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RESEARCH ARTICLE

Molecular characterization of microbial communities and quantification of *Mycobacterium immunogenum* in metal removal fluids and their associated biofilms

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Abstract A number of human health effects have been associated with exposure to metal removal fluids (MRFs). Multiple lines of research suggest that a newly identified organism, *Mycobacterium immunogenum* (MI), appears to have an etiologic role in hypersensitivity pneumonitis (HP) in case of MRFs exposed workers. However, our knowledge of this organism, other possible causative agents (e.g., Pseudomonads), and the microbial ecology of MRFs in general, is limited. In this study, culture-based methods and small subunit ribosomal RNA gene clone library approach were used to characterize microbial communities in MRF bulk fluid and associated biofilm samples collected from fluid systems in an automobile engine plant. PCR amplification data using universal primers indicate that all samples had bacterial and fungal contaminated. Five among 15 samples formed colonies on the *Mycobacteria* agar 7H9 suggesting the likely presence of *Mycobacteria* in these five samples. This observation was confirmed with PCR amplification of 16S rRNA gene fragment using *Mycobacteria* specific primers. Two additional samples, Biofilm-1 and Biofilm-3, were positive in PCR amplification for *Mycobacteria*, yet no colonies formed on the 7H9 cultivation agar plates. Real-time PCR was used to quantify the abundance of *M. immunogenum* in these samples, and the data showed that the copies of *M. immunogenum* 16S rRNA gene in the samples ranges from 4.33×10^4 copy/ml to 4.61×10^7 copy/ml. Clone library analysis revealed that *Paecilomyces* sp. and *Acremonium* sp. and *Acremonium*-like were dominant fungi in MRF samples. Various bacterial species from the major phylum of proteobacteria were found and *Pseudomonas* is the dominant bacterial genus in these samples. *Mycobacteria* (more specifically *MI*) were found in all biofilm samples, including biofilms collected from inside the MRF systems and from adjacent environmental surfaces, suggesting that biofilms may play an important role in microbial ecology in MRFs. Biofilms may provide a shield or sheltered microenvironment for the growth and/or colonization of *Mycobacteria* in MRFs.

Keywords Metal removal fluids · Microbial community · Biofilm · Pathogens · *Mycobacterium immunogenum* · Shelter

Introduction

Metal removal fluids (MRFs) are used to lubricate machining contact surfaces, cool the working zone, prevent corrosion, and remove metal chips in metalworking processes, i.e., metal deformation and removing. They play a pivotal role in modern industries related to metal cutting and metal forming, which includes the automotive, aerospace, defense, and construction sectors. They have high annual consumption volume, with over 2 billion gallons used per year. About one million US workers are exposed annually to MRFs ((NIOSH) 1999). During their lifecycle, water-based MRFs are prone to microbial growth, impacting fluid integrity as well as giving rise to the emergence of opportunistic pathogens, creating risk of health problems, including respiratory illnesses, cancers, and dermatitis (Bennett 1972; Bennett and Bennett 1987; Mattsby-Baltzer et al. 1989; Mattsby-Baltzer et al. 1990; Tolbert et al. 1992; Savonius et al. 1994; Rossmoore 1995;

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Eisen et al. 1997; Simpson et al. 2003). Some reports have suggested that exposure to the aerosolized microbial antigens present in used fluids may cause respiratory disorders such as asthma, hypersensitivity pneumonitis, bronchitis, and other respiratory symptoms (Kennedy et al. 1989; Greaves et al. 1997; Gupta and Rosenman 2006). The presences of Pseudomonads and *Mycobacteria* in MRFs have been implicated in respiratory disorders such as hypersensitivity pneumonitis in the exposed machine workers (Mattsby-Baltzer et al. 1989). Pseudomonads and their endotoxins were initially considered the main cause of HP (Bernstein et al. 1995), whereas later reports linked *Mycobacteria* with HP(Kreiss and CoxGanser 1997; Shelton et al. 1999; Wilson et al. 2001; Wallace et al. 2002). In the past decade, more than two hundred hypersensitivity pneumonitis (HP) cases have been reported among workers in MRF processes and most of them have been associated with *Mycobacterium immunogenum* (MI) (Moore et al. 2000; Wilson et al. 2001; Gordon 2004; Gordon et al. 2006; Thorne et al. 2006). These reports highlight the need for more detailed analyses of these fluids and their aerosols for total microbial load including both culturable and nonculturable microorganisms.

