

Metagenomic analysis of metalworking fluids from three different automotive manufacturing facilities

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ABSTRACT

Metalworking fluids are used in metal machining and cutting as lubricants and coolants, and sometimes for their anti-corrosion properties. Metalworking fluids are commonly used in automotive manufacturing plants where potential contamination by bacteria poses public health concern. This study was conducted to evaluate the usefulness of metagenomics in determining the identity of bacteria and the diversity and richness of bacterial communities in metalworking fluids. Five metalworking fluid samples from three different plants, located in two different U.S. states, were obtained. A protocol was optimized to isolate DNA from metalworking fluids. A metagenomic analysis was performed by sequencing the V3-V4 regions of the 16S rRNA gene. Bacterial communities were analyzed and the diversity and richness of the samples were determined. Genera such as *Mycobacterium* and *Pseudomonas* were relatively abundant in samples from some plants, whereas other genera such as *Comamonas* and *Acinetobacter* were more abundant in other samples. All five samples were found to have bacterial communities that included opportunistic pathogens, emphasizing the importance of surveillance and treatment of metalworking fluids as important steps in addressing a potential public health concern for automotive manufacturing workers.

Keywords: metalworking fluids, metagenomics, *Mycobacterium*, *Pseudomonas*

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INTRODUCTION

The microbiology of metalworking fluids used in industrial manufacturing remains poorly understood in the automotive industry, where metalworking fluids play many different roles in metalworking, machining, and metal-forming processes as coolants, lubricants, and for metal chip removal at the metal-tool interface (1, 2). There are many metalworking fluid formulations, and water-based semi-synthetic fluids are most widely used (1, 3). Water-based semi-synthetic fluids are mostly composed of water, which dissolves components such as refined petroleum, emulsifiers, chlorine, phosphorus, biocides, and fatty acid derivatives. These chemicals enhance the lubricity and high temperature/pressure endurance of metalworking fluids. The various components of metalworking fluids can provide nutrients for microbial communities to become established, grow, and spread in metalworking fluid systems (1).

An interest in microbial contamination of metalworking fluids began in 1993 after 10 cases of hypersensitivity pneumonitis originated in machinists exposed to aerosolized metalworking fluids at an industrial plant in the United States (4). In 1995, the sputum of infected workers was found to have acid-fast bacteria. After further analysis, it was suspected that infections were associated with a new species of mycobacteria, *Mycobacterium immunogenum* (4). Additional cases of hypersensitivity pneumonitis were identified at eight additional automotive plants in the 1990s (5). This discovery piqued interest in how metalworking fluids can become contaminated, which other microbial populations are supported by their composition, as well as how we can protect the health of workers by preventing exposure to pathogenic microorganisms. Individuals in close contact with metalworking fluids, such as those who are working on a machining production line or those in charge of maintaining or treating metalworking fluids, are the individuals most at risk for negative health consequences. This exposure can either be in the form of inhalation of aerosols of contaminated metalworking fluids or by direct skin contact (2, 6).

The presence and growth of microorganisms in metalworking fluids originate in different ways, including initial contamination routes as well as continued support of the microbial communities from ineffective routine cleaning procedures (3). For example, metalworking fluids are most commonly diluted with tap water which typically serves as the baseline introduction of microorganisms into industrial systems and reservoirs. Other routes include contamination from the industrial settings, workers, and residual biofilms that can

continually reestablish microbial communities (3). Metalworking fluids are known to host a wide diversity of microorganisms including *Legionella*, enteric bacteria, pseudomonads, fungi, and *Mycobacterium* sp. (1). Exposure to pathogens in metalworking fluids has been found to increase the risk of developing hypersensitivity pneumonitis, cancer, dermatitis, asthma, respiratory illness, and reduced pulmonary function (1, 2, 6).

Metagenomics is the study of all genomes of microorganisms isolated from a bulk sample. It is particularly useful for studying communities of microorganisms in various environments, such as human stools or water samples (7). Development in next generation sequencing and bioinformatics tools have allowed researchers to use these techniques for the analysis of complex microbial communities (7). A few recent studies utilized metagenomic approaches to assess bacterial and fungal diversity of metalworking fluids from sites in Russia, China, and Switzerland (8, 9, 10). However, it does not appear that metagenomics has been utilized to analyze the microbial communities of metalworking fluids used specifically in automotive manufacturing facilities.

