Title: Prospective Study of Biofilms, Mycobacteria and Pseudomonads in Metalworking Fluids

Investigator: Chuanwu Xi, Ph.D., Associate Professor, Department of Environmental Health Sciences, University of Michigan School of Public Health, Ann Arbor, MI Email: cxi@umich.edu

Alfred Franzblau, M.D., Professor, Department of Environmental Health Sciences

Affiliation: University of Michigan

State: MI

Telephone: (734) 615-7594

Award Number: 5-R21-OH-009306-02

Start & End Dates: 8/1/2009 to 7/31/2011

Program Area: Occupational Health

Final Report Abstract:

Abstract:

A number of human health effects have been associated with exposure to metal working fluids (MWFs), including various respiratory diseases, skin conditions, and cancers. Hypersensitivity pneumonitis (HP) in relation to metalworking fluids has been reported and there is strong evidence that the causative agent (or agents) is microbial contamination of the fluids. Multiple lines of research suggest that a newly identified organism, *Mycobacterium immunogenum*, appears to have an etiologic role. The goal of this study is to understand the microbial ecology of MWFs, with special focus on biofilms and Mycobacteria including M. immunogenum, using molecular techniques in addition to traditional culture-based approaches. Beginning in October, 2009, we started a one year monthly sampling campaign of seven MWF systems in two automobile plants. Both bulk fluid samples and associated biofilm samples from the same system were collected and a one-time aerosol sample was collected both inside and outside of one plant. Operational parameters were also recorded during the sampling campaign. Both traditional culture-based methods and quantitative real-time PCR methods were used to quantify total bacteria and *Mycobacterium immunogenum* from all collected samples. In addition, DNA pyro-sequencing was used to characterize microbial populations in metalworking fluids in selected samples from three systems in two plants. We found that all the samples had bacterial growth and *M. immunogenum* was also detected in most samples. The data showed that the relative abundance of *M. immunogenum* was significantly higher in biofilms than in bulk fluid samples. The DNA pyro-sequencing analysis revealed complex and different bacterial communities in metalworking fluids in different systems. Statistical analysis showed that common operational parameters except pH and oil content, including temperature, trap oil, type of coolants may not be able to explain the variability of relative abundance of *M. immunogenun* in these samples. The research team has shared their findings with stakeholders including UAW, two automobile plants via five data meetings and also disseminated data to the public via conference presentations. Our findings would be very useful information for metalworking fluid suppliers to improve their design of metalworking fluids and for plants to improve their routine management practice to save cost and improve occupational health.

Significant (Key) Findings

- Culture based method may not be accurate and less than 10% of bacterial cells can be cultured.

- All the samples had bacterial growth. Total bacterial concentrations were in the range of 10^5 to 10^7 cells/ml in bulk fluids from Company 1 (three systems mainly soluble oil) and 10^4-10^6 cells/ml in Company 2 (four systems mainly semi-synthetic or synthetic oil) based on realtime PCR quantification data. While in biofilm samples, total bacterial concentrations were similar and in the range of 10^4-10^5 cells/cm^2.
- *M. immunogenum* was also detected in most samples. Interestingly, the concentrations were very similar in all systems and in the range of 10^3-10^5 cells/ml in bulk fluid samples and 10^2-10^3 cells/cm^2 in biofilm samples except from one system in Company 2 which had 10^5 cells/cm^2 in biofilm samples. Surprisingly, the relative abundance of *M. immunogenum* could reach as high as 70% of total bacteria.
- On average, the relative abundance of *M. immunogenum* in biofilm samples was close to 100 times higher than that in bulk fluid samples.
- Statistical analysis found that common operational parameters except pH and oil content, including temperature, trap oil, may not be able to explain the variability of relative abundance of *M. immunogenum* in these samples.
- One DCR (drain, clean and recharge) event was monitored in Company 1. Our findings were in agreement with previous observations, bacterial counts increased very shortly after DCR and reached the same level in about a week in bulk fluid.
- The DNA pyro-sequencing analysis revealed complex and different bacterial communities in metalworking fluids in different systems and bacterial communities can be clustered and separated based on DNA sequencing data.

Translation of Findings

Our data found that all metalworking fluids had microbial contamination and *M. immunogenium* was ubiquitous in all systems examined. In order to monitor microbial contamination more precisely, molecular based detection methods should be developed and standardized. For better control of microbial contamination of metalworking fluids, biofilms that develop in systems should be a primary focus of management. We have communicated these findings to our stakeholders and expect that these would encourage them to improve their management approach. However, we have not done health risk assessment in this project and it is critical that we receive further support to perform health risk studies.

Outcomes/ Impact

Our findings provide useful information for metalworking fluid suppliers to improve their design of metalworking fluids and for plants to improve their routine management practice to save cost and improve occupational health. The outcome from this study and future projects will help users of MWFs to identify the right targets, i.e. biofilms, and develop novel approaches, i.e. molecular based methods, to monitor, control, reduce and/or prevent exposures to MWF-associated microbes that are suspected to cause adverse human health effects. Ultimately, this would serve to reduce the risk of adverse health events related to inhalation exposure to microbes or microbial products in MWFs.

Scientific Report

Background for the project

Metal working fluids (MWFs) 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 U.S. workers are exposed annually to MWFs [Sheehan 1999]. During their lifecycle water-based MWFs 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 [Simpson, et al. 2003, Rossmoore 1995, Bennett 1972, Tolbert, et al. 1992, Savonius, et al. 1994, Mattsby-Baltzer, et al. 1990, Mattsby-Baltzer, et al. 1989, Eisen, et al. 1997, Bennett and Bennett 1987]. 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, Gupta and Rosenman 2006, Greaves, et al. 1997]. The presences of pseudomonads and mycobacteria in MWFs have been implicated in respiratory disorders such as hypersensitivity pneumonitis in 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 HP with mycobacteria[Kreiss and CoxGanser 1997, Shelton, et al. 1999, Wallace, et al. 2002, Wilson, et al. 2001]. In the past decade, more than two hundred hypersensitivity pneumonitis (HP) cases have been reported among workers in MWF processes and most of them have been associated with M. immunogenum [Wilson, et al. 2001, Gordon 2004, Gordon, et al. 2006, Moore, et al. 2000, 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.