Monitoring and regulation of the microbiology relevant to MRFs has relied primarily on culture-based analysis that specifically target classical pathogens. Although culture can be successful for assessment of some microbes, a large body of DNA sequence-based studies has shown that standard enrichment techniques significantly underestimate the actual quantity and diversity of microorganisms in a wide variety of environments (Pace 1997). Therefore, the goal of this study was to better understand the microbial composition in MRFs, with special focus *Mycobacteria* including *M. immunogenum* and the role of biofilms, using molecular techniques in addition to traditional culture-based approaches.

Materials and methods

MRFs sampling

A set of fifteen samples were collected on the same day from six different MRF systems in an automobile engine plant including ten bulk fluid samples and five biofilm samples (list of 15 samples are summarized in Table 1). Two biofilm samples (biofilm 1 and biofilm 4) were collected by scraping biofilms from the floor close to the machining and other three biofilm samples were collected by scraping biofilms from inside surfaces of MRF tanks. Two types of soluble oils, semi-synthetic, and synthetic oil, were used in those systems. An isothiazolone class of biocide had been added to all fluids. The collected samples were kept on wet ice and transported to the laboratory at the University of Michigan for immediate analysis within 24 h of collection.

Quantification of microorganisms using culture-based method

MRF samples were serially diluted with 1×PBS buffer and seeded onto agar plates. The total bacterial viable counts were estimated as CFU on nutrient-rich Tryptic Soy Agar plates (0.1 g liter^{-1} cycoheximide to inhibit fungal growth) after aerobic incubation at 30 °C for 5 to 7 days. The total viable *Mycobacteria* counts were estimated as CFU on Middlebrook 7H9 agar plates after incubation at 30 °C for 2 weeks.

DNA Extraction and PCR Amplification

One milliliter of each MRF sample was taken for DNA extraction using UltraClean Soil DNA Kit (Cambio Ltd., Cambridge, UK) according to the manufacturer's protocol. DNA was finally eluted with 50 μL of water. The quality and concentration of DNA was measured spectrophotoscopically using Nanodrop ND-100 (NanoDrop Technologies, Wilmington, Delaware, USA). DNA extracted from MRFs samples were amplified with the universal primer pairs 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 1391R (5′-GACGGGCGGTGWGTRCA-3′) targeting small ribosomal RNA gene of bacteria and fungi (Angenent et al. 2005). PCR reactions were carried out in 50 μL volumes containing 400 nM of each primer, 1 μL of DNA samples, 10 μL of 5×PCR buffer, 4 μL of dNTPs mix (2.5 mM of each dNTPs), and 0.25 μL of GoTaq DNA polymerase (Promega, Madison, WI). PCR was conducted with a Mastercycler gradient Machine (Eppendorf, Westbury, NY) by running 20 cycles with a gradient from 65 to 45 °C (92 °C for 30s, 65 °C to 1 °C/cycle for 30 s, 72 °C for 90 s) and an additional 20 cycles at a 45 °C annealing temperature (92 °C for 30 s, 45 °C for 90 s, 72 °C for 90 s) before a final extension at 72 °C for 20 min. *Mycobacteria* genus-specific amplification were conducted with the primer pair targeting Mycobacterial 16S rRNA gene, 515F and 1027R (5′-GCACACAGGCCACAAGGG-3′) (Angenent et al. 2005). MI species-specific amplification was conducted with the primer pair targeting *MI* genes pMycImmF (5′-GGGGTACTCGAG TGGCGAAC-3′) and pMycImmR (5′-GGCCGGCTACCCGT TGTC-3′) (Veillette and Thorne 2005). PCR reaction mixtures were the same to that mentioned above. These two sets of PCR were conducted with an initial denaturation at 94 °C for 2 min followed by 30 cycles of the following incubation pattern: 94 °C for 30 s, 63 °C for 30 °C, and 72 °C for 40 s. A final extension at 72 °C for 10 min concluded the reaction.

16S rRNA gene clone library construction and sequencing

The PCR products amplified with universal primers were checked by electrophoresis on a 2 % agarose gel, stained with SYBR Green I (Cambrex, Rockland, ME. USA), and visualized with a UV transilluminator. The approximately 0.8 and 1.2-kb

Table 1 Summary of data on the microbial characterization of fluid and biofilm samples collected from different systems in an automotive engine plant using plate counting and PCR amplification methods