Currently, routine microbial testing of metalworking fluids mostly consists of cultivation methods, which require long incubation periods and may not correctly identify all opportunistic pathogenic bacteria (2). Consequently, the goal of this study was to evaluate whether DNA can be isolated from automotive metalworking fluids and then processed for further metagenomic analysis. Our objective was to conduct a metagenomics analysis on the isolated DNA using 16S rRNA DNA sequencing, and to assess the bacterial taxonomy of the metalworking fluids. The overall aim was to develop a robust approach that can be utilized by stakeholders to evaluate bacterial communities, including pathogens, in metalworking fluids.

MATERIALS AND METHODS

SAMPLING SITES

Samples (1 L each) of water-soluble semi synthetic metalworking fluid were collected by local employees from five different reservoirs in three automotive plants located in two midwestern U.S. states (Table 1). All metalworking fluids tested are used for machining automotive parts. The samples were collected in sterile polypropylene plastic bottles from the bulk fluid of the reservoirs in September and October 2025 and immediately shipped to the lab for analysis.

TABLE 1.*Sample sites for metalworking fluid and their use.*

Plant	Reservoir	Location	Used for:
1	1A	Ohio	Machining automotive discs and drums
1	1B	Ohio	Machining automotive transmission parts
2	2A	Indiana	Machining automotive engine parts
2	2B	Indiana	Machining automotive engine parts
3	3	Indiana	Machining automotive transmission parts

DNA ISOLATION

To concentrate suspended microorganisms, 200 mL of each metalworking fluid was thoroughly mixed and centrifuged (4,100 X g, VWR MegaStar 1.6). The concentrated samples were further centrifuged in microtubes (10,000 X g, Fisher Accuspin MicroR). Pellets ranging from 250-450 µg were obtained through this process from each reservoir. DNA isolation was performed on the concentrated metalworking fluid samples (pellets) using the FastDNA Spin Kit for Soil (MP Biomedicals) and the recommended protocol from the manufacturer with some modifications. In brief, this consisted of lysing the bacterial cells in the pellets using a lysing matrix solution and a homogenizer (BeadBug Microtube Homogenizer) for 80 seconds. This was followed by protein precipitation, DNA binding to a spin filter, washing steps, and DNA

elution as per the manufacturer protocol. The concentration and quality of the DNA was measured at 260 nm and 280 nm using a Jenway 7315 spectrophotometer. Low DNA purity (low A_{260}/A_{280}) was a common issue with these samples. To increase DNA purity, the duration of protein precipitation step in the FastDNA kit was doubled.

METAGENOMICS ANALYSIS

A DNA library of the V3-V4 regions of the 16S rRNA gene was constructed and sequenced by CD-Genomics (Shirley, NY) using a paired-end (PE) method on the Illumina NovaSeq X platform and published primers (11). The sequencing data were processed using the QIIME 2 platform (11). Determination of the bacterial composition of each sample was performed by read merging, Amplicon Sequence Variant

TABLE 2.*Data statistics of the quality control of the DNA sequences from each metalworking fluid sample.*

Sample	Raw PE ^a	Combined PE ^b	Qualified PE ^c	Nochime PE ^d	Base(nt)	AvgLen (nt) ^e	GC ^f	Q20 ^g	Q30 ^g
Reservoir 1A	104072	103408	101888	100074	42548688	425.17	53.80%	98.90%	95.98%
Reservoir 2A	112822	112196	110291	108226	44403784	410.29	56.95%	98.92%	95.96%
Reservoir 3	106161	105648	104095	103970	42517134	408.94	57.47%	99.04%	96.37%
Reservoir 1B	104190	103115	100990	94647	40645857	429.45	51.05%	98.58%	94.88%
Reservoir 2B	73931	73467	72181	61527	25395940	412.76	54.71%	98.88%	95.85%

^aRaw PE: Unprocessed Paired-End (PE) reads obtained from the sequencing platform.