A biofilm is a well-organized community of microorganisms that adheres to surfaces and is embedded in an extracellular slime layer. Biofilms are ubiquitous in natural and industrial environments, and it is now thought that biofilms are the primary habitat for many microorganisms as biofilms can protect microbes from harsh environments such as the presence of antibiotics and biocides [Costerton, et al. 1995, Hall-Stoodley, et al. 2004, O'Toole, et al. 2000]. Biofilms have been associated with a wide range of problems both in industry and in medicine, and it is very difficult to eradicate them. Previous studies have suggested that biofilms play a critical role in the microbial ecology of MWFs [Mattsby-Baltzer, et al. 1989, Skerlos, et al. 2001, Thorne, et al. 1996]. Biofilms may provide a shield or sheltered microenvironment for the growth and survival of Mycobacteria and act as seeding sources for circulating MWFs. Study of biofilms associated with MWFs may contribute significantly to the understanding of growth and persistence of microbial contaminants in MWF systems and ultimately may provide a basis for control of exposures of workers to harmful microbes in MWF systems.

Monitoring and regulation of the microbiology relevant to MWFs 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 gene 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 ecology of MWFs, with special focus on Mycobacteria including *M. immunogenum* and the role of biofilms, using molecular techniques.

Specific aims

The goal of this study is to understand the microbial ecology of MWFs, with special focus on biofilms and Mycobacteria including *M. immunogenum*, using molecular techniques in addition to traditional culture-based approaches. We will carry out a prospective study to characterize the prevalence and dynamics of the growth of Mycobacteria in a variety of MWF environments (e.g., different fluids, different plants and processes, different biocides), including the bulk fluids, MWF aerosols, and MWF-associated biofilms, along with characterization of chemical and physical properties of the systems. Statistical tools will be used to identify parameters, in addition to biofilms, that are associated with promotion of the growth and survival of Mycobacteria in MWFs.

Our specific aims include the following:

Specific aim 1: Characterize microbial communities in MWFs and associated biofilms using molecular approaches. We will use the 16S/18S rRNA clone library approach to investigate microbial communities in MWFs and associated biofilms from 20 selected samples under regular operational conditions. These samples will be taken from a minimum of 2 MWF systems in each of 2 plants. The samples will be selected to include bulk fluid samples, biofilm samples and area aerosol samples from systems in two plants using different types of fluids (soluble oils, semi-synthetic fluid and synthetic fluid), and with or without addition of biocides. A tap water sample used to dilute MWFs and an ambient air sample outside of the plants will be included in the analyses as control. We will track operational parameters, chemical and physical properties of the samples including pH, temperature, additives, biocides, tramp oil contamination and organic particulates. We anticipate revealing new findings concerning the microbial ecology of MWFs and finding strong evidence to support the important role of biofilms in the microbial ecology of MWFs.

Specific aim 2: Monitor the prevalence and dynamics of Mycobacteria in MWFs using real-time PCR and correlate and identify parameters that promote the growth of *M. immunogenum*. We will carry out a prospective study of the prevalence and dynamics of the growth of Mycobacteria in a variety of MWF environments (e.g., different fluids, different plants and processes, different biocides), including the bulk fluids, MWF aerosols, and MWF biofilms, along with characterization of chemical and physical properties of the systems. We will routinely collect samples from a minimum of 2 MWF systems in each of 2 plants covering the above mentioned MWF environments on a biweekly basis for 12 months. Traditional culture-based analysis and real-time PCR quantification will be used to quantify the abundance of Mycobacteria in all collected samples. Mixed-effect regression analysis will be used to model and identify associations among physical, chemical and biological parameters, in addition to biofilms, that are associated with the promotion of the growth of Mycobacteria in MWFs.

Methodology

MWFs sampling

Beginning in October, 2009, we started a one year monthly sampling campaign of seven MWF systems in two automobile plants (Table 1) including 170 bulk fluid samples and 170 biofilm samples. Biofilm samples were collected were collected by scraping biofilms from inside surfaces of MWF tanks. Biofilm samples collected were mixtures of fluids and solid biomass. The collected samples were kept on wet ice and transported to the laboratory at the University of Michigan for immediate analysis within 24 hours of collection.

Total cell counts and bacterial culturing

The following microbiological analysis was performed on each sample immediately upon arrival of the samples in the laboratory (within 24 hours). Samples was serially diluted when necessary. Total cell counts was performed by staining the sample with SYBO Green I (a dye stains double strand DNA, Invitrogen) using an Olympus Fluorescence Microscope. Diluted samples was also inoculated onto 2% malt extract agar (MEA, for fungi), tryptic soy agar (TSA, for bacteria), and Middlebrook 7H9 agar (for Mycobacteria). All plates will be incubated at 250 C. For aerobic bacteria and fungi, plates are checked in 5 to 7 days. For mycobacteria, plates will be checked twice a week for a month. Selected colonies from 7H9 agar for mycobacteria from selected samples was subcultured and purified for identification and speciation.

DNA Extraction and PCR Amplification

One milliliter of each MWF sample was taken for DNA extraction using UltraClean Soil DNA Kit (MO BIO, Carlsbad, CA) according to the manufacturer's protocol. DNA was finally eluted with 50 µL of water. The quality and concentration of DNA was measured by spectrophotoscopy using Nanodrop ND-100 (NanoDrop Technologies, Wilmington, Delaware, USA). DNA extracted from MWF samples were amplified with the universal primer pairs 515F (5'-GTGCCAGCMGCCGCGGTAA) and 1391R (5'-GACGGGCGGTGWGTRCA) targeting bacterial 16S rRNA genes and fungal 18S rRNA genes, respectively [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 (2.5mM of each dNTPs), and 0.25 µL of GoTaq DNA polymerase (Promega, Madison, Wis.). PCR was conducted with a Mastercycler gradient Machine (Eppendorf, Westbury, NY) by running 20 cycles with a gradient from 65oC to 45 oC (92 oC for 30 s, 65 oC to 1 oC/cycle for 30 s, 72 oC for 90 s) and an additional 20 cycles at a 45 oC annealing temperature (92 oC for 30 s, 45 oC for 90 s, 72 oC for 90 s) before a final extension at 72 oC for 20 min. Mycobacteria genus-specific amplification was conducted with the primer pair 515F and 1027R (5'-GCACACAGGCCACAAGGG) targeting mycobacteria 16S rRNA genes [Angenent, et al. 2005]. M. immunogenum species-specific amplification was conducted with the primer pair targeting M. immunogenum 16S rRNA genes pMycImmF (5'-GGGGTACTCGAGTGGCGAAC) and pMycImmR (5'-GGCCGGCTACCCGTTGTC) [Veillette M 2005]. PCR reaction mixtures were the same as that mentioned above. These two sets of PCR were conducted with an initial denaturation at 94oC for 2 min followed by 30 cycles of the following incubation pattern: 94 oC for 30 s, 63 oC for 30 oC, and 72 oC for 40 s. A final extension at 72 oC for 10 min concluded the reaction.