System	MWFs	Sample[a]	Culture[b] TSA	Culture[c] 7H9	PCR Bacteria/Fungi	PCR[d] Myco/MI	Q-PCR[e] MI	Q-PCR[e] Bacteria	Ratio of MI/bacteria
S1	Soluble oil-1	Fluid-1	−	−	+	−	ND	1.24×10^{9}	
		Fluid-2	+	−	+	−	ND	5.45×10^{8}	
S2	Semi-synthetic	Fluid-3	+	−	+	−	ND	9.15×10^{7}	
		Fluid-4	−	−	+	−	ND	8.80×10^{6}	
S3	Washer+Synthetic	Biofilm-1	+	−	+	+	1.64×10^{5}	2.24×10^{8}	0.73‰
S4	Soluble oil-1	Fluid-5	+	−	+	−	ND	2.20×10^{10}	
		Fluid-6	+	−	+	−	ND	1.36×10^{10}	
		Biofilm-2	+	+	+	+	1.23×10^{5}	4.29×10^{9}	0.03‰
S5	Semi-synthetic	Fluid-7	+	−	+	−	ND	2.91×10^{10}	
		Fluid-8	+	−	+	−	ND	1.05×10^{10}	
		Biofilm-3	+	−	+	+	1.8×10^{5}	2.00×10^{10}	0.01‰
		Biofilm-4	+	+	+	+	4.61×10^{7}	1.18×10^{10}	3.9‰
S6	Soluble oil-2	Fluid-9	−	+	+	+	1.3×10^{6}	2.90×10^{10}	0.045‰
		Fluid-10	−	+	+	+	4.33×10^{4}	1.75×10^{10}	0.002‰
		Biofilm-5	+	+	+	+	5.91×10^{6}	2.52×10^{10}	0.24‰

[a] Samples Biofilm-1 and Biofilm-4 were taken from the floor and Biofilm-2, Biofilm-3, and Biofilm-5 were taken from the surfaces in the tank where bulk MWFs circulated

[b, c] Cultivation of bacteria and *Mycobacteria* from each sample on TSA and 7H9 plates, respectively. Data not expressed in colony forming units due to inadequate dilutions of some samples and colonies grown on plates were too many to count

[d] PCR amplification using both *Mycobacteria* (Myco) and *M. immunogenum* (MI)-specific primers

[e] The unit is copies of 16S rRNA gene per ml of sample. Biofilm samples collected were mixtures of fluids and solid biomass. *ND* not detected

heterologous 16S rRNA and 18S rRNA gene products were excised from the gel, and the PCR products were purified from the gel slices by using a Wizard PCR prep kit (Promega, Madison, Wis.). The gel-purified PCR products were cloned into the pGEM-T easy cloning vector as specified by Promega (Promega, Madison, WI) and transformed into calcium chloride-competent *Escherichia coli* JM109 cells according to the manufacturer's instructions and standard techniques (Promega, Madison, WI). Plasmid DNA was isolated from individual clones by using a Wizard Plus SV minipreps DNA purification system (Promega, Madison, WI). Aliquots from a subset of purified plasmid DNA were digested with the restriction enzyme *Eco*R I (TaKaRa, Otsu, Shiga, Japan) for 4 h at 37 °C, and the digested products were separated by electrophoresis on a 2 % agarose gel. After being stained with SYBR Green I, the bands were visualized using a UV transilluminator to select clones containing the appropriately sized insert. The clones with the correct plasmid insert were then used for DNA sequencing. Sequencing was performed by The University of Michigan's DNA Sequencing Core using ABI PRISM dye terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase FS and an ABI model 3730 DNA sequencer (Applied Biosystems, Foster, CA. USA). All sequences were deposited in the NCBI sequence database (http://www.ncbi.nlm.nih.gov/) under access numbers from EU118986 to EU119153.

Sequence analyses and phylogenetic tree analysis

Primary screening of these clone libraries for the presence of chimeric sequences was done using Mothur program (Schloss et al. 2009). The screening results were further analyzed with MEGA (Molecular Evolutionary Genetics Analysis, version 3.1) (Kumar et al. 2004) to dereplicate the libraries of 16S rRNA and 18S rRNA gene sequences for subsequent analyses by comparing all the sequences in a data set to each other, grouping sequences with ≥97 and ≥90 % identity together, and outputting a representative sequence from each group. The all-rRNA gene sequences of each group were first compared with those in the GenBank database using the basic local alignment search tool BLAST (http://www.ncbi.nlm.nih.gov/BLAST/BLAST.cgi). A 97 to 100 % match of the unknown sequence with the GenBank data set was considered an accurate identification to the species level, 93 to 96 % similarity was accepted as genus-level identification, and an 86 to 92 % match was considered an accurate identification of a related organism (Stackebrandt and Goebel 1994). The sequence data for reference strains were obtained from the GenBank database. Phylogenetic trees were constructed using neighbor-joining method and graphically represented with MEGA software.