^bCombined PE: PE reads where both ends of a single DNA sequence are sequenced.

^cQualified PE: PE reads that have been reviewed by the prediction algorithm.

^dNochime: Clean Tags (no chimeras) following QC.

^eAvgLen: the average length of the Clean Tags.

^fGC: the percentage of guanine (G) and cytosine (C) base pairs.

^gQ20 and Q30: the percentage of bases with a quality score equal to or higher than 20 (error rate <1%) and 30 (error rate <0.1%).

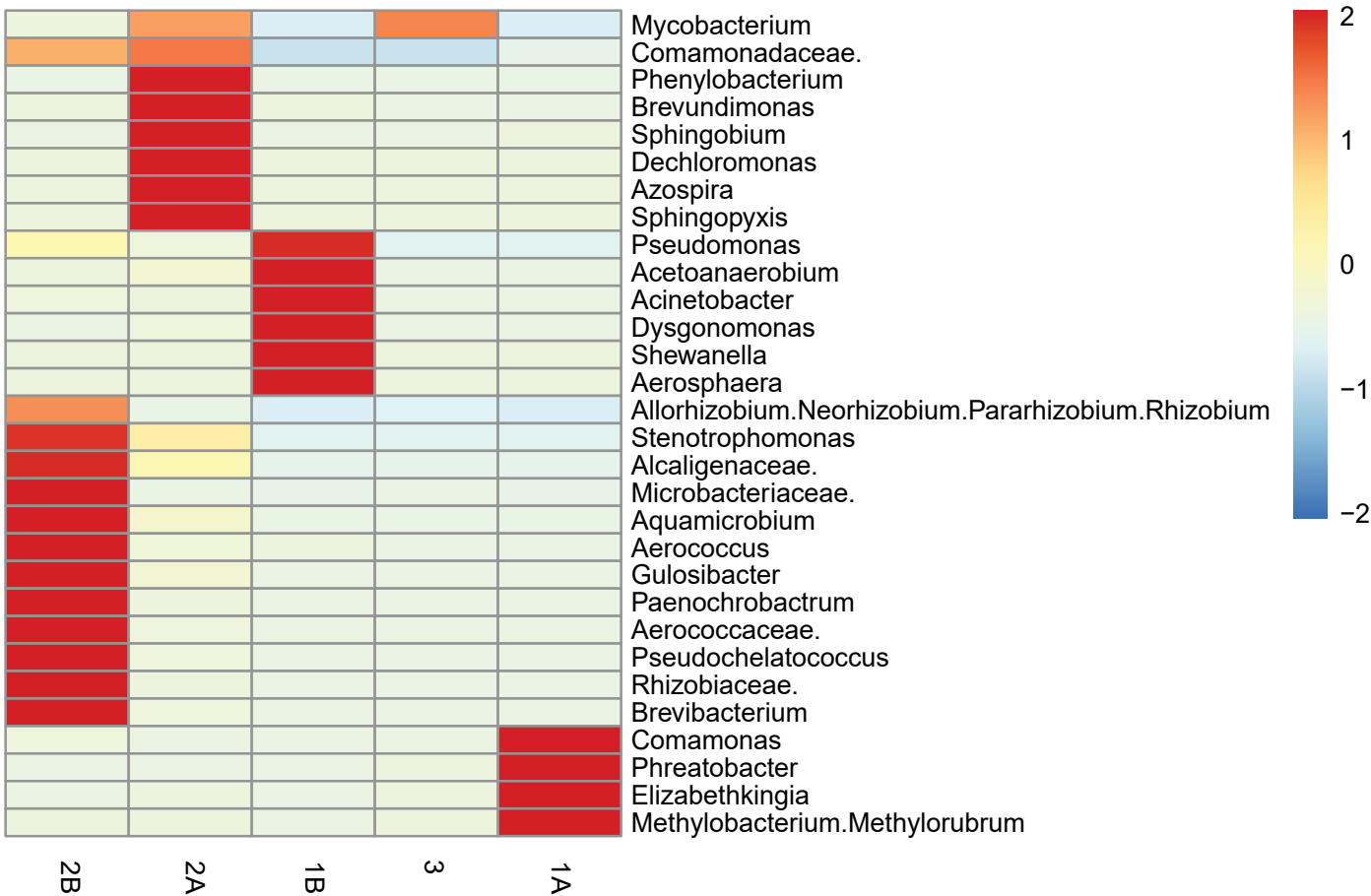
(ASV) detection, species annotation using a pre-trained Naïve Bayes classifier (trained on the Silva 138 99% ASVs), and abundance analysis (12). Four different indices were calculated to assess alpha diversity: Chao1 and Ace estimate the number of species in a community; Shannon and Simpson account for both richness and evenness of a community (13). Beta diversity analysis used three algorithms, binary Jaccard, Bray-Curtis dissimilarity, and unweighted UniFrac, to calculate the phylogenetic distance between bacterial communities and to obtain the β value between samples (14). Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was performed with the R language tool (version 3.6.2) to assess the similarity of bacterial composition between samples based on pairwise similarities. The clustering results were displayed using the web-based ETE Toolkit Phylogenetic tree (newick) viewer (v. 3.1.3) (<https://etetoolkit.org/treeview/>).

