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ORIGINAL ARTICLE

Identification of dichloroacetic acid degrading *Cupriavidus* bacteria in a drinking water distribution network model

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Abstract

Aims: Bacterial community structure and composition of a drinking water network were assessed to better understand this ecosystem in relation to haloacetic acid (HAA) degradation and to identify new bacterial species having HAA degradation capacities.

Methods and Results: Biofilm samples were collected from a model system, simulating the end of the drinking water distribution network and supplied with different concentrations of dichloroacetic and trichloroacetic acids at different periods over the course of a year. The samples were analysed by culturing, denaturing gradient gel electrophoresis (DGGE) and sequencing. Pipe diameter and HAA ratios did not impact the bacterial community profiles, but the season had a clear influence. Based on DGGE profiles, it appeared that a particular biomass has developed during the summer compared with the other seasons. Among the bacteria isolated in this study, those from genus *Cupriavidus* were able to degrade dichloroacetic acid. Moreover, these bacteria degrade dichloroacetic acid at 18°C but not at 10°C.

Conclusions: The microbial diversity evolved throughout the experiment, but the bacterial community was distinct during the summer. Results obtained on the capacity of *Cupriavidus* to degrade DCAA only at 18°C but not at 10°C indicate that water temperature is a major element affecting DCAA degradation and confirming observations made regarding season influence on HAA degradation in the drinking water distribution network.

Significance and Impact of the Study: This is the first demonstration of the HAA biodegradation capacity of the genus *Cupriavidus*.

Introduction

Chlorination is the most widespread approach of disinfection used to limit microbial growth in municipal drinking water distribution networks (American Water Works Association 2003; Serodes *et al.* 2003; Health Canada 2008). Besides being economic, chlorine disinfection possesses the advantage of preserving a residual effect to avoid proliferation of pathogenic microorganisms in the networks. However, the reaction of chlorine with organic matter naturally present in water may promote the emergence of various chlorination by-products (CBPs) that can potentially affect human health, such as trihalomethanes and haloacetic acids (HAAs) (Rook 1974; Symons *et al.* 1975; Serodes *et al.* 2003). The maximum acceptable concentration for total HAAs in drinking water set by Health Canada is 0·08 mg l^{-1} (Health Canada 2010). Regulated HAAs comprise monochloroacetic acid

(MCAA), dichloroacetic acid (DCAA), trichloroacetic acid (TCAA), monobromoacetic acid (MBAA) and dibromoacetic acid (DBAA). The potential health effects of HAAs vary depending on the specific compound. MCAA and MBAA are unclassifiable or considered unlikely to be carcinogenic to humans based on insufficient data from animal studies. DCAA, TCAA and DBAA are considered to be probable carcinogens to humans based on limited or sufficient evidence from animal studies. In all cases, there is not enough data to come to a definite conclusion regarding the danger to human health of long-term exposure to these compounds (Canada Health Canada 2008).

Biofilms form on a majority of natural and artificial materials such as rocks, catheters, probes, prostheses, as well as live biological surfaces such as enamel of teeth (dental plaque) (Costerton *et al.* 1995; Costerton 2007). The properties of biofilm vary according to micro-organisms involved, the surface of support as well as the environment of formation (Schaule *et al.* 2007; Goller and Romeo 2008; McBain 2009). It generally consists of several micro-organisms such as bacteria, protozoa and fungi embedded into an organic polymeric matrix.

In the case of drinking water distribution networks, biofilms are typically found on the inner surface of pipes, particularly at the network extremities (Serodes *et al.* 2003). Several studies revealed that an important fraction of biofilm found in drinking water distribution networks belongs to the phylum of *Proteobacteria* (Bayless and Andrews 2008; Zhang *et al.* 2009a; Henne *et al.* 2012; Liu *et al.* 2012). It has been observed that the HAA concentrations are reduced as the water moves through the drinking water network (Serodes *et al.* 2003; Rodriguez *et al.* 2004). The observed decreases of HAA concentrations in the distribution network, in particular DCAA, has been attributed to biodegradation by bacteria (Fetzner 1998; Martiny *et al.* 2003; Serodes *et al.* 2003; McRae *et al.* 2004; Schaule *et al.* 2007; Bayless and Andrews 2008; Tung and Xie 2009; Zhang *et al.* 2009a,b), likely initiated by a dehalogenation reaction of the molecules (Fetzner 1998; McRae *et al.* 2004). Moreover, some bacteria from the *Proteobacteria* phylum found in drinking water distribution pipes have the capacity to biologically degrade HAAs (Zhang *et al.* 2009a).

As most HAAs diminish as they pass through the system, most likely because of bacterial activity, more information is needed on biofilm formation and bacterial diversity in this network. This is a required initial step to understand the effects produced by the establishment of this kind of biofilm. Such information could eventually contribute to the better management of the presence of CBPs in drinking water. In this context, samples of biofilm were collected from a model system, simulating the end of the drinking water distribution system in Quebec City (Canada), supplied with different HAA concentration ratios (e.g. DCAA/TCAA) at different periods over the course of more than a year (Pluchon *et al.* 2013). These samples were analysed by culture through different nutritive media and by molecular biology approaches involving denaturing gradient gel electrophoresis (DGGE) and sequencing. In the present study, the biofilm forming on pipes wall was thus characterized in regards to its bacterial community composition and a bacterial genus able to degrade HAA was identified.

Materials and methods

Water source and treatment

The water used in the study was originated from the main drinking water production plant of Quebec City (QC, Canada) that supplies approximately 240 000 people. The raw water was provided from the Saint-Charles River in Quebec City. Coagulation/flocculation, sedimentation, filtration and interozonation are used to treat the water. A postchlorination step is conducted to ensure the presence of residual chlorine in the distribution system.

Experimental drinking water distribution system model

The experimental model consisted of four independent pipe systems (networks A, B, C and D) made with opaque high-density polyethylene (HDPE) pipes that were all constructed as shown in Fig. 1. The systems differed in their length, diameter and flow rate (Table 1). The concentrations and ratios of DCAA and TCAA also differed in the networks and over time (Table 1). The model was located at the extremity of Quebec City water distribution network to obtain low concentrations of residual chlorine (Pluchon *et al.* 2013). The residence time of water at the network location providing water to the experimental model was approximately 8 h (Simard 2008). Every network model possessed at its end a rack supporting HDPE coupons and located inside a clear pipe. Networks A and B possessed an additional rack located between the source of water and the main pipe (Pluchon *et al.* 2013). The clear plastic pipes containing coupons were covered with aluminium foil to limit light exposure and to avoid algal growth. Metal pipes were avoided for the construction of the experimental model as the iron and the zinc can react with the HAAs (Hozalski *et al.* 2001; Wang and Zhu 2010), while plastic pipes were considered as nonreactive (Hallam *et al.* 2002).