Microbial community similarity comparison

Microbial community similarity comparison was conducted using UniFrac program on the website (http://bmf.colorado.edu/unifrac/index.psp). Briefly, sequences from the four libraries were aligned and a single phylogenetic tree containing all sequences was created using MEGA software and exported in Newick format. Then, a text file was created that contained the name of each sequence, the name of the environment that it came from, and the number of times each clone was observed. Then, the PCoA and Jackknife Cluster option were selected to perform UniFrac analysis.

Real-time PCR quantification of total bacteria and MI

Total bacteria quantification was carried out by using real-time PCR according to Einen et al. (Einen et al. 2008). PCR reactions mixture were in 20 μL volumes containing 500 nM of primer Eu338 (5′-ACTCCTACGGGAGGCAGCAG-3′) and Eu518 (5′-ATTACCGCGGCTGCTGG-3′), about 10 ng of template DNA, and 10 μL of 2×SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). PCR program was as follow: 95 °C for 15 min, 40 cycles of denaturing (15 s at 94 °C), annealing (30 s at 61 °C), and extension (30 s at 72 °C). The cycling was followed by a final extension at 72 °C for 7 min, and a melting curve analysis from 65–95 °C with a plate read every 0.5 °C. Quantification of MI was accomplished by a TaqMan assay according to Veillette et al. (Veillette et al. 2008). Primers pMycImmF/pMycImmR and a dual-labeled probe (FAM-5′-CCGCATGCTTCATGGTGTGTGGT-3′-BHQ1) were used for specifically detection of MI. PCR mixture comprises 1×QuantiTect probe PCR buffer (Qiagen, Valencia, CA), 400 nM of each primer and 100 nM of probe. The program consisted of 15 min hot start at 94 °C, followed by 40 cycles of 3 s at 94 °C and 1 min at 61.5 °C. Fluorescence was acquired by detection of FAM (excitation, 495 nm; emission, 520 nm). As calibration standard, a dilution series of quantitative standard DNA was used as template (Ochsenreiter et al. 2003; Kemnitz et al. 2005). It was prepared from strain *E. coli* K-12 or *M. immunogenum* ATCC 200506, respectively. The standard DNA solution was then diluted to 10^8 copy of target DNA molecule per microliter and stored at −20 °C.

Results

Viable bacteria and *Mycobacteria* from MRFs samples

In 11 out of 15 samples bacterial colonies formed on the TSA plates. Five samples (Biofilm-2, Biofilm-4, Fluid-9, Fluid-10 and Biofilm-5) formed colonies on the *Mycobacteria* cultivation agar 7H9 suggesting the likely presence of *Mycobacteria* in these five samples (Table 1). The number of colony-forming units on the TSA plates and 7H9 plates were not counted because of inconsistencies of these numbers with serial dilutions of the original fluid samples, which may be due to some components in the fluids may have inhibitory factors.

