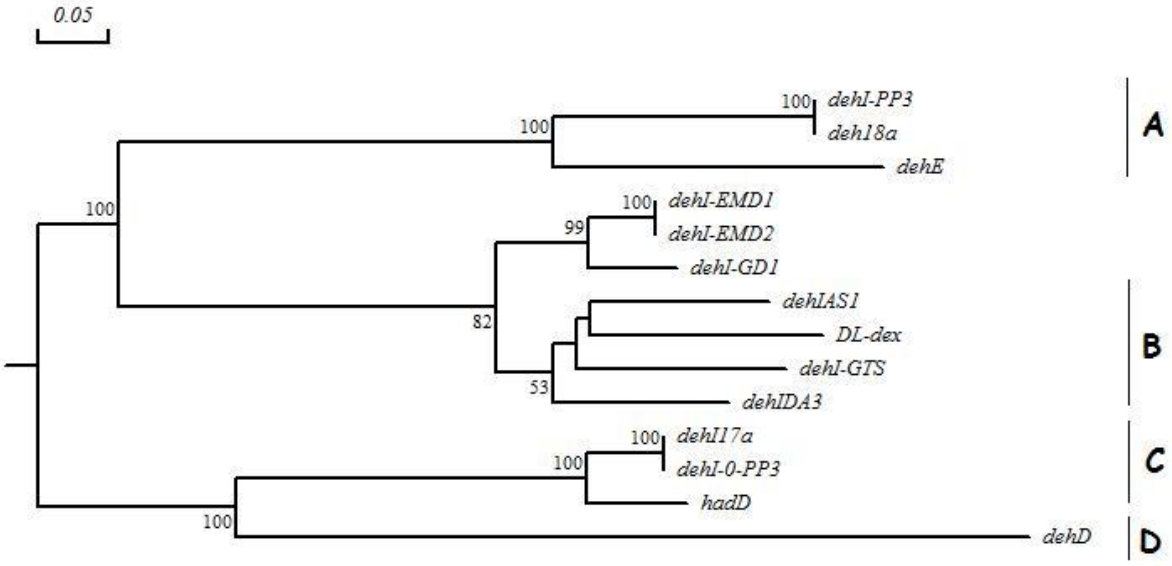

Figure 2.2. Dendogram illustrating the phylogenetic relationships between several group I *deh* sequences (adapted from Zhang et al., 2009a and Hill et al., 1999). DNA sequences of ~ 270 bp were aligned with the DNAMAN software (version 7; Lynnon Corp., Quebec, Canada) and the phylogenetic trees were constructed with the same program using the Jukes-Cantor algorithm (Jukes and Cantor, 1969). Bootstrap values are shown for nodes with > 50% probability of 500 replicates. The scale bar indicates an estimated change of 5%. A, B, C, and D are subdivisions based on nucleotide sequence identities that are higher than 55%.

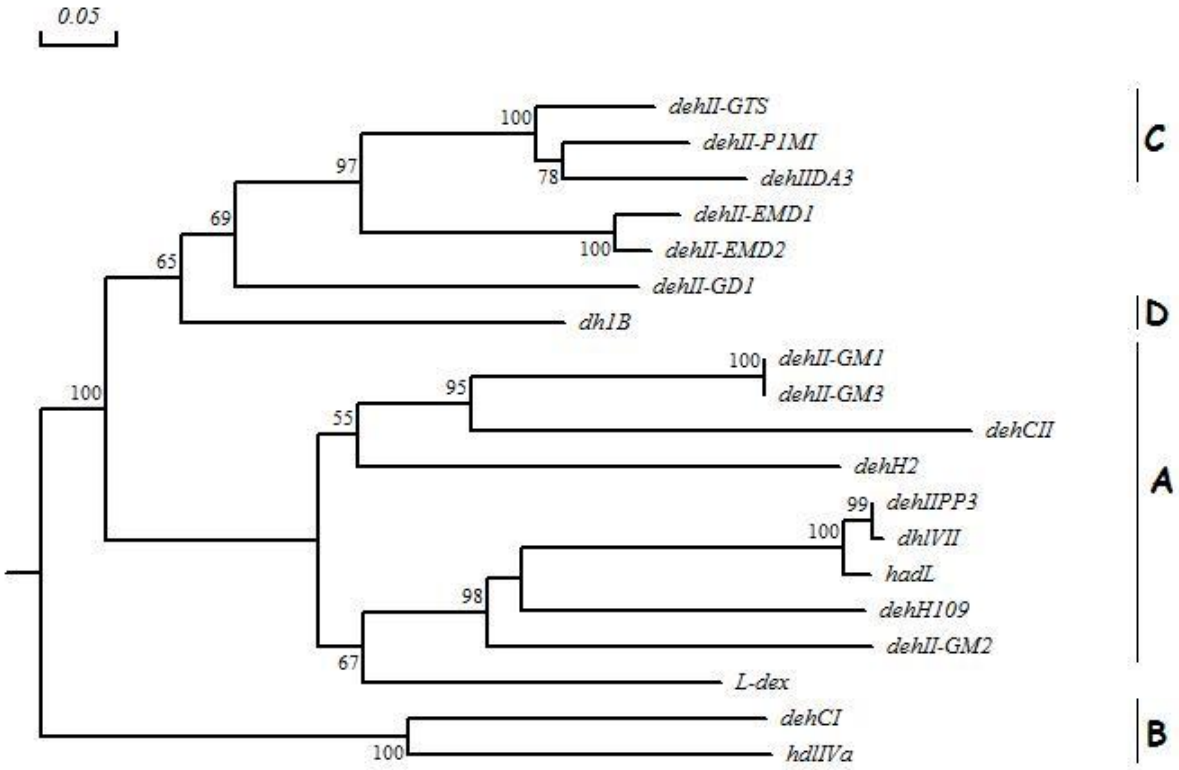

Figure 2.3. Dendogram illustrating the phylogenetic relationships between several group II *deh* sequences (adapted from Zhang et al., 2009a and Hill et al., 1999). DNA sequences of ~ 420 bp were aligned with the DNAMAN software (version 7; Lynnon Corp., Quebec, Canada) and the phylogenetic trees were constructed with the same program using the Jukes-Cantor algorithm (Jukes and Cantor, 1969). Bootstrap values are shown for nodes with > 50% probability of 500 replicates. The scale bar indicates an estimated change of 5%. A, B, C, and D are subdivisions based on nucleotide sequence identities that are higher than 55%.

Bacterial isolates carrying halocarboxylic acid dehalogenase genes can often metabolize a broad substrate range of mono-, di- and trihalogenated short chain carboxylic acids (Zhang et al., 2009a & b; Kerr & Marchesi, 2006). Although there are no studies relating the presence of either *dehI* or *dehII* genes with the degradation of specific halocarboxylic acid substrates, there is some evidence to suggest that only the drinking water isolates having a *dehI* gene could degrade trihalogenated acetic acids

(Zhang et al., 2009a). Also, organisms that possess both a *dehI* and *dehII* gene had a broad substrate range and were capable of biodegrading all chlorinated and/or brominated HAAs that were tested (i.e. up to 7 different HAAs) (Zhang et al., 2009a).

2.5 DETECTION AND ENUMERATION OF HAA-DEGRADING BACTERIA IN DRINKING WATER SYSTEMS

2.5.1 Drinking water distribution systems as microbial "black boxes"

Drinking water systems are intentionally harsh environments for microorganisms, in order to protect public health by inhibiting the survival of pathogens. This is achieved by maintaining high disinfectant residuals and very low nutrient concentrations (Srinivasan & Harrington, 2007; Volk & LeChevallier, 2000; Lu et al., 1999). Nevertheless, many bacteria adapted to live in low nutrient conditions (Egli, 2010) and became resistant to the disinfectant residuals present in drinking water systems by growing in biofilms on the pipe walls (Lehtola et al., 2004; Ollos et al., 2003; Zhang et al., 2002; Niquette et al., 2000; LeChevallier et al., 1987). Bacterial densities on the pipe walls can be up to 10^7-10^8 cells/cm^2 of pipe (Lehtola et al., 2004). Although tap water bacterial concentrations are generally very low (e.g., < 500 CFU/mL to comply with USEPA drinking water regulations), bacterial numbers can increase substantially (e.g., over two orders of magnitude) in the distribution systems at locations where chlorine residuals are very low (e.g., high residence time locations) (Carter et al., 2000; Kerneis et al., 1995), due to a preference of bacterial cells to detach from surfaces (Srinivasan et al., 2008). Information regarding the phylogenetic diversity of the bacteria found in drinking water systems has only recently started to appear in the literature, showing that Gram positive and α-, β- and γ-*Proteobacteria* are the predominant bacterial groups (Tokajian

et al., 2005; Williams et al., 2004). Microbial ecology data is still very scarce for drinking water systems and the general understanding about microbial life in such aquatic environments is very limited (Berry at al., 2006).

2.5.2 Biologically active filters as controlled systems for HAA removal

Sand and granular activated carbon (GAC) filters, which are frequently used in the process of drinking water treatment, can accumulate high densities of bacterial cells. For example, typical bacterial densities in biologically active GAC filters are between 10^7 and 10^{10} cells/cm^2 of filter grain (Velten et al., 2007; Magic-Knezev & van der Kooij, 2004). Moreover, if prechlorination is used in the water treatment process, generating high HAA concentrations, GAC filters are expected to allow the development of HAA-degrading bacteria because the residual chlorine is readily dissipated in the GAC filter (Fairey et al., 2007). In fact, biodegradation of HAAs in biologically-active filters has been confirmed in pilot-scale experiments (Xie and Zhou, 2002; Zhou and Xie, 2002) and full-scale monitoring studies (Kim and Kang, 2008; Tung et al., 2006). In addition, such filters have the advantage of controlling operating parameters (e.g., empty bed contact time and temperature), which can result in a better removal of HAAs (Wu and Xie, 2005).

2.5.3 Enumerating HAA degraders in drinking water systems

No precise information is currently available regarding the abundance and the diversity of HAA-degrading bacteria in drinking water systems. Zhang et al. (2009a & b), however, obtained several HAA-degrading bacterial isolates from pipe wall and granular activated carbon biofilms. The isolation of those HAA-degrading bacteria from water systems was very difficult and time-consuming (Zhang et al., 2009a & b). Moreover, it is

possible that the obtained HAA-degrading isolates are not the environmentally relevant HAA degraders in drinking water systems, due to the cultivation bias (Marchesi et al., 2003; Dunbar et al., 1997). Thus, more research is needed to find the predominant HAA degraders in drinking water systems, which could be used as indicators for quantifying the HAA-degrading biomass in these systems.

Leach et al. (2009) attempted to obtain information on the abundance of HAA degraders in drinking water systems by developing a quantitative polymerase chain reaction (qPCR) method targeting the *dehI* and *dehII* gene groups in tap water samples. The authors showed that *dehI* and *dehII* genes could be amplified from various drinking water samples using the highly degenerate primers developed by Hill et al. (2009). These degenerate primers, however, could not be used for quantification purposes due to nonspecific amplification. Thus, the authors developed specific *deh* primers for qPCR using HAA degraders isolated from wastewater as targets. Those isolates carried only the *dehII* gene and could be detected in drinking water samples only if an additional nested PCR step was used. Therefore, the qPCR method developed by Leach et al. (2009) needs further optimization as more information about the presence of HAA degraders in drinking water systems becomes available.

2.6 APPLICABILITY OF THE HAA BIODEGRADATION PROCESS FOR DRINKING WTAER TREATMENT

The biodegradation of HAAs is a potential useful process for decreasing HAA concentrations in drinking water systems. A recent study by Grigorescu and Hozalski (2010) proposed a kinetic model for the estimation of HAA loss due to biodegradation in

biologically-active filters and distribution systems. The authors showed that the quantity of HAA-degrading biomass is a critical parameter controlling HAA removal (Figure 2.4). The authors, however, pointed out that HAA-degrading biomass in drinking water systems are not known because of the difficulty in obtaining biofilm samples, especially from distribution system pipes, and because of the lack of a rapid and reliable method for quantifying HAA degraders in the samples. It is therefore important to have more precise information about HAA-degrading bacteria levels in drinking water systems. Thus, more research is needed concerning the identification and enumeration of major HAA degraders in drinking waters systems.

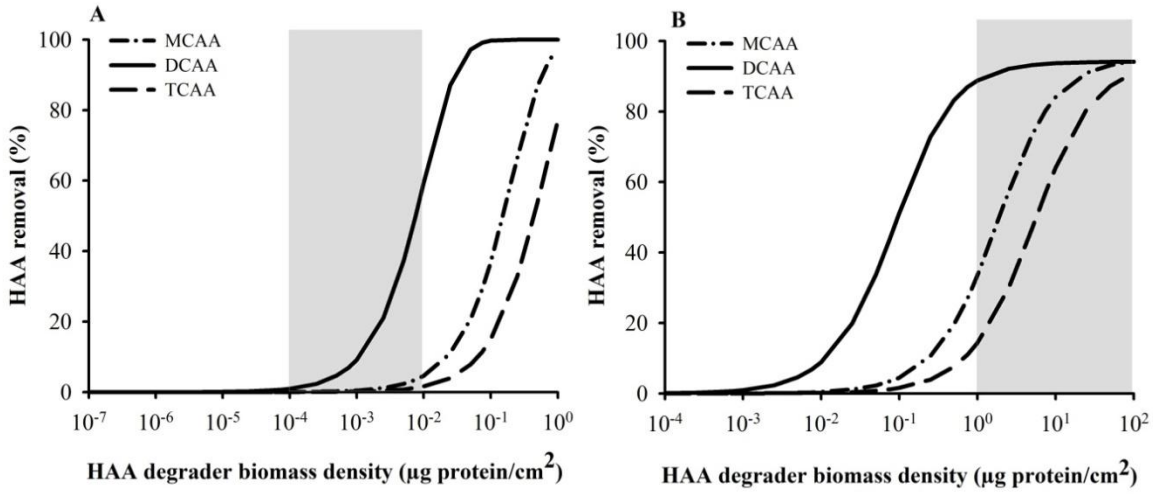

Figure 2.4. Effect of HAA-degrading biomass density on the biodegradation of MCAA, DCAA and TCAA in drinking water distribution systems (A) and biologically activated filters (B) (adapted from Grigorescu and Hozalski, 2010). The grey boxes represent HAA removals obtained with calculated HAA-degrading biomass densities based on the typical ranges of total bacterial cells in US drinking water systems (i.e., 10^4-10^6 cells/cm^2 of pipe wall and 10^8-10^{10} cells/cm^2 of filter grain).

2.7 CONCLUSIONS

There is concern over the potential deleterious health effects of chlorinated disinfection byproducts (DBPs), such as HAAs, in drinking water. As HAAs are easily biodegraded, modeling studies suggest that biodegradation could be exploited as a means to effectively remove HAAs during water treatment. In fact, biodegradation of HAAs in biologically-active filters has been confirmed in pilot-scale experiments and full-scale monitoring studies. Furthermore, HAA biodegradation is likely the major loss process occurring in drinking water distribution systems. Although drinking water distribution systems are harsh environments for microorganisms, studies have shown that HAA degraders are present in such environments. Future research is needed to develop quantification methods for estimating the HAA-degrading biomass in drinking water systems to improve estimates of the HAA biodegradation potential in these systems.

Chapter 3 : INFLUENCE OF THE ENVIRONMENT ON THE PHYLOGENETIC DIVERSITY OF BACTERIAL SPECIES ISOLATED ON HALOACETIC ACIDS AND THEIR CORRESPONDING *DEH* GENES

Most of the 2-haloalkanoic acid (2-HA)-degrading bacteria isolated to date are Gram negative and belong to the *Proteobacteria* group. A few Gram positive isolates have been obtained; however, no dehalogenase (*deh*) gene was detected in those isolates. In this study, a direct plating technique, involving haloacetic acid (HAA)-amended agar plates, was used to isolate 2-HA degraders from different environments (i.e., river water, tap water and agricultural soil). The obtained isolates included several Gram positive and Gram negative bacteria that were not isolated in the previous studies related to the biodegradation of 2-HAs: *Mycobacterium* sp., *Streptomyces* sp., *Stenotrophomonas* sp. and *Pantoea* sp. Moreover, additional species such as *Bradyrhizobium* sp., *Bosea* sp. and *Mycobacterium* sp., were isolated from drinking water systems. Furthermore, a *deh* gene was detected for the first time in a Gram positive bacterial species. This *deh* gene had almost 90% nucleotide sequence similarity with the *dehII* sequence from an α-*Proteobacterium* (*Bradyrhizobium* sp. St. Paul TCAA3), suggesting that lateral gene transfer of *deh* genes across unrelated bacterial species occurred.

3.1 INTRODUCTION

Microbial dehalogenation plays a key role in mitigating the anthropogenic inputs of halogenated organic compounds to the environment because microorganisms possess a wide variety of dehalogenase genes that can accommodate different dehalogenating pathways (de Jong & Dijkstra, 2003; Janssen et al., 2001 and 1994). One such dehalogenating pathway is the hydrolytic cleavage of 2-haloalkanoic acids (2-HAs) (Kurihara & Esaki, 2008). Haloacetic acids (HAAs) are a class of 2-HAs that are released in the environment through different human activities such as disinfection of drinking and wastewater by chlorination (Sirivedhin & Gray, 2005; Krasner et al., 1989), paper bleaching (Juuti et al., 1995), direct use of some HAAs as herbicides (Ashton & Crafts, 1973), as intermediates in the chemical industry and as food preservatives (Reinmann et al., 1996). HAAs are of concern because they are phytotoxic (Hanson & Solomon, 2004; Norokorpi & Frank, 1995) and hepatocarcinogenic (Herren-Freund et al., 1987). The major concern for human health is the presence of HAAs in drinking water (Krasner et al., 1989) allowing direct exposure of the human population to these chlorinated chemicals.

Microorganisms that are able to degrade HAAs and other 2-HAs have been isolated from soil (Olaniran et al., 2004; Hill et al., 1999), activated sludge (Kerr & Marchesi, 2006; McRae et al., 2004) and drinking water systems (Zhang et al., 2009a & b). Many of the enzymes responsible for the hydrolytic dehalogenation of 2-HAs in these organisms were characterized and the corresponding genes were classified into 2 phylogenetically unrelated groups, called the group I and group II *deh* genes (Hill et al., 1999). Both *deh* gene groups are characterized by high intra-group phylogenetic diversity

(Zhang et al., 2009a; Marchesi et al., 2003; Hill et al., 1999). The 2-HA dehalogeneases generally have a broad substrate range, degrading both short-carbon-chain 2-HAs such as HAAs and halopropionic acids and longer-carbon-chain 2-HAs such as halobutyric or halodecanoic acids (Liu et al., 1994; van der Ploeg et al., 1991).

Although useful information about 2-HA degraders, catalytic enzymes and the corresponding *deh* genes is available, there are still many questions about these organisms and the *deh* genes they carry. For example, most of the HAA-degrading bacteria isolated so far are strains of *Afipia* spp., *Rhizobium* spp. , *Xanthobacter* spp., *Burkholderia* spp., *Delftia* spp. and *Pseudomonas* spp., which belong to the α, β and γ-*Proteobacteria* subdivisions. Moreover, the *Afipia* spp. seem to be the predominant HAA degraders in drinking water systems (Chapter 4; Zhang et al., 2009a & b). A few Gram positive bacterial strains (i.e., *Bacillus* sp., *Enterococcus* sp. and *Corynebacterium* sp.) with haloalkanoic acid dehalogenating abilities (including the degradation of some HAAs) were also isolated but neither the enzymes nor the corresponding *deh* genes were characterized (Kerr & Marchesi, 2006; Olaniran et al., 2004). Hill and Weightman (2003) demonstrated that many *deh* genes are carried by mobile genetic elements that can easily be transferred between different bacterial hosts, allowing the dissemination of the *deh* genes in the environment. Furthermore, Marchesi and Weightman (2003) showed that the metagene pool of *deh* genes in the environment is different and more diverse than the gene pool that was analyzed in the isolated strains. Thus, the authors suggested that the isolates obtained so far by enrichment cultures might not be the environmentally relevant 2-HA degraders and that the inability to obtain more diverse 2-HA degraders might be due to the enrichment culture bias (Dunbar et al., 1997).

The goal of this study was to enlarge the existing database of 2-HA degraders by isolating novel degraders and characterizing their *deh* genes. For this, the direct plating technique was used instead of enrichment cultures to allow for growth of both slow and fast degraders (Dunbar et al., 1997). Source samples were obtained from different environments, likely to be contaminated with HAAs (i.e., river water, tap water and agricultural soil). HAAs (i.e., monochlororacetic acid (MCAA), dichloroacetic acid (DCAA) or trichloroacetic acid (TCAA)) were thus used as sole carbon source instead of the previously used enrichment substrates (i.e., dalapon and 2-chloropropionic acid) to select for novel 2-HA degraders.

3.2 MATERIALS AND METHODS

3.2.1 Sample collection and bacterial strain isolation on HAAs

Samples were collected from different locations that were likely contaminated with HAAs (i.e., tap water, agricultural soil and the Mississippi River). Tap water samples were obtained from two locations within the Minneapolis and Saint Paul, MN, distribution systems. Both cities use the Mississippi River water as source and apply a chlorination/chloramination disinfection strategy, thus resulting in the formation of HAAs due to the reaction between the natural organic matter (NOM) and the free chlorine. HAA_5 (i.e., the sum of MCAA, DCAA, TCAA and mono- and dibromoacetic acids) concentrations within the Minneapolis distribution system range between not detected and 54.7 µg/L (Minneapolis Water Quality Reports, 2009 & 2008) and within the Saint Paul distribution system range between 6.6 and 27.4 µg/L (SPRWS Water Quality Reports, 2011 & 2009). The agricultural soil samples were obtained from a corn field in Blairsburg, Iowa, where the herbicides metolachlor and alachlor had been applied

in the past. These two chemicals can be photodegraded into MCAA (Wilson & Malbury, 2000) and alachlor can be biodegraded into MCAA (Mangiapan et al., 1997). The Mississippi River water samples were collected in Minneapolis, MN, close to the shore. In river water, HAA concentrations typically vary from a few ng/L to a few µg/L for individual HAAs, the main sources of these HAAs being wastewater effluents and industrial water discharges that are contaminated with HAAs (Hashimoto et al., 1998)

Eighteen liters of tap water were aseptically collected from Minneapolis and St. Paul and immediately placed on ice until being processed. Within an hour of collection, these tap water samples were filtered through sterile nylon membrane filters (3L per filter) (47 mm diameter, 0.2 µm pore size, Millipore Corp., Billerica, MA). The filters were placed onto agar plates supplemented with 10 mM HAAs (i.e., MCAA, DCAA or TCAA). The agar plates contained basal medium at pH 7.2 (Zhang et al., 2009a & b), 1.5 % Noble agar (BD, Sparks, MD, USA) and one HAA as the sole carbon source. A pH indicator, phenol red (EM Industries, Inc., Gibbstown, N.J.), was added to the agar at a concentration of 0.03 % (w/v), which yielded a yellow color at pH $\leq$ 6.6 and dark red at pH $\geq$ 8 (Kerr and Marchesi, 2006). The pH indicator was added to detect whether HAA biodegradation was taking place, as dechlorination of HAAs results in an acidification of the medium. The plates were incubated in the dark at room temperature (~21ºC) for up to 30 days, until colonies developed and a change in the pH indicator color was noticed (from red to yellow). Single colonies of presumed HAA degraders were selected and streaked onto fresh plates. This process was repeated one or two more times for strain purification. The same procedure was used for the Mississippi River water samples, except that only 50 mL of water was filtered. The soil bacteria were first dislodged in

phosphate-buffered saline (PBS- 10 mM, pH 7.2) by placing 1 g of soil in 9 mL of sterile PBS and vortexing for 5 min. Three serial dilutions of the supernatant were prepared and 100 µL of each serial dilution were spread on separate HAA plates. Delvocid salt (DSM Food Specialties, Delft, Netherlands), containing 50% natamycin, was added to the agar at a concentration of 0.1 % (w/v) to inhibit fungal growth from the soil samples. To rapidly generate biomass for making frozen stocks and extracting DNA, the isolates were grown on agar plates supplemented with 10 mM acetate. For each isolate, the biomass grown on acetate plates was collected with sterile cotton swabs and part of it was placed in 15% sterile glycerol solution and stored at -55°C and the rest in 1 mL lysis buffer (120 mM Na_2HPO_4 and 5 % sodium dodecyl sulfate) and was used for genomic DNA extraction and PCR amplification of 16S rRNA genes and *deh* genes as described below.

3.2.2 Genomic DNA extraction and nucleotide analysis of 16S rRNA gene and groups I and II of *deh* genes

Cells that were placed in lysis buffer were lysed by performing three freeze-thaw cycles followed by incubation at 70°C for 90 min. DNA extractions were then performed with a FastDNA®Spin extraction kit (MP Biomedicals LLC, Irvine, CA) according to the manufacturer's instructions.

The 16S rRNA gene was PCR amplified using primers 8F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1522R (5'-AAG GAG GTG ATC CAG CCG CA-3'). The PCR reactions contained 1 × GoTaq™ reaction buffer (Promega, Madison, WI, USA), 0.5 µM of each primer, 80 µM deoxynucleoside triphospates (dNTPs), 1.25 units of Go *Taq* polymerase (Promega) and approximately 50 ng of genomic DNA as template. The PCR program included a 5 min initial denaturation step at 95°C, followed by thirty-five

cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and a final extension for 10 min at 72°C. Amplified products were resolved by electrophoresis on 1% (wt/vol) agarose gels stained with 1 µg/mL ethidium bromide. The PCR products were then purified with a GeneCleanII kit (QBiogene, Carlsbad, CA, USA) according to the manufacturer's protocol. The purified PCR products were submitted for sequencing at the BioMedical Genomics Center (BMGC) at the University of Minnesota using primers 338F (5-ACT CCT ACG GGA GGC AGC AG -3) and 907R (5'-CCG TCA ATT CCT TTR AGT TT-3'). Electropherograms of both 16S gene strands were aligned, analyzed and edited with the CodonCode Aligner software (version 3.5.6, CodonCode Corp.) in order to determine the correct 16S sequences.

