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The Detection of Airborne Sulfate-Reducing Bacteria from Metalworking Fluids

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Summary

In this presentation, the detection of airborne sulfate-reducing bacteria from contaminated metalworking fluids was examined. The study was conducted on-site, in a metalworking plant, in and around three separate machining lines and their central reservoirs using liquid, solid, and modified impingement air samplers. All of the samples were taken at room temperature during the machining line operation.

Results indicated that sulfate-reducing bacteria do survive in the airborne state in particles of contaminated metalworking fluid, provided it possesses a source population.

I. Introduction

The sulfate-reducing bacteria are just one group of several that have been isolated from metalworking fluids. Evidence indicates that sulfate-reducers require the presence of aerobic species in order to sustain viability. Bennett & Guynes (1959) demonstrated a synergistic relationship existed between these two groups which facilitated fluid spoilage, and Postgate (1979) implicated aerobic forms in the production of redox potentials suitable for sulfate-reducer viability. Various groups of fungi have also been routinely isolated from contaminated fluids, but only in conjunction with previously high aerobic bacterial counts, followed by their reduction after germicide additions to the fluid (Rossmore & Holtzman 1974). It is evident that all three groups of organisms, fungi, aerobic and sulfate-reducing bacteria, can inhabit a given metalworking fluid during the course of its use, and each has some effect upon the other. Sulfate-reducing bacteria appear in fluids after aerobic bacterial activity. Bennett & Guynes (1959) demonstrated sulfate-reducing bacteria in metalworking fluids required the presence of aerobic bacteria for two purposes: first, the aerobic species were able to oxidize and produce nutrients; and second, they served to lower the redox potential of the fluid to a point more suitable for sulfate-reducer activity.

Previously published studies indicated aerobic species, e.g. *Pseudomonas aeruginosa* (Hill & Al-Zubaidy 1979), *Klebsiella pneumoniae*, *Enterobacter aerogenes* and *Citrobacter freundii* (Rossmore *et al.* 1976), did survive and retain viability in airborne particles from contaminated fluids. The presence of inactive sulfate-reducers in airborne fluid particles could also be assumed based on studies conducted by Postgate (1956), and Hardy & Hamilton (1981), who identified the activity of cytochrome C₃ (Postgate 1979, 1956), superoxide dismutase and catalase in these organisms, as well as evidence of aerotolerance.

In view of the presence of sulfate-reducing bacteria in contaminated metalworking fluids, this study was initiated to detect their occurrence in airborne fluid particles.

II. Materials and Methods

Plate Counts and Sulfate-Reducing Bacteria Detection

Bacteria and fungi counts were performed on metalworking fluid samples following standard plate count techniques, on Trypticase Soy Agar (TSA-BBL) and Trypticase Soy Agar plus 1 ml Gentamicin (TSA-GENT).

Preliminary Air Sampling

The preliminary air sampling was conducted with a liquid (Millipore Corp., Model M000-000-01) and solid (Andersen Co., model) impingment air sampler. Both samplers were operated using a calibrated vacuum pump aspirating 28 l/minute. For liquid impingment, a recovery fluid was used containing an antifoaming agent and a nutrient substrate. After sampling was completed, the impingment fluid was filtered through a 0.45 μm filter. The filter was then transferred to a 50 x 12 mm locklid petri plate containing a pad soaked with TSA-broth and incubated at 30 °C. The solid impingment air sampler was composed of two stages, one sampling particles larger than 5.0 μm and the other particles smaller than 5.0 μm . The resulting plate counts for both bacteria and fungi were made using TSA and TSA-GENT respectively and incubated at 30 °C.

Air Sampling Methods for Recovery of Sulfate-Reducing Bacteria

The recovery of sulfate-reducing bacteria was conducted using a modified impingment sampler in which the impingment fluid also served as the recovery medium. Before air sampling was actually undertaken, three media were evaluated. The three media were set up in 500 ml screw-capped Ehrlenmeyer flasks with no air space and inoculated with 1 ml of metalworking fluid known to contain viable sulfate-reducing bacteria.