PCR-based detection of *Mycobacteria* and *M. immunogenum* in MRFs samples

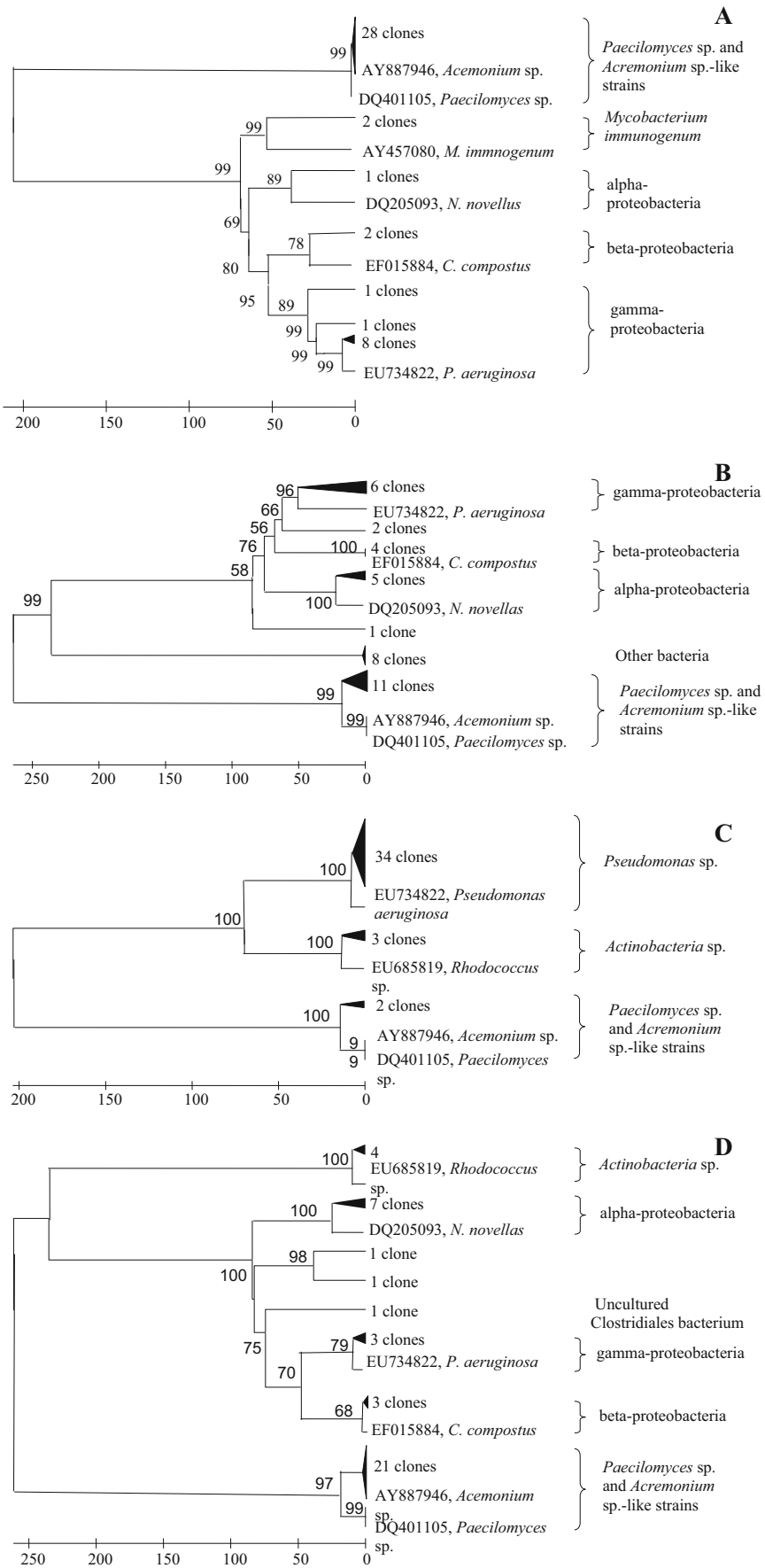
Detection of *Mycobacteria* and *M. immunogenum* in the collected MRF samples were performed using the *Mycobacteria* genus-specific primers 515F/1027R and species-specific primiers pMycImmF/pMycImmR, respectively. Among the 15 samples, the presence of *Mycobacteria* in the five samples detected by using culture-based method was confirmed with PCR amplification of 16S rRNA gene fragment using *Mycobacteria* genus-specific primers (data not shown). Two additional samples, Biofilm-1 and Biofilm-3, were positive in PCR amplification with expected 513 bp DNA fragment for *Mycobacteria*, although no colonies were formed on the 7H9 cultivation media. All seven samples that had positive PCR amplicons of expected 220 bp DNA fragment using *Mycobacterium* genus-specific primers also had positive amplicons using *M. immunogenum* species-specific primers (confirmed by DNA sequencing). PCR analyses based on genus-specific PCR were summarized in Table 1.

Bacterial and fungal diversity in MRFs

To understand the composition of microorganisms in MRFs samples, partial SSU rRNA gene libraries of four samples (Fluid-7 and Biofilm-3 from System 5 and Fluid-9 and Biofilm-5 from System 6) were prepared by cloning PCR products amplified with universal primers 515F/1391R targeting SSU rRNA gene (Angenent et al. 2005). Analysis of libraries based on universally conserved primers provided an estimate of the overall phylogenetic diversity in the samples. Fifty clones were randomly picked up from each SSU rRNA gene library for further DNA sequencing. Five small *Mycobacteria* SSU rRNA gene libraries using *Mycobacteria* genus-specific primers were constructed from *Mycobacteria*-genus PCR positive samples (Biofilm-1, Biofilm-2, Biofilm-3, Biofilm-4, Biofilm-5, Fluid-9, and Fluid-10) and 10 clones from each clone library were randomly selected for DNA sequencing.