The pipes were continuously supplied with drinking water for many months before beginning the experiments and for at least 4 weeks between each subsequent trial. To maintain a constant flow, the water was pumped

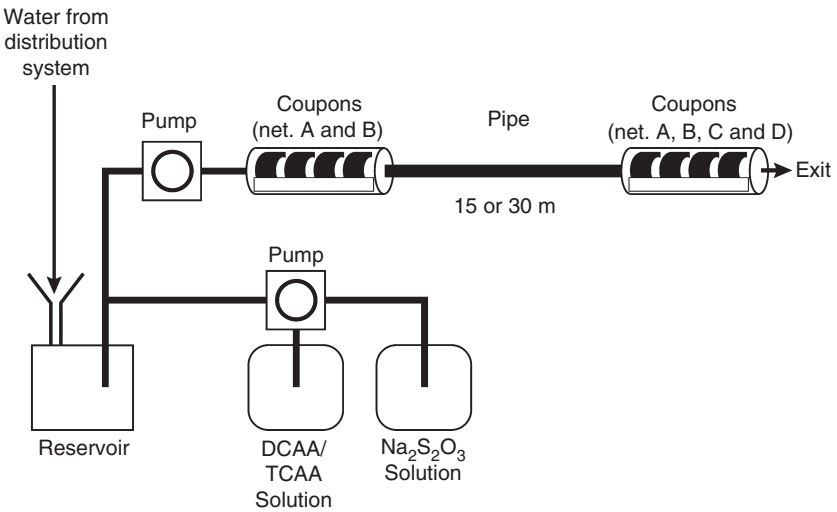

Figure 1 Schematic representation of the experimental model. Water from distribution system was accumulated in a reservoir where solutions of sodium thiosulfate and various concentrations of HAA were added by pumps. Only networks A and B possessed a rack located between the source of water and the main pipe. The length of the main pipe was 15 or 30 m depending of the model (see Table 1).

Table 1 Modelled network characteristics

				DCAA (μg l^{-1})		TCAA (μg l^{-1})	
Networks	Length (m)	Pipe lumen diameter (mm)	Flow rate (ml min^{-1})	Weeks 1 and 2	Week 3	Weeks 1 and 2	Week 3
A	30	12·7	60	30	20	30	40
B	30	12·7	60	60	20	60	80
C	15	25·4	120	30	20	30	40
D	15	25·4	120	60	20	60	80

using a peristaltic pump (Masterflex, Cole-Palmer, Vernon Hills, IL, USA) from a reservoir supplied continuously by distribution system water and equipped with an overflow. The water was dechlorinated using a solution of sodium thiosulfate ($Na_2S_2O_3$) (Fisher Scientific, Fair Lawn, NJ, USA). This solution was injected continuously using a multihead peristaltic pump (Masterflex, Cole-Palmer) at a rate of 1 ml min^{-1} (A and B) or 2 ml min^{-1} (C and D). The sodium thiosulfate concentration was adjusted according to the chlorine concentration in the network water (0–250 mg l^{-1} of $Na_2S_2O_3$). This same pump also served to inject the solution spiked with DCAA and TCAA during the campaigns (feeding periods, see Table 1).

Samples collection and preparation

Four feeding and sampling campaigns took place between October 2008 and September 2009 distributed as follows: campaign 1 (20 October to 11 November 2008), campaign 2 (9 February to 3 March 2009), campaign 3 (6–28 April 2009) and campaign 4 (17 August to 8 September 2009). For each campaign, coupon samples were collected within a 3-week period (two coupons at different time points; T = 0, 1, 7, 14, 21 days). The moment immediately before the beginning of a DCAA/TCAA feeding period represents T = 0. Each sampling campaign was separated by a period of 4 weeks minimum during which time no HAA was fed to the networks. Water flowed continuously in all the networks throughout the campaigns, the pause between the campaigns and for 3 months before the first experiment to allow biofilm formation.

The bacteria found on the coupons were analysed by culturing and molecular biology techniques. Biofilm was scraped from the coupons using a rubber policeman and was suspended in 6 ml of a 0·1% Tween 20 solution (Sigma, Oakville, ON, Canada). No additional steps were performed prior to plating, and only a few centrifugation steps were performed prior to extracting the DNA as described below. Preliminary biofilm recovery assays showed that this approach is more effective than

sonication based on the results of real-time PCR, which was performed to evaluate the quantity of recovered bacteria (data not shown). This is likely due to the smooth surface of the coupons, which can be efficiently scraped using rubber policemen.

For bacterial culture assays, biofilm biomass from samples collected after 21 days of exposure in the experimental models was recovered from coupons (one coupon per network) and cultured on three different media: Tryptic soy agar (TSA) (Becton Dickinson, Sparks, MD, USA), BD Difco R2A agar (Becton Dickinson) and defibrinated sheep blood agar (Becton Dickinson) with appropriate dilutions to obtain isolated colonies. Petri dishes were incubated at 25°C between 24 and 48 h before further analyses.

For molecular analyses, the biomass found on coupons of all time points was scraped using the same technique in 6 ml of 0·1% Tween 20 solution. The biofilm biomass was first pelleted by a centrifugation at 4000 × ***g*** for 20 min. The pellets were conserved in approximately 1·5 ml of 0·1% Tween 20 solution and then centrifuged at 16 000 × ***g*** for 5 min. Then, the pellets were analysed after appropriate DNA extraction. DNA from all biofilm samples, and selected isolates were extracted using QIAamp DNA Mini kit (Qiagen, Mississauga, ON, Canada). The protocol for the extraction of bacterial DNA was applied based on the appendix D of the manufacturer's recommendations.