Real-Time PCR Quantification of total Bacteria and Mycobacteria

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) and Eu518 (5'-ATTACCGCGGCTGCTGG), about 100 pg of template DNA, and 10 µL of 2 × SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). PCR program was as follow: 95 oC for 15 min, 40 cycles of denaturing (15 s at 94 oC), annealing (30 s at 61 oC), and extension (30 s at 72 oC). The cycling was followed by a final extension at 72 oC for 7 min, and a melting curve analysis from 65–95 oC with a plate read every 0.5 oC. 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 oC, followed by 40 cycles of 3 s at 94 oC and 1 min at 61.5 oC. 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 Mycobacterium immunogenum ATCC 200506, respectively, and the DNA quality and quantity was measured using the Nanodrop ND-100 spectrophotometer. The DNA solution was then diluted to 108 copy of target DNA molecule per microlitre and stored at -20oC.

The copy number of 16S rRNA genes were calculated using the standard curve and converted to cell number assuming the average 16S rRNA gene copy number in bacteria is 4 and MI is 2 (calculated using the data from the rrnDB, http://ribosome.mmg.msu.edu/rrndb/).

DNA pyrosequencing and analysis

To determine complex bacterial populations in MWFs, selected total DNA samples were subject to PCR amplification. PCR amplification was conducted in triplicate using Roche 454 titanium compatible primers targeting bacterial 16S rRNA genes as described earlier [Pinto and Raskin 2012]. Triplicate PCR products were pooled and purified using a QiaQuick PCR purification kit (Qiagen Inc., Valencia, CA). The amount of PCR product from each sample is quantified in triplicate using a Quant-iT dsDNA assay kit (Invitrogen, Carlsbad, CA) on a Nanodrop 3300 (Thermo Scientific, Wilmington, DE). The PCR products of each sample were further combined for sequencing. These samples was sequenced at the DNA Sequencing Core at University of Michigan.

DNA sequencing data processing was conducted using the Mothur program [Schloss, et al. 2009]. A total of 5-10 thousand sequences are expected to obtain for each samples and the sequences were trimmed to remove primers and barcodes, quality filtered, and chimera checked as defined previously [Pinto and Raskin 2012]. The sequences was clustered using the average neighbor approach [Quince, et al. 2009] to form operational taxonomic units (OTUs) at 97% sequence similarity cutoff (3% sequence divergence). All samples were normalized to ensure equal number of sequences in each sample, prior to further analyses. Phylogenetic trees were constructed using the Clearcut program [Sheneman, et al. 2006] and the parsimony test [Schloss and Handelsman 2006] were performed to determine significance of structural similarity among communities across sampling groups. Classification of reads were performed using an RDP training set [Werner, et al. 2012] using a confidence level cutoff of 75%.

Statistical analysis

Initially, bivariate relationships between log-transformed total bacteria and *M. immunogenum* concentration and each of the potential parameters affecting the abundance of total bacteria and *M. immunogenum* was explored for data within each environment. The relative abundance of MI to total bacteria was expressed as log difference of total bacteria and *M. immunogenum.* Because the data was collected over a year at multiple times, in order to account for the potential serial correlation of the data over time within each system, mixed-effect regression model was used to assess the bivariate relationships. The dependent variable will be the log-transformed concentration, and autoregressive correlation (order 1) was used as a correlation structure among the repeated measurements. Potential parameters included all measured variables including physical, chemical and biological parameters, in addition to biofilms. Specifically, they include MWF temperature, MWF pH, and Oil concentration as continuous variables. MWF type was a categorical variable (soluble oils, semi-synthetic fluid and synthetic fluid) and was included initially as category dummy variables.

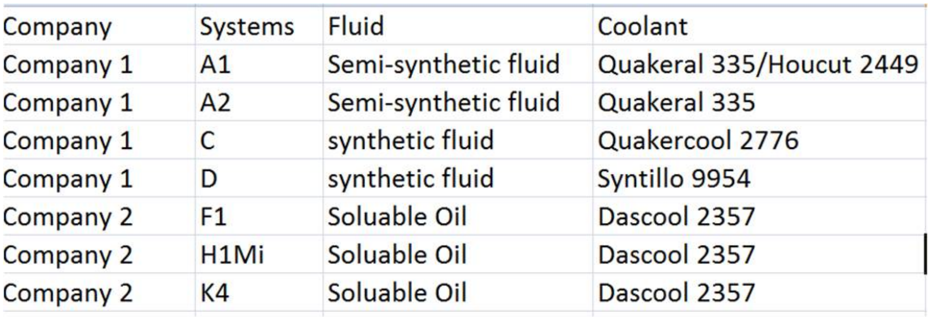
A separate mixed-effect regression model was developed for data in each of the three environments to evaluate important physical, chemical and biological parameters describing the machining systems and MWF characteristics affecting the abundance of *M. immunogenum*. We first explored the trends in the concentration of *M. immunogenum* graphically by plotting the bacterial concentration in each of the three environments over time to look for any cyclical nature of the concentration. This included checking for periodicity, seeing if the cyclical pattern corresponds with any event such as the timing of the use of biocide and checking for outliers. If any periodicities or trend over time such as a sudden decrease followed by a linear increase in concentration after a certain event was found, we modeled this in our final mixed-effect model. In the mixed-effect model, concentration (log-transformed if needed) was the dependent variable, system was included as a random-effect, and correlation was modeled with an auto-regressive correlation structure. Other correlation was considered including compound symmetry and Toeplitz, and the preferred correlation structure will be determined based on various information criteria. All selected potential predictors of concentration were included as fixed covariates. We graphically assessed the relationships between concentration data and parameters across the different systems to visualize if the relationship was similar across the different systems. We pooled the data across the environments and modeling environment as a fixed covariate. All analyses was carried out using SAS (SAS institute Inc., Cary, NC).

Results and discussion

Sample collected

Beginning in October, 2009, we started a one year monthly sampling campaign of selected seven MWF systems in two automobile plants (Table 1), which is located within 2 hours of driving distance of Dr. Xi's laboratory at the University of Michigan.

Table 1. Systems selected for this study and coolants used.