Sequences were compared with sequences in the public database, and phylogenetic trees were generated using neighbor-joining method for each sample (Fig. 1). DNA sequencing of 50 clones from each library revealed that *Paecilomyces* sp. and

Fig. 1 Neighbor-joining phylogenetic tree based on nearly complete ▶ SSU rRNA gene sequences from four samples (A, Fluid-9, B, Fluid-7, C, Biofilm-5, and D, Biofilm-3). *Bootstrap values* were shown at the major branches. The scale at the bottom indicates sequence divergence. The numbers of clones that have the same sequences are indicated


A
28 clones
AY887946, Acemonium sp.
DQ401105, Paecilomyces sp.
Paecilomyces sp. and Acremonium sp.-like strains
2 clones
AY457080, M. immnogenum
Mycobacterium immunogenum
1 clones
DQ205093, N. novellus
alpha-proteobacteria
2 clones
EF015884, C. compostus
beta-proteobacteria
1 clones
1 clones
8 clones
EU734822, P. aeruginosa
gamma-proteobacteria
200 150 100 50 0
B
6 clones
EU734822, P. aeruginosa
gamma-proteobacteria
2 clones
4 clones
EF015884, C. compostus
beta-proteobacteria
5 clones
DQ205093, N. novellas
alpha-proteobacteria
1 clone
8 clones
Other bacteria
11 clones
AY887946, Acemonium sp.
DQ401105, Paecilomyces sp.
Paecilomyces sp. and Acremonium sp.-like strains
250 200 150 100 50 0
C
34 clones
EU734822, Pseudomonas aeruginosa
Pseudomonas sp.
3 clones
EU685819, Rhodococcus sp.
Actinobacteria sp.
2 clones
AY887946, Acemonium sp.
DQ401105, Paecilomyces sp.
Paecilomyces sp. and Acremonium sp.-like strains
200 150 100 50 0
D
4
EU685819, Rhodococcus sp.
Actinobacteria sp.
7 clones
DQ205093, N. novellas
alpha-proteobacteria
1 clone
1 clone
1 clone
Uncultured Clostridiales bacterium
3 clones
EU734822, P. aeruginosa
gamma-proteobacteria
3 clones
EF015884, C. compostus
beta-proteobacteria
21 clones
AY887946, Acemonium sp.
DQ401105, Paecilomyces sp.
Paecilomyces sp. and Acremonium sp.-like strains
250 200 150 100 50 0

Acremonium sp. and *Acremonium*-like were dominant fungal genera in all samples. More than 50 different bacterial species from the main phylum of proteobacteria were found in these four samples, and *Pseudomonas* was the dominant bacterial genus in these samples (>11 %). The microbial compositions of the different samples were highly variable. Among the clones sequenced, 185 (92.5 %) showed ≥97 % rRNA sequence identity to a database sequence, indicating a species-level relationship between the environmental organisms and the database reference organisms.

Similarity comparison of microbial community in MRFs samples

UniFrac analysis was used to determine whether or not these microbial compositions are significantly different (Lozupone and Knight 2005; Lozupone et al. 2006). Figure 2 shows a UniFrac clustering diagram comparing the communities from four different samples and two different systems. Sample Fluid-7 and Biofilm-3 from System5 clearly cluster together and samples Fluid-9 and Biofilm-5 from System 6 are separated from the above two samples. The data suggest that microbial compositions in the suspended fluid samples and biofilm samples from the same system are more similar to each other than those from different systems.

Quantification of *M. immunogenum* and bacteria in MRFs samples

In order to monitor the abundance of *M. immunogenum* in different MRFs samples, a quantitative PCR assay that allows estimating the relative amount of 16S rDNA of this species in the collected samples was developed. For this purpose, two methods (Taqman and SYBR real-time PCR) specific for MI and general bacterial domain, respectively, were used. Based on the calibration curves generated for MI and bacteria from known amounts of template DNA, the copy number of MI and bacterial 16S rRNA genes were calculated in each collected sample. This allowed determination of the relative abundance of MI in the different MRFs samples. The results are summarized in Table 1. The data show that the number of copies of total bacterial 16S rRNA genes in all samples ranges from 10^6 to 10^{10} copy/ml, and the number of copies of *M. immunogenum* 16S rRNA gene in 7 out of 15 samples ranges from 4.3×10^4 copy/ml to 4.6×10^7 copy/ml. The ratio of *M. immunogenum* to total bacteria, indicating the relative abundance of *M. immunogenum*, ranges from 0.002 to 4‰. The relative abundance of *M. immunogenum* in biofilm samples is high, especially in samples Biofilm-1 and Biofilm-4 collected from the floor adjacent to the MRF system. The relative abundance of *M. immunogenum* in