Storage and identification of bacterial isolates

Bacterial isolates were incubated in 5 ml of Difco tryptic soy broth (TSB) (Becton Dickinson) at 25°C between 24 and 48 h. After bacterial growth, 2 ml was transferred into a microtube, and bacteria were pelleted at 5000 × ***g*** for 1 min. Broth was removed and replaced by a solution of sterile TSB with glycerine 10%. The suspension was transferred into a cryogenic tube and stored at −80°C. The remaining liquid culture (3 ml) was used for DNA extraction as previously described. Identification of isolates was performed by sequencing the 16S rRNA gene PCR products on both strands using 63F and 1387R primers (Table 2). Prior to sequencing, 16S rRNA gene of each isolate was amplified in 50 μl PCRs containing: 1 × GoTaq PCR buffer, 1·25 U GoTaq polymerase (Promega, Madison, WI, USA), 3 mmol l^{-1} of $MgCl_2$, 200 μmol l^{-1} dNTP, 0·5 μmol l^{-1} of each primer and 5 μl of extracted DNA. The amplification program used on the DNA Engine DYAD thermocycler (Bio-Rad, Mississauga, ON, Canada) was the following: 5 min hot start at 94°C followed by 45 cycles of 60 s at 95°C, 60 s at 57°C, 90 s at 72°C and a final extension step of 5 min at 72°C (Marchesi *et al.* 1998). PCR products were visualized by 1% agarose gel electrophoresis and sequenced by the Centre Hospitalier de l'Université Laval sequencing platform. Sequences were analysed by means of the Chromas lite software (Technelysium Pty Ltd; www.technelysium.com.au/chromas.html) and BioEdit Sequence Alignment Editor version 7.0.9.0 (Hall 1999). Each DNA sequence obtained was compared with sequences available in databases, using BLASTN (Altschul *et al.* 1990) from the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences affiliation to known genus or species was assessed based on their similarity.

DGGE analysis

The variable V3-V5 regions of 16S rRNA gene sequences found in biofilm biomass were amplified by PCR with GC-341f and 907r primers (Table 2) in a 50 μl PCR mixture containing 1 × GoTaq PCR buffer, 0·25 μmol l^{-1} of each primer, 2·5 mmol l^{-1} of $MgCl_2$, 200 μmol l^{-1} of each dNTP, 2% DMSO, 1·25 U of GoTaq polymerase (Promega) and 5 μl of DNA extract. The amplification program used was the following: 5 min at 94°C; 9 touchdown cycles of 30 s at 94°C, 30 s at 56°C (decrease of 0·5°C at every cycle), 1 min at 72°C; 24 cycles of 30 s at 94°C, 30 s at 51°C, 1 min at 72°C; and a final elongation step of 5 min at 72°C. After gel electrophoresis (1·5% (w/v) agarose gel) of 5 μl of the PCR products, the amount of amplified DNA was quantified by comparing band intensities to standard curves obtained with an EZ Load Precision Molecular Mass Ruler (Bio-Rad). Band intensities were measured with GeneTools analysis software (SynGen, Cambridge, UK). DGGE profiles of the amplified 16S rRNA gene sequences were produced as described (Muyzer *et al.* 1993). PCR products (100 ng) were loaded onto 6% polyacrylamide gel in 0·5 × TAE buffer (Bio-Rad) with 30–55% denaturant gradient (100% denaturant was 7 mol l^{-1} urea and 40% (v/v) deionized formamide). The electrophoresis was carried out in 0·5 × TAE buffer at 60 V for 16·5 h at 60°C. The DNA fragments were stained for 20 min in 0·5 × TAE buffer with SYBR Gold (Molecular Probes, Eugene, OR, USA). Gels were washed twice in 0·5 × TAE buffer for 20 min. Images of the gels were acquired using the imaging system ChemiGenius 2 (SynGen) and the imaging software GeneSnap (SynGen). DNA from bands was picked using a sterile tip and transferred into a 50 μl PCR mixture. Components in the PCR mix were the same as for the PCR-DGGE, except for primer 341f that was without GC-clamp. The amplification program used was described earlier. PCR products were visualized by 1% agarose gel electrophoresis and directly sequenced. Each DNA sequence obtained was compared with sequences available in databases, using BLASTN (Altschul

Table 2 Primers used in this study

Primers†	Target	Sequence	References
Primers used for the PCR-DGGE analysis			
341f‡	Bacteria 16S rDNA	5′-CCT ACG GGA GGC AGC AG-3′	Muyzer *et al.* (1993)
907r	Bacteria 16S rDNA	5′-CCG TCA ATT CCT TTG AGT TT-3′	Yu and Morrison (2004)
Primers used for the identification of bacterial isolates			
63f	Bacteria 16S rDNA	5′-CAG GCC TAA CAC ATG CAA GTC-3′	Marchesi *et al.* (1998)
1387r	Bacteria 16S rDNA	5′-GGG CGG WGT GTA CAA GGC-3′	Marchesi *et al.* (1998)

FAM, 6-carboxyfluorescein; BHQ-1 and IB™FQ, Black Hole Quencher-1 and Iowa Black Fret Quencher (Integrated DNA Technologies, Coralville, IA, USA).

†All the primers used in this study were purchased from IDT (Coralville, IA, USA).

‡A GC-clamp was attached on 5′ side of the forward primer 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3′ (Muyzer *et al.* 1993) when required.

et al. 1990) from the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences affiliation to known genus or species was assessed regarding their similarity.

Fingerprinting II Informatix Software version 3.0 (Bio-Rad) was used to normalize and compare all the DGGE profiles using hierarchical clustering to join similar profiles into groups (Fromin *et al.* 2002). For this purpose, all the images of DGGE gels were matched using a custom-made molecular DGGE ladder. A tolerance in the band position of 1% was applied during clustering, using bands showing intensity at least 8% of the most intense band on the lane. The similarity among profiles was calculated with the Pearson product–moment correlation coefficient, and the clustering was performed with the unweighted pair-group method using arithmetic averages (UPGMA). Dendrograms were constructed to evaluate the influence of each individual parameter (network, season, feeding time) on the bacterial community structure.

Bacterial growth capacity in the presence of DCAA

Isolates were grown on SM 1/5 agar plates (0·2 g l^{-1} yeast extract, 2 g l^{-1} bactopeptone, 0·2 g l^{-1} K_2HPO_4, 0·44 g l^{-1} KH_2PO_4, 0·2 g l^{-1} $MgSO_4 \bullet 7H_2O$, 2 g l^{-1} glucose, 20 g l^{-1} bactoagar) at 21°C prior to their inoculation on SM 1/5 agar plates containing various concentrations of DCAA from 486 μg l^{-1} to 7·6 g l^{-1}. The isolates were streaked on DCAA agar plates, and the amount of bacterial growth was recorded after a 7-day incubation at 21°C.