Viability was determined by microscopic examination at 1000x for characteristic motile vibrios. The inoculated flasks were incubated at 30 ° C and observed daily for sulfate reduction, as indicated by ferrous sulfide precipitation in the media. When this occurred, the transpired days were recorded and a sample was examined as previously indicated. Positive results were indicated when motile vibrios were present. The media used in this portion of the study are presented in Table 1. The modified impingment sampler was composed of an 8-oz. narrow-necked prescription bottle, a double-holed rubber stopper, 100 cm of 3mm Pyrex glass tubing, 1.0 g fine iron wire, 2.0 cm pipe cleaner, and 60 ml of impingment/culture medium. The unit was assembled by breaking the glass tubing into two equal lengths and placing one in each hole of the rubber stopper. At this time, 60 ml of impingment/culture medium was added, along with the amounts of iron wire and pipe cleaner previously specified. The rubber stopper was put in place and the entire unit was sterilized at 121°C, 15 atms. for 35 minutes. The sampler was then allowed to cool to room temperature prior to actual air sampling.

Air samples were all taken in the same manner. With each medium, one sample was taken at each system during the five week study. Sampling devices were connected to the calibrated vacuum pump and collected for 30 min. in the case of machine side samples, and 45 min. in the conference room samples. When sampling was completed, the sampling devices were disconnected from the vacuum pump and immediately filled to the top with fresh recovery medium. At this time, a 2.0 mm layer of sterile mineral oil was added and one carbon dioxide releasing tablet. The vessel was then sealed by securing the screw cap and set aside for transport back to the lab. The recovery vessels were incubated at 30°C and observed daily for sulfate reduction. Positive results were indicated as previously stated.

Aerobic air sampling studies, aerobic plate counts, S-R deep tests, and controls were also established at this time to ensure that positive results did not arise from other causes. It was assumed that in order for sulfate-reducers to be isolated from airborne fluid particles, aerobic organisms and sulfate-reducing bacteria had first to be detectable in the actual fluid. The controls served as positive and negative tests which ensured the ability of the media to support the growth of these organisms and that subsequent detection of them in the recovery vessels resulted from airborne transmission in fluid particles, and not from contamination. The air sample taken in the conference room served as a control to demonstrate that sulfate-reducers could not be isolated from an airborne state without first being present in another liquid environment.

Table 1 Sulfate-reducing bacteria growth media

Component	Medium R	Medium B	Medium E
Sodium lactate	5.2g	3.5g	3.5g
Yeast extract	1.0g	1.0g	
Bacto agar	0.75g		
Thioglycollic acid		1.0g	1.0g
Ascorbic acid	0.1g	1.0g	
Potassium diphosphate			0.5g
Dipotassium phosphate	0.01g		
Ammonium chloride		1.0g	1.0g
Calcium chloride. 6H ₂ O			1.0g
Sodium chloride	10.0g		
Magnesium chloride. 7H ₂ O			2.0g
Calcium sulfate		1.0g	
Magnesium sulfate. 7H ₂ O		3.0g	
Sodium sulfate			1.0g
Ferrous sulfate. 7H ₂ O		0.5g	0.5g
Ferrous ammonium sulfate	0.2g		
Sodium sulfite	0.6g		
Tap water			1.0l
Distilled water	1.0l	1.0l	

The media in the above table were prepared using standard microbiological methods for media containing thioglycollic acid and pH was adjusted after autoclaving.

Controls for Sulfate-Reducing Bacteria Studies

Growth support controls were set up following the same technique used for the growth evaluation study. The contamination controls were set up in the same manner as the air samplers after sampling was completed, and the conference room controls were handled just as the sampler/recovery vessels were. In all cases, the medium used for the controls was a portion of the medium which was used in the air sampling devices.

Study Site

The study was conducted at a metalworking plant which manufactured automobile engines. It focused on three separate machining lines, 30-35 machines each, and their central reservoirs (see Table 2). The systems ranged in size from 4.4×10^4 l to 2.2×10^5 l and were operated on a 24-hour, 5-day schedule. During weekend shutdowns, the main body of fluid was pumped only within the central reservoir. Air samplers were positioned approximately 1.5 m from the floor at the transfer line level, equal distance between adjoining machines. In the case of the millipore impingement samplers (Millipore Corp.), a second site was used. This site was located at a tool storage area associated with the machining line. Samples in this instance were placed 0.5 m from the floor on the work-bench surface, approximately 4 m from the actual line itself.

System A - Con-Rod Machining Line

This operation was involved in drilling, tapping, and boring the appropriate holes in the piston assembly. The parts were made out of steel, and a soluble oil metalworking fluid was used. The machines were arranged in a linear fashion with a parts transfer line running between them. Fluid application was by flood nozzles directed at the cutting tools.