The group I and group II of *deh* genes were amplified following the PCR methods described by Hill et al. (1999). The group I *deh* genes were amplified using the primer set $dehI_{forI}$ (5'-ACG YTN SGS GTG CCN TGG GT-3') and $dehI_{revI}$ (5'-AWC ARR TAY TTY GGA TTR CCR TA-3'), which generated a fragment of ~270 bp. The group II *deh* genes were amplified using the primer set $dehII_{forI}$ (5'-TGG CGV CAR MRD CAR CTB GAR TA-3') and $dehII_{revI}$ (5'-TCS MAD SBR TTB GAS GAN ACR AA-3'), which generated a fragment of ~420 bp. These *deh* gene fragments were sequenced at BMGC with the same primer sets used for the PCR amplification. The sequence results were analyzed as described for the 16S genes.

3.2.3 Bacterial strain identification and phylogenetic analysis of *deh* sequences

Strain identification was carried out by comparing the partial 16S rRNA gene sequences determined in this study with all sequences from GenBank, EMBL, DDBJ and PDB databases by using the Blastn software (Zhang et al., 2000) available online at

http://www.ncbi.nlm.nih.gov/. Bacterial strain identification was based on nucleotide sequence identity higher than 98% with species from the mentioned gene databases.

The partial nucleotide sequences of group I and group II *deh* genes obtained here (~270 bp of *dehI* fragments and ~420 bp of *dehII* fragments) were optimally aligned with the DNAMAN software (version 7; Lynnon Corp., Quebec, Canada) using the Clustal W program (Thompson et al., 1994). Phylogenetic trees were constructed with the same program using the neighbor-joining method (Saitou & Nei, 1987) together with the Jukes-Cantor algorithm (Jukes and Cantor, 1969). In order to determine the phylogenetic relationship of the newly obtained *deh* sequences, the sequences were compared with group I and group II *deh* genes available in the GenBank database. The group I and group II *deh* gene phylogenetic subdivisions that were previously identified by Hill et al. (1999) were marked with capital letters from A to D. These phylogenetic subdivisions were defined by Hill et al., 1999, based on > 55% nucleotide sequence identity and are supported by high bootstrapping values.

3.2.4 GenBank accession numbers

The 16S rRNA gene, *dehI* and *dehII* nucleotide sequences of 12 isolates obtained during this study (i.e., *Afipia* sp. Minneapolis MCAA1, *Bradyrhizobium* sp. Minneapolis DCAA1, *Bosea* sp. Minneapolis TCAA1, *Bosea* sp. Minneapolis TCAA2, *Afipia* sp. St. Paul MCAA1, *Afipia* sp. St. Paul TCAA2, *Bradyrhizobium* sp St. Paul TCAA3, *Afipia* sp. Soil 1 DCAA6, *Burkholderia* sp. Soil 2 MCAA6, *Pseudomonas* sp. Mississippi MCAA11, *Stenotrophomonas sp.* Mississippi MCAA16, *Burkholderia* sp. Mississippi DCAA10) have been deposited in the GenBank database under accession numbers HQ130463 to HQ130469. Other GenBank accession numbers for group I of *deh* genes

used in this study are as follows: *dehI* from *Afipia* sp. GD1 (FJ417105), *dehI* from *Afipia* sp. GTS (FJ417106), *dehI* from *Afipia* sp. EMD1 (FJ417107), *dehI* from *P. putida* PP3 (AY138113), *dehI-0* from *P. putida* PP3 (AJ133461), *had* from *P. putida* (M81841), *dehI17a* from *Pseudomonas* sp. (AJ133457), *dehI18a* from *Pseudomonas* sp. (AJ133458), *DL-DEX* from *Pseudomonas* sp. 113 (U97030), *dehD* from *Rhizobium* sp. (X93597), *dehE* from *Rhizobium* sp. (Y15517), *dehIDA1* from Beta-proteobacterium strain DA1 (AJ133455), *dehIDA2* from *Bradyrhizobium* sp. (AJ133456), *dehIDA3 Bradyrhizobium* sp. (AJ430683), *dehIAS1* from *Xanthobacter* sp. (AJ511295), *dhlC* from *Achromobacter xylosoxidans* (X77610) and for group II *deh* genes: *dehII* from *Burkholderia sp.* GM1 (FJ417109), *dehII* from *Herminiimonas sp.* GM2 (FJ417110), *dehII* from *Burkholderia sp.* GM3 (FJ417111), *dehII* from *Afipia* sp. GD1 (FJ417112), *dehII* from *Afipia* sp. P1MI (FJ417114), *hdlIVa* from *Burkholderia cepacia* strain MBA4 (X66249), *dehH2* from *Delftia acidovorans* (D90423), *dehIIPP3* from *P. putida* PP3 (AJ133462), *dehH109* from *P. putida* No. 109 (D17523), *L-dex* from *Pseudomonas* sp. YL (S74078), *dehCI* from *Pseudomonas* sp. strain CBS3 (M62908), *dehCII Pseudomonas* sp. strain CBS3 (M62909), *hadL* from *P. putida* (M81841), *dhlVII* from *P. fluorescens* (X94147), *dh1B* from *Xanthobacter autotrophicus* (M81691), *dehIIDA3* from *Bradyrhizobium* sp. strain DA3 (AJ133463).

3.2.5 Analysis of inferred amino acid sequences of *deh* gene fragments

The inferred amino acid sequences of the *deh* genes obtained in this study were aligned and compared with the amino acid sequences of *dehI* and *dehII* genes that were previously shown to have conserved amino acid residues with essential role in the catalytic reaction. The alignment was done with the DNAMAN software (version 7;

Lynnon Corp., Quebec, Canada). For the group I *deh* sequences, the following amino acids, which are found within the 270 bp frame analyzed here, are known to be essential in the catalytic process: T65, E69, N117 and Y120 together with the amino acid motif YGNPKY (Hill et al., 1999; Nardi-Dei et al., 1997). For group II *deh* sequences, the following amino acids, which are found within the 420 bp frame analyzed here, are known to be essential in the catalytic process: R41, S118, K151, Y157, S175 and N177 (Kurihara et al., 1995).

3.3 RESULTS AND DISCUSSION

3.3.1 Bacterial strain isolation

In this work, thirty five isolates were obtained from four different environments that had the potential to naturally select for HAA-degrading bacteria (Table 3.1). About half of these isolates (i.e., 18 isolates) were obtained from drinking water collected from two different distribution systems (i.e., Minneapolis, MN and St. Paul, MN), 9 isolates were obtained from the Mississippi River water and 8 isolates from agricultural soil.

The isolates obtained from drinking water systems were predominantly α-*Proteobacteria* (~83%), while this phylogenetic subdivision was the least abundant in the Mississippi River water (~11%) and less abundant than β-*Proteobacteria* in agricultural soil (~25% α-*Proteobacteria* compared to ~37% β-*Proteobacteria*) (Figure 3.1). α-*Proteobacteria* seem to be more resistant in water environments carrying a chlorine residual, because they became more abundant in a simulated distribution system, following exposure to free-chlorine or chloramine (Williams et al., 2004). The majority of isolates obtained from the Mississippi River water and agricultural soil were β- and γ-*Proteobacteria* (Figure 3.1). *Proteobacteria*, especially β-*Proteobacteria*,

are the predominant bacteria in both planktonic river water and soil (Roesch et al., 2007; Zwart et al., 2002).

Table 3.1. List of organisms isolated in this study and detection of group I and group II *deh* genes by PCR amplification of short *deh* fragments.

Host strain	**Phylogenetic afiliation**	**Group I *deh***	**Group II *deh***
Isolates from drinking water systems			
Afipia sp. Minneapolis MCAA1	α-*Proteobacteria*	-**	+*
Bradyrhizobium sp. Minneapolis DCAA1	α-*Proteobacteria*	-	+
Mycobacterium sp. Minneapolis DCAA5	*Actinobacteria*	-	+
Mycobacterium sp. Minneapolis DCAA6.1	*Actinobacteria*	-	-
Bosea sp. Minneapolis TCAA (4)***	α-*Proteobacteria*	-	+
Bosea sp. Minneapolis TCAA2	α-*Proteobacteria*	-	+
Afipia sp. St. Paul MCAA (5)	α-*Proteobacteria*	+	+
Mycobacterium sp. St. Paul DCAA1	*Actinobacteria*	-	ND
Afipia sp. St. Paul TCAA2	α-*Proteobacteria*	+	+
Bradyrhizobium sp. St. Paul TCAA3	α-*Proteobacteria*	+	+
Afipia sp. St. Paul TCAA4	α-*Proteobacteria*	ND	+
Isolates from Mississippi River water			
Delftia sp. Mississippi MCAA5	β-*Proteobacteria*	-	-
Serratia sp. Mississippi MCAA10	γ-*Proteobacteria*	-	-
Pseudomonas sp. Mississippi MCAA11	γ-*Proteobacteria*	+	+
Delftia sp. Mississippi MCAA15	β-*Proteobacteria*	-	-
Stenotrophomonas sp. Mississippi MCAA16	γ-*Proteobacteria*	+	+
Sphingomonas sp. Mississippi DCAA6	α-*Proteobacteria*	-	-
Burkholderia sp. Mississippi DCAA10	β-*Proteobacteria*	+	+
Pantoea sp. Mississippi TCAA3	γ-*Proteobacteria*	-	-
Delftia sp. Mississippi TCAA7	β-*Proteobacteria*	-	-
Isolates from agricultural soil			
Afipia sp. Soil 1 DCAA6	α-*Proteobacteria*	+	+
Burkholderia sp. Soil 2 MCAA6	β-*Proteobacteria*	-	+
Streptomyces sp. Soil 2 MCAA2	*Actinobacteria*	-	-
Stenotrophomonas sp. Soil 4 MCAA6	γ-*Proteobacteria*	-	+
Burkholderia sp. Soil 4 MCAA7	β-*Proteobacteria*	-	+
Burkholderia sp. Soil 4 MCAA8	β-*Proteobacteria*	-	+
Afipia sp. Soil 4 DCAA2	α-*Proteobacteria*	-	-
Klebsiella sp. Soil 4 DCAA6	γ-*Proteobacteria*	-	-

*(+) indicates that the presence of group I or group II of *deh* genes; the detected genes were confirmed by sequencing

**(-) indicates that group I or group II of *deh* genes could not be detected by PCR amplification

*** the number in parenthesis indicates the number of identical isolates obtained under the same isolating conditions (i.e., same location and HAA used as carbon source)

ND-sequence not determined

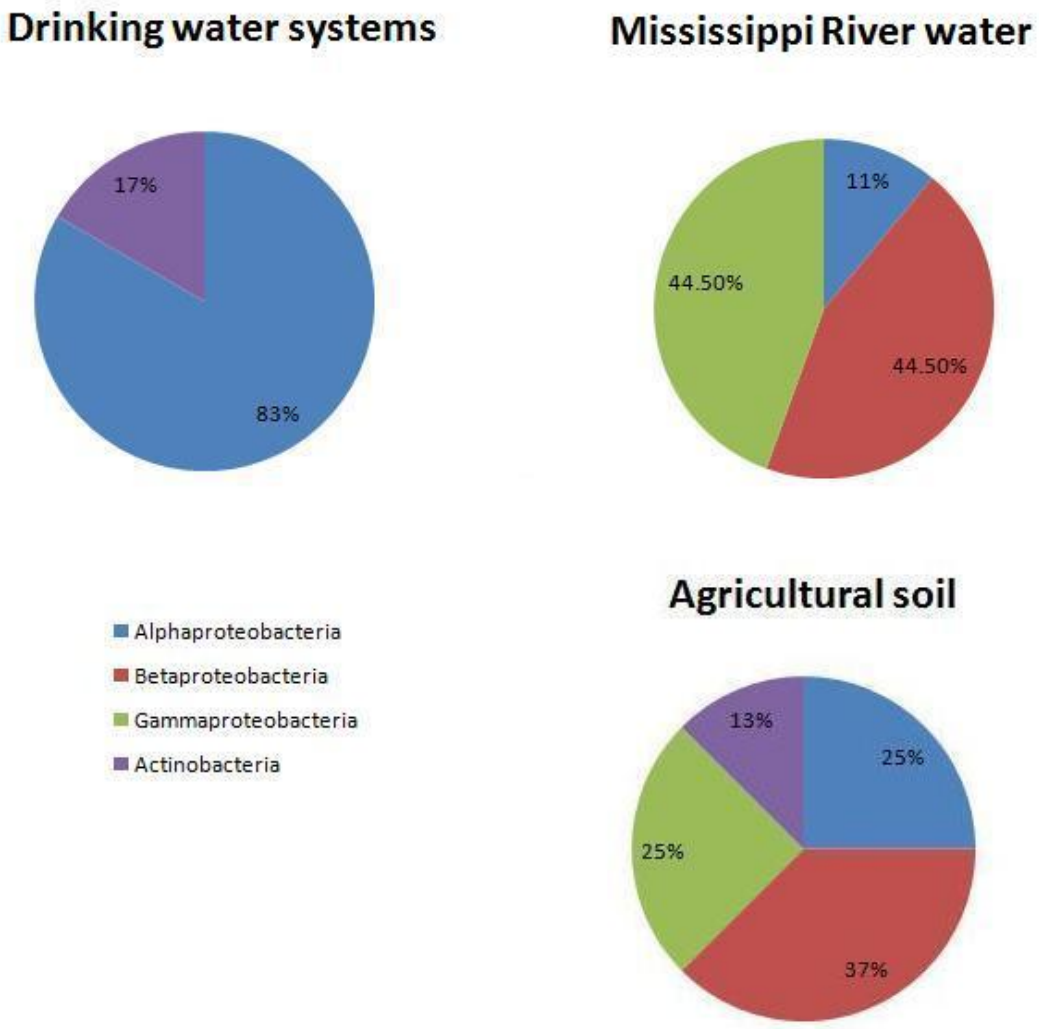

Figure 3.1. Phylogenetic subdivisions of the isolates obtained from drinking water systems, agricultural soil and Mississippi River water.

As expected, a greater phylogenetic diversity was found for the soil isolates as compared to the isolates obtained from drinking water or the Mississippi River. Thus, four different phylogenetic subdivisions were identified for the soil isolates, compared to only three phylogenetic subdivisions for the Mississippi River water isolates and two phylogenetic subdivisions for the drinking water isolates (Figure 3.1). This diversity, however, seemed low given the fact that it is estimated that the number of bacterial

species per gram of soil can be as high as 8.3 million for uncontaminated soils (Gans et al., 2005) and that other phylogenetic groups, such as the *Bacteroidetes* subdivision, are also abundant in soils (Roesch et al., 2007). Therefore, more research is still needed to demonstrate whether the HAA-degrading abilities are restricted to a few bacterial genera or if the well known culturing bias is the major obstacle preventing the isolation of other degraders.

Most of the α-*Proteobacteria* isolates obtained from drinking water in this study belonged to the genera of *Afipia* and *Bosea*, which are amoeba-resisting bacteria (ARB) that were previously detected in hospital water networks (Thomas et al., 2007; LaScola et al., 2000). ARBs are afforded protection from the chlorine residual by residing inside different amoeba (Thomas & Ashbolt, 2011). *Afipia* sp. and *Bosea* sp. were suspected to be agents responsible for nosocomial infections such as pneumonia (Berger et al., 2006; LaScola et al., 2003) and *Afipia* sp. used to be considered the causative agent of cat scratch disease (Brenner et al., 1991). *Afipia* spp., however, were recently shown to have the beneficial ability to degrade HAAs and appear to be the predominant HAA degraders in drinking water systems as demonstrated by both culturing and culture-independent studies (Chapter 4; Zhang et al, 2009a & b). Thus, it was not surprising that in this work, *Afipia* sp. was isolated from tap water under selective conditions for HAA degradation. One MCAA-degrading *Bosea* strain (i.e., *Bosea vestrisii*) was previously isolated from industrial wastewater on 2,3-dichloropropionic acid (Song et al., 2003). This is the first report of potential HAA-degrading *Bosea* sp. isolated from drinking water.

Here, a few Gram positive isolates belonging to the *Actinobacteria* phylogenetic division were also obtained from drinking water (i.e., *Mycobacterium* sp.) and soil

samples (*Streptomyces* sp.). This is the first study to report these two members of the *Actinobacteria* group isolated on HAAs. The only researchers who had previously obtained other Gram positive isolates with HAA dehalogenase activity were Kerr and Marchesi (2006) and Olaniran et al. (2004). Thus, Kerr and Marchesi obtained three isolates of the *Firmicutes* phylogenetic division (i.e., *Bacillus cereus*, *Bacillus thuringiensis* and *Enterococcus faecium*) that could degrade MCAA and DCAA, while Olaniran and coworkers (2004) isolated a *Corynebacterium* strain that was able to degrade MCAA and TCAA.

It was not surprising to find *Mycobacterium* sp. in the tap water samples as this is a bacterial species that is frequently found in drinking water systems, often living in association with amoeba (Thomas & Ashbolt, 2011; Corsaro et al., 2010). Moreover, *Mycobacteria* were shown to have the ability to dehalogenate haloalkanes (Jesenska et al., 2000), but not HAAs.

In this work, a *Streptomyces* sp. was isolated from an agricultural soil where the herbicide metolachlor was applied in the past. *Streptomyces* is a soil bacterium primarily known for the production of the antibiotic streptomycin, but it has also been shown to degrade metolachlor (Liu et al., 1991). *Streptomyces* sp. Soil 2 MCAA2, however, was isolated here under selective conditions for the biodegradation of MCAA.

3.3.2 Phylogenetic analysis of group I and group II *deh* genes

The PCR methods developed by Hill et al. (1999) were used for the detection of group I and group II *deh* genes in the isolated strains. Group I *deh* genes were detected in 12 of the 35 isolates and group II *deh* genes were detected in 25 of the 35 isolates obtained in this study. No *deh* gene from either group was detected by PCR

amplification in ten of the isolates (Table 3.1). The PCR-amplified fragments were obtained using highly degenerate primers (Hill et al., 1999), which could easily amplify unspecific products (Leach et al., 2009; Kerr and Marchesi 2006). In order to verify whether the amplified fragments corresponded to group I or group II *deh* sequences, those fragments were aligned with *deh* sequences from GenBank (Figures 3.2 & 3.3).

All group I *deh* sequences from the strains isolated here fell within known phylogenetic subdivisions of group I *deh* sequences (Figure 3.2), namely the B and C subdivisions described by Hill et al. (1999). Based on the phylogenetic analysis in Figure 3.2, it seemed that *dehI* sequence diversity depended on the location where the isolates were obtained from and the bacterial strain identification. For example, the *Afipia* sp. isolated from St. Paul tap water had a different *dehI* sequence than the *Afipia* sp. isolated from agricultural soil (only 80.5% nucleotide sequence similarity). The *dehI* sequences from the Mississippi River isolates did not seem related to the *dehI* sequences from the tap water or the soil isolates. Thus, the *dehI* sequences from the γ-*Proteobacteria* isolates (i.e., *Pseudomonas* sp. Mississippi MCAA11 and *Stenotrophomonas sp.* Mississippi MCAA16) from the Mississippi River clustered in the C subdivision and had less than 50% sequence similarity with the *dehI* sequences from the St. Paul tap water and soil isolates. High sequence divergence was also noticed for *dehI* sequences in isolates coming from the same location (e.g., Mississippi River water) but belonging to different phylogenetic subdivisions. Thus, the *dehI* sequence of the β-*Proteobacteria* isolate from the Mississippi River (i.e., *Burkholderia* sp. Mississippi DCAA10) was more related to the *dehI* sequences of the tap water and soil isolates (69.5% nucleotide sequence similarity) than the *dehI* sequences of the two

γ-*Proteobacteria* isolates from the Mississippi River (only 53% nucleotide sequence similarity). This is also reflected in the fact that it clustered in the same subdivision B with the *dehI* sequences from the tap water and soil isolates and not in the C subdivision like *dehI* sequences of the two γ-*Proteobacteria* isolates (Figure 3.2).

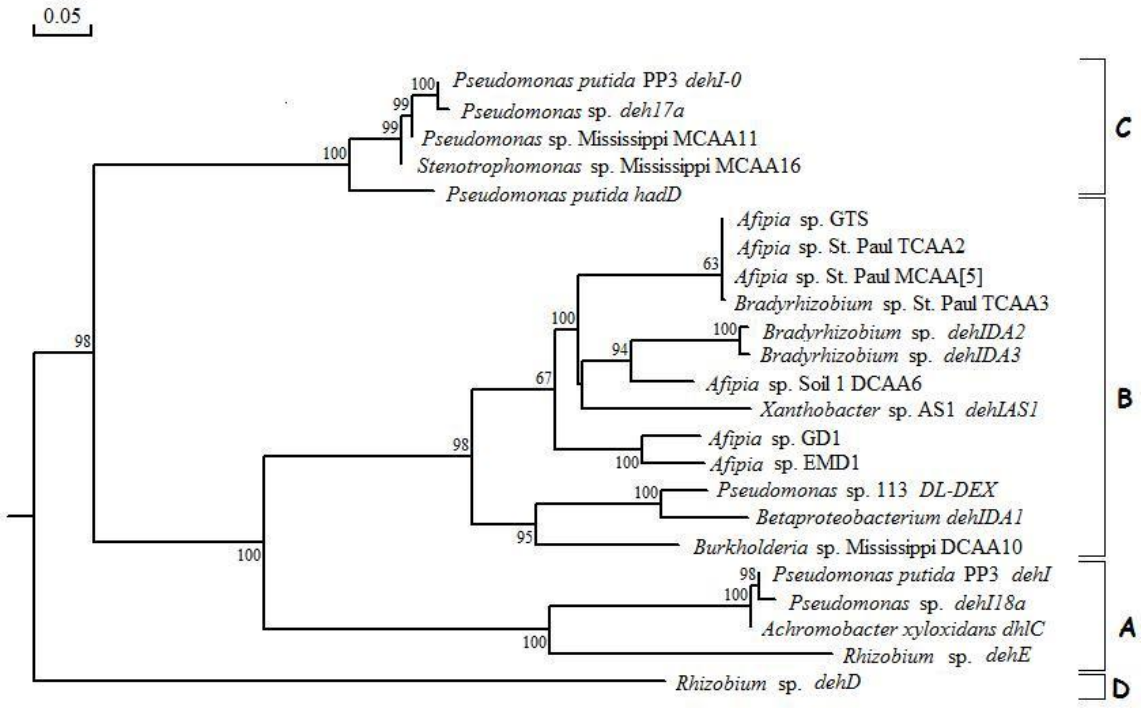

Figure 3.2. Dendrogram illustrating the phylogenetic relationship between group I of *deh* genes from different bacterial strains isolated in this study (i.e., the isolates listed in Table 3.1) and in previous studies. DNA sequences of ~ 270 bp were aligned with the DNAMAN software (version 7; Lynnon Corp., Quebec, Canada) and the phylogenetic trees were constructed with the same program using the neighbor-joining method (Saitou & Nei, 1987) and the Jukes-Cantor algorithm (Jukes and Cantor, 1969). Bootstrap values are shown for nodes with > 50% probability of 500 replicates. The scale bar indicates an estimated change of 5%. A, B, C, D are subdivisions as previously defined by Hill et al. (1999). Gene identifications are provided for those genes that were characterized in previous studies, if available.

The majority of group II *deh* sequences from the strains isolated here grouped within known phylogenetic subdivisions of group II *deh* sequences (Figure 3.3), namely the A and C subdivisions described by Hill et al., 1999. Based on Hill's et al. (1999) definition of phylogenetic subdivisions (i.e., one phylogenetic subdivision includes sequences with > 55% nucleotide sequence similarity), it was found here that the A subdivision should include at least two phylogenetically different pools of *dehII* sequences, named A1 and A2. For the group II *deh* sequences, sequence diversity was again associated with the location where isolates were obtained from and the bacterial strain identification. Thus, similar to the *dehI* sequences, the *dehII* sequences from *Afipia* sp. isolated from drinking water had only 79% nucleotide sequence similarity with the *dehII* sequence from the soil *Afipia* sp. isolate. Moreover, the *dehII* sequences from the *Bosea* isolates obtained from Minneapolis drinking water had less than 67% nucleotide sequence similarity with the *dehII* sequences from the other isolates obtained from the same place (i.e., *Mycobacterium* sp. Minneapolis DCAA5, *Bradyrhizobium* sp. Minneapolis DCAA1 and *Afipia* sp. Minneapolis MCAA1). These *dehII* sequences from the *Bosea* isolates clustered together with the *dehII* sequence from *Afipia* sp. GD1 previously isolated by Zhang et al. (2009 a & b), thus potentially forming a new phylogenetic subdivision. Here, it was shown for the first time that a Gram positive bacterium, the *Mycobacterium* sp. Minneapolis DCAA5, carries a group II *deh* sequence. The *dehII* sequence identified in the *Mycobacterium* sp. Minneapolis DCAA5 had 89.4% nucleotide sequence similarity with the *dehII* sequence from the *Bradyrhizobium* sp. St. Paul TCAA3, suggesting that lateral gene transfer across unrelated bacterial species occured.