Company	Systems	Fluid	Coolant
Company 1	A1	Semi-synthetic fluid	Quakeral 335/Houcut 2449
Company 1	A2	Semi-synthetic fluid	Quakeral 335
Company 1	C	synthetic fluid	Quakercool 2776
Company 1	D	synthetic fluid	Syntillo 9954
Company 2	F1	Soluable Oil	Dascool 2357
Company 2	H1Mi	Soluable Oil	Dascool 2357
Company 2	K4	Soluable Oil	Dascool 2357

All together, we have collected 85 fluid samples and 85 biofilms samples from each system. During each sampling campaign, we collected operational data including pH, oil content, including temperature, and trap oil. We also collected once aerosol samples from each plant.

Quantification of total bacteria, fungi and Mycobacteria using selective media

Traditional culture based methods were used to culture total bacteria, fungi, Pesudomonads and Mycobacteria from all collected samples upon arrival within 24 hours. Data were compiled as shown in Figure 1.

Figure 1. Plate counting of total bacteria, Psudononads, fungi and Mycobacteria in seven systems using selective media. Total cell counts were determined by light microscopic examination.

All the samples collected had bacteria growth and the numbers were low in a few samples which may due to inhibitor presented. Culture based method may not be accurate and less than 10% of bacterial cells can be cultured.

Quantification of *M. immunogenum* and bacteria in MWFs samples using real time PCR

In order to monitor the abundance of *M. immunogenum* in different MWFs 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, a Taqman assay (primer pMycImmF, pMycImmR, and a Taqman probe) specific for M. immunogenum [Veillette, et al. 2008] and a SYBR Green real time PCR (primer Eu338 and Eu518) specific for general bacteria [Einen, et al. 2008], respectively, were used. Based on the calibration curves generated for *M.*

immunogenum and *E. coli* K-12 from known amounts of template DNA, the copy number of *M. immunogenum* and bacterial 16S rRNA genes were calculated in each collected sample. This allowed determination of the relative abundance of *M. immunogenum* in the different MWFs samples. Data was summarized in Figure 2.

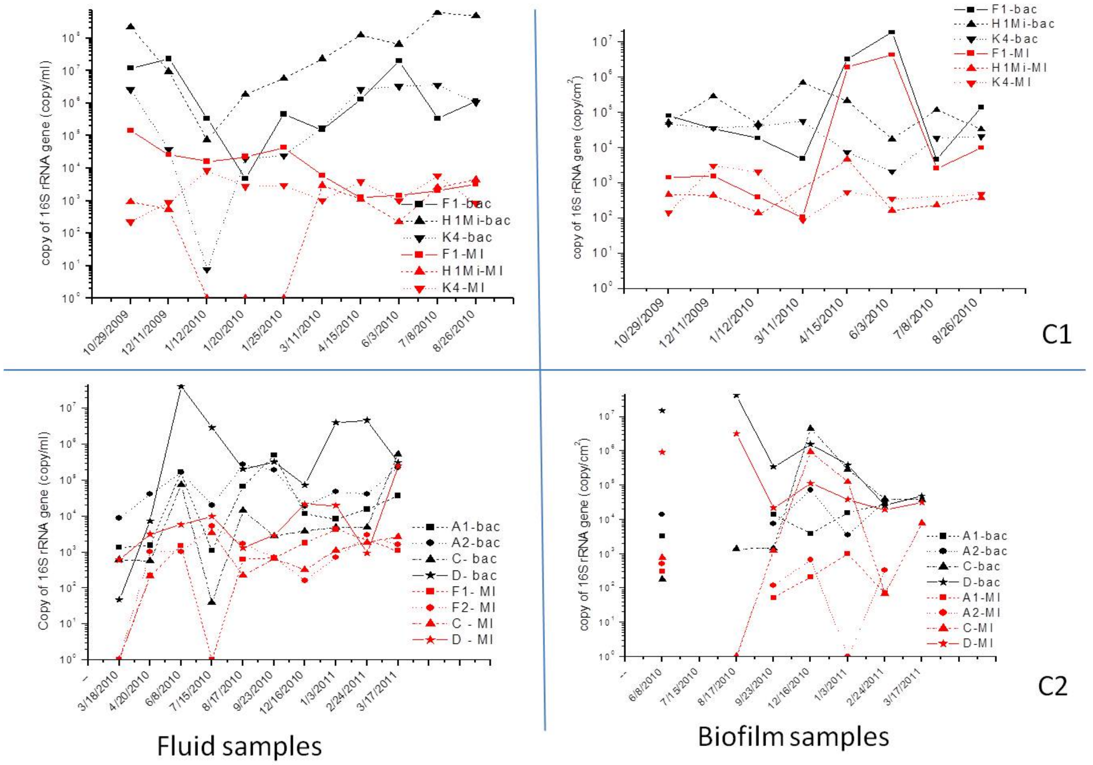


Figure 2. Quantification of total bacteria and *M. immunogenum* in fluid and biofilm samples collected from seven systems in two companies using quantitative PCR method.

All the samples had bacterial growth. Total bacterial concentrations were in the range of 10^5 to 10^7 cells/ml in bulk fluids from Company 1 (three systems mainly soluble oil) and 10^4-10^6 cells/ml in Company 2 (four systems mainly semi-synthetic or synthetic oil) based on realtime PCR quantification data. While in biofilm samples, total bacterial concentrations were similar and in the range of 10^4-10^5 cells/cm2.

M. immunogenum was also detected in most samples. Interestingly, the concentrations were very similar in all systems and in the range of 10^3-10^5 cells/ml in bulk fluid samples and 10^2-10^3 cells/cm2 in biofilm samples except from one system in Company 2 which had 10^5 cells/cm2 in biofilm samples. Surprisingly, the relative abundance of *M. immunogenum* could reach as high as 70% of total bacteria.

One DCR (drain, clean and recharge) event was monitored in Company1. Our findings were in agreement with previous observations, bacterial counts increased very shortly after DCR and reached the same level in about a week in bulk fluid.

Microbial communities in selected fluid and biofilm samples

Six samples from two companies were selected for DNA pyrosequencing. The data revealed complex and different bacterial communities in metalworking fluids in different systems (Figure 3) and bacterial communities can be clustered and separated based on DNA sequencing data (Figure 4).