System B - Front-Cover Machining Line

This operation was responsible for drilling, tapping, and boring the holes of the front cover assembly for the water pump housing. The part was composed of 380 series aluminum, and the machines were arranged in a square. Parts entering the square were moved counterclockwise from machine to machine via a transfer line. Finished parts exited the line at the opposite end they entered. The system used initially a semisynthetic metalworking fluid, but was switched to soluble oil due to bacterial and fungal problems. Fluid application at the machines was via flood lines directed at the cutting tool.

System C - Intake-Manifold Machining Line

This operation bored, drilled, and tapped the holes in the intake manifold. The machines were arranged in a linear fashion and a soluble oil metalworking fluid was used. A transfer line moved the parts from machine to machine, and the fluid was applied in a floodtype manner.

In all three cases, the flooding of the cutting tool with the metalworking fluid resulted in the generation of a fine mist of airborne particles. The air samplers used in the study were placed in locations at all three systems to facilitate sampling of this mist.

III. Results and Discussion

Table 2, Part A, presents the results of the preliminary re-examination of the microbial flora of two different types of metalworking fluids i.e., a soluble oil and a synthetic. The results indicate that both supported large populations of bacteria and fungi with detectable levels of sulfate-reducing bacteria. Part B of Table 2 indicates bacteria and fungi were also isolated from airborne particles of these fluids. The presence of viable bacteria in airborne fluid particles (Rossmore *et al.* 1976; Hill & Al-Zubaidy 1979) and their isolation from droplets smaller than $5.0 \mu\text{m}$ is well documented in the literature (Hickey & Reists 1975).

Table 2 Preliminary aerobic bacteria studies.

System	Fluid Type	(PART A)				(PART B)			
		Aerobic & Sulfate-Reducing Bacteria Results		Sulfate-Reducing Bacteria		Andersen Sampler		Air Sampling Data ^a	
		Mold	Bacteria	^b Sulfate-Reducing Bacteria	<5.0µm Bact./Mold	>5.0µm Bact./Mold	Milipore Sampler Machining Line	Milipore Sampler Storage Bench	
A	Soluble Oil	1 x 10 ²	4 x 10 ¹⁰	+	4.2 x 10 ⁷ /27	2.6 x 10 ⁷ /37	3.3 x 10 ³	2.7 x 10 ³	
B	Synthetic	2 x 10 ⁴	8 x 10 ⁹	-	2.7 x 10 ⁷ /78	3.1 x 10 ⁷ /51	2.4 x 10 ³	3.3 x 10 ³	

^aAir sampling data are presented in units of organisms per m³ of air sampled.

^bThe sulfate-reducing bacteria were detected using the S-R Deep Test Method described previously.

^cPlate count data are presented in units of organisms per ml.

Table 3 Results of study conducted at System A.

FLUID TEST	METALWORKING FLUID DATA				
	1	2	3	4	5
Aerobic plate count	5 x 10 ⁷	5 x 10 ⁷	5 x 10 ⁷	5 x 10 ⁷	5 x 10 ⁷
S-R deep test	+	+	+	+	-

AIR SAMPLING RESULTS

Sulfate-reducer characteristics	Medium B Weeks					Medium E Weeks					Medium R Weeks				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Vibrio morphology	-	-	+	-	-	-	-	-	-	-	+	+	+	+	-
Motility	-	-	+	-	-	-	-	-	-	-	+	+	+	+	-
Ferrous sulfide precipitation	-	-	+	-	-	-	-	-	-	-	+	+	+	+	-
Hydrogen sulfide detected	-	-	+	-	-	-	-	-	-	-	+	+	+	+	-

Typical vibrio morphology and motility were observed as a result of microscopic examination using a phase contrast microscope at 1000x. In all cases, whether air sampling media were positive for sulfate-reduction or not, other morphological species were detected via the same technique. Aerobic plate count results were determined by diluting samples to 1 x 10⁶ and counting bacterial colonies according to standard practice.