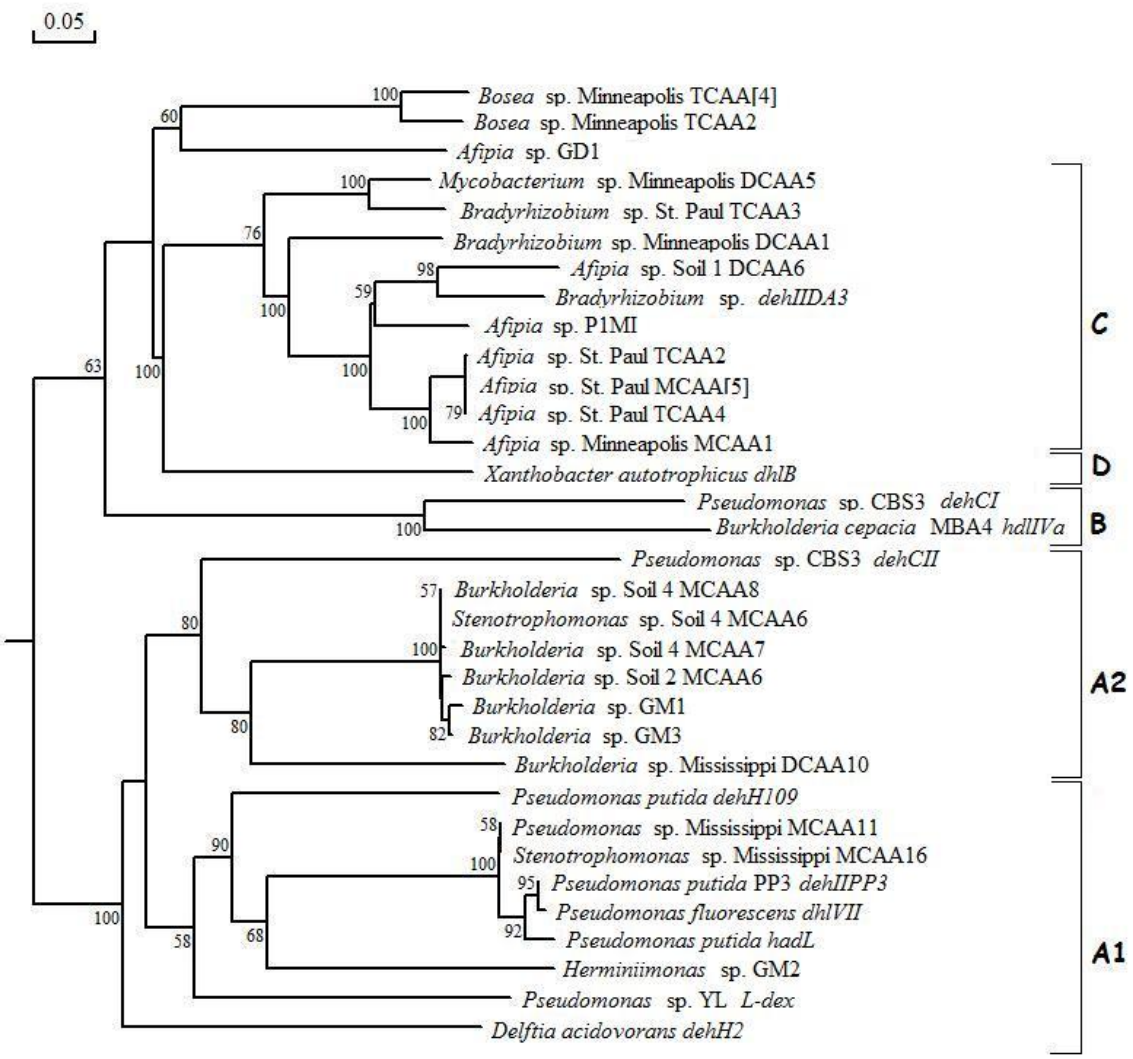

Figure 3.3. Dendrogram illustrating the phylogenetic relationship between group II of *deh* genes from different bacterial strains isolated in this study (i.e., the isolates listed in Table 3.1) and in previous studies. DNA sequences of ~ 420 bp were aligned with the DNAMAN software (version 7; Lynnon Corp., Quebec, Canada) and the phylogenetic trees were constructed with the same program using the neighbor-joining method (Saitou & Nei, 1987) and the Jukes-Cantor algorithm (Jukes and Cantor, 1969). Bootstrap values are shown for nodes with > 50% probability of 500 replicates. The scale bar indicates an estimated change of 5%. A, B, C, D are subdivisions as previously defined by Hill et al. (1999). A1 and A2 are two different subgroups of the subdivision A, as identified in this study. Gene identifications are provided for those genes that were characterized in previous studies, if available.

3.3.3 Analysis of inferred amino acid sequences of *deh* gene fragments

The inferred amino acid sequences of the the new *deh* fragments obtained in this study were analyzed for the presence of conserved amino acids that are implicated in the enzymatic processes (Appendix A), in order to confirm that these fragments belonged to group I and group II *deh* sequences. All *dehI* and *dehII* nucleotide sequences obtained in this study were translated into the corresponding amino acid sequences and were first verified that they represented open reading frames free of stop codons. The inferred amino acid sequences were then aligned with the translated sequences of other *deh* genes from GenBank (Appendix A), which were known to have conserved amino acids in the catalytic region (Hill et al., 1999).

All new group I sequences obtained in this study presented the following amino acid residues identified as essential for catalysis by Nardi-Dei et al. (1997): T65, E69, N117 and Y120 (Figure A1, Appendix A). The positions of the conserved amino acids are based on the *DL-DEX* gene from *Pseudomonas* sp. 113 (Nardi-Dei et al., 1997). Other conserved residues such as D28, D194, T219 and D250 (Nardi-Dei et al., 1997) could not be identified because they were outside the PCR-amplified region (only ~270 bp fragments were amplified). All group I sequences also presented the conserved amino acid motif YGNPKY (Hill et al., 1999; Nardi-Dei et al., 1997).

All new group II sequences obtained in this study presented the following amino acid residues identified as essential for catalysis by Kurihara et al. (1995): R41, S118, K151, Y157, S175 and N177 (Figure A2, Appendix A). The positions of the conserved amino acids are based on the *L-dex* gene from *Pseudomonas* sp. YL (Kurihara et al., 1995). Other conserved residues such as D10, T14 and D180 (Kurihara et al., 1995) could

not be identified because they were outside the PCR-amplified region (only ~420 bp fragments were amplified).

These findings strengthen the hypothesis that all new sequences identified in this study belong to the group I and group II *deh* sequences and that they potentially encode for active haloalkanoic acid dehalogenases.

3.4 SUMMARY

In this study, isolates belonging to four different phylogenetic subdivisions were obtained from four different environments (i.e., Minneapolis and St. Paul distribution systems, agricultural soil and Mississippi River). The isolates obtained from drinking water systems presented the least phylogenetic diversity, suggesting that these systems primarily select for α-*Proteobacteria*. None of the α-*Proteobacteria* species isolated from tap water samples (i.e., *Afipia* sp., *Bosea* sp. and *Bradyrhizobium* sp.), however, were isolated from the Mississippi River water, which is the source water for the drinking water systems analyzed here. This suggests that drinking water systems have a unique microbial life that is adapted to low nutrient levels and presence of a disinfectant residual.

This study confirmed that the phylogenetic diversity of group I and group II *deh* genes is high and that there are even more phylogenetic subdivisions than those initially defined by Hill et al. (1999) (Figures 3.2 & 3.3). One disadvantage of the phylogenetic analysis performed here is that only short *deh* fragments were analyzed (i.e., fragments of ~270 bp for the group I of *deh* genes and fragments of ~420 bp for the group II *deh* of genes), which might not have provided enough sequence information. Moreover, although highly degenerate primers were used to amplify fragments of both groups of *deh* genes, these primers could not confirm the presence of *deh* genes in 10 out of the 35

isolates obtained in this study (Table 3.1). It is therefore unclear if these strains do not have a group I or a group II *deh* gene or whether the PCR reactions did not work. Additional experiments such as Southern analysis or genomic clone library screening would be necessary to confirm the presence or absence of *deh* genes in those strains but were not done here.

All *deh* gene fragments obtained here correspond to open reading frames and present conserved amino acids that are essential for catalysis suggesting that they encode for active enzymes, but more characterization is needed for the obtained isolates, such as performing degradation kinetic studies, to confirm whether these isolates can degrade HAAs.

The major accomplishment of this study is that the database of 2-HA degraders and *deh* genes was expanded through the isolation of a few additional genera under selective conditions for HAA degradation. Thus, *Mycobacterium* sp. was isolated from drinking water, *Pantoea* sp. from Mississippi River water, *Streptomyces* sp. from agricultural soil and *Stenotrophomonas* sp. from both Mississippi River water and agricultural soil. Moreover, additional species such as *Bradyrhizobium* sp., *Bosea* sp. and *Mycobacterium* sp. were isolated from drinking water systems where *Afipia* spp. were previously detected as the predominant HAA degraders (Zhang et al., 2009 a & b). Last but not least, a *dehII* gene was detected for the first time in a Gram positive bacterium (i.e., *Mycobacterium* sp. Minneapolis DCAA5 isolated from tap water). This gene had almost 90% nucleotide sequence similarity with the *dehII* sequence from an α-*Proteobacterium*, *Bradyrhizobium* sp. St. Paul TCAA3, suggesting that lateral gene transfer of *deh* genes across unrelated bacterial species occurred.

Chapter 4 : HALOACETIC ACID-DEGRADING BACTERIAL COMMUNITIES IN DRINKING WATER SYSTEMS AS DETERMINED BY TERMINAL RESTRICTION FRAGMENT LENGTH POLYMORPHISM (TRFLP) OF PCR-AMPLIFIED HALOACID DEHALOGENASE GENE FRAGMENTS

Haloacetic acids (HAAs) are carcinogenic disinfection byproducts formed during the chlorination of drinking water. Some evidence suggests that HAAs are susceptible to biodegradation in drinking water distribution systems, but relatively little is known about the organisms responsible for the degradation. In this study, terminal restriction fragment length polymorphism (tRFLP) of two classes of haloacid dehalogenase genes (*dehI* and *dehII*) was used to fingerprint the HAA-degrading bacterial communities in drinking water systems. Because this tRFLP method targeted relatively short gene fragments (i.e., ~270 bp for *dehI* genes and ~420 bp for *dehII* genes), *in silico* analysis was used to identify *MspI* and *Bfu*CI as the optimal restriction enzymes for discerning different *dehI* and *dehII* gene fragments, respectively. Substantial similarities were observed among the tRFLP patterns of *dehI* and *dehII* gene fragments in drinking water samples obtained from three different cities (Minneapolis, MN; St. Paul, MN and Bucharest, Romania) and from one biologically-active granular activated carbon filter (Hershey, PA). The dominant fragment in the tRFLP profiles of *dehI* genes from the drinking water samples matched the pattern from *Afipia* spp. that were previously isolated from drinking water. In contrast, the dominant fragments in the tRFLP profiles of *dehII* genes did not match any previously characterized *dehII* gene fragment.

4.1 INTRODUCTION

Haloacetic acids (HAAs) are a major class of halogenated disinfection byproducts (DBPs) formed during the chlorination of drinking water and of wastewater (Krasner et al., 2006). Due to the potential toxic and carcinogenic effects of HAA consumption (USEPA, 2003a & b; Swan et al., 1998; Swan and Waller, 1998; Kühn and Pattard, 1990), the United States Environmental Protection Agency (USEPA) began regulating HAAs in tap water in 1998 under the Stage 1 Disinfectants and Disinfection Byproducts Rule (USEPA, 1998). At present, the maximum contaminant level is 60 µg/L for the sum of five HAAs (HAA_5: monochloroacetic acid (MCAA), dichloroacetic acid (DCAA), trichloroacetic acid (TCAA) and mono- and dibromoacetic acid).

HAAs can be biodegraded by numerous bacteria isolated from soil (Olaniran et al., 2002; Olaniran et al., 2001; Hill et al., 1999), activated sludge (Kerr & Marchesi, 2006; McRae et al., 2004) and drinking water systems (Zhang et al., 2009a & b). The initial step in the HAA biodegradation pathway involves a substitutive dehalogenation reaction mediated by α-halocarboxylic acid dehalogenases, which replace the halogen atom with a hydroxyl group (Ellis et al., 2001). Two phylogenetically unrelated families of α-halocarboxylic acid dehalogenase genes, the group I and group II *deh* genes, have been identified (Hill et al., 1999). Both *deh* groups are characterized by high intra-group phylogenetic diversity (Zhang et al., 2009a; Marchesi et al., 2003; Hill et al., 1999).

Relatively little is known regarding the abundance and the diversity of HAA-degrading bacteria in drinking water systems. The isolation of HAA-degrading bacteria from water systems is very difficult and time-consuming (Zhang et al., 2009a & b) and could result in the selection of HAA degraders that are not environmentally relevant due

to the well-known cultivation bias (Marchesi et al., 2003; Dunbar et al., 1997; Amann et al., 1995). Leach et al. (2009) attempted to quantify the two *deh* gene groups in tap water samples by developing a quantitative polymerase chain reaction (qPCR) method targeting indicator bacterial strains. The authors, however, used HAA degraders isolated from wastewater as targets for qPCR. Those isolates carried only the *dehII* gene and could be detected in drinking water samples only if an additional nested PCR step was used.

The goal of the present study was to analyze both *deh* gene groups amplified from samples collected from drinking water systems by using terminal restriction fragment length polymorphism (tRFLP). tRFLP is much faster than cultivation-dependent methods and is not limited to those bacteria that are cultivable under specific laboratory conditions. tRFLP is a molecular technique that has been extensively used for analyzing the phylogenetic diversity of bacterial communities, primarily based on the amplification of 16S rRNA genes (Li et al., 2007; Sánchez et al., 2006; Blackwood et al., 2003; Brodie et al., 2002; Kitts, 2001). tRFLP has also been used to analyze the phylogenetic diversity of functional genes encoding nitrogen fixation (*nifH*) (Noda et al., 1999), mercury resistance (*merR*) (Bruce, 1997) and ammonia oxidation (*amoA*) (Horz et al., 2000). Unlike previous tRFLP methods, which were based on the amplification of nearly-complete genes, the method described here involves the amplification of short *deh* gene fragments (i.e., ~270 base pairs (bp) for *dehI* and ~420 bp for *dehII*), thus requiring careful optimization to ensure that the technique has sufficient resolution to provide useful information.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial isolates and consortia, plasmids and culturing conditions

Eight HAA-degrading isolates obtained previously from drinking water systems by Zhang et al. (2009a & b) were used here for developing a tRFLP method for *dehI* and *dehII* gene analysis. The drinking water isolates were grown at room temperature (~21°C) in mineral salt medium (Zhang et al., 2009a & b), supplemented with 1 mM HAAs (i.e., MCAA, DCAA or TCAA) until the optical density at 600 nm was 0.1. The cells were then harvested and resuspended in 1 mL lysis buffer (120 mM Na_2HPO_4 and 5 % sodium dodecyl sulfate) for DNA extraction and subsequent analysis.

Two plasmids, pYW2 carrying the cryptic *dehI*^{0} gene from *P. putida* PP3 (Hill et al., 1999) and pAWT6 carrying the *dehI* gene from *P. putida* PP3 (Thomas et al., 1992), were used as positive controls for *dehI* analysis. Three mixed consortia that were obtained by enriching wastewater activated sludge samples on MCAA, DCAA and TCAA as described by Leach et al. (2009) were also used as positive controls for applying the tRFLP method developed here on mixed bacterial communities.

Twelve new HAA-degrading isolates obtained from tap water, agricultural soil and the Mississippi River as described in the previous chapter of this thesis as well as other such isolates obtained in previous studies were used for an *in silico* tRFLP analysis as described below. All isolates and their corresponding *dehI* and the *dehII* genes that were analyzed in this study are listed in Table 4.1.

Table 4.1. List of organisms used in this study and their corresponding group I and group II *deh* genes.

Host strain	group I *deh*	group II *deh*	Reference
Isolates from drinking water systems			
Burkholderia sp. GM1		*dehII*	Zhang et al., 2009a & b
Herminiimonas sp. GM2		*dehII*	Zhang et al., 2009a & b
Afipia sp. GD1	*dehI*	*dehII*	Zhang et al., 2009a & b
Afipia sp. GTS	*dehI*	*dehII*	Zhang et al., 2009a & b
Afipia sp. EMD1	*dehI*	*dehII*	Zhang et al., 2009a
Afipia sp. P1MI		*dehII*	Zhang et al., 2009a & b
Afipia sp. Minneapolis MCAA1		*dehII*	Chapter 3
Bradyrhizobium sp. Minneapolis DCAA1		*dehII*	Chapter 3
Bosea sp. Minneapolis TCAA1		*dehII*	Chapter 3
Bosea sp. Minneapolis TCAA2		*dehII*	Chapter 3
Afipia sp. St. Paul MCAA1	*dehI*	*dehII*	Chapter 3
Afipia sp. St. Paul TCAA2	*dehI*	*dehII*	Chapter 3
Bradyrhizobium sp St. Paul TCAA3	*dehI*	*dehII*	Chapter 3
Isolates from other environments			
Afipia sp. Soil 1 DCAA6	*dehI*	*dehII*	Chapter 3
Burkholderia sp. Soil 2 MCAA6		*dehII*	Chapter 3
Pseudomonas sp. Mississippi MCAA11	*dehI*	*dehII*	Chapter 3
Stenotrophomonas sp. Mississippi MCAA16	*dehI*	*dehII*	Chapter 3
Burkholderia sp. Mississippi DCAA10	*dehI*	*dehII*	Chapter 3
Rhizobium sp.	*dehD, dehE*		Cairns et al., 1996; Stringfellow et al., 1997
Pseudomonas sp. PP3	*dehI, dehIcryptic*	*dehII*	Thomas et al., 1992; Hill et al., 1999
Achromobacter sp. ABIV	*dhlC*		Brokamp et al. 1997
Pseudomonas sp.	*deh18a*		Hill et al., 1999
Xanthobacter sp. AS1	*dehIAS1*		Marchesi & Weightman. 2003
Bradyrhizobium sp. DA3	*dehIDA3*	*dehIIDA3*	Marchesi & Weightman. 2003;Hill et al., 1999
Pseudomonas sp. AJ1	*hadD*	*hadL*	Barth et al. 1992 ; Jones et al., 1992
Pseudomonas sp.	*deh17a*		Hill et al., 1999
Pseudomonas sp. 113	*DL-DEX*		Motosugi et al., 1982
Delftia acidovorans strain B		*dehH2*	Kawasaki et al., 1992; Sota et al., 2002
Pseudomonas sp. No.109		*dehH109*	Kawasaki et al., 1994
Pseudomonas sp. YL		*L-dex*	Liu et al., 1994
Pseudomonas sp. ABVII		*dhlVII*	Unpublished
Pseudomonas sp. CBS3		*dehCI, dehCII*	Schneider et al., 1991
Xanthobacter sp. GJ10		*dh1B*	Van der Ploeg et al., 1991
Pseudomonas sp. MBA4		*hdlIVa*	Murdiyatmo et al., 1992

4.2.2 Sample collection from drinking water systems

A granular activated carbon (GAC) sample was obtained from a prechlorinated GAC filter at Hershey Water Treatment Plant (Hershey, PA, USA). This GAC filter was biologically active and actively removing HAAs (Zhang et al., 2009a). The GAC sample was shipped on ice and was placed at -20°C upon arrival. For DNA extraction, the GAC was thawed and 3 g (wet weight) were scooped from the surface, placed in 6 mL of lysis buffer and stored at -20°C until subsequent DNA extraction.

Drinking water samples were collected from different distribution systems (i.e., Bucharest, Romania, Minneapolis and Saint Paul, MN, USA) to provide a range of water quality and treatment procedures. Thus, Minneapolis and St. Paul use river water as source and a chlorination/chloramination disinfection strategy. Bucharest also uses river water combined with a chlorination disinfection strategy. Several samples were collected from the Minneapolis distribution system, at different locations along the system and at different time periods, to evaluate spatial and temporal variability of the HAA-degrading bacterial communities in one distribution system. All water samples were collected during the months of July through November when the water temperature in the distribution system was relatively high (i.e., 15 to 20°C) and the biomass was more abundant. Only planktonic biomass was collected from distribution systems because the process of collecting and filtering tap water was much easier than extracting pipes and scraping biofilm samples from the interior pipe walls. Planktonic cells, however, generally result from the growth and detachment of biofilm cells (Van der Wende et al., 1989) or from the detachment of biofilm cells under certain conditions (e.g., lower chlorine residual) (Srinivasan et al., 2008). Thus, the same predominant bacterial groups

have been detected in both biofilm and planktonic cells (Srinivasan et al., 2008). Ten to twenty liters of tap water were aseptically collected in sterile 1L polypropelene Nalgene bottles (Rochester, NY, USA) and immediately placed on ice to inhibit microbial growth. Within 30 minutes of water collection, the water samples were filtered through sterile membrane filters (47 mm diameter, 0.2 µm pore size, Millipore Corp., Billerica, MA). Each filter was placed in 1 mL of lysis buffer and stored at -20°C until subsequent DNA extraction.

4.2.3 Genomic extraction

All DNA extractions were performed with a FastDNA Spin extraction kit (MP Biomedicals LLC, Irvine, CA) according to the manufacturer's instructions. For the tap water samples, an additional purification step was performed in order to concentrate the DNA extracted from multiple membrane filters used for filtering each tap water sample.

4.2.4 Optimization of *deh* PCR conditions for tRFLP analysis

The *dehI* and *dehII* genes were amplified following the PCR methods described by Hill et al. (1999), with some modifications intended to optimize PCR product formation versus the formation of primer-dimers. The *dehI* gene was amplified using the primer set $dehI_{forI}$ (5'-ACG YTN SGS GTG CCN TGG GT-3') and $dehI_{revI}$ (5'-AWC ARR TAY TTY GGA TTR CCR TA-3'). The *dehII* gene was amplified using the primer set $dehII_{forI}$ (5'-TGG CGV CAR MRD CAR CTB GAR TA-3') and $dehII_{revI}$ (5'-TCS MAD SBR TTB GAS GAN ACR AA-3'). The forward primers were labeled with hexachlorofluorescein (HEX) for tRFLP detection. The primer concentrations were varied over a range of 0.5 µM to 2 µM to obtain the optimum value; the concentration of

$MgCl_2$ was varied over a range of 0 to 2 mM to obtain the optimum value. The optimized PCR mixtures contained 1 × GoTaq reaction buffer (Promega, Madison, WI, USA), 80 µM dNTPs, 1 µg/ µL bovine serum albumin (BSA), 1.25 U of GoTaq polymerase and ~ 1 ng of genomic DNA. The PCR programs were as described by Hill et al. (1999), except that the elongation step was performed at 72°C instead of 75°C and that the number of PCR cycles was increased from 20 to 25 for *dehI* and from 36 to 40 for *dehII.* In order to obtain sufficient PCR product for tRFLP from the drinking water samples, several PCR reactions were performed simultaneously and the pooled DNA was concentrated using a GeneCleanII kit (QBiogene, Carlsbad, CA, USA). All PCR products were initially resolved by agarose gel electrophoresis to verify that PCR products were the correct size.

4.2.5 tRFLP sample preparation and analysis

The PCR products were first purified with a GeneCleanII kit (QBiogene, Carlsbad, CA, USA) and then resolved on a 1% agarose gel along with the Lambda/*Hind*III DNA marker (Promega, Madison, WI, USA) to quantify the amount of PCR products. LabWorks Image Acquisition and Analysis Software, (ver. 4.6, UVP BioImaging Systems, Upland, CA, USA) was used to quantify light intensities.

Restriction digests were set up using about 40 to 50 ng of the purified PCR product and 10 U of either *Msp*I (i.e., for *dehI* digestion) or *Bfu*CI (i.e., for *dehII* digestion) according to the manufacturer's instructions (New England Biolabs, Ipswich, MA, USA). The restriction digest reactions were incubated overnight at 37°C. The enzymes were then heat-inactivated at 65°C (i.e., *Msp*I) or at 80°C (i.e., *Bfu*CI). Fragment analysis was performed at the Biomedical Genomics Center (BMGC) at the University of Minnesota using an ABI 3130xl capillary electrophoresis analyzer (Applied

Biosystems Inc., Foster City, CA, USA). A ROX500 standard was used to determine the fragment lengths. Electropherograms were visualized with the GeneMapper software (ver. 4.0 Applied Biosystems Inc., Foster City, CA, USA). The peak detection limit was set at 100 relative fluorescent units (RFU). The peaks having an area of less than 1% of the total peak area were excluded from the tRFLP profiles. Samples that had not been cut with restriction enzymes were also analyzed as a control to test for the quality of the PCR purification (i.e., elimination of primer-dimers). These tRFLP profiles corresponding to the undigested *dehI* PCR products contained one single peak at ~ 270 bp for all samples (data not shown), confirming that there were no primer-dimers or non-specific PCR products that could affect the interpretation of the results. For *dehII*, the primary fragment was of the expected 1ength of ~ 420 bp, but a few minor peaks were detected for some samples (i.e., with a peak area of less than 5% of the total peak area) corresponding to fragment lengths between 42 and 135 bp.

4.2.6 In silico restriction digestion analysis

Theoretical tRFLP profiles were predicted for all *dehI* and *dehII* genes presented in Table 4.1 by performing *in silico* restriction digests using the NEBcutter software (ver. 2.0, www.neb.com). Only frequent-cutting enzymes (i.e., restriction enzymes which recognize short DNA patterns, of four nucleotides) were included in this analysis.

4.2.7 Clone library

A clone library was constructed of PCR-amplified *dehII* gene fragments from the GAC sample. Purified PCR products were ligated into the pGEM-T Easy cloning vector (Promega, Madison, WI, USA) and transformed into *E. coli* JM109 (Stratagene, Cedar

Creek, TX). Plasmids were extracted and purified using the alkaline lysis procedure (Sambrook et al., 1989) from twenty colonies that were initially identified as having an insert via blue-white screening. Inserts were screened by PCR-amplifying the inserts with M13f and M13r primers and digesting the amplified fragments with *Bfu*CI. Seven inserts giving different restriction digest profiles were sequenced at BMGC using M13f and M13r as sequencing primers.

4.3 RESULTS

4.3.1 PCR optimization

The *dehI* and *dehII* genes were successfully amplified from drinking water samples following the PCR methods described by Hill et al. (1999). The amplification, however, was weak for some of the samples and generated substantial quantities of primer-dimers that interfered with the tRFLP analysis. The formation of primer-dimers was caused by the fact that the primers used for the amplification of both groups of *deh* genes were highly degenerate and used at high concentrations (i.e., 2 µM). Several attempts to remove these primer-dimers were unsuccessful using two different commercially-available kits intended for purifying PCR products (data not shown). PCR conditions, therefore, were empirically optimized to provide the necessary quantities of PCR products while avoiding interfering quantities of primer-dimers.