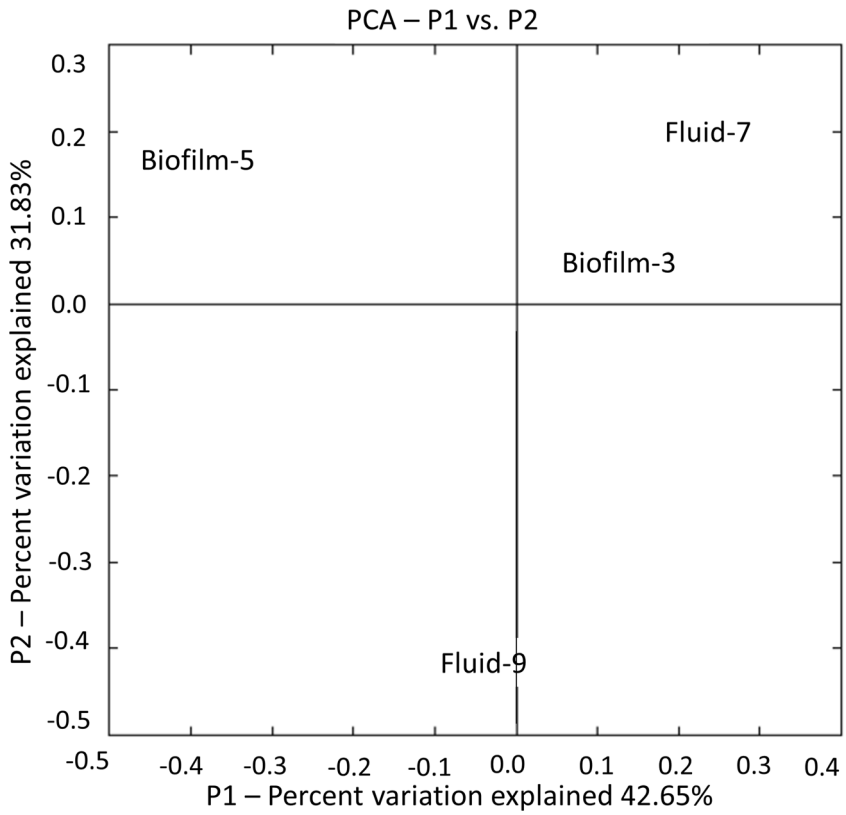


Fig. 2 Distribution of community samples in UniFrac principal coordinates analysis (PCoA). Unweighted UniFrac was used in the comparison. Samples Fluid-7 and Biofilm-3 from System5 clearly cluster together and samples Fluid-9 and Biofilm-5 from System 6 are separated from the above two samples from System 6

biofilm sample (Biofilm-5) is 5 or more times higher than that of *M. immunogenum* in the corresponding fluid samples (Fluid-9 and Fluid-10).

Discussion and conclusion

The present study, though limited in the number of samples collected, documents that traditional culture-based methods underestimate the full diversity of the microbial composition in MRFs. Culture-based and PCR methods were used to check the presence of microorganisms in MRFs samples. Although four out of 15 samples had no bacterial growth on TSA plates, all samples had bacterial and fungal detected by PCR method when the universal primers, targeting 16S rRNA gene of bacteria or 18S rRNA gene of fungi, were used. In addition, five samples formed colonies on *Mycobacteria* cultivation agar 7H9 suggesting the likely presence of *Mycobacteria* in these five samples. The presence of *Mycobacteria* in these five samples was confirmed with PCR amplification of 16S rRNA gene fragment using *Mycobacteria* specific primers. Two additional samples, Biofilm-1 and Biofilm-3, were positive in PCR amplification for *Mycobacteria*, yet no colonies formed on the 7H9 cultivation media. These five *Mycobacteria*-contaminated samples also contained *M. immunogenum* cells (which confirmed by DNA sequenceing of clone library). In addition, analysis of libraries based on *Mycobacteria*-specific primers provided an estimate of the overall phylogenetic diversity of *Mycobacteria* in the samples. The results showed all the DNA sequences amplified with *Mycobacteria*-specific primers had high identities to the 16S rRNA gene of *M. immunogenum* (>99 %) (total of 50 clones were sequenced, data not shown). These data imply that *M. immunogenum* was the dominant *Mycobacteria* species in all five *Mycobacteria*-contaminated samples.