HAA biodegradation assays

The bacteria were cultivated 24 h before the assay at 21°C on TSA agar plates (EMD Biosciences, Inc., La Jolla, CA, USA). For each isolate or group of isolates tested, multiple flasks with 150 ml of SM 1/5 broth supplemented with 40 μg l^{-1} of DCAA or TCAA were then inoculated with one colony per isolate or with a mix of various bacteria and were incubated for up to 72 h at 10, 18 or 21°C with agitation (200 rpm). Two samples of 50 ml were collected at 0, 6, 24, 48 and 72 h or at 0, 24 or 72 h depending on the experiments (one flask per sampling time). The moment immediately before the addition of bacteria represents T = 0. The samples were centrifuged 16 000 × ***g*** for 10 min, filtered (0·2 μm) and stored at 4°C prior to DCAA or TCAA extraction. The extraction and quantification of DCAA and TCAA was done as previously described (Pluchon *et al.* 2013). Briefly, HAAs were measured using the EPA 552.2 method (USEPA 1995) with a gas-phase chromatograph equipped with an electron capture detector (GC-ECD). As control, no interference of the broth with the extraction and quantification procedure was observed (data not shown).

Results

PCR-DGGE fingerprint profiles

DGGE profiles from all coupons samples were obtained and compared by clustering analysis (Fig. 2). Specific network profiles did not group, suggesting that pipe diameter, HAA ratios and flow rate had little influence on community structure. However, campaign 4 (August–September 2009) samples clustered, with similarities higher than 75% between profiles for networks A, B and C. This suggests that season had an impact on biofilm organization inside the pipe, with summer profiles being different than those observed the rest of the year. The results were the same for the duplicates in the same networks. Also, the position of the racks at the beginning or at the end of the network (networks A and B) did not influence the result obtained between coupons (data not shown).

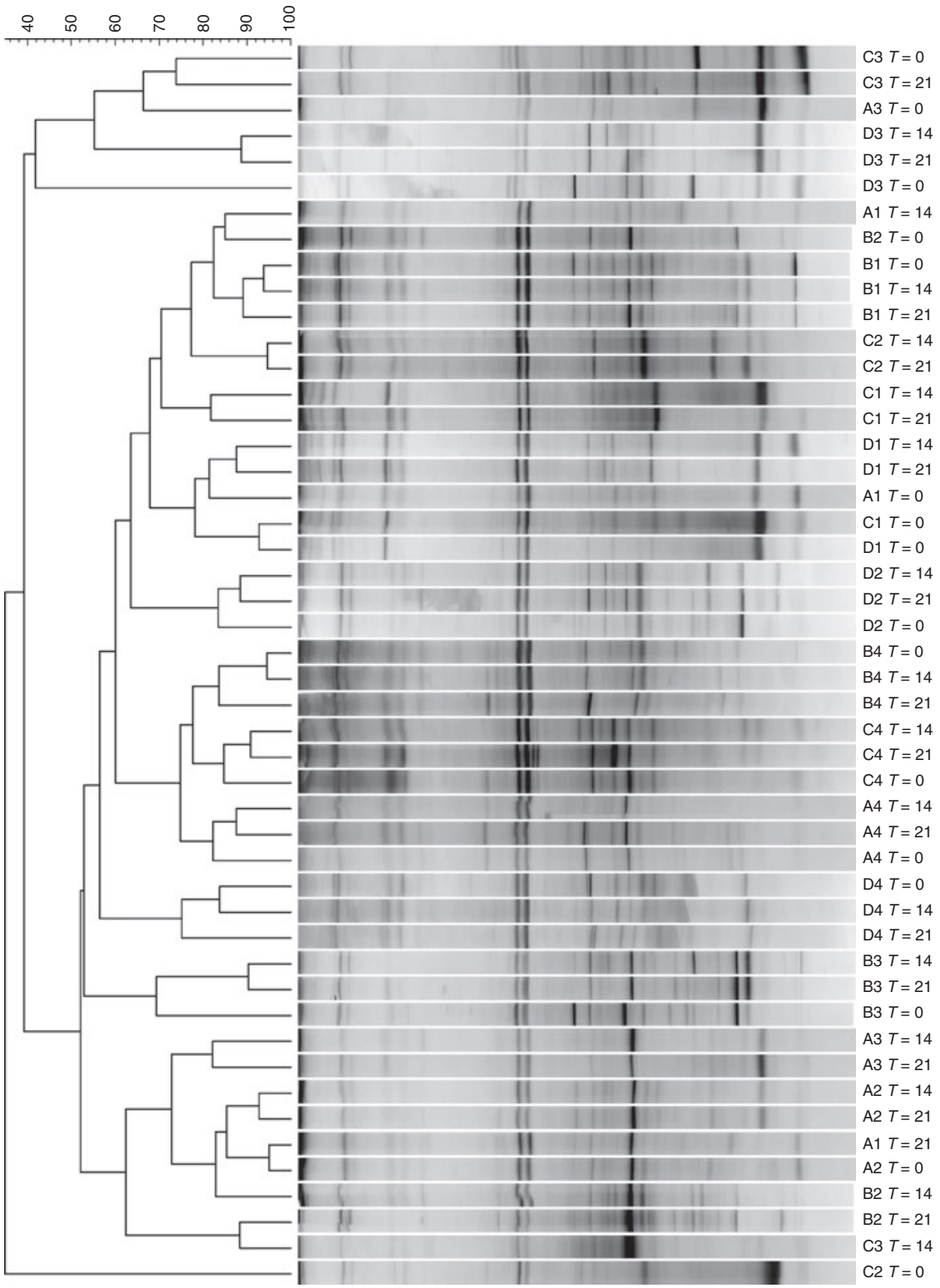

Figure 2 Dendrogram of DGGE profiles from simulated drinking water networks fed with DCAA and TCAA. The dendrogram was built with UPGMA method using Pearson's correlation (minimum profiling 8% of relative maximum value of lane). Profiles identification refers to Network (A, B, C, D), campaign number (1 [October–November 2008], 2 [February–March 2009], 3 [April 2009], 4 [August–September 2009]) and sampling time (T = 0–21 days). As the results were the same for the duplicates and the rack at the beginning of the network (networks A and B), only the results for one rack per network were shown to simplify the figure.