Thus, primer concentrations were reduced from 2 µM to 0.5 µM for the amplification of *dehI* (Figure B1, Appendix B) and from 2 µM to 1 µM for the amplification of *dehII* (data no shown). The concentrations of $MgCl_2$ were also optimized for both reactions, identifying 1 mM (final concentration) as the best concentration for amplifying *dehI* gene fragments (Figure B2, Appendix B), whereas additional $MgCl_2$

enhanced the production of primer-dimers during the amplification of *dehII* gene fragments. Finally, the addition of bovine serum albumin to a final concentration of 1 μg μL^{-1} improved the amplification of both *dehI* and *dehII* gene fragments.

4.3.2 *In silico* tRFLP profiles

An *in silico* analysis was performed to select the optimal restriction enzymes for generating tRFLP profiles for *dehI* and *dehII* gene fragments. The goal was to detect restriction enzymes generating *dehI* and *dehII* fragments of different sizes for phylogenetically different *deh* genes. This posed a challenge compared to previous studies that targeted large gene fragments (i.e., gene fragments > 1,000 bp; for examples, see refs Li et al., 2007; Blackwood et al., 2003). In this study, only small fragments of the *dehI* (~270 bp) and *dehII* (~420 bp) genes were amplified, which roughly corresponds to the rate of cutting for frequent-cutting restriction enzymes (i.e., an enzyme recognizing a four-nucleotide DNA pattern should cut, on average, every 256 nucleotides).

The theoretical terminal restriction fragment (TRF) lengths, therefore, were considered for 11 frequent-cutting restriction enzymes against 21 *dehI* gene fragments (Table 4.2) and 29 *dehII* gene fragments (Table 4.3) assuming that the forward primer was fluorescently-labeled. *Msp*I was identified as the best enzyme for tRFLP for *dehI* gene fragments because it was capable of cutting 17 out of 21 *dehI* sequences, all of which had a length greater than 35 bp (i.e., the shortest fragment length in the ROX500 size standard). *Bfu*CI was selected as the best enzyme for tRFLP for *dehII* gene fragments because it could cut 27 out of 29 *dehII* sequences, all of which were longer than 35 bp. Moreover, these two enzymes generated TRFs of different sizes for *deh* genes belonging to different phylogenetic subgroups or organisms. A similar analysis was performed,

assuming that the reverse primer was fluorescently-labeled, but this approach failed to produce better results, often because many of the predicted terminal fragment lengths were less than 35 bp.

Table 4.2. PCR fragment length (in bp) of *dehI* genes and *in silico* prediction of the TRF sizes (in bp) resulting from the enzymatic digestion of the *dehI* PCR fragments. Only the TRFs corresponding to the forward PCR primer are provided. The TRFs generated by the restriction enzyme that was selected for tRFLP profiles are shown in bold. Phylogenetic subgroups are as determined in Chapter 3 (Figure 3.2).

Group I *deh* gene	**PCR fragment**	**Phylogenetic *sub*group**	***Aci*I**	***Alu*I**	***Bfu*CI**	***Bst*UI**	***Hae*III**	***Mse*I**	***Msp*I**	***Nla*III**	***Rsa*I**	***Taqα*I**	***Tsp* 509I**
dehE	272	A	78	272	272	8	76	236	**194**	96	11	33	272
***dehI*-PP3**	272	A	78	129	272	8	272	172	**272**	74	187	200	212
dhlC	272	A	78	129	272	8	272	172	**272**	74	187	200	212
deh18a *	272	A	78	129	272	134	272	172	**272**	74	187	200	212
dehIAS1 *	272	B	33	183	118	35	156	73	**64**	272	149	142	54
dehIDA3 *	274	B	121	274	123	35	274	245	**64**	165	274	97	46
***dehI*-Soil 1 DCAA6**	272	B	88	272	123	22	272	245	**64**	272	272	215	54
***dehI*-GTS**╬	272	B	52	183	123	35	79	188	**151**	42	272	272	138
***dehI*-GD1**╬	272	B	77	183	123	272	76	272	**152**	165	272	97	46
***dehI*-EMD1**╬	272	B	77	272	123	70	76	272	**152**	17	272	97	46
***dehI*-St. Paul MCAA1**╬	272	B	52	183	123	35	79	188	**151**	42	272	272	138
***dehI*-St. Paul TCAA2**╬	272	B	52	183	123	35	79	188	**151**	42	272	272	138
***dehI*-St. Paul TCAA3**╬	272	B	52	183	123	35	79	188	**151**	42	272	272	138
***dehI*-Mississippi DCAA10**	272	B	33	183	92	35	155	272	**151**	42	272	50	272
DL-DEX	272	B	6	222	29	35	166	224	**163**	17	62	50	272
***dehI*-Mississippi MCAA11**	281	C	37	150	134	85	54	281	**51**	74	281	65	281
***dehI*-Mississippi MCAA16**	281	C	37	150	134	85	54	281	**51**	74	281	65	281
hadD	280	C	37	149	133	280	54	280	**51**	74	280	109	280
***dehIcryptic*-PP3**	281	C	37	150	134	85	54	281	**51**	74	281	65	281
dehI17a *	281	C	37	150	134	85	54	281	**51**	74	281	65	281
dehD	278	D	120	278	167	51	278	278	**278**	223	96	5	278

* Genes missing short sequences from the 5'-end or/and the 3'-end of the PCR fragment. It was assumed that there were no restriction sites in those fragments.
╬ *dehI* genes from drinking water system isolates.

Table 4.3. PCR fragment length (in bp) of *dehII* genes and *in silico* prediction of the TRF sizes (in bp) resulting from the enzymatic digestion of the *dehII* PCR fragments. Only the TRFs corresponding to the forward PCR primer are provided. The TRFs generated by the restriction enzyme that was selected for tRFLP profiles are shown in bold. ND- not determined. Phylogenetic subgroups are as determined in Chapter 3 (Figure 3.3).

Group II *deh* gene	**PCR fragment**	**Phylogenetic *sub*group**	***AciI***	***AluI***	***BfuCI***	***BstUI***	***HaeIII***	***MseI***	***MspI***	***NlaIII***	***RsaI***	***TaqαI***	***Tsp 509I***
***dehII*-GM2**[ǂ]	422	A	3	117	**45**	70	361	422	422	98	323	62	57
L-dex	422	A	3	15	**114**	82	422	56	99	29	22	17	422
***dehII*-Mississippi DCAA10**	422	A	3	15	**227**	82	70	422	175	136	22	62	422
dehH2	422	A	33	422	**227**	384	133	422	175	45	52	17	422
***dehII*-GM1**[ǂ]	422	A	3	111	**241**	132	422	422	74	399	52	62	422
***dehII*-Soil 2 MCAA6**	422	A	3	15	**241**	132	422	422	39	399	52	62	422
***dehII*-PP3**	422	A	4	185	**255**	191	216	422	422	376	422	63	286
hadL	422	A	3	184	**254**	347	47	422	422	45	422	119	13
dhlVII	422	A	3	184	**254**	190	215	422	422	375	422	62	285
***dehII*-Mississippi MCAA16**	422	A	3	15	**254**	190	215	422	422	375	422	62	285
***dehII*-Mississippi MCAA11**	422	A	3	15	**254**	190	215	422	422	375	422	62	285
dehH109	422	A	48	15	**305**	187	47	422	175	45	422	17	422
dehCII	422	A	3	15	**422**	103	361	107	422	419	22	194	227
hdlIVa	422	B	33	15	**117**	94	128	422	138	365	154	17	160
dehCI	422	B	33	15	**126**	33	110	203	212	29	189	17	422
***dehII*-St. Paul MCAA1**[ǂ]	416	C	3	15	**68**	75	110	416	272	29	22	119	416
***dehII*-St. Paul TCAA2**[ǂ]	416	C	3	15	**68**	75	110	416	272	29	416	119	416
***dehII*-St. Paul TCAA3**[ǂ]	416	C	3	15	**68**	75	115	416	175	105	416	134	267
***dehII*-P1MI**[ǂ]	416	C	3	216	**135**	77	275	416	175	147	416	134	416
dehIIDA3[*]	416	C	210	216	**135**	170	110	326	99	147	416	119	416
***dehII*-Soil 1 DCAA6**	420	C	3	15	**135**	190	110	420	272	147	154	278	284
***dehII*-Minneapolis MCAA1**[ǂ]	416	C	3	15	**165**	75	110	416	272	147	22	119	416
***dehII*-GTS**[ǂ]	416	C	3	15	**165**	75	110	416	272	29	22	119	416
***dehII*-Minneapolis DCAA1**[ǂ]	416	C	3	15	**221**	130	88	416	175	147	22	134	416
dh1B	416	D	3	15	**416**	35	88	416	118	249	365	290	416
***dehII*-Minneapolis TCAA2**[ǂ]	416	ND	3	15	**45**	35	79	416	175	249	22	86	416
***dehII*-GD1**[ǂ]	416	ND	3	15	**104**	75	199	416	175	42	22	134	370
***dehII*-Minneapolis TCAA1**[ǂ]	416	ND	3	15	**140**	35	47	416	138	249	22	86	416
***dehII*-EMD1**[ǂ]	415	ND	3	15	**163**	75	108	415	173	42	22	132	415

[*] Genes missing short sequences from the 5'-end or/and the 3'-end of the PCR fragment. It was assumed that there were no restriction sites in those fragments.
[ǂ] *dehII* genes from drinking water system isolates.

4.3.3 Experimental tRFLP profiles

tRFLP was performed on *dehI* gene fragments (Figure 4.1) and *dehII* gene fragments (Figure 4.2) amplified from selected haloacetic acid-degrading bacterial isolates, up to three different enrichment cultures, five tap water samples and the biologically active GAC filter from the Hershey drinking water treatment facility.

The tRFLP profiles of *dehI* gene fragments matched the predicted profiles for all of the analyzed drinking water isolates (i.e., *Afipia* sp. strains GTS, GD1 and EMD1) and for the two *Pseudomonas putida dehI* genes carried by the pYW2 and pAWT6 plasmids (Figure 4.1 and Table 4.2). Only the tRFLP profile for the isolate GD1 is shown in Figure 4.1 because it was identical to the profiles of the other two drinking water isolates (i.e., all tRFLP profiles consisted in one peak corresponding to a fragment length of ~150 bp). A single TRF of ~150 bp was also detected in a culture enriched on TCAA (note: *dehI* genes were not detected in cultures enriched on MCAA or DCAA), in which *Afipia* sp. was predicted to be present through a *dehII* indicator-specific PCR amplification (Leach et al. 2009).

Four tap water samples collected from the Minneapolis, MN and St. Paul, MN distribution systems as well as the biologically-active GAC filter revealed similar tRFLP profiles containing one prominent peak at ~150 bp, suggesting that *dehI* genes similar to those detected in numerous *Afipia* spp. were also common in these samples (Figure 4.1). This finding was also supported by the *in silico* analysis of the *dehI* genes from two new *Afipia* isolates from drinking water (i.e., *Afipia* sp. St. Paul MCAA1 and *Afipia* sp. TCAA2), which predicted a similar tRFLP profile consisting in one TRF of 151 bp (Table 4.2).

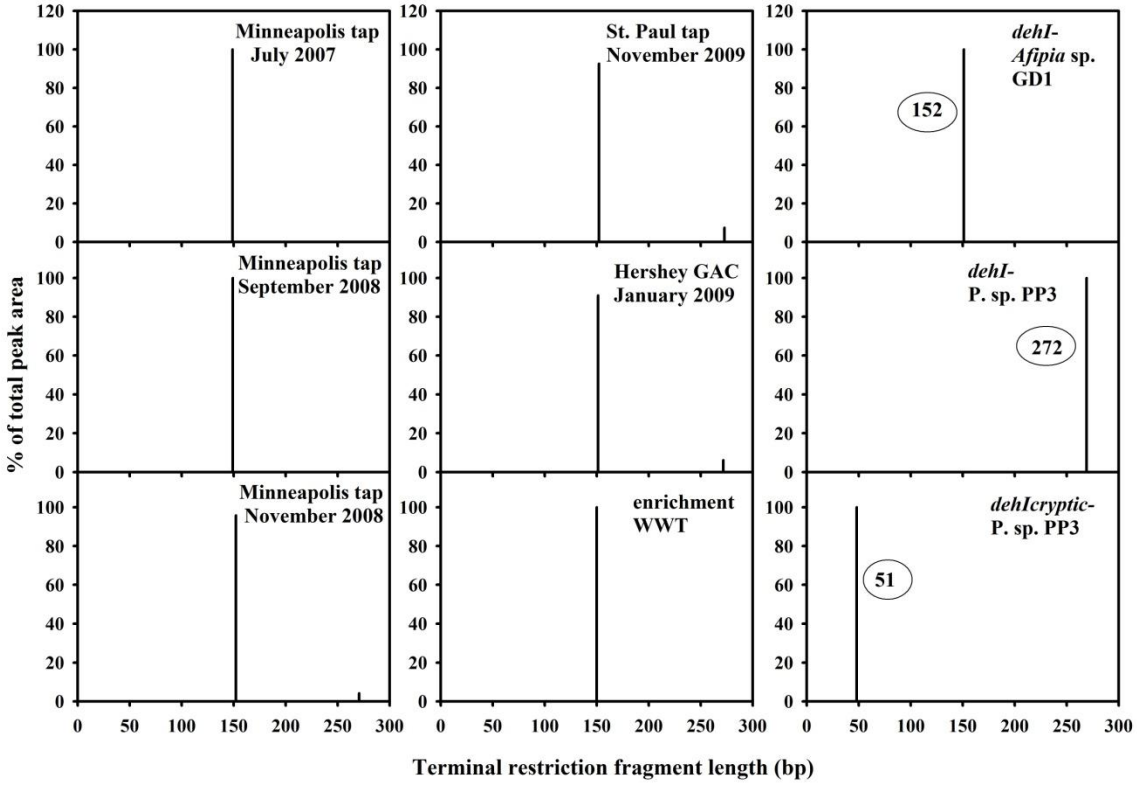

Figure 4.1. Terminal restriction fragment length profiles of *dehI* fragments obtained from drinking water systems, one wastewater enrichment culture and selected bacterial isolates, using *Msp*I. Tap water samples are labeled as "Minneapolis July 2007", "Minneapolis September 2008", "Minneapolis November 2008" and "St. Paul November 2009". Sample "Hershey GAC" represents a sample collected from a biologically-active granular activated carbon filter used to treat drinking water. Sample "enrichment WWT" was a culture enriched from municipal wastewater on trichloracetic acid. The rest of the samples are bacterial isolates that are described in more detail in Table 4.1. Circled numbers represent the expected TRF sizes as predicted in Table 4.2.

Some of the four analyzed tap water samples as well as the GAC filter sample also had a second, minor peak of a length of ~270 bp, corresponding to the length of the original PCR product. Although this could have been the result of incomplete digestion with the restriction enzyme, a peak of this length likely reflects the presence of *dehI* genes from organisms without a target site, as shown in Table 4.2.

The tRFLP profiles of *dehII* gene fragments of the drinking water isolates analyzed in this study (i.e., *Burkholderia* sp. strain GM1, *Herminiimonas* sp. strain GM2 and *Afipia* sp. strains GD1, GTS and P1MI), contained fragments predicted by the *in silico* analysis (Table 4.3), but also other fragments that were not predicted (Figure 4.2). The tRFLP profiles of the undigested *dehII* gene fragments amplified from these isolates consistently had only one peak of the expected size (i.e., ~420 bp), which excludes the possibility of nonspecific fragment amplification as a cause for the presence of unpredicted fragments on the digested tRFLP profiles. Multiple fragments were also detected in three different cultures enriched on MCAA, DCAA, or TCAA, each of which produced a unique *dehII* tRFLP profile (Figure 4.2). Some of the peaks detected in these samples could be explained by the presence of certain bacterial species that were previously identified by Leach et al. (2009). Thus, based on the *in silico* predictions from Table 4.3, the presence of *Afipia* sp. in the TCAA-enriched sample (WWT) could explain the detection of the ~165 bp and the ~100 bp peaks on the tRFLP profile. Similarly, the presence of *Pseudomonas* sp. in the MCAA and DCAA-enriched samples (WWM and WWD) could explain the detection of the ~300 bp peaks on the corresponding tRFLP profiles. Other peaks, however, were present in these *dehII* tRFLP profiles, which likely reflect the presence of other HAA degraders.

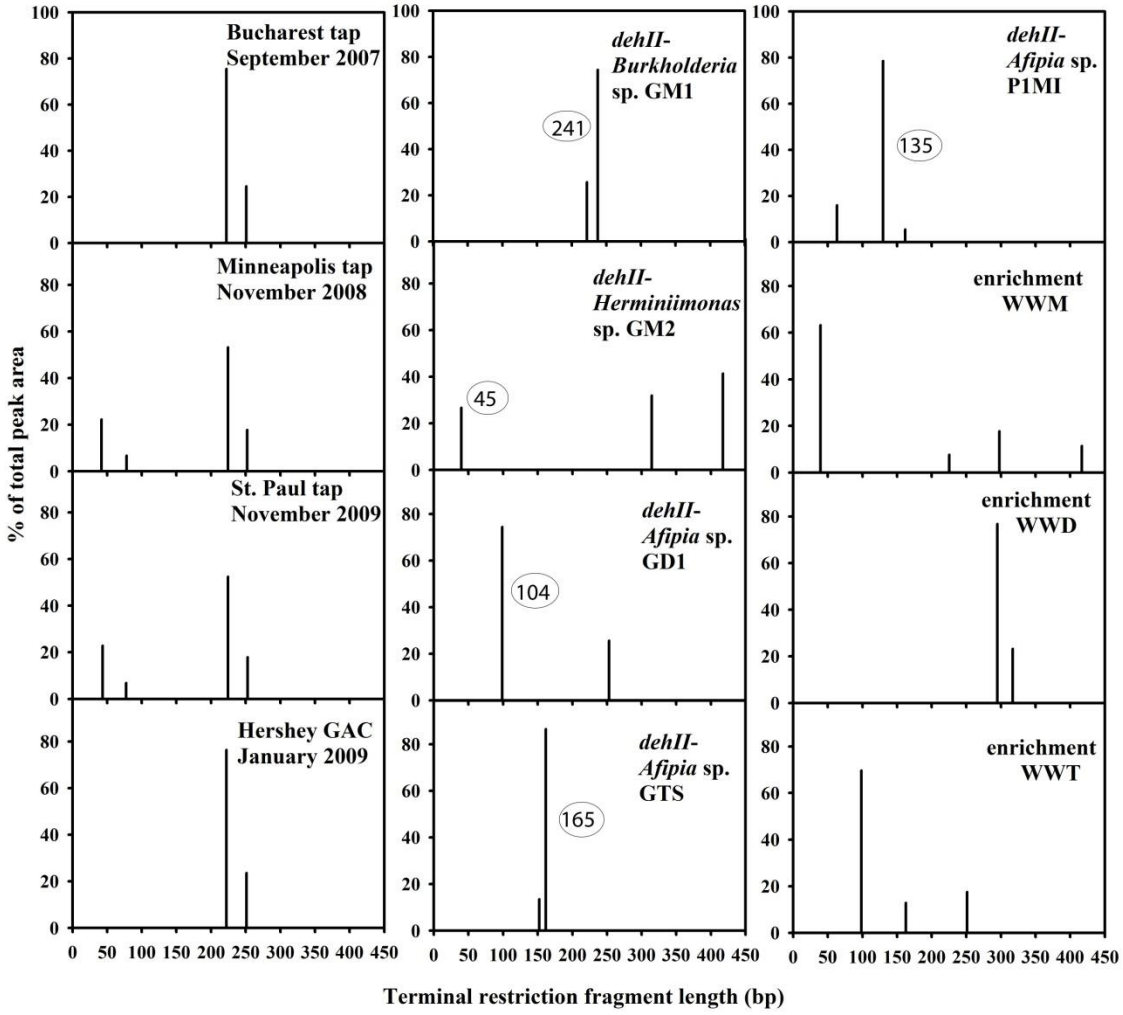

Figure 4.2. Terminal restriction fragment length profiles of *dehII* fragments obtained from drinking water systems, wastewater enrichment cultures and selected bacterial isolates, using *Bfu*CI. Tap water samples are labeled as "Bucharest September 2007", "Minneapolis November 2008" and "St. Paul November 2009". Sample "Hershey GAC" represents a sample collected from a biologically-active granular activated carbon filter used to treat drinking water. Samples "enrichment WWM", "enrichment WWD" and "enrichment WWT" are cultures enriched from municipal wastewater on monochloroacetic acid, dichloroacetic acid and trichloracetic acid. The rest of the samples are bacterial isolates that are described in more detail in Table 4.1. Circled numbers represent the expected TRF sizes as predicted in Table 4.3.

Curiously, the tRFLP profiles of *dehII* gene fragments from different drinking water systems (i.e., the tap water samples from Bucharest, Minneapolis and St. Paul and the GAC sample from Hershey) had very similar patterns consisting of two major peaks

at fragment lengths of ~225 bp and ~250 bp (Figure 4.2). These profiles, however, did not match any of the profiles of drinking water isolates and enrichment cultures analyzed here. These profiles also did not match the *in silico* profiles predicted for seven new isolates obtained from the water collected in St. Paul and Minneapolis (Table 4.3 and Figure 4.2). Based on Table 4.3, TRFs of ~225 bp and ~250 bp were predicted for strains of *Burkholderia*, *Delftia*, *Pseudomonas* and *Stenotrophomonas*, but which were isolated from other environments than drinking water systems. The profiles from the Minneapolis and St. Paul water samples also contained two minor peaks at ~45 bp (~20% of the total) and ~78 bp (~5 % of the total). The first of these peaks matches the *in silico* prediction for *Herminiimonas* sp. GM2, whereas no bacterial isolate in our collection generated a *dehII* fragment of 78 bp.

To characterize the major peaks from the *dehII* fingerprints from drinking water samples, a clone library was constructed of *dehII* gene fragments amplified from the biologically-active GAC sample. Seven inserts from this clone library were sequenced, six of which matched known *dehII* genes from GenBank. The theoretical tRFLP predictions for those six *dehII* sequences gave TRFs of 165 bp, 221 bp and 227 bp. No TRF of ~250 bp was found in this clone library that could explain the presence of the second major peak in the tRFLP profile of the GAC sample (Figure 4.2). A TRF of ~165 bp was predicted for the clone library but was not detected in the tRFLP profile of the GAC sample (Figure 4.2), suggesting that the organisms giving the respective TRF were not predominant species. Given the fact that the resolution of tRFLP profiles is not perfect, resulting in detected fragments that could be a few nucleotides away from the theoretical predictions, the major ~225 bp peak detected in the tRFLP profile of the GAC

sample most likely corresponds to either the 221 bp or the 227 bp fragments that were predicted from the clone library. The *dehII* insert from the clone library that generated a predicted TRF of 221 bp best matched the *dehII* gene fragment from the isolate *Bradyrhizobium* sp. Minneapolis DCAA1, with 75% nucleotide sequence similarity. Three *dehII* inserts from the clone library that had a predicted TRF of 227 bp had less than 68% gene identity with any of the *dehII* genes analyzed here. The best match was with the *dehII* gene fragments from *Burkholderia* sp. strain Mississippi DCAA10 and *Delftia acidovorans*. These findings suggest that the most prominent *dehII* gene fragments in the drinking water samples analyzed in this study, which generated TRFs of ~225 bp and ~250 bp, belong to novel organisms that have yet to be cultivated.

4.4 DISCUSSION

In this work, the tRFLP technique was successfully optimized to fingerprint *dehI* and *dehII* genes and was validated with single isolates and mixed cultures (i.e., cultures enriched on MCAA, DCAA and TCAA). The optimized tRFLP technique was capable of distinguishing between the major phylogenetic subgroups of *deh* genes (as described in Tables 4.2 and 4.3), demonstrating that relatively short PCR products (i.e., ~270 bp for *dehI* and ~420 bp for *dehII*) are suitable for this technique as long as there is sufficient sequence variation for fingerprint analysis. Conversely, the application of tRFLP to such small gene fragments was necessitated by the relatively high variation in haloacid dehalogenase gene sequences, which limits the number of conserved targets for which PCR primers could be designed (Hill et al., 1999). Even so, the successful development of the tRFLP approach described herein required careful consideration of the known *deh* gene sequences and their respective restriction enzyme targets.

The primary goal of this study was to develop a method to fingerprint HAA-degrading bacteria in drinking water systems. Although HAAs may be toxic and/or carcinogenic and are regulated in drinking water by the USEPA, there is relatively little known about their fate in drinking water distribution systems. In some cases, HAA concentrations decline as a function of distance in a water distribution system (LeBel et al., 1997), suggesting that *in situ* biodegradation might be an important mechanism to suppress HAA concentrations. The HAA loss due to biodegradation can be evaluated if information is available about the HAA-degrading biomass in water systems (Grigorescu and Hozalski, 2010). A previous study by Leach et al. (2009) showed that in order to quantify the HAA-degrading biomass in drinking water systems it is needed to determine the major HAA degraders in these systems that could be used as indicator strains. The tRFLP method developed herein, therefore, provides a useful tool to better understand HAA-degrading bacteria in drinking water systems and their population dynamics.

The present research makes a substantial advance in our knowledge of HAA-degrading bacteria in drinking water. Our present results suggest, albeit based on a relatively small number of samples, that different drinking water systems contain the same few *dehI* and *dehII* genes regardless of the time of year or of geographic location.