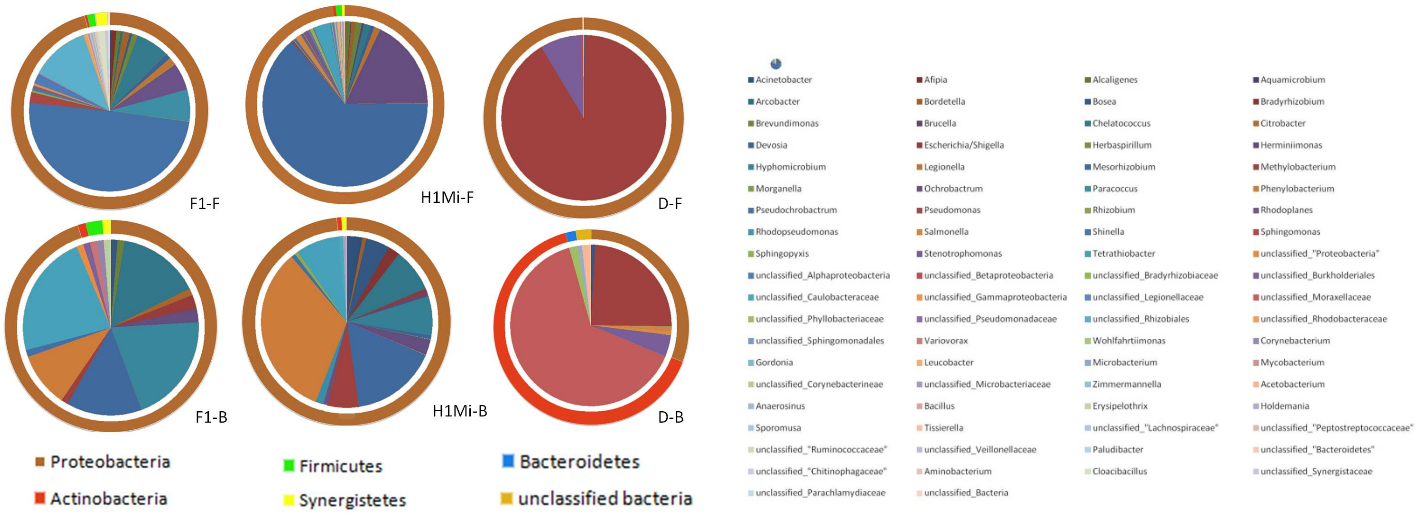


Figure 3. Complex microbial community in MWFs revealed by DNA pyrosequencing. The pie showed distribution of bacterial populations at the phylum and genus level.

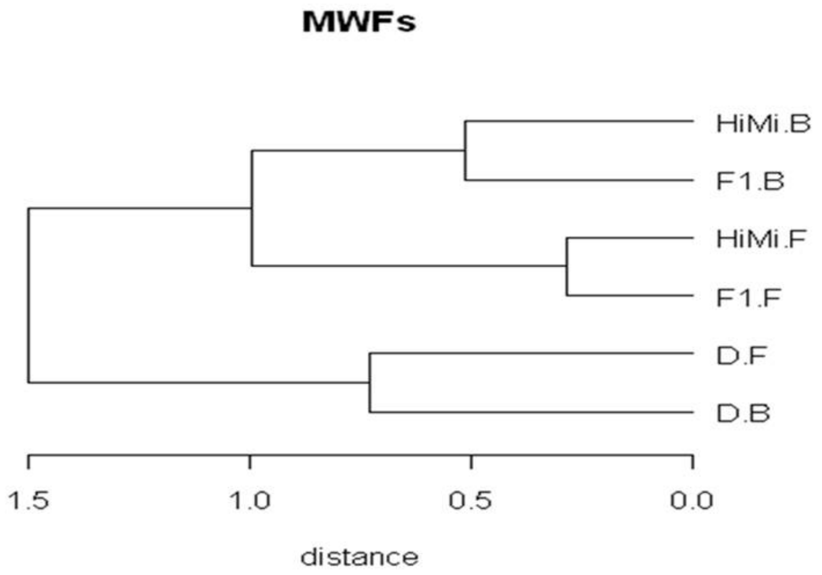
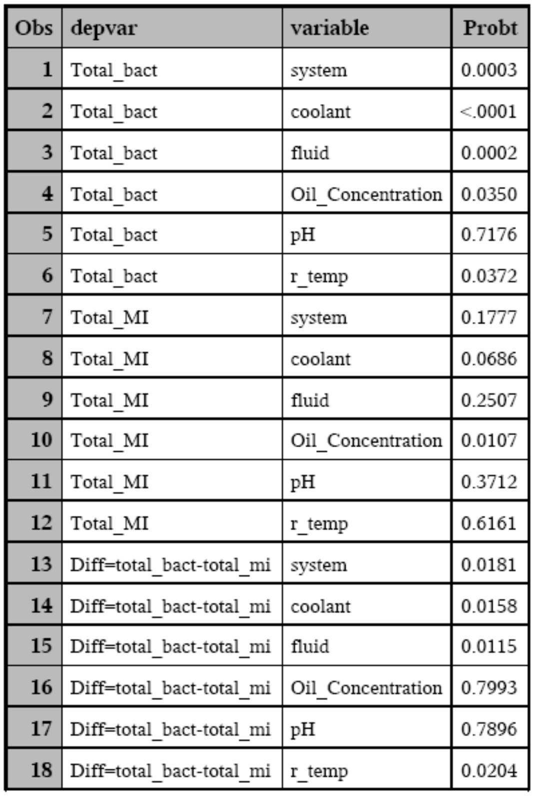


Figure 4. Clustering of MWF samples based on bacterial populations revealed by DNA pyrosequencing.

The clustering analysis indicates that MWF of different systems had distinct bacterial communities. Bacterial communities in biofilms were more similar to fluid samples in the same systems suggesting biofilms may act as a seeding source of fluids for bacterial regrowth.

Statistical analysis

We have performed comprehensive statistical analysis on the data collected and it was found that bacterial populations in bulk fluid and biofilms respond differently to operational conditions. In bulk fluid, the oil concentration was a critical factor that affects bacterial growth. The higher the concentration the more bacterial and Mycobacteria growth was observed but does not significantly impact the relative abundance of *M. immunogenum* (Figure 6)



Obs	depvar	variable	Probt
1	Total_bact	system	0.0003
2	Total_bact	coolant	<.0001
3	Total_bact	fluid	0.0002
4	Total_bact	Oil_Concentration	0.0350
5	Total_bact	pH	0.7176
6	Total_bact	r_temp	0.0372
7	Total_MI	system	0.1777
8	Total_MI	coolant	0.0686
9	Total_MI	fluid	0.2507
10	Total_MI	Oil_Concentration	0.0107
11	Total_MI	pH	0.3712
12	Total_MI	r_temp	0.6161
13	Diff=total_bact-total_mi	system	0.0181
14	Diff=total_bact-total_mi	coolant	0.0158
15	Diff=total_bact-total_mi	fluid	0.0115
16	Diff=total_bact-total_mi	Oil_Concentration	0.7993
17	Diff=total_bact-total_mi	pH	0.7896
18	Diff=total_bact-total_mi	r_temp	0.0204

Figure 5. Correlation analysis of different operational factors with total bacteria and *M. immunogenum* and relative abundance of *M. immunogenum* (ratio of MI/total bacteria expressed as Log difference of total bacteria and MI, the higher the difference the lower abundance of MI) in bulk fluids.