RESULTS

The DNA purity (A_{260}/A_{280}) of the five metalworking fluid samples was relatively low, ranging from 1.03 to 1.39, whereas DNA concentrations varied from 12.50 to 80.50 $\mu\text{g}/\text{mL}$ (date not shown). The DNA purity (A_{260}/A_{280}) of a soil sample used as a control was 1.45 using the same protocol. Despite low purity (an ideal A_{260}/A_{280} ratio should be closer to 1.8), all DNA samples passed quality control at CD-Genomics and the 16S rRNA gene was sequenced for each sample. Between 73931 and 112822 raw reads were generated by sequencing (Table 2). The values of Q20 and Q30 in Table 3 show that read confidence overall was high, with values near or above 95%. The average length of the clean tags ranged from 408.94 to 429.45 nucleotides (Table 2). Out of the five metalworking fluid samples tested, the sample with the least

FIGURE 1.

Taxa abundance heatmap of samples based on standardized Z-scores. The Z-score calculates how many standard deviations a value is from the mean of its row. Color represents the abundance of genera, and the horizontal clustering indicates the similarity of the abundance of different genera between samples.



confidence was from Reservoir 1B, which had a Q20 value of 98.58% and Q30 of 94.88%. The sample with the highest confidence was from Reservoir 3 with a Q20 of 99.04% and Q30 of 96.37% (Table 2).

A Z-score heatmap is a graphical representation of clustering using color gradients to represent the relative abundance of similar genera in a sample based on read counts. The Z-score calculates how many standard deviations a value is from the mean of its row. For example, a score of +2 indicates that the abundance of that genus at a given site is two standard deviations greater than the mean abundance of that genus across all sites (Figure 1). The heatmap results show that the relative abundance of bacterial genera varied significantly between samples (Figure 1). For example, *Mycobacterium* was very abundant in two of the five samples (Reservoir 2A and Reservoir 3) but less abundant in the other three samples, especially in Reservoirs 1A and 1B as shown by the blue color (Figure 1). Other genera, such as *Sphingobium*, have a high Z-score in sample from Reservoir 2B (Figure 1). On the other

hand, *Pseudomonas* and *Acinetobacter* had high Z-scores in the Reservoir 1B sample indicating relatively high abundance levels in that sample (Figure 1). These data were further analyzed to tabulate the percentages of bacterial genera or families detected from each site based on read counts to visualize within-group comparisons (Table 3). Once again, *Mycobacterium* was very abundant in Reservoirs 2A and 3, representing 87.09% and 99.72% of the sequences, respectively (Table 3). *Mycobacterium* was also abundant at site 2B at 28.12% but was mostly absent from reservoirs 1A and 1B (Table 3). *Pseudomonas* (9.26%) and *Rhizobiaceae* (29.54%) were abundant at reservoir 2B (Table 3). Reservoir 1A mostly included bacteria from the genera *Comamonas* (64.80%) and *Elizabethkingia* (24.59%), whereas Reservoir 1B mostly consisted of *Acinetobacter* (76.45%) and *Pseudomonas* (18.66%) (Table 3).