The *dehI* gene amplified from different tap water samples (i.e., from Minneapolis and St. Paul) and one GAC sample (i.e., from Hershey, PA) presented a similar pattern consisting in one peak corresponding to a TRF of ~150 bp. Moreover, there was no shift in the tRFLP pattern over time (i.e., from July 2007 to November 2008) and space (i.e., each sample was collected at a different location along the distribution system) for the Minneapolis distribution system (Figure 4.1), suggesting that distribution systems select

for particular *dehI* genotypes. The ~150 bp TRF was also detected in four *Afipia* spp. isolated from drinking water systems and in one enrichment culture sample in which *Afipia* sp. was predicted to be present (Figure 4.1), suggesting that *Afipia* spp. could be predominant HAA degraders in drinking water systems. A TRF of ~150 bp, however, was also theoretically predicted for *dehI* gene fragments from other bacterial strains such as *Bradyrhizobium* sp. St. Paul TCAA3 and *Burkholderia* sp. Mississippi DCAA10 (Table 4.2). Thus, more work is needed to determine whether other species generating a similar tRFLP pattern with that detected for *Afipia* spp. could be important HAA degraders in drinking water systems.

The *dehII* gene amplified from drinking water systems (i.e., Bucharest, Minneapolis and St. Paul tap water samples and Hershey GAC samples) also presented a pattern (i.e., a ~225 bp peak and a ~250 bp peak), which was not detected in the drinking water isolates or the wastewater enrichment cultures analyzed here. This suggests that the HAA degraders carrying *dehII* genes that were isolated by a cultivation-based method (Zhang et al., 2009a) are not the predominant species in drinking water systems. The tRFLP profiles corresponding to single isolates contained more than one expected peak, thus indicating that HAA degraders usually carry multiple *dehII* gene copies. Schneider et al. (1991) described the presence of two *deh* genes on the chromosome of *Pseudomonas* sp. CBS3 that were later classified as *dehII*-type genes (Hill et al., 1999). Six *dehII* genes were identified in the recently annotated genome sequence of an *Afipia* sp. isolated from subsurface sediments contaminated with mixed radioactive and nitrate waste (Green et al., 2010; GenBank NZ_ADVZ00000000). The predicted tRFLP profile of these six *dehII* genes consisted of four peaks corresponding to fragment sizes of 120

bp, 255 bp, 302 bp and 419 bp (Figure 4.3). The same *Afipia* isolate had only one *dehI* gene giving a predicted TRF of 152 bp, similar to those identified in the drinking water *Afipia* isolates. This indicates that it was reasonable to expect the presence of multiple *dehII* genes with high phylogenetic diversity for single isolates. The presence of multiple peaks on the *dehII* tRFLP profiles corresponding to drinking water samples could thus be related either to the presence of several predominant HAA degraders in these systems or to the presence of a single predominant HAA degrader carrying multiple copies of *dehII* genes. These HAA degraders are yet to be cultivated.

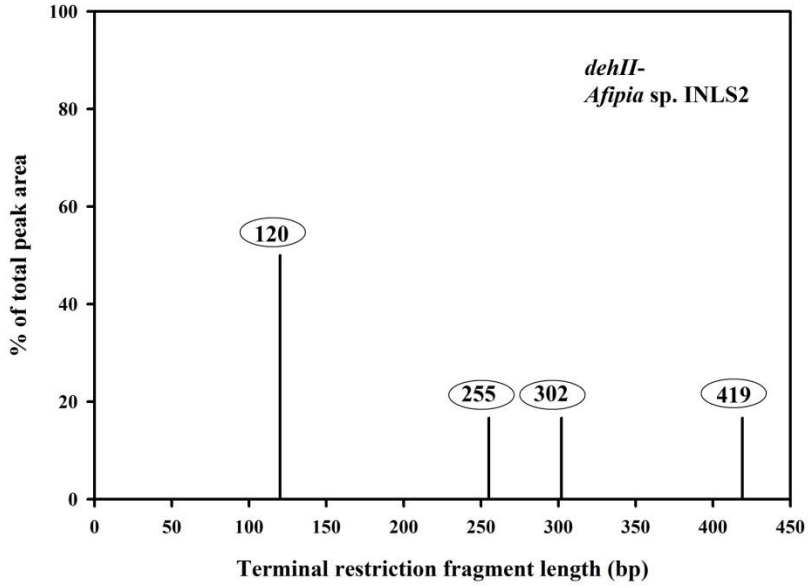

Figure 4.3. Predicted terminal restriction fragment length profile of *dehII* gene fragments from *Afipia* sp. INLS2, using *Bfu*CI. Circled numbers represent the predicted TRF sizes.

Both groups of *deh* genes could not always be amplified from each drinking water sample. For example, the *dehI* gene was not detected in the tap water sample from Bucharest (Figure 4.1). The *dehII* gene, on the other hand, was detected in all water

samples analyzed here, but the tRFLP analysis failed for a few of the Minneapolis tap water samples due to a very weak amplification of this gene (Figure 4.2). The *dehII* gene produced a longer PCR fragment (~420 bp compared to only ~270 bp for *dehI*), thus making it a better target for fingerprinting the HAA-degrading bacterial communities in drinking water systems. The tRFLP profiles generated in this study, however, suggest that it is easier to analyze the fingerprints for the *dehI* gene because it gives more consistent results with the theoretical predictions. Moreover, the results of this tRFLP study showed that the HAA degraders in drinking water systems that carry *dehI* genes are different bacterial species than those carrying *dehII* genes. This study, therefore, pointed out that both *dehI* and *dehII* genes provide important information about the HAA degraders in drinking water systems that should be considered when developing quantitative methods for estimating the total HAA-degrading biomass in these systems.

The tRFLP method described here is relatively fast and easy but has some limitations. One limitation is that the highly degenerate PCR primers used to amplify *dehI* and *dehII* genes generated some nonspecific fragment amplification for some samples, which was also demonstrated by the detection of an insert in the GAC clone library that did not match any known *dehII* sequence. Most of the tRFLP peaks generated by nonspecific amplification, however, were not greater than 1% of the total signal and were excluded from the analysis. Another limitation is that the tRFLP profiles corresponding to mixed bacterial communities are sometimes difficult to interpret because *deh* genes from different organisms can generate the same pattern (e.g., the *dehI* gene fragments from *Afipia* sp., *Burkholderia* sp. and *Bradyrhizobium* sp. give the same theoretical TRF of ~150 bp with *Msp*I (Table 4.2)). However, other restriction enzymes

could further be used for distinguishing between different bacterial species. For example, the *dehI* gene fragment from *Burkholderia* sp. gives a different predicted TRF with *Hae*III than those generated by the *dehI* gene fragments from *Afipia* sp. and *Bradyrhizobium* sp. (Table 4.2).

In summary, the present study demonstrated that an optimized tRFLP technique was capable of fingerprinting both of the known classes of haloacid dehalogenase genes in drinking water systems. The tRFLP profiles of *dehI* gene fragments indicated that *Afipia* spp., many of which have been previously isolated, are likely to be the predominant haloacetic acid-degrading bacteria carrying *dehI* genes in drinking water systems. In contrast, the tRFLP profiles of *dehII* gene fragments indicated that the predominant haloacetic acid-degrading bacteria carrying *dehII* genes have yet to be isolated.

Chapter 5 : MODELING HALOACETIC ACID BIODEGRADATION IN BIOFILTERS AND WATER DISTRIBUTION FILTERS

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A 1-dimensional plug flow reactor equation with pseudo-first order biodegradation kinetics was used to model the fate of haloacetic acids (HAAs) in biologically-active filters and water distribution systems. Overall, the model calculations suggest that biodegradation is likely to lead to significant HAA removals in biologically-active filters but not in most distribution systems. Relatively high total biomass densities (i.e., > 10^5 cells/cm^2) are needed for significant HAA removals (i.e., > 10%) in both systems. Such biomass densities are unlikely in U.S. distribution systems where relatively high total chlorine residuals inhibit the development of biofilm on the pipe walls. Biodegradation of HAAs is possible in distribution systems with intentionally low total chlorine residuals (as in some European systems) or where the residual has been depleted, such as high residence time locations or dead ends. Biologically-active filters can be very efficient at removing HAAs, however, because these filters typically contain relatively high biomass densities.

5.1 INTRODUCTION

Haloacetic acids (HAAs) represent a major class of halogenated disinfection byproducts (DBPs) among several hundred DBPs formed as a result of the chlorination of water and wastewater (Krasner et al., 2006). Because of the potential toxic and carcinogenic effects of HAA consumption (Swan et al., 1998; Swan and Waller, 1998; Kühn and Pattard, 1990), the US Environmental Protection Agency began regulating HAAs in tap water in 1998 (USEPA, 1998). Currently, the maximum contaminant level (MCL) is 60 µg/L for the sum of five HAAs or HAA_5 (i.e., monochloroacetic acid

(MCAA), dichloroacetic acid (DCAA), trichloroacetic acid (TCAA), monobromoacetic acid (MBAA) and dibromoacetic acid (DBAA)).

HAAs in tap water result from HAAs formed in the water treatment plant or the distribution system due to reactions between residual chlorine and organic matter and the hydrolysis of other DBPs (e.g., haloacetonitriles) (Li et al., 2000; Glezer et al., 1999). There is also evidence that HAAs are degraded in water treatment systems such as biologically-active filters (Xie and Zhou, 2002; Zhou and Xie, 2002) and in water distribution systems (Tung and Xie, 2009; Sung et al., 2000; Chen and Weisel, 1998; LeBel et al., 1997; Williams et al., 1997).

HAAs undergo abiotic reductive dehalogenation in the presence of zero valent iron (Zhang et al., 2004; Hozalski et al., 2001) or iron minerals (Chun et al., 2007), hydrolysis (i.e., decarboxylation) (Zhang and Minear, 2002), and biodegradation (Zhang et al., 2009a & b; McRae et al., 2004). Decarboxylation, however, is generally slow at room temperature and neutral pH (the half-life of TCAA is 2,190 days according to Zhang and Minear, 2002) and reduction via zero valent iron is only possible in unlined cast or ductile iron water mains and mainly for trihalogenated HAAs containing one or more bromine atoms (Zhang et al., 2004; Hozalski et al., 2001). Thus, aerobic biodegradation is believed to be the dominant HAA degradation process in natural waters (Ellis et al., 2001), soils (Matucha et al., 2003), biologically-active sand or granular activated carbon (GAC) filters (Kim and Kang, 2008; Tung et al., 2006; Wu and Xie, 2005; Xie and Zhou, 2002; Zhou and Xie, 2002), and water distribution systems (Tung and Xie, 2009; Zhang et al., 2009a & b; Bayless and Andrews, 2008).

Information concerning aerobic HAA biodegradation (i.e., pathways and kinetics) largely comes from work with enrichment cultures and isolates obtained from soil and wastewater environments. Several HAA-degrading bacterial strains such as *Burkholderia* sp., *Bradyrhizobium* sp. and *Pseudomonas* sp., have been isolated from soil (Hill et al., 1999) and other strains such as *Xanthobacter* sp., *Sphingomonas* sp. and *Chrysobacterium* sp. were isolated from activated sludge (McRae et al., 2004). All HAA degraders isolated so far belong to the group of *Proteobacteria.* The initial step in the HAA biodegradation pathway is a substitutive dehalogenation in which the halogen atom is replaced by a hydroxyl group (Ellis et al., 2001).

Recent efforts in the authors' research group have been targeted at characterizing HAA-degrading bacteria from drinking water treatment and distribution systems (Zhang et al., 2009a & b; Leach et al., 2009). Zhang and colleagues (2009b) generated HAA-degrading enrichment cultures and isolated several novel HAA-degrading bacterial strains from drinking water systems including *Afipia* spp. The isolates showed a wide range of HAA biodegradation kinetics (Table 5.1), with the fastest DCAA degrader, *Afipia felis* EMD2, having a 27 times greater affinity for DCAA than the slowest DCAA degrader, *Methylobacterium fujisawaense* PAWDI. The enrichment cultures generally had higher substrate affinities and biodegradation potentials than the isolates (Zhang et al., 2009b).

In this work, computer simulations were performed to predict the fate of three HAAs (MCAA, DCAA and TCAA) along a water distribution system and within a biologically-active filter. Sensitivity analyses were performed to investigate the effects of

physical parameters (e.g., fluid velocity) and biological parameters (e.g., biodegradation kinetics, biomass density) on HAA removal.

Table 5.1. Summary of kinetic parameter values used in the model calculations

Enrichment culture or isolate	Bacterial source*	k (µg HAA/ µg protein/ day)	K_M (µg/L)	k_r (L/ day/µg protein)
EXPERIMENTALLY DETERMINED VALUES **				
MCAA kinetics				
WWM	AS enriched on MCAA	8.4 ± 1.5	97.7 ± 44.8	0.086
DCAA kinetics				
WWD	AS enriched on DCAA	47.0 ± 1.8	26.5 ± 5.6	1.77
PAWD	PA tap water enriched on DCAA	9.36 ± 0.48	7.79 ± 5.82	1.2
PAWDI	Isolate from PAWD on DCAA	6.48 ± 0.48	77.91 ± 16.14	0.083
EMD2E	UK tap water enriched on DCAA	32.88 ± 0.72	4.38 ± 2.22	7.51
EMD2	Isolate from EMD2E on DCAA	23.28 ± 0.72	10.42 ± 3.61	2.23
TCAA kinetics				
WWT	AS enriched on TCAA	6.6 ± 0.6	210.7 ± 37.9	0.03
ESTIMATED VALUES ***				
MCAA kinetics				
PAWM	MCAA-degrading enrichment	-	-	0.0588
PAWMI	MCAA-degrading isolate	-	-	0.0041
EMM2E	MCAA-degrading enrichment	-	-	0.37
EMM2	MCAA-degrading isolate	-	-	0.11
TCAA kinetics				
PAWT	TCAA-degrading enrichment	-	-	0.0204
PAWTI	TCAA-degrading isolate	-	-	0.0014
EMT2E	TCAA-degrading enrichment	-	-	0.13
EMT2	TCAA-degrading isolate	-	-	0.038

***** Abbreviations: AS-activated sludge, PA-Pennsylvania, UK-United Kingdom

****** The average k and K_M values were used for calculating the k_r values.

******* The k_r values were calculated by multiplying the calculated k_r values for the DCAA-enrichment cultures and isolates by $k_{r,WWM}/k_{r,WWD}$ (0.049) or by $k_{r,WWT}/k_{r,WWD}$ (0.017).

5.2 METHODS

5.2.1 Model development

The loss of HAAs in distribution systems and in biologically-active filters was modeled using the integrated form of the 1-dimensional plug flow reactor equation:

$$\frac{C_x}{C_{x=0}} = \exp\left(-\frac{k_{overall}x}{u}\right) \quad (1)$$

in which C_x is the HAA concentration at location x (µg HAA/L), $C_{x=0}$ is the influent HAA concentration (µg HAA/L), x is distance (miles), u is the water velocity (ft/s), and $k_{overall}$ is the overall reaction rate constant (s^{-1}). For a packed bed, u is the pore velocity (m/h) which is related to the filtration rate (v, m/h) and the bed porosity (ε, %) according to Eq 2:

$$u = \frac{v}{\varepsilon} \quad (2)$$

For these calculations, the authors assumed that biodegradation by planktonic bacteria was negligible so that HAA biodegradation occurred only in biofilms. This heterogeneous reaction is governed by two processes occurring in series: mass transfer of the solutes (i.e., HAAs) from the bulk liquid to the biofilm and biodegradation within the biofilm. Therefore, the overall reaction rate constant ($k_{overall}$) can be calculated as in Eq 3:

$$k_{overall} = \frac{1}{\frac{1}{k_{ma}} + \frac{1}{k_{ra}}} \quad (3)$$

in which k_{ma} (s^{-1}) is the mass transfer rate constant (k_m, m/s) multiplied by the specific surface area (i.e., the surface area per unit volume) (a, m^{-1}) and k_{ra} (s^{-1}) is the pseudo-first order biodegradation rate constant (k_r, Table 5.1) multiplied by the biofilm density and by

the specific surface area. The expressions for computing the specific surface areas for a pipe and packed bed of spherical media are given in Eqs 4 and 5, respectively:

$$\text{Pipe: } a = \frac{4}{d} \tag{4}$$

$$\text{Packed bed: } a = \frac{6(1-\varepsilon)}{d_p} \tag{5}$$

in which d is the pipe diameter (inches), d_p is the grain size (mm) and ε is the bed porosity (%).

The mass transfer rate constants for the distribution system and filter were estimated using Sherwood number (*Sh*) correlations for a smooth pipe and packed bed, respectively (Crittenden et al., 2005; Zhang et al., 2004). Table 5.2 summarizes the equations used to compare the mass transfer rate constants for the distribution system and the biologically active filter.

Table 5.2. Summary of equations used to compute the mass transfer rate constants for the distribution system and biologically-active filter

Distribution system	**Biologically-active filter**	**Notation**
$Sh = 0.023\text{Re}^{0.83} Sc^{0.33}$ $k_m = \frac{ShD_w}{d}$ $Sc = \frac{\mu_w}{\rho_w D_w}$ $\text{Re} = \frac{du\rho_w}{\mu_w}$	$Sh = 1.09\varepsilon^{-2/3}\text{Re}^{1/3} Sc^{1/3}$ $k_m = \frac{ShD_w}{d_p}$ $Sc = \frac{\mu_w}{\rho_w D_w}$ $\text{Re} = \frac{d_p v\rho_w}{(1-\varepsilon)\mu_w}$	d = pipe diameter d_p = filter media grain diameter D_w = solute diffusion coefficient in water k_m = mass transfer rate constant Re = Reynolds number Sc = Schmidt number Sh = Sherwood number u = water flow velocity v = filtration rate ε = bed porosity μ_w = water viscosity at 20°C ρ_w = water density at 20°C

HAA biodegradation can be described by the Monod model shown in Eq 6:

$$-\frac{dC}{dt} = \frac{kXC}{K_M + C} \quad (6)$$

in which $-\frac{dC}{dt}$ is the biodegradation rate (µg HAA/µg protein/day), k is the maximum specific utilization rate (µg HAA/µg protein/day), K_M is the substrate concentration where the biodegradation rate is half of the maximum rate (µg HAA/L), X is the biomass concentration (µg protein/L), and C is the substrate concentration (µg HAA/L). For the case of low substrate concentrations (i.e., $C << K_M$), the Monod equation can be simplified to a pseudo-first order model as shown in Eq 7:

$$-\frac{dC}{dt} = k_r XC \quad (7)$$

in which k_r is the pseudo-first order biodegradation rate constant (i.e., $k_r = \frac{k}{K_M}$).

5.2.2 Model input parameters

5.2.2.1 *Biodegradation kinetics*

The biodegradation kinetic parameters used in the model calculations (Table 5.1) were obtained from batch experiments with HAA-degrading enrichment cultures and isolates as described elsewhere (Zhang et al., 2009b). The batch experiments were performed under conditions that were likely to favor HAA biodegradation: room temperature, pH 7.2, and no chlorine present. Furthermore, the HAAs served as sole carbon and energy source and were supplied at concentrations ranging from 12 to 300 µg/L. Kinetic parameters obtained with drinking water bacteria were available for DCAA biodegradation only. Therefore, pseudo-first order rate constants for biodegradation of

MCAA and TCAA were estimated by multiplying the pseudo-first order rate constants for DCAA biodegradation by drinking water bacteria by the ratios of $k_{r,MCAA}$ to $k_{r,DCAA}$ (0.049) and $k_{r,TCAA}$ to $k_{r,DCAA}$ (0.017) obtained from experiments using wastewater enrichment cultures.

5.2.2.2 *General parameters*

The parameters values that were held constant for all simulations are listed in Table 5.3. The water temperature was assumed to be 20°C, which fixed the physical properties of water (i.e., viscosity and density). This temperature was selected because the kinetic parameters were measured at room temperature and because it simulates typical water temperatures in northern climates during the summer months when biological activity is most favorable. The aqueous diffusion coefficients of MCAA, DCAA and TCAA also are given in Table 5.3.

Table 5.3. General parameter values used for the model calculations

Parameter	**Symbol**	**Value**	**References/ Observations**
Water temperature	T	20°C	simulate summer conditions
Water viscosity	$\mu_{w,20°C}$	1.0087×10^{-3} kg m^{-1} s^{-1}	Reynolds and Richards (1996)
Water density	$\rho_{w,20°C}$	998.2 kg m^{-3}	
Diffusion coefficient of MCAA in water	$D_{w,\ MCAA}$	1.12×10^{-9} m^2 s^{-1}	Zhang et al. (2004)
Diffusion coefficient of DCAA in water	$D_{w,\ DCAA}$	1.02×10^{-9} m^2 s^{-1}	Zhang et al. (2004)
Diffusion coefficient of TCAA in water	$D_{w,\ TCAA}$	9.75×10^{-10} m^2 s^{-1}	Zhang et al. (2004)

5.2.2.3 *Distribution system parameters*

The parameters used to simulate HAA fate in water distribution systems are summarized in Table 5.4. In general, an urban drinking water distribution system has hundreds to thousands of miles of water mains ranging from 2 to 96 inches (0.05 to 2.4 m) in diameter (McGhee, 1991; Rhoades, 1986). In this research, the pipe length was varied from zero to 100 miles (160 km) but most simulations were performed with a fixed pipe length of 10 miles (16 km). The pipe diameter was varied from 2 to 36 inches (0.05 to 0.9 m), but a pipe diameter of 6 inches (15.24 cm) was used as the baseline value for most of the simulations. A diameter of 6 inches is the typical size for the bulk of water mains serving residential neighborhoods (McGhee, 1991).

Table 5.4. Parameter values used to simulate HAA fate in water distribution systems

Parameter	Symbol	Range	References
Total bacterial density on the pipe wall	ρ	10-10^8 cells/cm^2, 10^7 cells/cm^2 for simulations where other parameters were varied	Silhan et al. (2006); Lehtola et al. (2004) ; Chang et al. (2003); Ollos et al. (2003); Zhang et al. (2002); Niquette et al. (2000); Donlan and Pipes (1988); LeChevalier et al. (1987)
Pipe diameter	d	2-36 inches, 6 inches for simulations where other parameters were varied	McGhee (1991); Rhoades (1986)
Water flow velocity	u	0.1-4 ft/s, 2 ft/s for simulations where other parameters were varied	McGhee (1991)
Pipe distance	x	0-100 miles, 10 miles for simulations where other parameters were varied	

Flow velocities of 0-10 fps (0-3 m/s) with an average of 2 fps (0.6 m/s) are typical in water distribution systems (Crittenden et al., 2005; McGhee, 1991; Rhoades, 1986). In this work, the water flow velocity was varied from 0.1 to 4 fps. For the ranges of pipe diameter and velocity used in the calculations, the Reynolds number (Re) ranged from 4,627 to 555,321, which is consistently in the turbulent flow regime (Re > 4,000).

The density of HAA-degrading bacteria attached to the pipe wall was estimated from reported values for the total bacterial density of $10\text{-}10^8$ cells/cm^2 (Silhan et al., 2006; Lehtola et al., 2004; Chang et al., 2003; Ollos et al., 2003; Zhang et al., 2002; Niquette et al., 2000; Donlan and Pipes, 1988; LeChevallier et al., 1987). The fraction of total bacteria that was capable of biodegrading HAAs was assumed to be equivalent to the ratio of HAA carbon to assimilable organic carbon (AOC). Typical AOC levels are 50-500 µg C/L (Lehtola et al., 2004). Total HAA concentrations detected in US drinking waters range from 5 to 130 µg/L in the plant effluent (Krasner et al., 2006) and between 14 and 106 µg/L in the distribution system (Chen and Weisel, 1998; Singer et al., 1995). For a total HAA concentration of 50 µg/L (10 µg C/L), the ratio of HAA to AOC ranges from 2% to 20%. A value of 10% was selected for the calculations. The concentration of HAA degraders was then converted to units of µg protein/cm^2 by assuming that one bacterial cell contains 100 femptograms (fg) of total protein, which is in the range reported for marine bacteria (60-330 fg/cell) by other researchers (Zubkov et al., 1999). Thus, the HAA-degrading biomass density on the pipe wall was varied from 10^{-7} to 1 µg protein/cm^2.

5.2.2.4 *Biologically-active filter parameters*

The parameter values used to simulate HAA fate in biologically-active rapid filters were adapted from other research (Crittenden et al. 2005) and are provided in Table 5.5. The media grains were assumed to be perfect spheres ranging in diameter from 0.1 to 1.2 mm. Typical filtration rates for rapid filters range from 5 to 15 m/h (2 to 6 gpm/sq ft), but some filters have been designed for higher filtration rates of up to 33 m/h (13.5 gpm/sq ft; Crittenden et al., 2005). A range of 1-25 m/h (0.4 to 6 gpm/sq ft) was used for the calculations in the current work. For the ranges of grain size and filtration rate used in the calculations, Re ranged from 0.35 to 8.65, which is consistently in the laminar flow regime that is typical of granular media (Re < 100).

Table 5.5. Parameter values used to simulate HAA fate in biologically-active filters

Parameter	Symbol	Value	References
Total biomass density on the filter media	ρ	10^4-10^8 cells/cm^2, 10^7 cells/cm^2 for simulations where other parameters were varied	Magic-Knezev and van der Kooij (2004); Velten et al. (2007)
Filter media grain diameter	d_p	0.1-1.2 mm, 0.75 mm for simulations where other parameters were varied	Crittenden et al. (2005)
Bed porosity	ε	30-70 %, 40 % for simulations where other parameters were varied	Crittenden et al. (2005)
Water filtration rate (superficial velocity)	v	1-25 m/h, 10 m/h for simulations where other parameters were varied	Crittenden et al. (2005)
Active bed depth	l	10 cm	Magic-Knezev and van der Kooij (2004)

Based on published results from other studies (Velten et al., 2007; Magic-Knezev and van der Kooij, 2004), the maximum bacterial density in a biologically-active filter was 10^{10} cells/cm^2 and the steady-state biomass level was between 10^7 and 10^8 cells/cm^2. Therefore, the total bacterial density was varied from 10^4 to 10^{10} cells/cm^2 in the calculations, with the lower end of this range simulating biomass levels after thorough filter backwashing procedures. These values were converted to μg HAA-degrader protein/cm^2 using the approach described previously. Here it was assumed that the active biomass was concentrated in the top 10 cm of the biologically-active filter (Magic-Knezev and van der Kooij, 2004). Therefore, although granular media filters are ~1 m deep, the bed depth used for the calculations was only 10 cm.