In fact, clustering analyses performed on samples from each network separately confirmed low similarity between winter and summer campaigns. A clustering analysis of DGGE profiles performed on all tested networks but after a fixed feeding time (T = 21 days) showed more clearly that summer's community structure was different than other seasons, with only 45% similarity between summer and winter profiles notwithstanding the network. Specific profiles clustering analysis also showed that DCAA/TCAA ratios and pipe diameter had minimal impact on bacterial community structure in the biofilm.

Analyses revealed high similarity (superior to 80%) between profiles within the same campaign, indicating that bacterial community remains relatively constant throughout a specific feeding period. However, analysis performed on individual networks did not allow the clustering of T = 0 samples of any of the four campaigns, with an average similarity of about 50% between these DGGE profiles. This suggests that the biofilm evolved during the study from when it was first established inside the pipes. It seems that there is no returning to initial bacterial community between campaigns. These observations support the existence of a dynamic population inside the biofilm that evolves through time. However, two bands corresponding to *Aquabacterium* spp. and to an uncultured bacterium were observed regardless of the season, suggesting that the season did not affect the entire bacterial community in the biofilm but only some populations.

Table 3 Affiliation† of micro-organisms found by DGGE and culture analyses. The identification of the micro-organisms was performed by sequencing the 16S rRNA gene PCR products. Results from all campaigns were combined. The bacteria genera that were common to both analytical approaches are indicated in bold

Phylum	DGGE	Culture
Actinobacteria	***Mycobacterium* sp.**	***Mycobacterium* sp.**
	Propionibacterium sp.	*Rhodococcus* sp.
		Arthrobacter sp.
		Microbacterium sp.
Firmicutes	*Geobacillus* sp.	*Bacillus* sp.
Proteobacteria		
Alpha	***Sphingomonas* sp.**	***Sphingomonas* sp.**
	Rhizobiales	*Methylobacterium* sp.
Beta	***Cupriavidus* sp.**	***Cupriavidus* sp.**
	***Delftia* sp.**	***Delftia* sp.**
	Thiobacillus sp.	*Ralstonia* sp.
	Aquabacterium sp.	*Burkholderia* sp.
		Acidovorax sp.
Gamma	*Nevskia* sp.	*Pseudomonas* sp.
	Escherichia sp.	*Acinetobacter* sp.
	Shigella sp.	*Xanthomonas* sp.
Bacteroidetes		*Chryseobacterium* sp.
		Elizabethkingia sp.
		Flavobacterium sp.

†Sequence length between 560 and 598 bp were used for the analysis. Only organisms with sequence identity higher than 90% are presented in this table for DGGE analyses.

Bacterial isolates identification

From each coupon, the most abundant culturable micro-organisms found on the plates were isolated and their 16S rRNA genes were sequenced on both strands. The genus of all sequenced DGGE bands and cultured bacteria isolated from the networks, are presented in Table 3. The complete list of the bacterial isolates with their identification to species (when possible) is detailed in Table 4. Even though the DGGE profiles displayed dynamic behaviour inside the pipes, most micro-organisms that we isolated were present during the ageing of the biofilm and were generally retrieved throughout the experiment. No micro-organisms that were identified based on the DGGE bands or that were cultured appeared to be specific to a network, a season or operational conditions during the present study. However, *Variovorax* spp., *Herminiimonas* spp. and *Flavobacterium* spp. were only cultured in campaign 3 and *Acidovorax* was only cultured in campaign 4. The variations in biomass (Pluchon *et al.* 2013) and the bacterial profiles determined by DGGE in all four campaigns indicated that the biofilms were very dynamic. As such, the bacterial diversity of the networks is presented as a whole to provide an overall picture of this kind of structure.

Identification of isolates able to degrade DCAA

From 2008 to 2010, DCAA but not TCAA degradation has been observed in the drinking water network model used in this study (Pluchon *et al.* 2013). Bacterial isolates were recovered from the model during the same period. Consequently, the second step of our analysis focused on the identification of bacteria able to degrade DCAA among these isolates. To facilitate this identification, the capacity of isolates to tolerate the presence of significant concentrations of this compound during their growth was tested. The highest concentration of DCAA on which all of the 48 isolates were able to grow was 61 mg l^{-1}. Only 15 isolates showed a good level of growth (++ or +++) at 304 mg l^{-1} of DCAA (Table 4). These isolates were chosen for DCAA degradation assays.

For the biodegradation assays, the 15 isolates were first tested in one group. A decrease of DCAA level was seen after 72 h (Table 5) suggesting that at least one of the 15 isolates was capable of DCAA degradation. Subgroups were then made with the isolates

Table 4 Identification of isolates obtained from culture and growth capacity in presence of DCAA. The identification of the isolates was performed by sequencing the 16S rRNA gene PCR products