Diversity indices such as Simpson and Shannon provide a measure of biodiversity by combining richness and evenness. Overall, samples from Plant 2 were found to have the greatest number of observed features (Operational Taxonomic Units)

TABLE 3.

The percentage relative abundance of the main bacterial genera or families detected in each metalworking fluid sample based on read counts.

Genus of Family	Percentage relative abundance (%) in each sample				
	Reservoir 1A	Reservoir 1B	Reservoir 2A	Reservoir 2B	Reservoir 3
<i>Acinetobacter</i>	- ^a	76.45	1.23	5.29	-
<i>Aerococcaceae</i>	-	-	0.03	4.86	-
<i>Aquamicrobium</i>	-	-	0.07	1.18	-
<i>Brevundimonas</i>	-	-	0.78	0.03	-
<i>Comamonas</i>	64.80	0.01	0.12	2.47	-
<i>Comamonadaceae</i>	0.16	-	0.99	1.11	-
<i>Dysgonomonas</i>	-	1.34	0.03	-	-
<i>Elizabethkingia</i>	24.59	-	-	-	-
<i>Methylobacterium</i>	5.10	-	-	-	-
<i>Microbacteriaceae</i>	-	-	0.05	4.49	0.02
<i>Mycobacterium</i>	0.02	-	87.09	28.12	99.72
<i>Paenochrobactrum</i>	-	-	0.02	5.74	-
<i>Phreatobacter</i>	4.76	-	-	-	-
<i>Pseudomonas</i>	0.04	18.66	1.70	9.26	-
<i>Rhizobiaceae</i>	-	0.04	0.33	29.54	0.04
<i>Sphingobium</i>	-	-	1.44	-	-
<i>Stenotrophomonas</i>	-	-	0.32	1.71	-
Others	0.53	3.50	5.83	5.76	0.21

^a Not detected

with Reservoir 2B having 114 different bacterial groups and Reservoir 2A having 367 groups (Table 4). Reservoir 2B had the highest diversity indices with a Simpson index of 0.909 and a Shannon index of 4.158. Table 3 also shows that Reservoir 2B had a relatively rich representation of different bacterial groups. As expected, Reservoir 3 had the lowest diversity indices with a Simpson index of 0.006 and a Shannon index of 0.040 (Table 4). This makes sense as the sample from Reservoir 3 was found to comprise 99.72% *Mycobacterium* (Table 3) and only 38 bacterial groups overall (Table 4). The other three samples had Shannon diversity indices ranging from 1.433 to 2.315 (Table 4).

Figure 2 shows species richness, diversity, and relatedness of the metalworking samples based on unweighted clustering (UPGMA). A UPGMA tree was constructed to visualize the differences among bacterial communities from the different reservoirs. A closer sample distance and a shorter branch indicate more similarity in bacterial composition between the samples. The large percentage of *Mycobacterium* in the sample from Reservoir 3 (Table 3), combined with its low diversity indices (Table 4), is the main reason it is the least related to any other sample. Samples collected from the same plants were seen to be phylogenetically related (Figure 2).

DISCUSSION

In the present study, DNA was isolated and purified from five different metalworking fluid samples and successfully sequenced as part of a metagenomic analysis using the V3-V4 regions of the 16S rRNA gene. Overall, a large number of raw PE reads were generated and the Q30 for all samples was approximately 95% or higher. Our metagenomic study provides

additional insights into the diverse bacterial communities present in automotive metalworking fluids and supports the notion that the presence of mycobacteria in some, but not all, metalworking fluids is significant.