The evaluation of the three media for the sulfate-reducer air sampling studies indicated all were capable of supporting growth of this group of bacteria. However, there were differences in the rate of sulfate reduction between the three. Medium R showed signs of sulfate reduction in 3 days after inoculation, while Medium B and Medium E took 5 and 7 days respectively. It was assumed that the microbial numbers in the inoculum used were basically the same and that differences in apparent growth rate of organisms were due to one medium's ability over another to facilitate growth. This is indicated in Postgate's (1979) work where Medium B was suggested as a medium for diagnostic purposes and maintenance of cultures, while Medium E was designated for enumeration and isolation of pure cultures of sulfate-reducing bacteria. In the case of Medium R, it is a modification of an existing sulfate-reducer medium with differences as indicated in Table 1. Increased levels of lactate and the presence of sodium sulfite in this medium may have resulted in production of an environment more suitable for sulfate reducer growth (Pankhurst 1971).

The air sampling studies for the recovery of sulfate-reducers were composed of two phases as stated in the materials and methods section. The first phase consisted of establishment of three controls previously described. In all cases, the controls indicated the results that were as expected. The growth ability controls were positive for weeks 1 - 5 of the study which indicated the medium used in the air samplers was normal and supportive of sulfate-reducing bacterial growth. Contamination controls indicated this was not a problem during the five weeks of the study, and the conference room air samples indicated sulfate-reducing bacteria were not present in areas where a source population was non-existent. The second phase involved actual air sampling studies. As stated in the materials and methods section, aerobic plate counts and S-R deep tests (Rossmore *et al.* 1984, this symposium) were established to determine the fluid microbial load and sulfate-reducer population prior to air sampling. The results indicated that when sulfate-reducing bacteria were present in the fluid, they also were isolated from its airborne particles. The results for System A are presented in Table 3.

Studies conducted on Systems B and C were supportive of the results presented in Table 3. In the case of System B, results showed plate count levels of $> 5 \times 10^7$ /ml existed during weeks one through five and detectable levels of sulfate-reducers were present during week one and two. Air sampling data indicate that Medium B was successful in recovering sulfate-reducers during week 2 of the study but not week 1. Medium E was not successful at all. Medium R recovered sulfate-reducers during both weeks they were isolated from the fluids. Studies conducted at System C indicated that, again, aerobic plate counts of $> 5 \times 10^7$ organisms/ml were seen and sulfate-reducing bacteria were detected in the fluid. Sulfate-reducing bacteria were isolated on weeks 1, 3 and 5 of this air sampling study. Air sampling results indicated that Medium B was ineffective in recovery of this group of organisms at this system and medium E was only successful at recovery of sulfate-reducers during week 3. Medium R, however, again showed positive signs of sulfate reduction during weeks when sulfate-reducing bacteria were isolated from the fluid. Thus, this evidence conclusively demonstrates that if sulfate-reducing bacteria are present in a metalworking fluid, they will also be present in airborne particles originating from it. It also suggests that Medium R is more favorable for recovery than either Medium E or B, in terms of this particular sampling method.

The recovery of sulfate-reducing bacteria from airborne metalworking fluid particles is not surprising based on studies conducted demonstrating aerotolerance of this group in seawater (Hardy & Hamilton 1981).

Sulfate-reducing bacteria have been characterized in the literature as a group of organisms that is anaerobic by nature in active cultures and requires low redox potentials to sustain viability (Postgate 1979, Pankhurst 1971). A minimum of -100 millivolts is required to stimulate growth and multiplication of sulfate-reducers in culture. The addition of iron wire was used to help in reaching this initial redox potential. In a study published by Starkey & Wright (1945), it was demonstrated that iron added to a medium composed of lactate and inorganic salts resulted in initial potentials of -280 to -400 mv in uninoculated media.

The addition of the carbon dioxide tablet primarily served to remove the trace amount of oxygen at the top of the sampler vessel prior to incubation. However, it also may have aided in providing better growth conditions in the medium based upon information published by Pankhurst (1971). Pankhurst demonstrated that carbon dioxide could be assimilated by sulfate-reducing bacteria and also function as a carbon source.

In conclusion, the successful detection of sulfate-reducing bacteria from airborne particles of contami-

nated metalworking fluids resulted from aerotolerance demonstrated by Hardy & Hamilton (1981) and the establishment of a recovery method which allows the recovered organisms to enter an environment which stimulates growth. These findings indicate that sulfate-reducing bacteria survive in the airborne state for brief periods of time, but it does not indicate to what degree. Further research is necessary to examine how airborne numbers compare to numbers found in the fluid and whether or not airborne survival can explain central reservoir cross contamination.

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