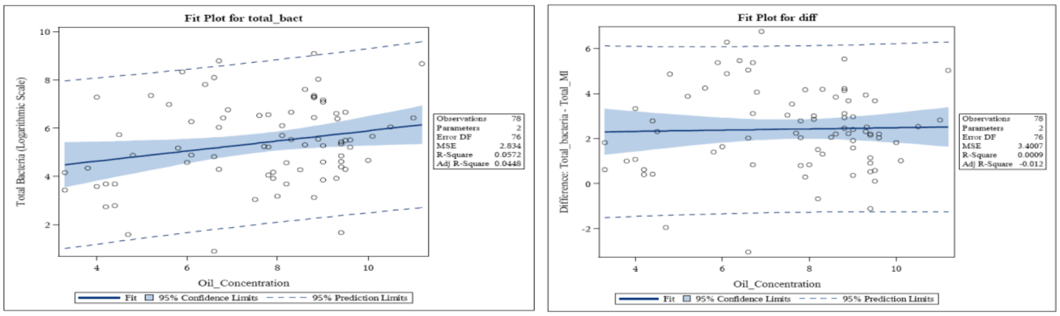
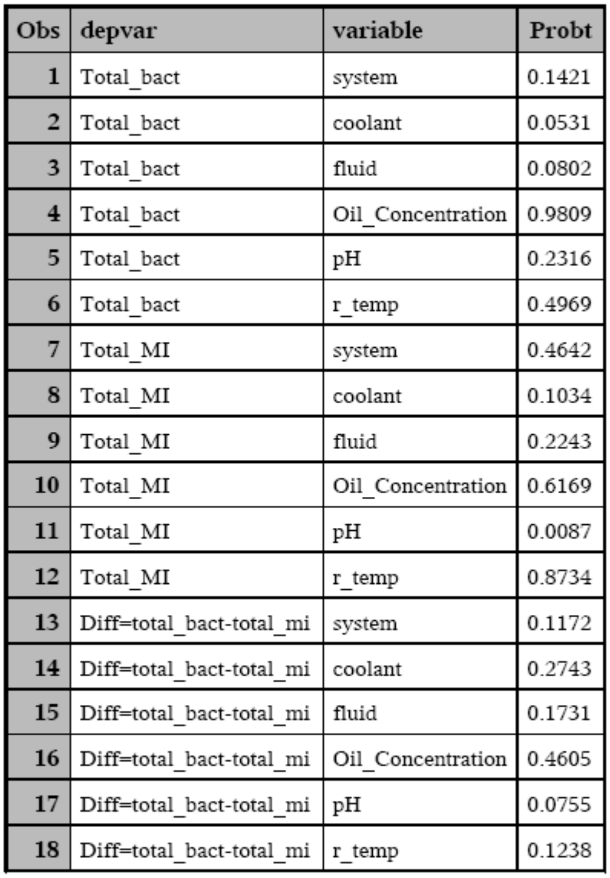


Figure 6. Plot of total bacteria and the relative abundance of MI (ratio of MI/total bacteria expressed as Log difference of total bacteria and MI, the higher the difference the lower abundance of MI) vs. oil concentration in bulk fluids.

While in biofilm samples, the pH is a critical factor that affects bacterial growth (Figure 7). The lower the pH the more growth of *M. immunogenum* in biofilm samples was observed across all samples. The pH also affects the relative abundance of *M. immunogenum* (Figure 8)



Obs	depvar	variable	Probt
1	Total_bact	system	0.1421
2	Total_bact	coolant	0.0531
3	Total_bact	fluid	0.0802
4	Total_bact	Oil_Concentration	0.9809
5	Total_bact	pH	0.2316
6	Total_bact	r_temp	0.4969
7	Total_MI	system	0.4642
8	Total_MI	coolant	0.1034
9	Total_MI	fluid	0.2243
10	Total_MI	Oil_Concentration	0.6169
11	Total_MI	pH	0.0087
12	Total_MI	r_temp	0.8734
13	Diff=total_bact-total_mi	system	0.1172
14	Diff=total_bact-total_mi	coolant	0.2743
15	Diff=total_bact-total_mi	fluid	0.1731
16	Diff=total_bact-total_mi	Oil_Concentration	0.4605
17	Diff=total_bact-total_mi	pH	0.0755
18	Diff=total_bact-total_mi	r_temp	0.1238

Figure 7. Correlation analysis of different operational factors with total bacteria and *M. immunogenum* and relative abundance of MI (ratio of MI/total bacteria expressed as Log difference of total bacteria and *M. immunogenum*, the higher the difference the lower abundance of MI) in biofilm samples.

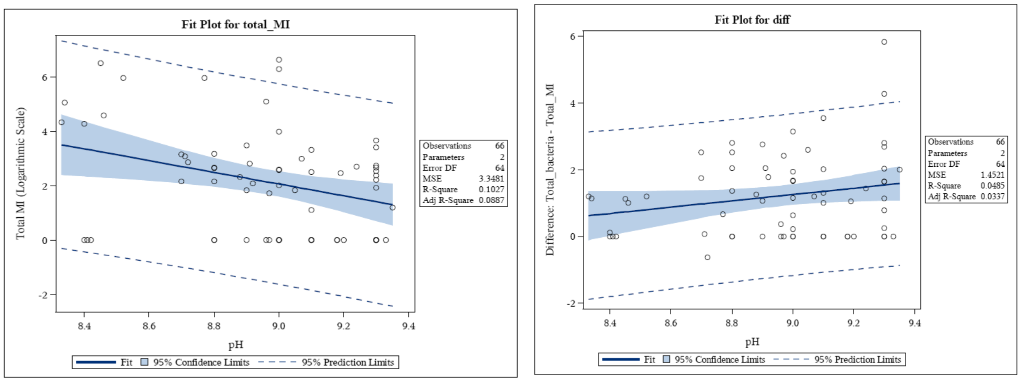


Figure 8. Plot of total bacteria and the relative abundance of *M. immunogenum* (ratio of MI/total bacteria expressed as Log difference of total bacteria and *M. immunogenum*, the higher the difference the lower abundance of MI) vs. oil concentration in biofilm samples.