As shown by the taxa abundance heatmap of samples, diversity indices, and clustering tree, there were significant differences between samples. The Shannon index for four of the five reservoirs ranged from 1.433 to 4.158, which is relatively similar to the Shannon index calculated (ranging from 1.86 to 2.93) in a metagenomic study conducted with spent metalworking fluids (8). Samples from the same plants (Reservoirs 1A and 1b; Reservoirs 2A and 2B) showed some relatedness between them, which is to be expected since similar products and environmental factors are present. Nevertheless, there were variations in bacterial communities between reservoirs as shown by the heatmap Z-scores which highlighted the relative abundance of certain genera at each site. For instance, Z-scores showed the relative abundance of mycobacteria in two of the five samples. The sample from Reservoir 3 yielded very low Simpson index (0.006) and Shannon index (0.040) in comparison to the other four samples. This might be due to the metalworking fluid at that site having been recently heavily treated with broad-spectrum sulfur containing antibacterial biocides (personal communication from the personnel), resulting in a fluid that contained very few types of bacteria. This would suggest that mycobacteria are especially resistant to this type of disinfection and would warrant additional evaluation. These results are similar to those of another study, in which relatively higher levels of *Mycobacterium* spp. were found in treated metalworking fluids than in untreated fluids (10). The current study is one of the first to use metagenomics to obtain a comprehensive analysis of bacterial communities

FIGURE 2.

UPGMA clustering tree based on unweighted UniFrac to visualize the relatedness of each individual metalworking fluid sample against one another. The support or bootstrap values are displayed at each node.

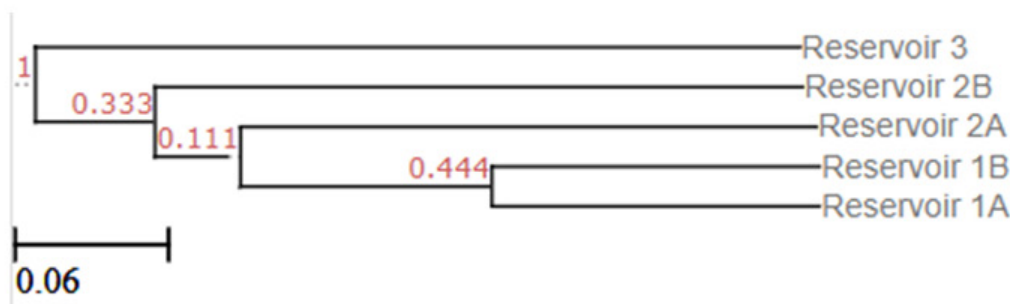


TABLE 4.

Statistical analysis of alpha diversity indices in each metalworking fluid sample.

Sample	Bacterial taxa	Ace	Chao1	Simpson	Shannon
Reservoir 1A	102	102	102	0.516	1.433
Reservoir 1B	64	64	64	0.738	2.315
Reservoir 2A	367	367	367	0.241	1.444
Reservoir 2B	114	114	114	0.909	4.158
Reservoir 3	38	38	38	0.006	0.040

in metalworking fluids. A recent Russian study used metagenomics to analyze metalworking fluids and identified a large number of bacterial and fungal genera including *Alcaligenes*, *Bacteroides*, and *Pseudomonas* (8). Two other studies, conducted in China and Switzerland, also used metagenomics to evaluate metalworking fluids from various industries (9, 10). Future metagenomic studies could provide additional insights into the microbiology of metalworking fluids in a variety of industries and under different manufacturing conditions. For example, evaluating changes in microbial communities with the use of specific biocides or disinfectants could be done using metagenomic analysis. Although metagenomics may not be appropriate for routine surveillance and monitoring of metalworking fluids, it is a powerful approach that may help address specific microbial contamination problems facing the automotive industry.

As in other studies (1, 8, 9, 10, 15, 16), many bacterial genera and families were detected in the five metalworking fluid samples analyzed in this study. *Mycobacterium* spp. and *Pseudomonas* spp. were found among the main identifiable bacterial groups in three of the five samples. The presence of *Pseudomonas* in metalworking fluids agrees with the results of other studies that have reported it as a predominant genus in metalworking fluids (8, 9, 10). At one site, Reservoir 3, *Mycobacterium* made up more than 99% of the bacterial composition, which warrants future research. The presence of mycobacteria is not surprising since *M. immunogenum* has been repeatedly found in metalworking fluids and is persistent in such systems. *Mycobacterium immunogenum* has been of increasing concern due to being linked to cases of hypersensitivity pneumonitis infections in industrial workers exposed to metalworking fluids (15). The detection of *Pseudomonas*, which is often associated with biofilms, in our bulk fluid samples suggests that sloughed-off biofilm cells are likely to contaminate metalworking fluids. Biofilm formation in metalworking fluid systems makes disinfection more challenging as