Campaign†	Isolate	Homology (%)‡	Bacterial identification	Growth capacity in the presence of 304 mg l^{-1} of DCAA§
1	1	99	*Pseudomonas fluorescens* AB680296.1	+
1	2	99	*Pseudomonas plecoglossicida* JN700130.1	+
1	3a	99	*Cupriavidus* sp. JN226398.1	++
		99	*Cupriavidus basilensis* GU220488.1	
1	3b	100	*Ralstonia insidiosa* FJ772078.1	+++
1	4	100	*Cupriavidus basilensis* GU220488.1	+/−
1	5	100	Uncultured bacterium JQ769981.1	+
		100	Uncultured bacterium JQ769972.1	
		100	*Muciloginibacter* sp. JX089330.1	
1	6	100	*Ralstonia pickettii* HE575958.1	+
1	8	100	*Pseudomonas reactans* JN411452.1	+
		100	*Pseudomonas syncyanea* AB680130.1	
1	9	100	*Cupriavidus basilensis* GU220488.1	+++
		100	*Cupriavidus* sp. JN226398.1	
1	10	99	*Rhodococcus qingshengii* HE820128.1	N/D
		99	*Rhodococcus erythropolis* JQ435727.1	
1	11	99	*Chryseobacterium* sp. FJ938215.1	+
		99	*Elizabethkingia miricola* EU375848.1	
1	12	100	*Pseudomonas* sp. JQ320089.1	+/−
1	13	99	*Bacillus thuringiensis* JQ342856.1	−
1	14	100	*Pseudomonas putida* JF928561.1	++
		100	*Pseudomonas* sp. JN871227.1	
1	15	99	*Acinetobacter johnsonii* JN409466.1	−
1	16	99	*Chryseobacterium* sp. FJ938215.1	+
		99	*Elizabethkingia miricola* EU375848.1	
1	17	93	Uncultured bacterium FJ229319.1	−
		92	*Elizabethkingia* sp. GU086415.1	
		92	*Chryseobacterium* sp. FJ938215.1	
1	18	99	*Ralstonia pickettii* HE575958.1	+
		99	*Ralstonia mannitolilytica* JN699058.1	
1	19	99	*Chryseobacterium* sp. FJ938215.1	−
		99	*Elizabethkingia miricola* EU375848.1	
1	20	95	*Bacillus thuringiensis* JQ342856.1	−
		95	*Bacillus cereus* JN700160.1	
2	21	99	*Cupriavidus basilensis* GU220488.1	N/D
2	22	98	*Pseudomonas gessardii* NR_024928.1	N/D
		98	*Pseudomonas fluorescens* AB680974.1	
2	23	100	*Pseudomonas* sp. EU082808.1	−
		100	*Pseudomonas fluorescens* AY092072.1	
2	24	99	*Arthrobacter chlorophenolicus* CP001341.1	N/D
		99	*Arthrobacter niigatensis* HQ236055.1	
2	25	99	*Pseudomonas* sp. AF205135.1	N/D
		99	*Pseudomonas fluorescens* AB680296.1	
2	26	99	*Delftia* sp. JF274927.1	−
2	27	100	*Pseudomonas* sp. JF901709.1	N/D
2	28	100	*Sphingomonas* sp. AB265150.1	+++
		100	*Alpha proteobacterium* AY162053.1	
2	29	100	*Methylobacterium* sp. AB600003.1	N/D
2	30	99	*Mycobacterium* sp. FJ605266.1	N/D
2	31	100	*Pseudomonas* sp. EU082808.1	+/−
		100	*Pseudomonas fluorescens* AY092072.1	
2	32	100	*Delftia tsuruhatensis* JN590248.1	−
2	33	100	*Mycobacterium* sp. FJ605266.1	N/D

Table 4 (*Continued*)

Campaign†	Isolate	Homology (%)‡	Bacterial identification	Growth capacity in the presence of 304 mg l^{-1} of DCAA§
2	35	99	*Cupriavidus basilensis* GU220488.1	+
2	36	99	*Delftia tsuruhatensis* JN590248.1	N/D
		99	*Delftia* sp. HQ327477.1	
2	37	99	*Rhodococcus* sp. HQ256820.1	+/−
2	38	99	*Cupriavidus basilensis* HQ647269.1	N/D
2	39	100	*Delftia* sp. JF274927.1	N/D
2	40	99	*Rhodococcus* sp. HQ256820.1	+/−
2	41	99	*Sphingomonas echinoides* AB649019.1	N/D
		99	*Alpha proteobacterium* HM163221.1	
2	42	100	*Sphingomonas* sp. AB265150.1	++
		100	*Alpha proteobacterium* AY162053.1	
2	43	99	*Mucilaginibacter* sp. HQ825034.1	N/D
		99	*Sphingobacteriaceae bacterium* AB545741.1	
2	44	99	*Cupriavidus basilensis* GU220488.1	N/D
2	45	99	*Pseudomonas* sp. EU082808.1	N/D
		99	*Pseudomonas fluorescens* AY092072.1	
2	46	99	*Pseudomonas fluorescens* AB680974.1	N/D
		99	*Pseudomonas gessardii* NR_024928.1	
2	47	99	*Pseudomonas fluorescens* AB680974.1	N/D
2	48a	99	*Cupriavidus basilensis* GU220488.1	N/D
2	48b	99	*Pseudomonas fluorescens* JQ236807.1	N/D
2	49	100	*Pseudomonas* sp. EU082808.1	N/D
		100	*Pseudomonas fluorescens* AY092072.1	
2	50	99	*Pseudomonas* sp. EU082808.1	N/D
		99	*Pseudomonas fluorescens* AY092072.1	
2	51	99	*Cupriavidus basilensis* GU220488.1	N/D
2	52	99	*Arthrobacter* sp. GU377123.1	N/D
2	53	99	*Delftia* sp. JF274927.1	N/D
3	54a	99	*Cupriavidus basilensis* GU220488.1	N/D
3	54b	100	*Bacillus cereus* JX077093.1	N/D
		100	*Bacillus anthracis* JX077088.1	
3	55	99	*Pseudomonas fluorescens* AB680974.1	N/D
		99	*Pseudomonas gessardii* NR_024928.1	
3	56	95	*Luteibacter anthropi* JF708871.1	N/D
3	57	99	*Sphingomonas* sp. AB495350.1	N/D
		99	*Sphingomonas yunnanensis* EU730917.1	
3	59	99	*Pseudomonas* sp. EU082808.1	N/D
		99	*Pseudomonas fluorescens* AY092072.1	
3	60	99	*Pseudomonas* sp. EU082808.1	N/D
		99	*Pseudomonas fluorescens* AY092072.1	
3	61a	99	*Cupriavidus basilensis* GU220488.1	N/D
3	61b	99	*Bacillus cereus* JX077092.1	N/D
		99	*Bacillus anthracis* JX077088.1	
3	63	99	*Microbacterium luteolum* JQ282808.1	N/D
		99	*Microbacterium* sp. FJ785511.1	
3	64	99	*Pseudomonas* sp. EU082808.1	N/D
		99	*Pseudomonas fluorescens* AY092072.1	
3	65	99	*Sphingopyxis witflariensis* JF459986.1	N/D
		99	*Sphingopyxis* sp. DQ112242.1	
3	66	99	*Variovorax paradoxus* FJ527675.1	N/D
3	67	99	*Herminiimonas glaciei* AB681896.1	N/D
		99	*Herminiimonas* sp. HE610501.1	
3	68	99	*Herminiimonas glaciei* AB681896.1	N/D
		99	*Herminiimonas* sp. HE610501.1	

Table 4 (*Continued*)