Real-time PCR was used to quantify the total bacteria and the abundance of *M. immunogenum* in these samples. The data show that the number of copies of total bacterial 16S rRNA genes in all samples ranges from 10^6 to 10^{10} copy/ml and the number of copies of *M. immunogenum* 16S rRNA gene in 7 out of 15 samples ranges from 4.3×10^4 copy/ml to 4.6×10^7 copy/ml. The ratio of *M. immunogenum* to total bacteria, indicating the relative abundance of *M. immunogenum*, ranges from 0.002 to 4‰ (Table 1). The relative abundance of *M. immunogenum* in biofilm samples is high, especially in samples Biofilm-1 and Biofilm-4 collected from the floor. The relative abundance of *M. immunogenum* in biofilm sample 5 (Biofilm-5) is five or more times higher than that of *M. immunogenum* in the corresponding fluid samples (Fluid-9 and Fluid-10). Compared to the data from the culture-based analyses, the much higher concentration of *M. immunogenum* in these samples revealed by real-time PCR indicates that real-time PCR is significantly more sensitive than culture-based techniques, and/or a certain fraction of *M. immunogenum* in MRFs might be uncultivable or dead.

The rDNA sequences reveal the basic characteristics of environmental microorganisms related to known organisms. Our analyses detected and identified a broad diversity of microbes, more than 50 different strains and species. Most of them belong to fungi and alpha-, beta-, gamma-, and other proteobacteria (Fig. 1). Among these strains, *Paecilomyces* sp. and *Acremonium* sp. and *Acremonium*-like strains were dominant in fungi (>67 %), and Pseudomonads were the dominant gamma-proteobacteria strains (>23 %). Only 2 *M. immunogenum* DNA sequences were detected in Fluid-9 while no *M. immunogenum* sequences were found in Biofilm-5 although Biofilm-5 was shown to have *M. immunogenum* revealed by PCR analysis. This observation indicates that many more clones should be sequenced in order to obtain a better representation of microbial diversity in the MRF environment, especially to detect microorganisms of low abundance. It was interesting that there were eight clones identified as uncultured bacterium clones in Fluid-7 (16 % of total microbes).

Moreover, in sample Fluid-7, we found some clones were linked to geochemical recycle related strains, such as a DNA sequence with high identity to SSU rRNA gene of *Rhodocyclus* sp. HOD 5 (AY691423, 99 %), which was isolated from hydrogen-oxidizing-denitrifying bioreactor for treatment of nitrate-contaminated drinking water (Smith et al. 2005); a DNA sequence with high identity to SSU rRNA gene of *Desulfuromonas acetexigens* (DAU23140, 99 %), which has the ability to reduce Fe(III) and S0 (Lovley et al. 1995); and a DNA sequence with high identity to SSU rRNA gene of an uncultured bacterium clone TANB77 (AY667263, 93 %), which was detected in a dechlorinating community (Macbeth et al. 2004). These results suggest that some bacteria in MRF bacterial communities have the ability to use the ingredients in MRFs, such as mineral oil, monoethanolamine, and surfactant, as their nitrogen and energy source to support their growth and further supply nutrients for the growth of other strains.

This study enables several important observations. First, *Mycobacteria* (more specifically *M. immunogenum*) were found in all biofilm samples, including samples from inside the MWF systems and samples from adjacent environmental surfaces. In addition, the relative abundance of *M. immunogenum* in biofilm samples was much higher than that in the corresponding fluid samples. These findings strongly support that biofilms play an important role in the microbial ecology of MRFs, which confirmed previous studies also suggested that biofilms play a critical role in the microbial ecology of MWFs (Mattsby-Baltzer et al. 1989; Thorne et al. 1996; Skerlos et al. 2001). Biofilms may provide a shield or sheltered microenvironment for the growth and survival of *Mycobacteria* and act as seeding sources for

circulating MRFs. To control growth of *Mycobacteria* in MRFs, the primary target may need to be biofilms.

Second, in one particular system in this study, S6, using one type of soluble oil, growth of *Mycobacteria* was found in both fluid and biofilm samples and the relative abundance of *M. immunogenum* in biofilm samples was much higher than that in its corresponding fluid samples. It had been previously reported by the company that *Mycobacteria* contamination was present in this MRF system about 6 months prior to obtaining the present samples. The old MRF was discharged; the whole system was cleaned and recharged with this new type of MRF (soluble oil, which is designed for effective control of microbial growth including *Mycobacteria*). This system would be a valuable target system in which to study specific components in this MRF that may promote the growth of *Mycobacteria* in fluids or associated biofilms (Veillette et al. 2004; Selvaraju et al. 2008), or other factors in the system, e.g., certain locations where biofilms are abundant in the system may provide seeding of *Mycobacteria* for the new fluid.

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Conflict of interest All authors disclaim no conflict of interest.

Compliance with ethical standards We are in compliance with all Ethical Standards.

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