5.3 RESULTS AND DISCUSSION

5.3.1 Comparison of HAA biodegradation in distribution systems and biologically-active filters

Spreadsheet calculations were performed using a one-dimensional plug-flow reactor model and experimentally-determined (and estimated) kinetic parameters to investigate the potential for HAA biodegradation in distribution systems and biologically-active filters. Using typical values for relevant parameters (e.g., biomass density, flow velocity), the model calculations suggested that biodegradation is a potentially important loss process for HAAs in both of these systems. For both systems, simulated removals ranged from < 1% to > 90%, depending on the HAA biodegradation rate and the level of HAA-degrader biomass. DCAA was removed with the highest efficiency, followed by MCAA, and then TCAA (Figures 5.1 and 5.2).

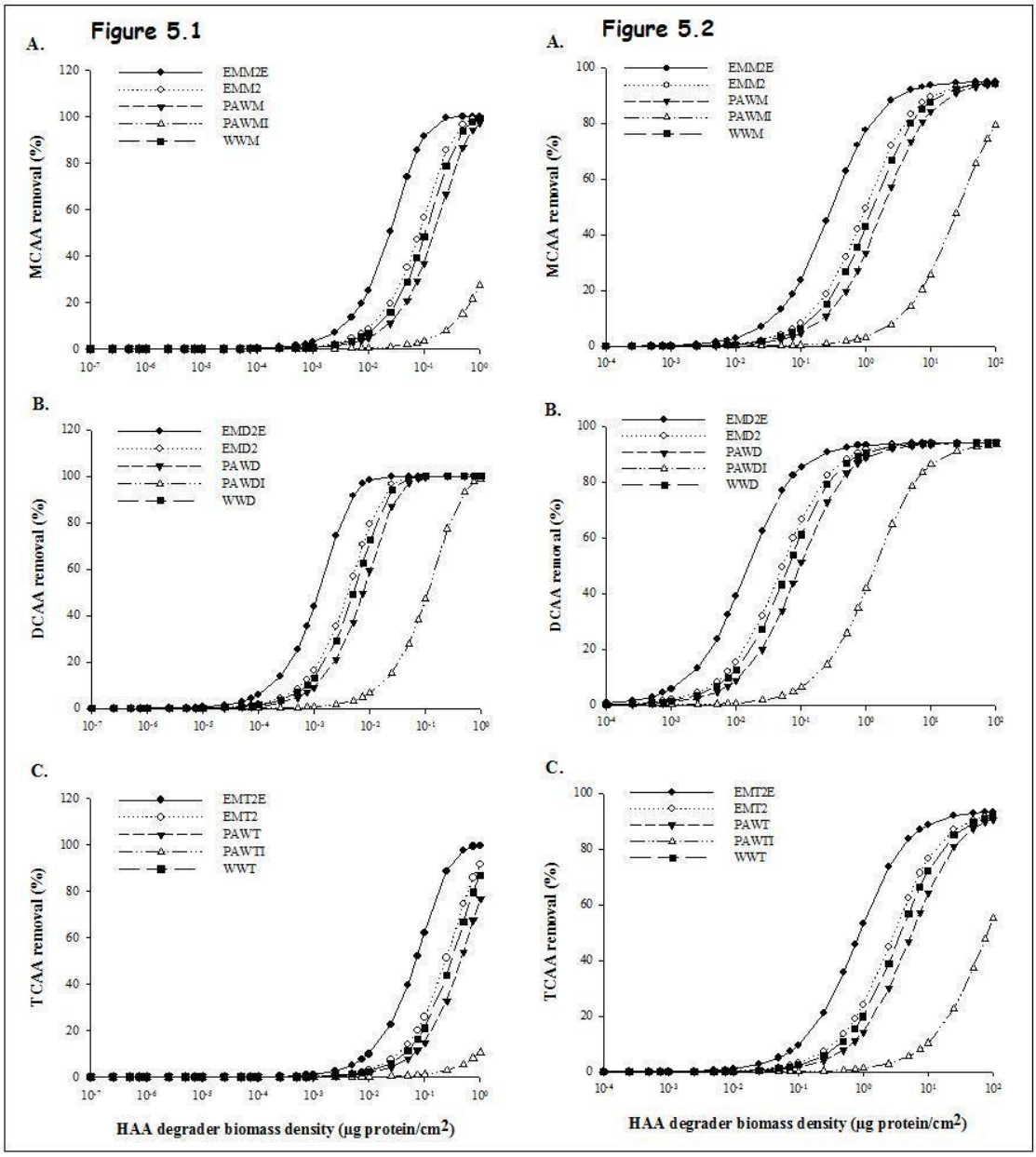

Figure 5.1. Effect of culture and HAA-degrader biomass density on the biodegradation of MCAA (A), DCAA (B) and TCAA (C) in a water distribution system. For these simulations, the pipe length was 10 m, the pipe diameter was 6 in. and the water flow velocity was 2 fps.

Figure 5.2. Effect of culture and HAA-degrader biomass density on the biodegradation of MCAA (A), DCAA (B) and TCAA (C) in a biologically-active filter. For these simulations, the bed porosity was 40 %, the diameter of the filter grain was 0.75 mm and the filtration rate was 10 m/h.

As expected, a biologically-active filter proved much more efficient than a water distribution system at removing HAAs. The specific surface area of a biologically-active filter (i.e., 3,000-36,000 m^{-1}) is much greater than that of a distribution system (i.e., 4.4-78.7 m^{-1}), which results in much greater biodegradation rate constants (k_{ra}) and mass transfer coefficients (k_{ma}). Therefore, to achieve similar HAA removals with the same HAA degrader biomass density, 1 mile (~1.6 km) of 6-in. pipe is approximately equivalent to only 10 cm of filter bed. The simulations performed here suggested that significant HAA removals (i.e., > 10%) could be achieved in both systems only for high HAA-degrader biomass densities (i.e., > 0.001 µg protein/cm^2, corresponding to a total cell density of 10^5 cells/cm^2). Such biomass densities are unlikely in distribution systems, however, because typical conditions (i.e., presence of a chlorine residual, high pH, and generally low nutrient levels) do not favor the growth of bacteria. Indeed, HAA concentrations might even increase in the distribution system when a free chlorine residual is present (Singer et al., 1995).

Biodegradation is likely to be the main HAA removal process in biologically-active filters and there is experimental evidence demonstrating that MCAA and DCAA can be completely removed by biodegradation in such filters (Xie and Zhou, 2002). TCAA was removed to a lesser extent (~80%; Xie and Zhou, 2002). With the model used in this research, predicted HAA removal efficiencies ranged from 3.2% to 77.5% for MCAA, from 41.6% to 93.4% for DCAA and from 1.1% to 53% for TCAA for a filtration rate of 10 m/h (4 gpm/sq ft) and a HAA-degrader biomass density of 1 µg protein/cm^2 (i.e., total bacterial cell concentration of 10^8 cells/cm^2), which is the steady-state biomass level in a typical biofilter (Velten et al., 2007; Figure 5.2). For lower

filtration rates, similar to those used in other research (i.e., 1.3 m/h or ~0.52 gpm/sq ft) (Xie and Zhou, 2002), the ranges of HAA removal efficiencies predicted by our model increased to between 21.9% and 99.9% for MCAA, ≥ 99.9% for DCAA and between 8.2% and 98.8% for TCAA for an HAA-degrader biomass density of 1 μg protein/cm^2. These calculations showed the effects of operating conditions such as the filtration rate on HAA removal efficiency and demonstrated that the model calculations were in reasonable agreement with experimental results.

HAA biodegradation in filters can occur only when chlorination is used ahead of the filters to generate HAAs followed by dechlorination to permit bacterial growth. Dechlorination occurs automatically, however, when the filter media is GAC (Fairey et al., 2007). Although prechlorination-dechlorination is not a common treatment strategy in the United States because of the need to rechlorinate before distribution, there are full-scale US treatment facilities that use this strategy (Zhang et al., 2009b). Furthermore, HAA biodegradation is also possible in residential point-of-use GAC filters commonly used for taste and odor removal (Xie and Zhou, 2002). More research is needed to demonstrate that HAA biodegradation occurs in such point-of-use filters.

5.3.2 Relative importance of mass transfer versus biodegradation rate

For the distribution system, the removal of MCAA and TCAA was biodegradation rate limited for all conditions simulated, and DCAA was biodegradation rate-limited under most conditions (Figure 5.3). This observation is in agreement with previous findings that biodegradable organic matter removal was not limited by mass transfer in annular reactors operated at 19 ± 2°C (Gagnon & Huck, 2001). For the distribution system, DCAA was mass transfer-limited for high HAA-degrader biomass densities (i.e., > 0.1

μg protein/cm^2) and low flow velocities (i.e., < 1 fps) (Figure 5.3). Again, such high biomass densities are unlikely in U.S. distribution systems where chlorine residuals often exceed 1 mg/L. For example, the biomass density on the pipe wall of a system with a total chlorine (primarily chloramine) residual of ~2.5 mg/L as Cl_2 ranged from 25 to 220 cfu/cm^2 (Zhang et al., 2002). Because culturable bacteria represented only a fraction of the total, the actual number of attached bacteria likely is greater by one to two orders of magnitude or more. Nevertheless, even 22,000 cells/cm^2 is several orders of magnitude less than the equivalent total bacteria density (~10^7 cells/cm^2) needed for DCAA to become mass transfer-limited.

For the biologically-active filter, DCAA was mass transfer-limited for HAA-degrader biomass densities higher than 0.3 μg protein/cm^2, and MCAA and TCAA were mass transfer-limited for HAA-degrader biomass densities higher than 10 μg protein/cm^2 (Figure 5.4). For an average HAA-degrader biomass density of 0.1 μg protein/cm^2, all HAAs remained biodegradation rate-limited, regardless of the filtration rate and the filter grain diameter. Thus, anything that significantly affects microbial kinetics, such as a change in temperature, is likely to have a significant effect on HAA loss rate in these systems.

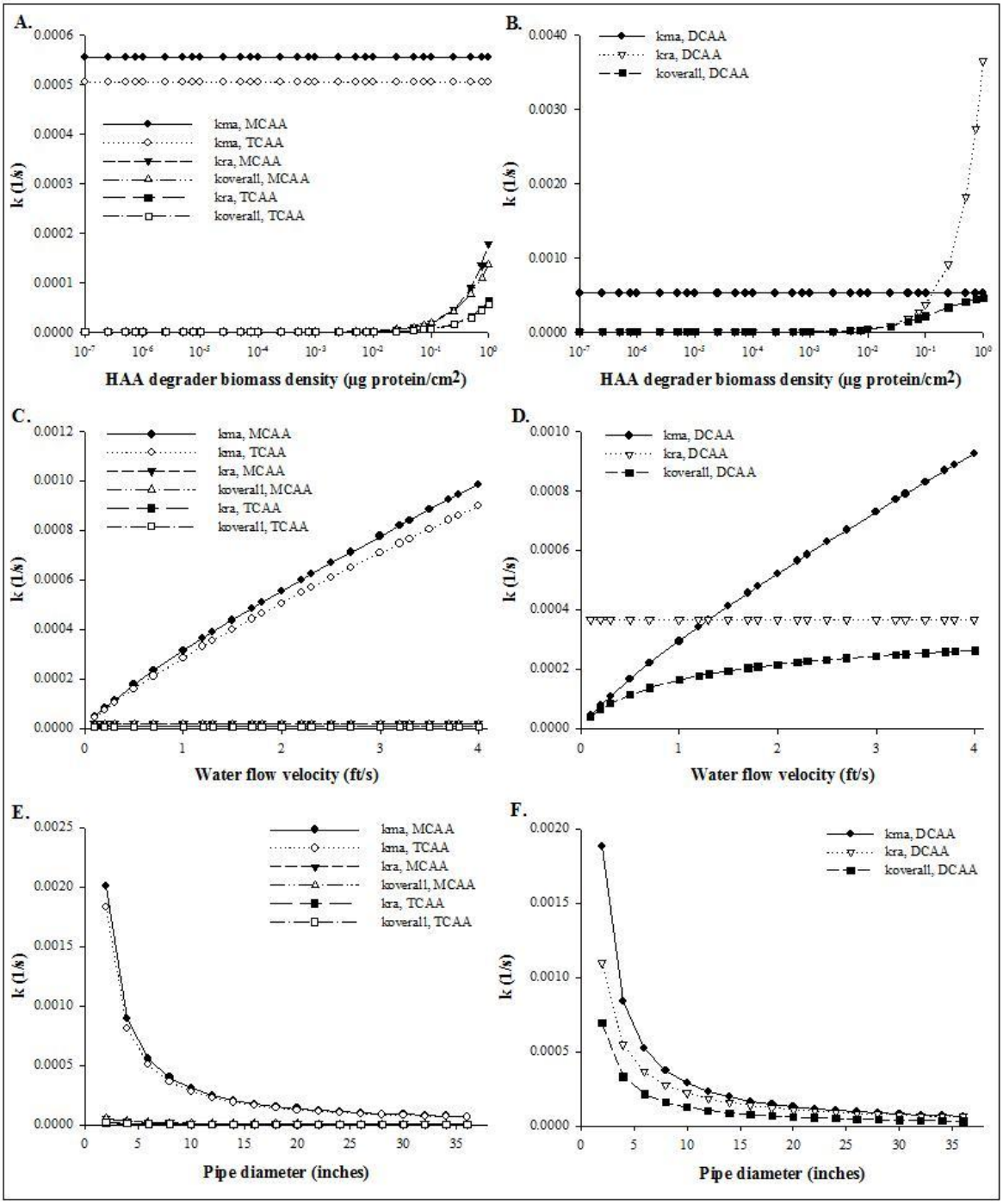

Figure 5.3. Effect of HAA-degrader biomass density (A, B), water flow velocity (C, D), and pipe diameter (E, F) on the biodegradation rate constant (k_{ra}), mass transfer rate constants (k_{ma}) and the overall rate constant ($k_{overall}$) for a water distribution system. Simulations were performed using the kinetic parameters for Pennsylvania tap water enrichment cultures (i.e., PAWM, PAWD, PAWT). For A & B, the water flow velocity was 2 fps and the pipe diameter was 6 in. For C & D, the HAA-degrader biomass density was 0.1 µg protein/cm^2 and the pipe diameter was 6 in. For E & F, the water flow velocity was 2 fps and the HAA-degrader biomass density was 0.1 µg protein/cm^2.

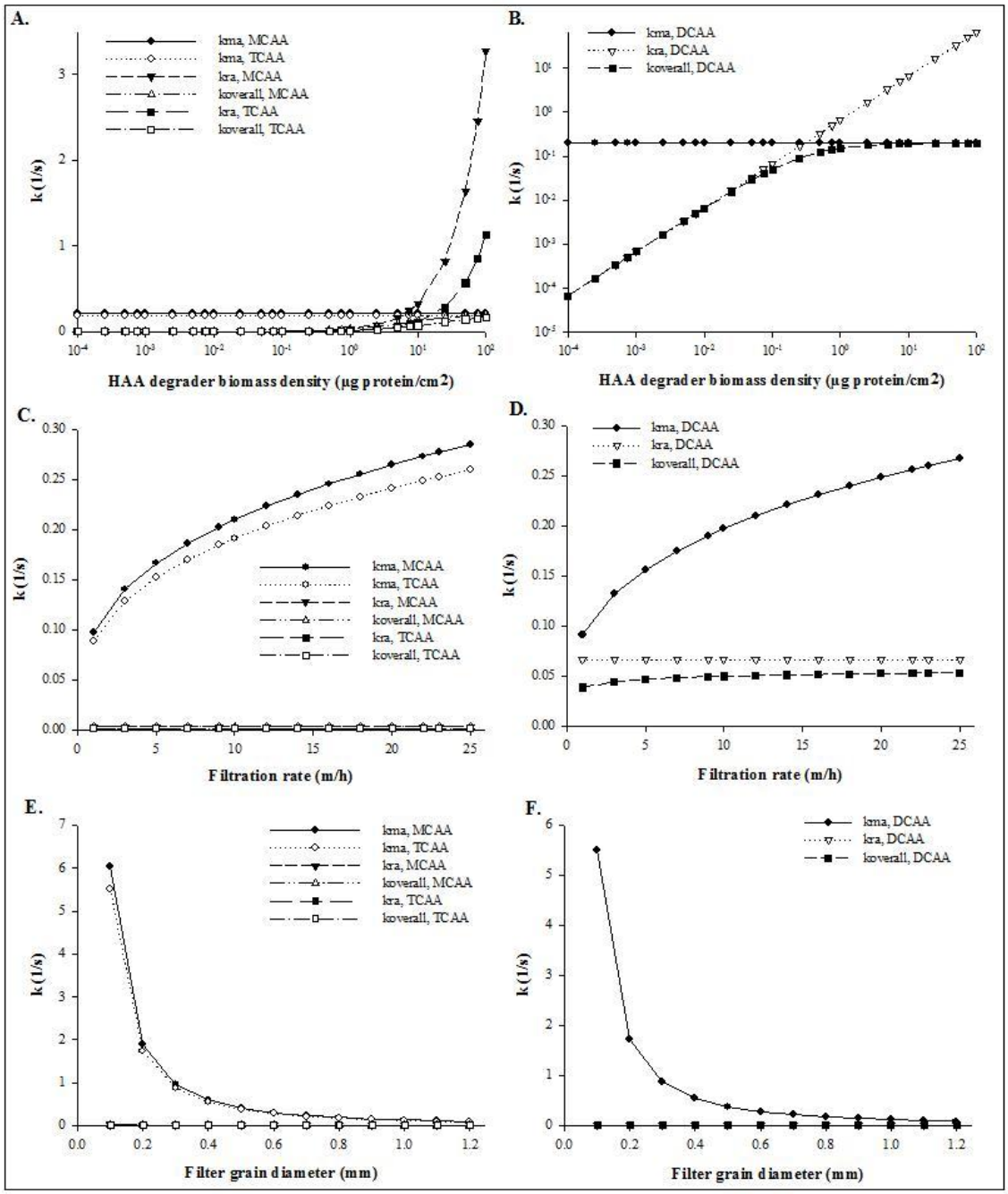

Figure 5.4. Effect of the HAA-degrader biomass density (A, B), the filtration rate (C, D) and the filter grain diameter (E, F) on the biodegradation rate constant (k_{ra}), the mass transfer rate constant (k_{ma}) and the overall rate constant ($k_{overall}$) for a biologically-active filter. Simulations were performed using the kinetic parameters for Pennsylvania tap water enrichment cultures (i.e., PAWM, PAWD, PAWT). For A & B, the filtration rate was 10 m/h and the filter grain diameter was 0.75 mm. For C & D, the HAA-degrader biomass density was 0.1 µg protein/cm^2 and the filter grain diameter was 0.75 mm. For E & F, the filtration rate was 10 m/h and the HAA-degrader biomass density was 0.1 µg protein/cm^2. For all simulations, the bed porosity was 40 %.

5.3.3 Effect of biodegradation rate constant on HAA removal

The pseudo-first-order DCAA biodegradation rate constant values used in the simulations were consistently 20 times greater than the MCAA biodegradation rate constants and 59 times higher than the TCAA biodegradation rate constants (Table 5.1). The same ranking in terms of biodegradation rate observed here for the wastewater enrichment cultures in the laboratory (DCAA > MCAA > TCAA) has been observed by other researchers (Ellis et al., 2001), although other biodegradation rankings have been reported (Bayless and Andrews, 2008; Zhou and Xie, 2002). Therefore, these estimates of the relative losses of the different HAAs may not represent the expected behavior for all systems. The essential point is that wide-ranging removals are possible and are highly dependent on the kinetics of the resident microbial population and the amount of biomass in the system. Although the authors simulated only the fate of MCAA, DCAA, and TCAA, results can be extrapolated to predict the fate of other HAAs. For example, all monohalogenated HAAs of relevance for drinking water systems (i.e., MCAA, MBAA, and monoiodoacetic acid) are biodegraded at similar rates (Zhang et al., 2009b; McRae et al., 2004).

For each compound (i.e., MCAA, DCAA, and TCAA) there was a substantial range of HAA removal, depending on the kinetic parameter values used (Figures 5.1 and 5.2). In particular, the pseudo-first-order DCAA degradation rate constant for isolate PAWDI (*Methylobacterium fujisawanse*) was substantially less than the rate constants for the other DCAA-degrading enrichment cultures and isolates. Methylobacteria isolated from chlorinated water are known to exhibit relatively slow biodegradation rates (Nishio et al., 1997). Given that *Afipia* spp. were frequently isolated from water systems (Zhang

et al., 2009a) and exhibited more rapid biodegradation kinetics (e.g., EMD2), use of the pseudo-first-order degradation rate constants for PAWDI, PAWMI, and PAWTI may result in underestimation of HAA losses in water systems. The enrichment culture from which PAWDI was isolated, however, had similar DCAA removal kinetics as the other DCAA-degrading enrichment cultures obtained from wastewater or drinking water inocula (Figures 5.1 and 5.2). This suggests that using kinetic parameters from enrichment cultures may be more universal than those from isolates. Moreover, PAWD was one of two enrichment cultures obtained from a US drinking water distribution system. Therefore, the kinetic parameters for PAWD, PAWM, and PAWT were used in the simulations where other parameters (e.g., biomass density, water flow velocity) were varied as discussed subsequently.

5.3.4 Effect of biomass density on HAA removal

The biomass density (i.e., density of HAA degraders) on the pipe wall or the filter media is a critical parameter controlling HAA removal in these systems (Figures 5.1 and 5.2). For example, using the kinetic parameters for PAWM, PAWD and PAWT, significant HAA removals (i.e. $\geq$ 10%) were obtained only when the HAA-degrader biomass density exceeded 0.075 µg protein/cm^2 for TCAA, 0.025 µg protein/cm^2 for MCAA, and 0.001 µg protein/cm^2 for DCAA. For a biomass density of 1 µg protein/cm^2, DCAA was completely removed within a 10-mi-long, 6-in.-diameter pipe, and ~89% of the influent DCAA was removed within the top 10 cm of a biologically-active filter. Under the same conditions, MCAA was nearly completely removed (~97%) within the distribution system and removed to a lesser extent within the biofilter (~33%); removals for TCAA were even lower (i.e., ~77% for the distribution system and ~14% for the

biofilter). It is important to mention that an HAA-degrader biomass density of 1 µg protein/cm^2 corresponds to a total bacterial cell density of 10^8 cells/cm^2. Although such high bacterial cell densities were found in some European distribution systems (Lehtola et al., 2004), total bacterial cell densities are expected to be much lower in US distribution systems (i.e., at least two or three orders of magnitude lower; Chang et al., 2003) because of the presence of relatively high chlorine concentrations. As mentioned previously, Zhang and co-workers (2002) reported bacterial densities of 25 to 220 cfu/cm^2 for a distribution system with a total (primarily monochloramine) residual of ~2.5 mg/L as Cl_2. Negligible HAA removal would be expected in such a system (i.e., < 0.2 % over 10 mi for DCAA). There is some ability to increase biomass levels in filter media by minimizing chlorine exposure during filtration and backwashing (Ahmad et al., 1998), but such strategies are obviously not practical for distribution systems in which low nutrient levels and a chlorine residual are desired to minimize regrowth and the risk of pathogen contamination. Indeed, mature biologically-active filters have bacterial concentrations as much as 10^{10} cells/cm^2 (Magic-Knezev and van der Kooij, 2004). At such total bacterial concentrations, the estimated HAA-degrader biomass density is 100 µg protein/cm^2, and all three HAAs are more than 90% removed within the top 10 cm of the biofilter (Figure 5.2).

5.3.5 Effect of physical parameters

The effects of pipe length, pipe diameter, and flow velocity on HAA removal in distribution systems were investigated for a fixed HAA-degrader biomass density of 0.1 µg protein/cm^2 (corresponding to a total bacterial cell density of 10^7 cells/cm^2). A high biomass density was selected for these simulations in order to illustrate the effects of the

physical parameters on HAA removal. For a pipe diameter of 6 in. and a flow velocity of 2 fps, approximately 8 mi of pipe was needed to achieve 99% DCAA removal (Figure 5.5). For MCAA and TCAA, the same removal would require 100 mi of pipe and 280 mi of pipe, respectively, given the same assumptions (Figure 5.5).