Most importantly it was found that the relative abundance of *M. immunogenum* in biofilm samples were significantly much higher (close to 100 times more, $p<0.0001$) that that in fluids samples (Figure 9), suggesting that biofilms may provide a shield or sheltered microenvironment for the growth and survival of Mycobacteria that can act as seeding sources for circulating MWFs.

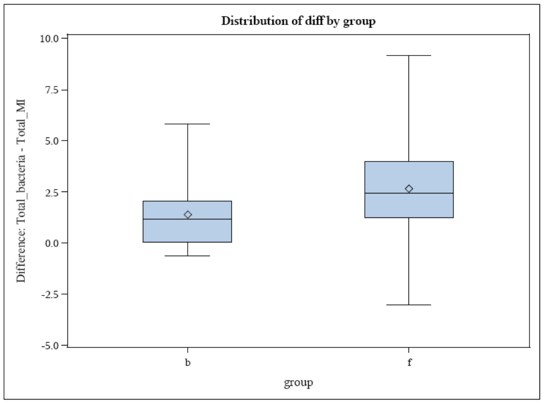


Figure 9. Relative abundance of *M. immunogenum* (ratio of MI/total bacteria expressed as Log difference of total bacteria and MI, the higher the difference the lower abundance of MI) in biofilm samples (b) vs. fluid samples (f).

Conclusions

Our data found that all metalworking fluids had complex and diverse microbial contaminations and *M. immunogenium* was ubiquitous in all systems examined. In addition, concentrations of total bacteria and *M. immunogenium* were not constant in different systems over time. The relative abundance of MI was affected more significantly by oil concentration in bulk fluids and by pH in biofilms. Most importantly it was found that the relative abundance of MI in biofilm samples were significantly much higher than that in fluids samples suggesting that biofilms may provide a shield or sheltered microenvironment for the growth and survival of Mycobacteria that can act as seeding sources for circulating MWFs. Culture based method for detection of bacterial and Mycobacterium contamination is not sensitive and molecular based detection methods should be developed and standardized for more better monitoring and exposure assessment. For better control of microbial contamination of metalworking fluids, biofilms that develop in systems should be a primary focus of management.

Additional support is necessary to perform a large scale survey study of hundreds of plants to confirm whether our initial findings hold the truth across most plants where metalworking fluids are being used. The data would be very useful information for metalworking fluid suppliers to improve their design of metalworking fluids and for plants to improve their routine management practice to save cost and improve occupational health.

We have not done health risk assessment, which is not within the scope of this project, and it is critical that we receive further support to perform health risk studies

Publications

Journal Article

Two manuscripts are under review or in preparation.

Proceedings

Xi, C., J. Wu, and A. Franzblau. 2011 Prevalence of Mycobacterium immunogenum and microbial ecology in Metalworking Fluids and its Associated Biofilms (Invited talk). 4th Symposium on Metal Removal Fluids: Global challenges. Barcelona, 14-16 September 2011

Xi, C. A. Franzblau. 2008. Molecular characterization of Microbial Communities and Quantification of Mycobacterium immunogenum in Metal Removal Fluids and its Associated Biofilms (Invited talk). 2008 MRF Symposium: From the Shop Floor to the Global Market. Dearborn, MI, October 5-8, 2008

Wu, J., A. Franzblau, and C. Xi. “Molecular characterization of Microbial Communities and Quantification of Mycobacterium immunogenum in Metal Removal Fluids and its Associated Biofilms”. The 107th ASM General Meeting. Toronta, Canada, May, 2007.

Inclusion of gender and minority study subjects

N/A

Inclusion of Children

N/A.

Materials available for other investigators

Detailed protocol for quantifying M. immunogenium from metalworking fluids are available and can be requested by email:cxi@umich.edu

References cited

1. Angenent LT, Kelley ST, St Amand A, Pace NR, Hernandez MT. 2005. Molecular identification of potential pathogens in water and air of a hospital therapy pool. Proceedings of the National Academy of Sciences of the United States of America 102: 4860-4865.
2. Bennett EO, Bennett DL. 1987. Minimizing Human Exposure to Chemicals in Metalworking Fluids. Lubrication Engineering 43: 167-175.
3. Bennett EO. 1972. Biology of Metalworking Fluids. Lubrication Engineering 28: 237-&.
4. Bernstein DI, Lummus ZL, Santilli G, Siskosky J, Bernstein IL. 1995. Machine operator's lung. A hypersensitivity pneumonitis disorder associated with exposure to metalworking fluid aerosols. Chest 108: 636-641.
5. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. 1995. Microbial biofilms. Annu Rev Microbiol 49: 711-745.
6. Einen J, Thorseth IH, Ovreas L. 2008. Enumeration of Archaea and Bacteria in seafloor basalt using real-time quantitative PCR and fluorescence microscopy. FEMS Microbiol Lett 282: 182-187.
7. Eisen EA, Holcroft CA, Greaves IA, Wegman DH, Woskie SR, Monson RR. 1997. A strategy to reduce healthy worker effect in a cross-sectional study of asthma and metalworking fluids. American Journal of Industrial Medicine 31: 671-677.
8. Gordon T, Nadziejko C, Galdanes K, Lewis D, Donnelly K. 2006. Mycobacterium immunogenum causes hypersensitivity pneumonitis-like pathology in mice. Inhalation Toxicology 18: 449-456.
9. Gordon T. 2004. Metalworking fluid - The toxicity of a complex mixture. Journal of Toxicology and Environmental Health-Part A-Current Issues 67: 209-219.
10. Greaves IA, Eisen EA, Smith TJ, Pothier LJ, Kriebel D, Woskie SR, Kennedy SM, Shalat S, Monson RR. 1997. Respiratory health of automobile workers exposed to metal-working fluid aerosols: respiratory symptoms. Am.J Ind.Med. 32: 450-459.
11. Gupta A, Rosenman KD. 2006. Hypersensitivity pneumonitis due to metal working fluids: sporadic or under reported? American Journal of Industrial Medicine 49: 423-433.
12. Hall-Stoodley L, Costerton JW, Stoodley P. 2004. Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol 2: 95-108.
13. Hamady M, Lozupone C, Knight R. 2010. Fast UniFrac: facilitating high-throughput phylogenetic analyses of microbial communities including analysis of pyrosequencing and PhyloChip data. The ISME journal 4: 17-27.
14. Hammer Ø, Harper DAT, Ryan PD. 2001. PAST: Paleontological Statistics Software Package for Education and Data Analysis. Palaeontologia Electronica 4.
15. Kemnitz D, Kolb S, Conrad R. 2005. Phenotypic characterization of Rice Cluster III archaea without prior isolation by applying quantitative polymerase chain reaction to an enrichment culture. Environ.Microbiol 7: 553-565.
16. Kennedy SM, Greaves IA, Kriebel D, Eisen EA, Smith TJ, Woskie SR. 1989. Acute pulmonary responses among automobile workers exposed to aerosols of machining fluids. Am.J Ind.Med. 15: 627-641.
17. Kreiss K, CoxGanser J. 1997. Metalworking fluid-associated hypersensitivity pneumonitis: A workshop summary. American Journal of Industrial Medicine 32: 423-432.
18. Lozupone C, Knight R. 2005. UniFrac: a new phylogenetic method for comparing microbial communities. Applied and environmental microbiology 71: 8228-8235.
19. Mattsby-Baltzer I, Edebo L, Jarvholm B, Lavenius B, Soderstrom T. 1990. Subclass distribution of IgG and IgA antibody response to Pseudomonas pseudoalcaligenes in humans exposed to infected metal-working fluid. J Allergy Clin.Immunol. 86: 231-238.