Campaign†	Isolate	Homology (%)‡	Bacterial identification	Growth capacity in the presence of 304 mg l^{-1} of DCAA§
3	69	98	*Pseudomonas* sp. EU082808.1	N/D
		98	*Pseudomonas fluorescens* AY092072.1	
3	70	99	*Pseudomonas* sp. EU082808.1	N/D
		99	*Pseudomonas fluorescens* AY092072.1	
3	71	99	*Pseudomonas* sp. EU082808.1	N/D
		99	*Pseudomonas fluorescens* AY092072.1	
3	72	100	*Pseudomonas* sp. AF205135.1	++
		99	*Pseudomonas koreensis* HM367599.1	
3	73	99	*Flavobacterium* sp. HE612100.1	N/D
3	74	99	*Methylobacterium* sp. AB600003.1	N/D
4	94	99	*Acidovorax facilis* JQ236816.1	–
		99	*Acidovorax* sp. AY623930.1	
4	95	99	*Cupriavidus basilensis* GU220488.1	+
4	96	99	*Mucilaginibacter* sp. HQ825034.1	–
4	97	100	*Uncultured bacterium* JF145422.1	–
		99	*Acidovorax radicis* JF915365.1	
		99	*Acidovorax* sp. GU086421.1	
4	98	100	*Microbacterium* sp. FJ605266.1	+++
4	99	100	*Pseudomonas* sp. HM755541.1	++
			Pseudomonas putida JN606325.1	
4	100	99	*Pseudomonas gessardii* NR_024928.1	–
		99	*Pseudomonas fluorescens* AB680974.1	
4	101	99	*Cupriavidus* sp. JN226398.1	++
		99	*Cupriavidus basilensis* GU220488.1	
4	102a	100	*Pseudomonas* sp. HM755541.1	++
		100	*Pseudomonas putida* JN606325.1	
4	102b	100	*Luteibacter anthropi* JF708869.1	+++
4	103	99	*Elizabethkingia miricola* EU375848.1	+
		99	*Chryseobacterium* sp. FJ938215.1	
4	104	99	*Sphingopyxis witflariensis* JF459986.1	–
		99	*Sphingopyxis* sp. DQ112242.1	
4	105	100	*Pseudomonas* sp. EU681017.1	++
		100	*Pseudomonas reactans* JN662499.1	
4	106	99	*Elizabethkingia miricola* EU375848.1	+
		99	*Chryseobacterium* sp. FJ938215.1	
4	108	99	*Acinetobacter* sp. JN713903.1	–
4	109	99	*Cupriavidus basilensis* GU220488.1	++
4	110	100	*Pseudomonas reactans* JN662499.1	+
		100	*Pseudomonas* sp. EU681017.1	
4	111	100	*Pseudomonas* sp. AF205135.1	+++
		99	*Pseudomonas koreensis* HM367599.1	
4	112	99	*Acinetobacter* sp. JX177713.1	–

§N/D, not determined.
+/–: Light bacterial growth at the beginning of the streak; +: Light bacterial growth over almost the entire Petri dish; ++: More abundant bacterial growth, especially in the first half of the Petri dish; +++: Abundant bacterial growth over the entire Petri dish.
†Campaign 1: October–November 2008, 2: February–March 2009, 3: April 2009, 4: August–September 2009.
‡Sequences of about 1324 bp were used for the analysis.

mainly based on their species. Of the five subgroups, two showed a complete biodegradation of the DCAA (Table 5). The bacteria in these groups were all *Cupriavidus* sp. bacteria. The five *Cupriavidus* isolates were then tested individually, and three of them were able to degrade DCAA after 48 h. These isolates were #3a, 9 and 101 (Table 6). TCAA degradation was also attempted with the *Cupriavidus* isolates over 72 h. No

degradation of TCAA was observed for all tested isolates (Table 6).

The weather in the area of Quebec City is changing over the year with very cold and relatively long winters and tempered summers. Consequently, water temperature in the network model varied considerably, from 3 to 18°C over the year. The effect of these temperature variations was tested on the DCAA degradation capacity of the *Cupriavidus* isolates #3a, 9 and 101. Bacteria were grown either at 10 or 18°C on agar plates without DCAA and then incubated in broth with DCAA at the same or the opposite temperature for the biodegradation assay. Sampling was made at 0, 6, 24, 48 h and remaining DCAA was quantified (Fig. 3). The growth of the bacteria was similar at 10 or 18°C. However the three *Cupriavidus* isolates displayed DCAA degradation at 18°C but not at 10°C regardless of the temperature of the preculture (Fig. 3) suggesting an important effect of the temperature on the DCAA degradation capacity of *Cupriavidus*.

Table 5 DCAA biodegradation assays with various groups of isolates. Groups of bacteria were cultivated in SM 1/5 broth with 40 μg l^{-1} of DCAA and incubated at 21°C for 72 h. The bacteria were pelleted and the remaining DCAA measured in the supernatant after filtration

Sample	Isolates†	Remaining DCAA after 72 h (%)
Control	None	100
1	3a, 3b, 9, 14, 28, 42, 72, 98, 99, 101, 102a, 102b, 105, 109, 111	14
2	3a, 9	0
3	14, 99, 102a	102
4	72, 102b, 105, 111	100
5	28, 42, 98	100
6	3b, 101, 109	0

†Isolate numbers refer to those in Table 4.

Table 6 DCAA and TCAA biodegradation assays with *Cupriavidus* isolates. The bacteria were cultivated in SM 1/5 broth with 40 μg l^{-1} of DCAA or TCAA and incubated at 21°C for 48 or 72 h, respectively. The bacteria were pelleted and the remaining HAAs measured in the supernatant after filtration

Isolates†	Remaining DCAA after 48 h (%)	Remaining TCAA after 72 h (%)
Control (no bacteria)	109	100
3a	0	108
3b	96	103
9	4	103
101	0	107
109	100	N/D‡

†Isolate numbers refer to those in Table 4.
‡Not determined.

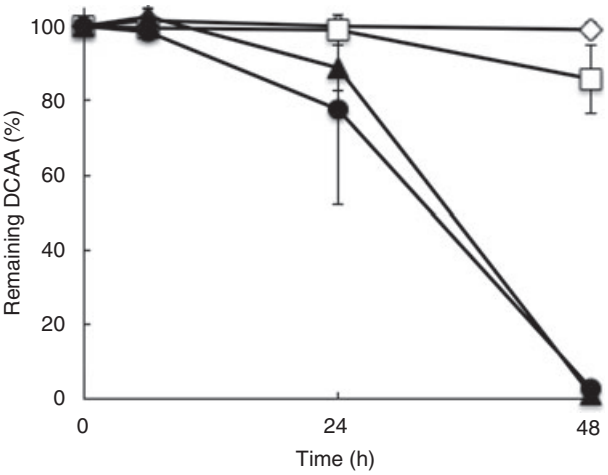

Figure 3 Influence of temperature on DCAA degradation by *Cupriavidus* isolates. Each curve represents the mean with the standard error of the mean of the remaining DCAA (%) over time for the three *Cupriavidus* isolates tested (3a, 9, and 101). The bacteria were grown either at 10 or 18°C for a preculture (temperature on the left of the arrow) and then incubated at the same or the opposite temperature for the biodegradation assay (temperature on the right of the arrow) (◇) 18°C; (□) 10°C; (▲) 18°C; (●) 10°C).