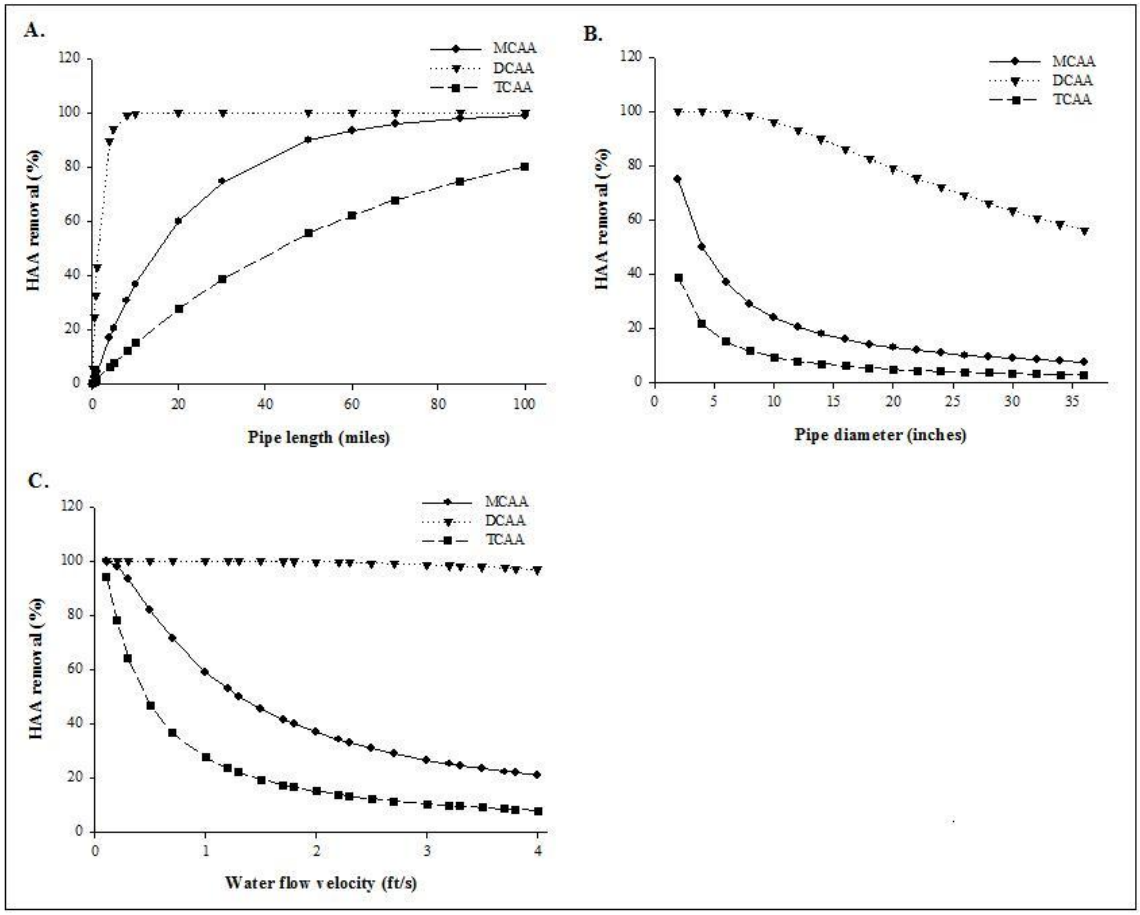

Figure 5.5. Effect of distance (A), pipe diameter (B) and water flow velocity (C) on the fate of MCAA, DCAA, and TCAA in a water distribution system. Simulations were performed using the kinetic parameters for Pennsylvania tap water enrichment cultures (i.e., PAWM, PAWD, PAWT). For A, the pipe diameter was 6 in., and the water flow velocity was 2 fps. For B, the water flow velocity was 2 fps, and the pipe length was 10 mi. For C, the pipe diameter was 6 in. and the pipe length was 10 mi. The HAA-degrader biomass density was 0.1 µg protein/cm^2 in all simulations.

When the biomass density was decreased by two orders of magnitude to 0.001 µg protein/cm^2, DCAA removal decreased to ~9 % after 10 mi of pipe, and removals of MCAA and TCAA were negligible over the same distance. Increasing the water flow velocity or pipe diameter decreased HAA removal (Figure 5.5). Although increasing the water flow velocity improves the rate of mass transfer of the HAA molecules from the bulk of water to the biofilm, it also decreases the hydraulic residence time, which means less time for the HAAs to be degraded by the bacteria. Thus, loss of DCAA because of biodegradation is expected in distribution systems in which conditions allow for the accumulation of biofilm (i.e., systems that use low residual chlorine levels or have very long residence time locations or dead ends in the system). Very little to no biodegradation of MCAA and TCAA is expected in distribution systems because of the slow biodegradation kinetics for these compounds. Baribeau and colleagues (2005) showed that in a simulated distribution system, in warm water (17-22°C) without a chlorine residual, DCAA was readily degraded by the biofilm bacteria while TCAA was not degraded. However, in cold water (12-14°C) or when chlorine residual was present such that the development of biofilms was hindered, neither HAA was removed.

Increasing the water flow velocity, pipe diameter, or filter media diameter decreased HAA removal (Figures 5.5 and 5.6). For example, in the case of DCAA removal by a biologically-active filter, the removal efficiency decreases between 1 and 5 % for each 1-m/h increase in filtration rate. On the other hand, an increase in the pipe diameter (Figure 5.3) or the filter media diameter (Figure 5.4) reduces the specific surface area and both the mass transfer rate constant (k_{ma}) and the biodegradation rate constant (k_{ra}).

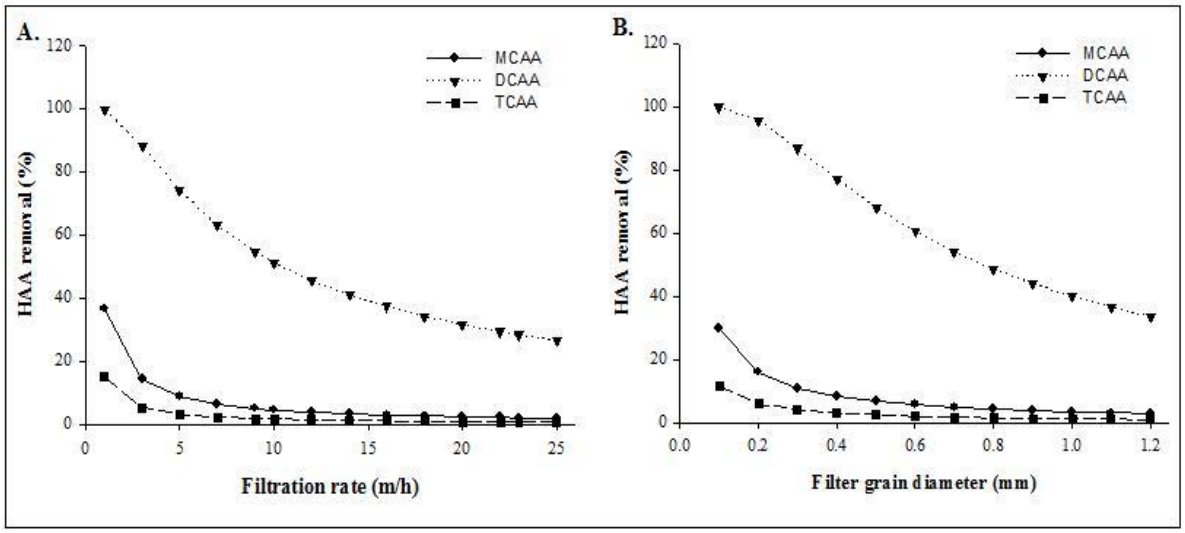

Figure 5.6. Effect of the filtration rate (A) and the filter grain diameter (B) on the fate of MCAA, DCAA and TCAA in a biologically-active filter. The simulations were performed with PAWM, PAWD and PAWT. For A, the filter grain diameter was 0.75 mm. For B, the water filtration rate was 10 m/h. The HAA-degrader biomass was 0.1 µg protein/cm^2 and the bed porosity was 40 % in all simulations.

5.3.6 Model limitations

The calculations performed in this research did not consider the effects of variations in temperature and pH or the presence of residual chlorine that may inactivate bacteria or inhibit bacterial activity. For example, the kinetic parameters were obtained from batch experiments performed at circumneutral pH (7.2) and room temperature (~20 °C), whereas distribution system pH values are typically higher (i.e., 8 to 9; Krasner et al., 1989), and water temperatures can vary widely (< 5 °C to > 25 °C). HAA biodegradation is not expected in prechlorinated sand or anthracite sand filters unless the water is dechlorinated prior to filtration. Because residual chlorine is rapidly dissipated in a GAC filter (Fairey et al., 2007), biological activity is expected in all GAC filters, including

those that are prechlorinated. In water distribution systems, biological activity and HAA biodegradation will likely be severely inhibited in regions where the total chlorine concentration exceeds 1 mg/L (Zhang et al., 2002; LeChevallier et al., 1990). The effects of temperature, pH, chlorine, and competing substrates should be considered in future models, but more research is needed to determine the effects of these variables on the attached biomass levels and on HAA biodegradation kinetics.

Another limitation of the simulations performed here is the lack of data on HAA-degrader biomass density on pipe walls and filter media. One significant issue is that it is difficult to characterize environmental bacterial communities using traditional culture-based methods because of the culturing bias (Dunbar et al., 1997). Furthermore, viable cell counts (such as those obtained using the heterotrophic plate count method) or total cell counts do not provide specific information on HAA degrader densities. Thus, the direct quantification of HAA degraders present within distribution systems and biofilters using molecular (i.e., DNA-based) methods would be more reliable for estimating HAA removals. Unfortunately, it is not currently possible to enumerate HAA degraders because quantitative molecular methods are not yet sufficiently optimized (Leach et al., 2009). Even if a viable method was available, the obvious difficulty of collecting biofilm samples from distribution system pipes remains a significant issue.

Finally, another limitation is the use of a pseudo-first-order kinetic model in which it is assumed that the HAA concentrations are much lower than the half-saturation coefficient (i.e., K_M) values. Some of the cultures and isolates from Table 5.1 (i.e., PAWD, EMD2, EMD2E), however, have low K_M values (e.g., 7.79 ± 5.82 µg/L for PAWD), whereas reported DCAA concentrations in distribution systems can exceed that

value (e.g., 9 to 60 µg/L; Singer et al., 1995). Therefore, use of the pseudo-first-order kinetic model might result in overestimation of the removal because of biodegradation, especially at high HAA concentrations.

5.4 CONCLUSIONS

A one-dimensional plug flow reactor equation with pseudo-first-order biodegradation kinetics was used to model the fate of HAAs in biologically-active filters and drinking water distribution systems. Because of the wide range of conditions in practice (e.g., chlorine residual, pipe material), HAA-degrader biomass density on the pipe walls in water distribution systems was expected to vary widely (up to eight orders of magnitude). As indicated by the simulations performed here, biomass density is an important parameter controlling HAA removal in both distribution systems and filters. Significant HAA removals (i.e., > 10%) were obtained only when HAA-degrader biomass densities exceeded 0.075 µg protein/cm^2 for TCAA, 0.025 µg protein/cm^2 for MCAA, and 0.001 µg protein/cm^2 for DCAA within a 10-mi-long 6-in.diameter pipe or within the top 10 cm of a biologically-active filter.

Removals of MCAA and TCAA were consistently less than that for DCAA because of slower biodegradation kinetics. For each HAA, however, a wide range of removals was possible, depending on the biodegradation rates of the isolates and enrichment cultures used in the simulations. In addition to biomass density and biodegradation kinetics, HAA removal rates were also sensitive to physical parameters such as flow velocity, pipe diameter, and filter media grain size. The HAA loss rate was biodegradation rate-limited for both MCAA and TCAA for all conditions simulated for distribution systems. The DCAA loss rate was mass transfer-limited for some conditions

(i.e., HAA-degrader biomass densities > 0.1 µg protein/cm^2 or flow velocities < 1 fps). In biologically-active filters, DCAA was mass transfer-limited at HAA degrader biomass densities > 0.3 µg protein/cm^2, and MCAA and TCAA were mass transfer-limited at HAA-degrader biomass densities > 10 µg protein/cm^2.

Overall, the model calculations suggested that biodegradation is likely to be an important loss process for HAAs in biologically-active filters and not likely to play a major role in most water distribution systems. The conditions needed for significant HAA removals in a distribution system (i.e., total biomass densities > 10^5 cells/cm^2 over long distances of pipe) are unlikely in the US water distribution systems where total chlorine residuals are typically high and thus inhibit the development of biofilm on the pipe walls. Losses of HAAs are possible in distribution systems where the total chlorine residual is intentionally low (as in some European systems) or in locations where the residual has been depleted, such as high residence time zones or at dead ends. In biologically-active filters, however, relatively high biomass densities are typical and such systems can be very efficient at removing HAAs.

Chapter 6 : SUMMARY AND CONCLUSIONS

The main achievements of this research are that it expanded the very limited information that was available on HAA degraders in drinking water systems and that it demonstrated that biodegradation could be exploited by water utilities as a means to effectively remove HAAs during water treatment. This was achieved by isolating and characterizing additional bacterial strains from drinking water systems and by developing a culture-independent technique to fingerprint the HAA-degrading bacterial communities in these systems. Furthermore, a kinetic model was developed to predict the loss of HAAs due to biodegradation in drinking water systems, thus showing that biodegradation could improve the removal of HAAs from drinking water.

At first, a direct plating technique, involving haloacetic acid (HAA)-amended agar plates, was used to isolate HAA degraders from different environments (i.e., river water, tap water and agricultural soil). The obtained isolates included several Gram positive and Gram negative bacteria that were not isolated in previous studies: *Mycobacterium* sp., *Streptomyces* sp., *Stenotrophomonas* sp. and *Pantoea* sp. Moreover, additional species such as *Bradyrhizobium* sp., *Bosea* sp. and *Mycobacterium* sp., were isolated from drinking water systems. The isolates obtained from drinking water systems presented the least phylogenetic diversity, suggesting that these systems have a unique microbial life that is adapted to low nutrient levels and presence of a disinfectant residual and that they primarily select for α-*Proteobacteria*. Furthermore, a *deh* gene was detected for the first time in a Gram positive bacterial species. This *deh* gene had almost 90% nucleotide sequence similarity with the *dehII* sequence from an α-*Proteobacterium*,

suggesting that lateral gene transfer of *deh* genes across unrelated bacterial species occurred.

A culture-independent technique, the terminal restriction fragment length polymorphism (tRFLP) of two classes of *deh* genes (*dehI* and *dehII*), was then used to fingerprint the HAA-degrading bacterial communities in drinking water systems. Substantial similarities were observed among the tRFLP patterns of *dehI* and *dehII* gene fragments in drinking water samples obtained from three different cities (Minneapolis, MN; St. Paul, MN and Bucharest, Romania) and from one biologically-active granular activated carbon filter (Hershey, PA). The dominant fragment in the tRFLP profiles of *dehI* genes from the drinking water samples matched the pattern from *Afipia* spp. that were previously isolated from drinking water. In contrast, the dominant fragments in the tRFLP profiles of *dehII* genes did not match any previously characterized *dehII* gene fragment.

A 1-dimensional plug flow reactor equation with pseudo-first order biodegradation kinetics was used to model the fate of haloacetic acids (HAAs) in biologically-active filters and water distribution systems. Overall, the model calculations suggested that biodegradation is likely to lead to significant HAA removals in biologically-active filters but not in most distribution systems. A sensitivity analysis showed that while the controlling parameter was the HAA-degrader biomass, physical parameters, such as water flow velocity and pipe length, also have an influence on the HAA removal. Relatively high total biomass densities (i.e., $> 10^5$ cells/cm^2) are needed for significant HAA removals (i.e., > 10%) in both systems. Such biomass densities are unlikely in U.S. distribution systems where relatively high total chlorine residuals inhibit

the development of biofilm on the pipe walls. Biologically-active filters can be very efficient at removing HAAs, however, because these filters typically contain relatively high biomass densities.

Substantial progress about the HAA-degrading bacteria in drinking water systems and the diversity of *deh* genes was made through this study. More research, however, is still needed to understand the specific functions of the two different groups of *deh* genes and to confirm which the major HAA degraders are in drinking water systems. This additional information would allow the development of specific molecular tools to quantify the HAA-degrader biomass in drinking water systems, which represents the key point for constructing a more robust kinetic model for predicting the fate of HAAs in these systems.

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APPENDIX A: Supporting information for Chapter 3.

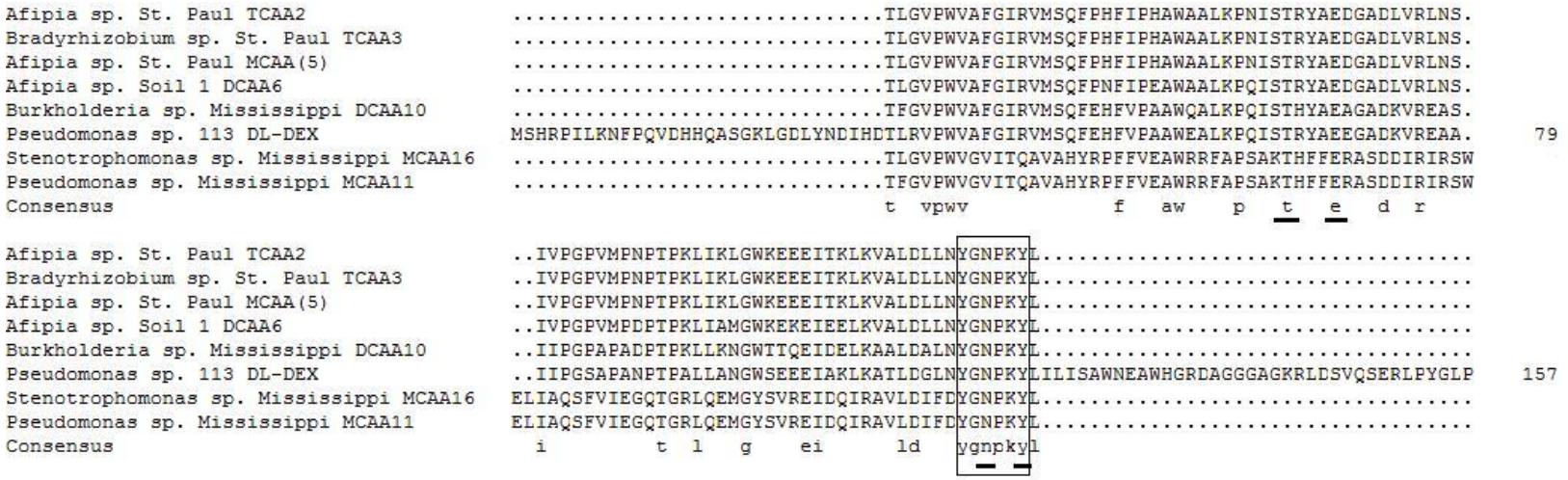


Figure A1. Alignment of derived amino acid sequences of partial group I deh gene sequences from isolates obtained in this study with the amimo acid sequence of the DL-DEX gene from Pseudomonas sp. 113. Sequences were optimally aligned using the multiple sequence editor of the DNAMAN software (version 7; Lynnon Corp., Quebec, Canada). Residues that were identified as essential for catalysis by Nardi-Dei et al. (1997) are underlined: T65, E69, N117 and Y120. The positions of the conserved amino acids are based on the *DL-DEX* gene from *Pseudomonas* sp. 113. The conserved amino acid motif YGNPKY is shown as boxed.

APPENDIX A-continued

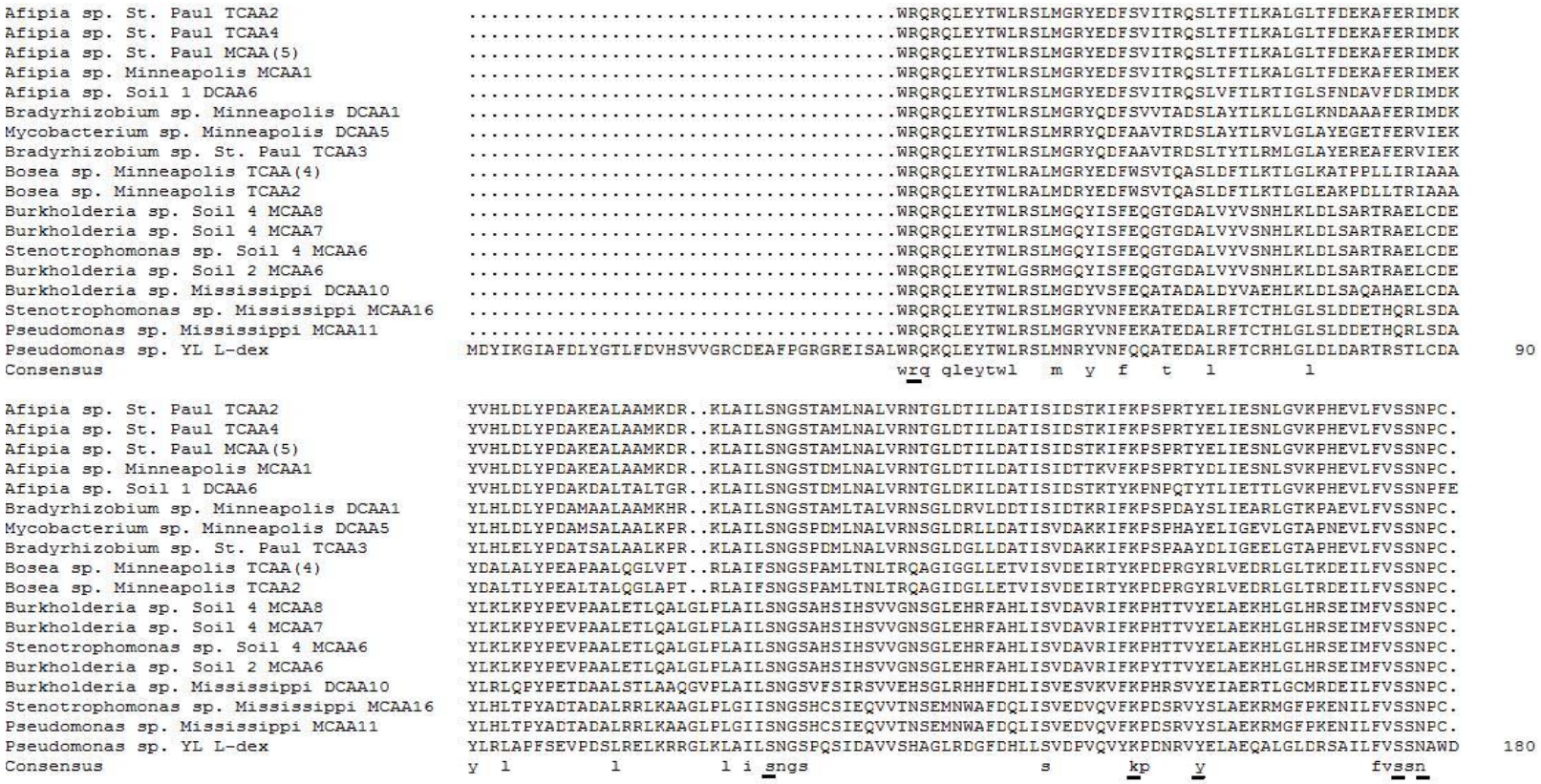


Figure A2. Alignment of derived amino acid sequences of partial group II deh gene sequences from isolates obtained in this study with the amino acid sequence of the L-dex gene from Pseudomonas sp. YL. Sequences were optimally aligned using the multiple sequence editor of the DNAMAN software (version 7; Lynnon Corp., Quebec, Canada). Residues that were identified as essential for catalysis by Kurihara et al. (1995) are underlined: R41, S118, K151, Y157, S175 and N177. The positions of the conserved amino acids are based on the *L-dex* gene from *Pseudomonas* sp. YL.

APPENDIX B: Supporting information for Chapter 4.

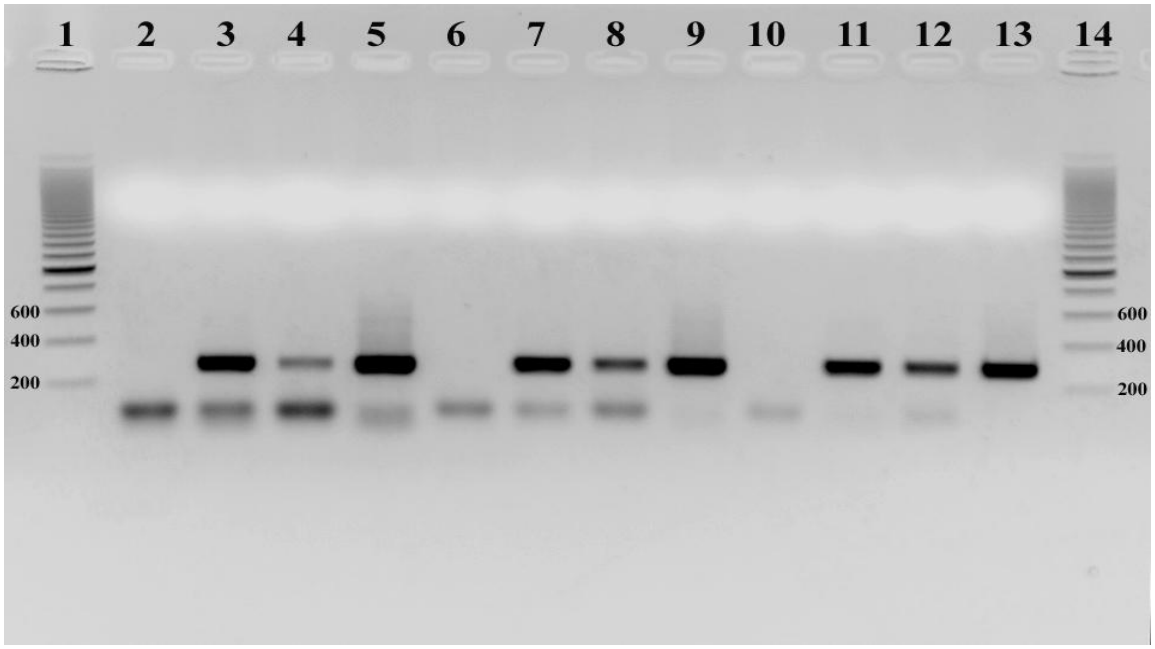


Figure B1. Optimization of primer concentrations for the PCR-amplification of dehI. Lanes: 1 & 14- 200 bp Step ladder (Promega, Madison, WI, USA); 2, 6 & 10- C(-)/ no DNA; 3, 7 & 11- *Afipia* sp. GTS; 4, 8 & 12- Hershey GAC; 5, 9 & 13- Enrichment WWT. *Afipia* sp. GTS is a drinking water isolate as described in Table 1. Sample "Hershey GAC" represents a sample collected from a biologically-active granular activated carbon filter used to treat drinking water. Sample "Enrichment WWT" was a culture enriched from municipal wastewater on trichloracetic acid. Primer concentrations: 2 µM in lanes 2-5; 1 µM in lanes 6-9; 0.5 µM in lanes 10-13. Other PCR components and the PCR program were as described in Materials & Methods.