biofilm bacteria are highly resistant to environmental stressors and chemical treatments (17). Therefore, the presence of *Mycobacterium* and *Pseudomonas* in biofilms could have implications on disinfection methods of metalworking fluid systems, as biofilms often remain even with the addition of biocides and general cleansing processes. In a 2008 study of a relatively small U.S. Midwest machining facility, *Alcaligenes faecalis* was identified from every sample collected during summer and winter sampling periods. However, *Mycobacterium immunogenum* was only detected from one machine in winter samples (22). In the present study, *Mycobacterium* was detected in samples collected during the fall, suggesting that future research should evaluate the effect of temperature and seasonality on the presence of mycobacteria in metalworking fluids. Additionally, *Alcaligenes* sp. was not found in any of our samples. Examples of other bacteria previously found in metalworking fluids, but not in this study, include *Bacteroides*, *Citrobacter*, *Legionella*, *Micrococcus* spp., *Propionibacterium* spp., *Pseudochrobactrum* spp., and *Staphylococcus capitis* (3, 8, 10, 16).

Several other bacterial groups were identified in our samples. The dominant taxonomic units vary, which is probably due to the unique conditions of each reservoir. For example, *Rhizobiaceae*, a family of soil bacteria, is most notable for its nitrogen fixing symbiotic relationship with plant roots (18). Its high presence in Reservoir 2B (at more than 29% of its overall composition) may indicate soil or sand contamination because the rough automotive parts machined using the metalworking fluid from Reservoir 2B come from a casting plant that uses sand and iron filings in the casting molds. Several large operations take place in Reservoir 2B, which may release some sand. This is not necessarily the case with other reservoirs. Both *Comamonas* and *Elizabethkingia* are genera of bacteria common to the environment and both with documentation of occasional virulence to humans (19). The level of *Comamonas* was especially high (almost 67%

of bacteria) in Reservoir 1A. Species of this genus are found not only in the natural environment but also in engineered conditions such as factory settings, where they play a role in nitrate reduction (19). *Comamonas* was also very abundant in metalworking fluids used for cutting aerospace materials at a research institute in Shanghai, China (9). *Elizabethkingia* is a genus commonly found in both the environment and notably, in hospital tap water. Historically, *Elizabethkingia* species have caused illness in immunocompromised patients. The most important of these is *E. meningoseptica*, which was responsible for a meningitis outbreak in the 1950s (20). The presence of both *Comamonas* and *Elizabethkingia* in Reservoir 1A indicates a need for further research to determine whether known pathogenic species are present. *Acinetobacter* spp. was found at a high percentage in Reservoir 1B (more than 76%) and to a lesser extent (more than 5%) in Reservoir 2B. Its presence is not surprising; *Acinetobacter* was first detected in metalworking fluids in 1987 and since then has been shown to be a very common contaminant of metalworking fluids (9, 21, 22). It is an opportunistic pathogen that can form biofilms and cause a variety of infections in blood, lungs, or wounds (23).

The results of this study indicate that our DNA isolation

procedure works well to obtain metagenomically analyzable DNA. However, a different metagenomic analysis (for example, full-length 16S rRNA sequencing) could be done to identify species and subspecies of bacteria. In general, a wide variety of taxonomic groups were found at each site with the exception of Reservoir 3. As in several other studies on metalworking fluids, all samples were found to have bacterial communities that include opportunistic pathogens (e.g., *Mycobacterium*, *Pseudomonas*, *Acinetobacter*), making further surveillance of metalworking fluids important to closely monitor pathogenic bacteria. The high percentages of *Mycobacterium*, *Acinetobacter*, and *Pseudomonas* in samples indicate that there may be biofilms composed of those bacteria present in metalworking fluids, making further research of the biofilms of interest, specifically at the species level. A limitation of this type of study, however, is that DNA detection does not necessarily imply that viable cells are present in a sample.

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