Discussion

Results obtained in the current study describe the bacterial community structure in the biofilm attached on the surface of a drinking water network model. The microbial diversity evolved throughout the experiment, but it was not influenced by pipe diameter or HAA ratios. DGGE profiles were more closely related during the summer, notwithstanding the network, during which season HAA degradation was observed (Pluchon *et al.* 2013).

Regarding the bacterial community structure, a majority of the isolates and DGGE bands belonged to *Alpha-*, *Beta-* and *Gamma-Proteobacteria*, but other groups of bacteria have been detected as well (Table 3). *Proteobacteria* are frequently identified in drinking water distribution systems (Eichler *et al.* 2006; Bayless and Andrews 2008; Zhang *et al.* 2009a; Henne *et al.* 2012; Liu *et al.* 2012). Some species detected during this study are frequently isolated from rivers and drinking water distribution system, such as *Acinetobacter* sp. (Simoes *et al.* 2007; Andersson *et al.* 2008), *Pseudomonas* sp. (Martiny *et al.* 2003; Andersson *et al.* 2008), *Methylobacterium* sp. (Simoes *et al.* 2007; Zhang *et al.* 2009a), *Mycobacterium* sp., *Sphingomonas* sp., *Burkholderia* sp. (Simoes *et al.* 2007), and *Afipia* sp. (Zhang *et al.* 2009a,b).

It has been reported that potential candidates for the biodegradation of HAAs are often members of the *Proteobacteria* phylum (Zhang *et al.* 2009a). The isolation of HAA-degrading bacteria from enrichment cultures by

cultivating bacteria on HAA supplemented agar plates was attempted previously (McRae *et al.* 2004). In this study, micro-organisms closely related to *Xanthobacter* sp. and *Sphingomonas* sp., both of the α-subdivision of *Proteobacteria* were found to be able to grow on monochloroacetic acid. *Xanthobacter autotrophicus* GJ10 was previously described as producing an haloacid dehalogenase (Janssen *et al.* 1985). McRae *et al.* (2004) also succeeded in isolating one organism able to grow on TCAA, which was affiliated to *Chryseobacterium* sp., a member of the *Cytophaga-Flavobacteria-Bacteroides* division. However, DGGE analyses suggested that these cultivated organisms constituted only a limited fraction of the entire community. As mentioned, no TCAA degradation was observed from 2008 to 2010 in the drinking water network model used in the present study, which corresponded to the period of the present study (Pluchon *et al.* 2013). However, significant TCAA degradation in the model was observed in summer 2011 (i.e. the subsequent summer) (Pluchon *et al.* 2013), suggesting that new species with the ability to degrade TCAA had entered the bacterial community over time.

Results obtained previously (McRae *et al.* 2004) and during the present study showed the importance of using bacterial cultures to complement the DGGE analysis. We identified 25 genera associated with biofilms in our model. Only four genera were found by both methods (*Cupriavidus* sp., *Mycobacterium* sp., *Sphingomonas* sp., and *Delftia* sp.). Interestingly, we observed greater diversity with the culture approach, with 14 genera specifically found using this method compared to 8 with DGGE. Despite their low proportion/incidence in the environment, it is sometimes possible to cultivate bacteria on selective media that are not detected by molecular biology techniques. Furthermore, micro-organisms isolated by culturing on selective media can be subsequently characterized in greater detail to investigate potential metabolic activities, including HAA degradation. In our case, this appeared to be very useful.

At this stage, it remains fairly difficult to identify the relative contribution of each parameter (bacterial community structure, available nutrients, water temperature, biomass quantity, organic carbon and water treatment operations) on HAA degradation. However, results were obtained on the capacity of *Cupriavidus* to degrade DCAA only at 18°C but not at 10°C. That correlates with the fact that DCAA degradation was observed only when water temperature in the model was elevated (Pluchon *et al.* 2013). However, the low biomass concentration found during winter and spring might also partially explain why DCAA was not degraded (Pluchon *et al.* 2013). On the other hand, DCAA degradation during fall, where water temperature was decreasing, was also lower than in summer even if the biofilm biomass was equivalently high for both seasons (Pluchon *et al.* 2013). Thus, the results presented here regarding the influence of water temperature on DCAA degradation combined with the conditions observed in fall (Pluchon *et al.* 2013) suggest that water temperature is really a crucial factor modulating DCAA degradation in the modelled network.

The present study revealed for the first time, to our knowledge, that *Cupriavidus* spp. can degrade DCAA. We found haloacid dehalogenase genes in the genomes of *Cupriavidus necator* (Poehlein *et al.* 2011) and *Cupriavidus taiwanensis* LMG19424 (Amadou *et al.* 2008). This suggests that some species of this genus have the capacity to degrade DCAA and probably other HAAs. The *Cupriavidus* genus belongs to the Proteobacteria phylum. Like *Cupriavidus* spp., other Proteobacteria such as *Afipia* spp. and the *Methylobacterium* spp. possess the capacity to degrade HAAs (Zhang *et al.* 2009a). In fact, members of the *Cupriavidus* genus are known as pollutant-degrading organisms. For example, *Cupriavidus necator* JMP134, a soil bacterium, degrades aromatic compounds, including halogenated ones (Perez-Pantoja *et al.* 2008). *Cupriavidus pauculus*, which was isolated from wastewater treatment system sludge, is an efficient degrader of 3,5,6-trichloro-2-pyridinol, another halogenated compound (Cao *et al.* 2012). Given that *Cupriavidus* spp. have been isolated from water and drinking water network biofilms by other researchers (Bai *et al.* 2010; Revetta *et al.* 2010, 2011), it is tempting to speculate that these bacterial species play an important role in the natural biodegradation of DCAA and perhaps other contaminants in drinking water networks. Further studies will be required to investigate the potential of these species for improving the quality of drinking water and in other applications.

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Conflict of interest

The authors declare no conflict of interest.

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