APPENDIX B-continued

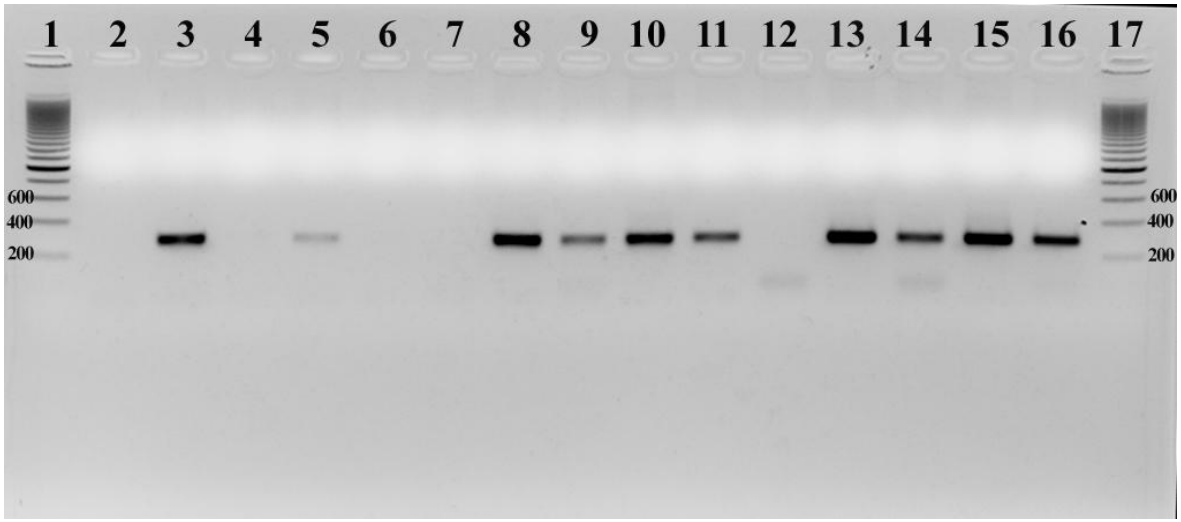


Figure B2. Optimization of MgCl2 concentration for the PCR-amplification of dehI. Lanes: 1 & 17- 200 bp Step ladder (Promega, Madison, WI, USA); 2, 7 & 12- C(-)/ no DNA; 3, 8 & 13-*Afipia* sp. GTS; 4, 9 & 14- Hershey GAC; 5, 10 & 15-Enrichment WWT; 6, 11 & 16-tap water sample. *Afipia* sp. GTS is a drinking water isolate as described in Table 1. Sample "Hershey GAC" represents a sample collected from a biologically-active granular activated carbon filter used to treat drinking water. Sample "Enrichment WWT" was a culture enriched from municipal wastewater on trichloracetic acid. $MgCl_2$ concentration: 0 mM in lanes 1-6; 1 mM in lanes 7-11; 2 mM in lanes 12-16. Other PCR components and the PCR program were as described in Materials & Methods.

APPENDIX C: FUNCTIONAL TESTS OF *DEH* GENES IN *AFIPIA* SP. GD1

C.1. INTRODUCTION

Some haloacetic acid (HAA)-degrading bacterial isolates obtained from drinking water systems exhibited a broad substrate range, including mono-, di- and trihalogenated acetic acids (Zhang et al., 2009a; Zhang et al., 2009b). One consistent feature of the isolates with a broad substrate range was the presence of both known classes of haloacid dehalogenase genes, the *dehI* and *dehII* genes (Zhang et al., 2009a). Isolates carrying only one of the classes of *deh* genes had a more limited substrate range. For example, isolates not carrying a *dehI* gene were unable to degrade trichloroacetic acid (TCAA) while with one exception (i.e., *Methylobacterium* sp. strain PAWDI), isolates possessing a *dehI* gene were capable of degrading TCAA (Zhang et al., 2009a). All isolates were able to degrade monohalogenated HAAs, whether they possessed a *dehI* gene, a *dehII* gene, or both genes. The connection between specific classes of *deh* genes and the ability of the isolates to degrade dihalogenated HAAs was not clear, as one isolate containing only a *dehII* gene was able to degrade DCAA but others were not (Zhang et al., 2009a). The purpose of this study was to determine the specificity of selected *dehI* and *dehII* genes obtained from a bacterium isolated from a granular activated carbon filter by Zhang and coworkers (2009a), namely *Afipia* sp. GD1. For this work, expression experiments were set up and knockout constructs were designed.

C.2. MATERIALS AND METHODS

C.2.1. Expression experiments

Afipia sp. GD1 was grown at room temperature in minimum medium (as described in the previous chapters) supplemented with 1 mM DCAA, 10 mM acetate or 10 mM acetate and 1 mM DCAA, until the optical density at 600 nm was 0.1-0.2. One to three mL from these cultures were used for RNA extraction with an RNAqueous- Micro kit (Ambion/Applied Biosystems, Austin, TX), according to the manufacturer's instructions. Seven µL of the RNA extraction were then used for transformation into cDNA with a DyNAmo cDNA synthesis kit (New England Biolabs, Ipswich, MA, USA), according to the manufacturer's instructions. The cDNA template was then used to amplify a fragment of the 16S rRNA gene in order to verify if the RNA extraction and the cDNA transformation worked. The 16S rRNA gene fragment was amplified using primers Eub341F (5'-CCT ACG GGA GGC AGC AG-3') and Eub534R (5'-ATT ACC GCG GCT GCT GGC-3'). The expression of the *dehI* and *dehII* genes was tested by amplifying fragments of these genes, using primers GD1-downstream1 (5'-GCT CTC AAG CCA CAT ATC TCG-3') and GD1-upstream (5'-CGT CTT CTT TCC AAC CCA TCG C-3') for the amplification of *dehI* and primers GD1-RTPCR-fwd (5'-GCG CTT TCC TAC ACG TTG AAG AC-3') and GD1-upstream2 (5'-CGC ATC CGG ATA GGG TTT CAG-3') for the amplification of *dehII*. The PCR program used for the amplification of all three gene fragments consisted of one initial denaturation step at 95°C for 5 minutes and 30 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, elongation at 72°C for 20 seconds and a final elongation step at 72°C for 10 minutes. Controls without reverse transcriptase were included for all three genes (i.e., using RNA template that was not converted into cDNA) to determine whether extracted

RNA was contaminated with genomic DNA. The amplified fragments were resolved on a 2% agarose gel.

C.2.2. Knockout constructs

All plasmids necessary for building the knockout constructs (i.e., pUC4K, pHP45omega and pSUP202), as well as the helper strain *E. coli* S17-1 were obtained from Prof. Michael Sadowsky at the Biotechnology Institute, University of Minnesota.

C.2.2.1. *dehI* knockout vector

A 1.5 kb fragment containing the nearly complete *dehI* gene from *Afipia* sp. GD1 and some upstream region was PCR-amplified using the *dehI*-F1-INLS2 (5'-GTT CGT GAA CGC CTG CTT C-3') and the *dehI*-R3-INLS2 (5'-GAG GAT GTC TTC AAG CAG TC-3') primers, which were designed based on the *dehI* sequence with upstream and downstream regions of *Afipia* sp. INLS2 (Green et al., 2010; GenBank NZ_ADVZ00000000). The following PCR program was used: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, elongation at 72°C for 2 minutes and a final elongation step at 72°C for 10 minutes. The size of the amplified fragment was verified on 1% agarose gel. The fragment was then cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) and was sequenced with primers M13 forward and M13 reverse. Based on the determined sequence, the restriction enzyme *Blp*I was found to cut only once in the middle of the *dehI* gene. Moreover, *Blp*I was a non-cutter for the pGEM-T easy vector. This enzyme was thus chosen to use for building the knockout construct (i.e., introducing the kanamycin resistance gene within the *dehI* gene, without destroying the pGEM-T easy vector).

The kanamycin resistance gene was amplified from the pUC4K vector, using the following primers that had flanking *Blp*I sites: Kan-forward (5'-GGG CTG AGC GGT CTG CCT CGT GAA GAA GG-3') and Kan-reverse (5'-GGG CTG AGC GGA AAG CCA CGT TGT GTC TC-3'). Prior to the amplification, it was verified that *Blp*I did not cut within the kanamycin fragment. The size of the amplified kanamycin fragment (i.e., 1.2 kb) was verified on 1% agarose gel. The fragment was then cloned into the pGEM-T easy vector to be able to digest its ends with *Blp*I (the PCR fragment could not be directly digested with *Blp*I because the enzyme needed longer end sequences to be able to perform the digestion).

As seen in Figure C1, the next step was to ligate the kanamycin fragment digested with *Blp*I into the *dehI* fragment cloned in the pGEM-T easy vector and also digested with *Blp*I. The last step was to transfer the knockout construct (i.e., the *dehI* gene disrupted by the kanamycin fragment) from the pGEM-T easy vector to the pSUP202 suicide vector, which is a common vector used for knockout experiments (Simon et al., 1983).

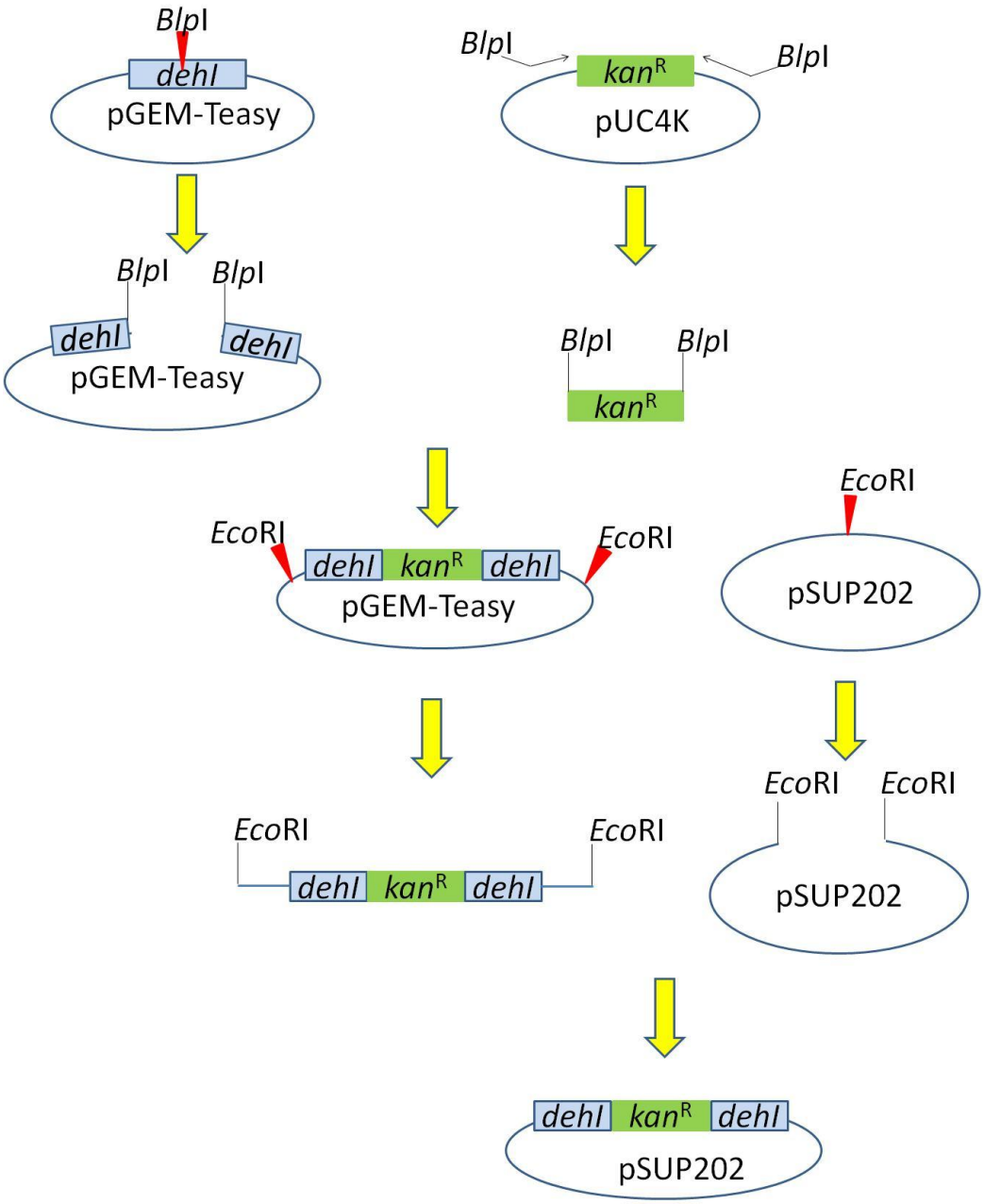


Figure C1. Strategy for building a vector to knockout the *dehI* gene in *Afipia* sp. GD1.

C.2.2.1. *dehII* knockout vector

The strategy used for building the *dehII* knockout vector was similar to that described for *dehI* (Figure C2). A 1.3 kb fragment containing the nearly complete *dehII* gene from *Afipia* sp. GD1 and some upstream region was also PCR-amplified using the *dehII*-F1-INLS2 (5'-GCG TTC TCC ATT GTT TCG AG -3') and the *dehII*-

R2-INLS2 (5’- AAT GCG TTC GTC AGG CTC TG -3’) primers. The same PCR program as previously described was used. The size of the amplified fragment was verified on 1% agarose gel. The fragment was then cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) and was sequenced with primers M13 forward and M13 reverse. Based on the determined sequence, the restriction enzyme *Sfo*I was found to cut only once in the middle of the *dehII* gene. This enzyme did not have any restriction site within the pGEM-T easy vector and was thus chosen to use for building the knockout construct (i.e., introducing the streptopmycin/spectinomycin resistance gene cassette within the *dehII* gene, without destroying the pGEM-T easy vector).

The streptopmycin/spectinomycin resistance gene cassette (i.e., omega fragment) was excised from the pHP45omega vector, using the restriction enzyme *Sma*I. As seen in Figure C2, the next step was to ligate the omega fragment digested with *Sma*I into the *dehII* fragment cloned in the pGEM-T easy vector and digested with *Sfo*I. Although the two fragments were digested with different enzymes, the ligation was possible because both enzymes generated blunt ends. The last step was to transfer the knockout construct (i.e., the *dehII* gene disrupted by the omega fragment) from the pGEM-T easy vector to the pSUP202 suicide vector. This was done by excising the knockout construct from the pGEM-T easy vector through digestion with *Eco*RI and ligating the excised fragment into the pSUP202 vector that was also digested with *Eco*RI.

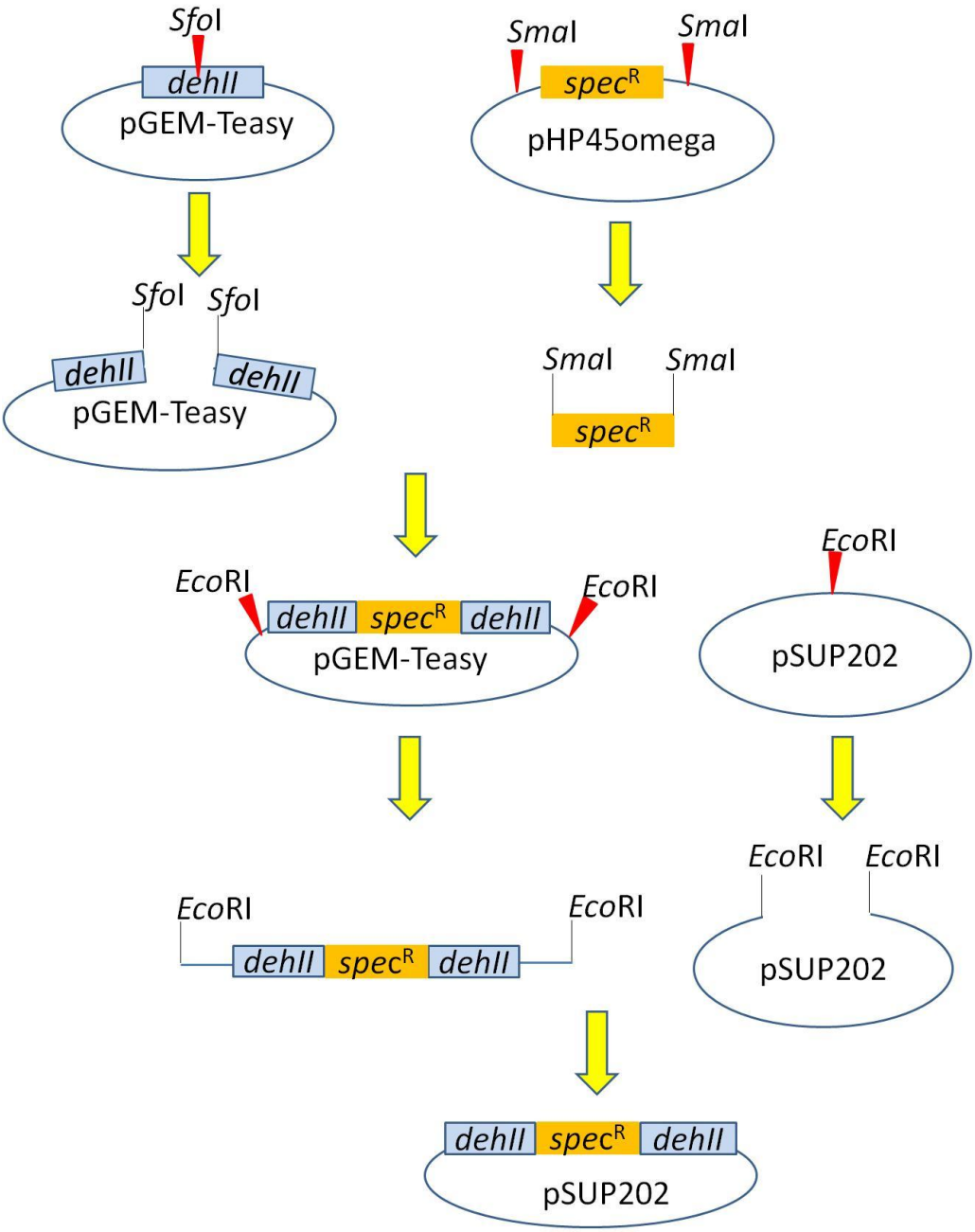


Figure C2. Strategy for building a vector to knockout the *dehII* gene in *Afipia* sp. GD1.

C.3. RESULTS AND DISCUSSION

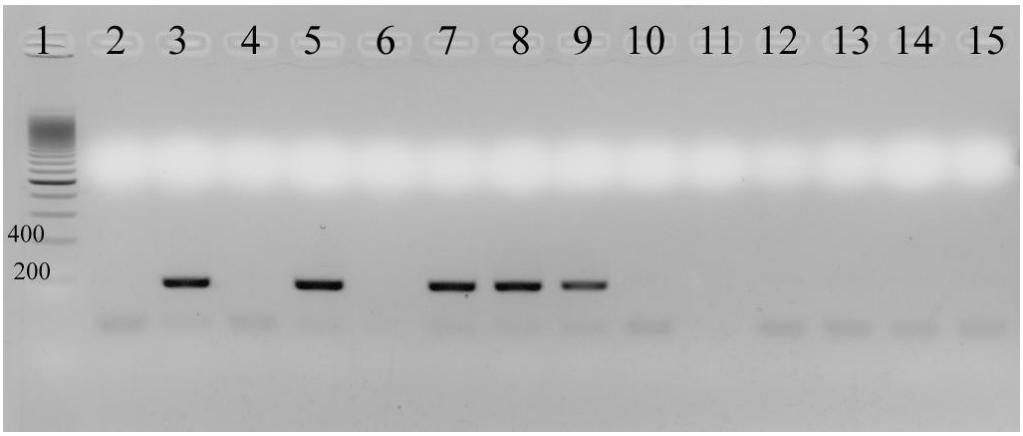
C.3.1. Expression experiments

A band of the expected size (~ 200 bp) was amplified for the 16S rRNA gene in four out of the six *Afipia* sp. GD1 samples (Figure C3A, lanes 3-8), suggesting that the RNA extraction and the conversion into cDNA was successful. Moreover, no band was obtained for the no reverse transcriptase control (Figure C3A, lanes 10-15), confirming that the extracted RNA was not contaminated with genomic DNA and that the 16S rRNA gene was expressed during cell culturing.

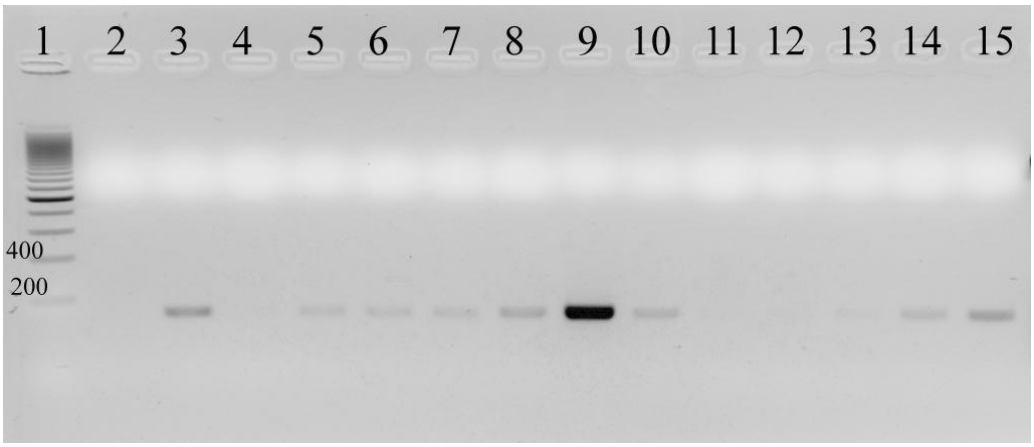
The expected bands for the amplification of *dehI* and *dehII* fragments were small (i.e., ~ 130 bp for *dehI* and ~ 100 bp for *dehII*) and small fragments often migrate similarily to primer dimers on agarose gels. Thus, it is hard to confirm whether the obtained bands in Figure C3, B & C, corresponded to *dehI* and *dehII* fragments or to primer dimers formed during the PCR reaction. Moreover, the bands obtained for the no reverse transcriptase control (Figure C3, B & C, lanes 10-15) suggest that either there was still some contamining DNA in the extracted RNA or that primer dimer formation interfered with the expected results. More optimization is thus needed in order to detect the expression of the *dehI* and *dehII* genes, including the design of new primers amplifying bigger *deh* fragments (~ 200 bp) that can be easily distinguished from primer dimers on agarose gels.

After optimizing the PCR conditions for the detection of the *dehI* and *dehII* genes, the expression of these genes under different growth conditions (e.g., growth on HAAs versus growth on yeast extract or acetate) will have to be compared by real-time quantitative PCR, in order to determine the conditions that induce the expression of these two genes.

A.



B.



C.

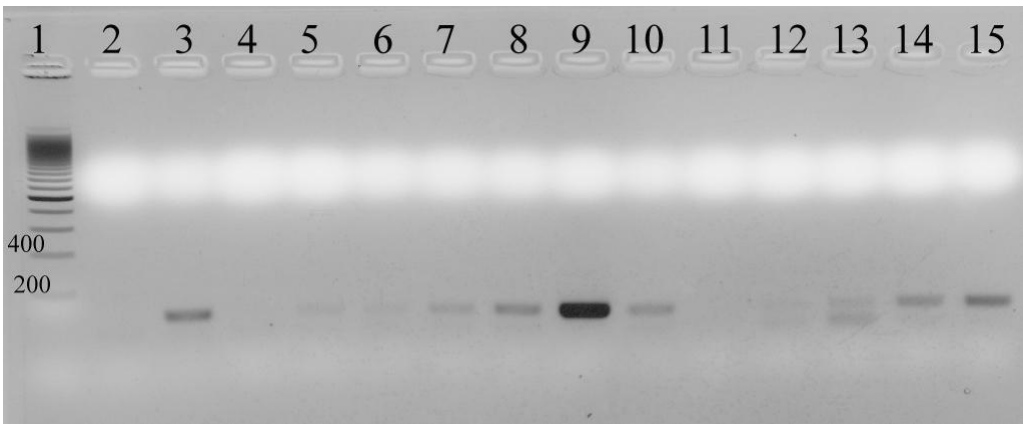


Figure C3. Detection of 16S rRNA (A), *dehI* (B) and *dehII* (C) genes, amplified from cDNA in *Afipia* sp. GD1. Lanes: 1- Step ladder (Promega, Madison, WI, USA); 2- C(-)/no DNA template; 3,4- *Afipia* sp. GD1 grown on DCAA; 5,6- *Afipia* sp. GD1 grown on acetate; 7,8- *Afipia* sp. GD1 grown on acetate & DCAA; 9- C(+)/genomic DNA. Lanes 10-15 represent no reverse transcriptase controls for lanes 3-8.

C.3.2. Knockout constructs

The *dehII* knockout vector was successfully obtained but was not delivered to *Afipia* sp. GD1. Future work will thus involve a triparental mating experiment between the carrier strain (i.e., the *E. coli* JM109 strain carrying the knockout vector), a helper strain (i.e., *E. coli* S17-1 carrying the pRK2013 plasmid, which mobilizes non-self-transmissible plasmids) and the recipient strain (i.e., *Afipia* sp. GD1), in order to deliver the *dehII* knockout vector to the *Afipia* sp. GD1 and create knockout mutants for the *dehII* gene. The knockout mutants will have to be tested for the ability of degrading HAAs. A loss of the ability of degrading any HAA will indicate a correlation between the presence of the *dehII* gene and the degradation of that HAA.

The *dehI* knockout vector, however, could not be obtained because the ligation between the kanamycin and the *dehI* genes digested with the *Blp*I enzyme did not work. This could be related to an incorrect insertion of the *Blp*I sites at the two ends of the kanamycin gene. Therefore, a better approach for obtaining the *dehI* knockout vector would be to use the same strategy as for the design of the *dehII* knockout vector (i.e., find an enzyme to excise the kanamycin gene from the pUC4K vector instead of amplifying it). Another option would be to blunt the sticky ends generated with *Blp*I to make them compatible for ligation.

The ultimate goal of this study is to obtain both single mutants for each of the two *deh* genes and double mutants (i.e., having both *deh* genes disrupted) and to correlate the results obtained from the expression experiments with those from the knockout experiments, in order to determine the role of the *dehI* and *dehII* genes in degrading mono-, di- and trihalogenated HAAs.