20. Mattsby-Baltzer I, Sandin M, Ahlstrom B, Allenmark S, Edebo M, Falsen E, Pedersen K, Rodin N, Thompson RA, Edebo L. 1989. Microbial growth and accumulation in industrial metal-working fluids. Appl.Environ.Microbiol 55: 2681-2689.
21. Moore JS, Christensen M, Wilson RW, Wallace RJ, Zhang YS, Nash DR, Shelton B. 2000. Mycobacterial contamination of metalworking fluids: Involvement of a possible new taxon of rapidly growing mycobacteria. American Industrial Hygiene Association Journal 61: 205-213.
22. Ochsenreiter T, Selezi D, Quaiser A, Bonch-Osmolovskaya L, Schleper C. 2003. Diversity and abundance of Crenarchaeota in terrestrial habitats studied by 16S RNA surveys and real time PCR. Environ.Microbiol 5: 787-797.
23. O'Toole G, Kaplan HB, Kolter R. 2000. Biofilm formation as microbial development. Annu Rev Microbiol 54: 49-79.
24. Pace NR. 1997. A molecular view of microbial diversity and the biosphere. Science 276: 734-740.
25. Pinto AJ, Raskin L. 2012. PCR biases distort bacterial and archaeal community structure in pyrosequencing datasets. PloS one 7: e43093.
26. Quince C, Lanzen A, Curtis TP, Davenport RJ, Hall N, Head IM, Read LF, Sloan WT. 2009. Accurate determination of microbial diversity from 454 pyrosequencing data. Nature methods 6: 639-641.
27. Rossmoore HW. 1995. Microbiology of Metalworking Fluids - Deterioration, Disease and Disposal. Lubrication Engineering 51: 113-118.
28. Savonius B, Keskinen H, Tuppurainen M, Kanerva L. 1994. Occupational asthma caused by ethanolamines. Allergy 49: 877-881.
29. Schloss PD, Handelsman J. 2006. Introducing TreeClimber, a test to compare microbial community structures. Applied and environmental microbiology 72: 2379-2384.
30. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and environmental microbiology 75: 7537-7541.
31. Sheehan MJ. 1999. Final Report of the OSHA Metalworking Fluids Standards Advisory Committee.
32. Shelton BG, Flanders WD, Morris GK. 1999. Mycobacterium sp. as a possible cause of hypersensitivity pneumonitis in machine workers. Emerg.Infect.Dis. 5: 270-273.
33. Sheneman L, Evans J, Foster JA. 2006. Clearcut: a fast implementation of relaxed neighbor joining. Bioinformatics 22: 2823-2824.
34. Simpson AT, Stear M, Groves JA, Piney M, Bradley SD, Stagg S, Crook B. 2003. Occupational exposure to metalworking fluid mist and sump fluid contaminants. Annals of Occupational Hygiene 47: 17-30.
35. Skerlos SJ, Rajagopalan N, DeVor RE, Kapoor SG, Sanford RA. 2001. Model of Biomass Concentration in a Metalworking Fluid Reservoir Subject to Continuous Biofilm Contamination during the Use of Membrane Filtration to Control Microorganism Growth. Transactions of NAMRI/SME XXIX: 229-234.
36. Thorne P, DeKoster J, Subramanian P. 1996. Environmental assessment of aerosols, bioaerosols, and airborne endotoxins in a machining plant. AMERICAN INDUSTRIAL HYGIENE ASSOCIATION JOURNAL 57: 1163-1167.
37. Thorne PS, Adamcakova-Dodd A, Kelly KM, O'Neill M E, Duchaine C. 2006. Metalworking fluid with mycobacteria and endotoxin induces hypersensitivity pneumonitis in mice. Am J Respir Crit Care Med 173: 759-768.

38. Tolbert PE, Eisen EA, Pothier LJ, Monson RR, Hallock MF, Smith TJ. 1992. Mortality Studies of Machining-Fluid Exposure in the Automobile-Industry .2. Risks Associated with Specific Fluid Types. Scandinavian Journal of Work Environment & Health 18: 351-360.
39. Veillette M PGTPSDC. 2005. Recovery and quantification of mycobacterium immunogenum DNA from metalworking fluids using dual-labeled probes. Journal of ASTM International 2 1-9.
40. Veillette M, Page G, Thorne PS, Duchaine C. 2008. Real-time PCR quantification of Mycobacterium immunogenum in used metalworking fluids. J Occup Environ Hyg 5: 755-760.
41. Wallace RJ, Zhang YS, Wilson RW, Mann L, Rossmoore H. 2002. Presence of a single genotype of the newly described species Mycobacterium immunogenum in industrial metalworking fluids associated with hypersensitivity pneumonitis. Applied and Environmental Microbiology 68: 5580-5584.
42. Werner JJ, Koren O, Hugenholtz P, DeSantis TZ, Walters WA, Caporaso JG, Angenent LT, Knight R, Ley RE. 2012. Impact of training sets on classification of high-throughput bacterial 16s rRNA gene surveys. The ISME journal 6: 94-103.
43. Wilson RW, Steingrube VA, Bottger EC, Springer B, Brown-Elliott BA, Vincent V, Jost KC, Jr., Zhang Y, Garcia MJ, Chiu SH, Onyi GO, Rossmoore H, Nash DR, Wallace RJ, Jr. 2001. Mycobacterium immunogenum sp. nov., a novel species related to Mycobacterium abscessus and associated with clinical disease, pseudo-outbreaks and contaminated metalworking fluids: an international cooperative study on mycobacterial taxonomy. Int J Syst Evol Microbiol 51: 1751-1764.