

The Identification of a Defined Microbial Inoculum for the Evaluation of Biocides in Water-Based Metalworking Fluids

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LUBRICATION ENGINEERING

Three bacterial species Pseudomonas aeruginosa, Proteus mirabilis and Klebsiella pneumoniae were dominant in more than 100 field samples of water-based metalworking fluid. They survived well together in 12 of 15 market fluids and in several ASTM test methods withstood fungal competition. They are recommended as candidate inoculum for evaluating biocides.

INTRODUCTION

In an earlier study (1), the value of a spoiled fluid as the basis for challenging the bioresistance of a water-based metalworking fluid was reported. This was done to establish the role of the inoculum in assessing reliability of a bioresistance test method being promulgated by Technical Committee L of ASTM sp D-2, based on ranking rather than absolute counts. The results seemed independent of the original source of the inoculum. This suggested that the same qualitative evaluation of bioresistance could be made at different locations making the concept of a universal inoculum both unnecessary and potentially feasible. This is no contradiction but rather a reflection of the different aims of two test methods. The ASTM D-2 L bioresistance test is aimed primarily at inplant evaluation for product selection; the other, originating from a task force of ASTM E35.15 is being promoted for the evaluation of biocides in water-based metalworking fluids. It is this requirement i. e. of a universal inoculum, that will grow in a very large number of biocide-free proprietary fluids (in the latter tests) for demonstrating biocide efficacy, that suggested the need for this research.

PROCEDURE

Prior to and during the course of this study, more than 100 field samples were evaluated microbiologically and the dominant species identified (Table 1), (the technique will be described below). This survey was considered sufficiently representative to serve as a repository for candidate organisms in the proposed universal inoculum. However, a number of species isolated had counterpart namesakes with pedigrees established by the American Type Culture Collection (ATCC). It was deemed appropriate to evaluate these in metalworking fluid for survival and/or growth characterization since their use had already been established for other biocide test systems (e. g. sanitizers, disinfectants).

ISOLATION AND IDENTIFICATION OF CULTURES

Metalworking fluid samples were streaked on a variety of selective and differential solid media to initially characterize the general taxonomic location of the isolate. Bacterial colonies with different reactions and morphologies were transferred to soy peptone casein digest agar, incubated 24 hours at 30°C and then identified. Fungal isolates were identified to genera by macroscopic and microscopic appearance.

Isolated and identified organisms used throughout these studies were maintained on soy peptone casein digest agar (bacteria) and potato dextrose agar (fungi) incubated at 30°C and transferred to fresh agar slants every ten days. Inocula for each test were prepared from fresh slants transferred into soy peptone casein digest broth for bacteria and Czapek-Dox broth for fungi. The cultures were incubated at 30°C with continuous shaking, 48 hours for bacteria and 120 hours for fungi.

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TABLE 1—FREQUENCY OF ISOLATION OF BACTERIAL METALWORKING FLUID SPECIES

SPECIES	FLUID TYPE:	
	SOLUBLE OIL	SYNTHETIC AND SEMI-SYNTHETIC
<i>Pseudomonas aeruginosa</i>	16	19
<i>Pseudomonas fluorescens</i>	2	1
<i>Pseudomonas cepacia</i>	0	2
<i>Pseudomonas stutzeri</i>	1	0
<i>Pseudomonas alcaligenes</i>	0	1
<i>Pseudomonas pseudomallei</i>	0	1
<i>Pseudomonas putida</i>	1	0
<i>Aeromonas hydrophilia</i>	1	1
<i>Proteus mirabilis</i>	2	3
<i>Proteus vulgaris</i>	1	1
<i>Proteus rettgeri</i>	1	1
<i>Enterobacter cloacae</i>	3	7
<i>Enterobacter agglomerans</i>	2	1
<i>Enterobacter gergoviae</i>	1	3
<i>Citrobacter freundii</i>	2	12
<i>Escherichia coli</i>	1	4
<i>Klebsiella pneumonia (oxytoca)</i>	6	8
<i>Klebsiella ozaenae</i>	3	0
<i>Serratia liquefaciens</i>	1	0

Several different types of tests were used including permutations of system size, treatment time, inoculum and incubation conditions. All evaluations of treatment conditions were based on the standard plate count method. Soy peptone casein digest agar was used for bacterial levels and Czapek-Dox agar with 50 mgm/ml gentamicin was used for fungi (2). In both the isolation and detection of survival of specific bacterial types violet red bile agar and eosin methylene blue agar, lead acetate agar, and cetrimide agar were used for coliform, (*Escherichia coli*, *Klebsiella sp.*, *Enterobacter sp.*, *Citrobacter sp.*), *Proteus mirabilis* and *Pseudomonas sp.*, respectively.

METALWORKING FLUIDS

Fifteen fluids from seven different manufacturers representing the three major categories, synthetic, semisynthetic and soluble oil were used in the study. Assurances were received from five of the companies involved that the products contained no biocides. Concentrates were diluted in Detroit City tap water at 1-20 or 1-40 as indicated.

System Size

- A. Fifty milliliter total volume; 45 ml of diluted metalworking fluid was sterilized in a 4-fluid-ounce screw-cap prescription bottle to which 5 ml of inoculum was added. Dilutions of inocula were made in sterile, distilled water to levels indicated in each experiment. Tests were carried out at 28°C with continuous shaking.
- B. One liter total volume—900 ml of diluted nonsterile metalworking was placed in a 32-ounce French square bottle containing 90 gm of 20 mesh iron to which 100 ml of inoculum was added (note: this brings the

liquid level to the very top and it was necessary to discard approximately 100 ml to lower the level to the shoulder). The systems were left open in the environment of a cabinet maintained at 28°C and aerated from a central compressed air source with individual sterile, plugged, 2.2 ml pipets. This latter step was a precaution to minimize excessive adventitious contamination from the compressed air lines. Aeration was carried out for 5 days with 2 days of quiescence.

Results and Discussion

In Table 2, the effect of inoculum size and fluid concentration on population survival is presented. Of the four species used, two were laboratory strains; one from Wayne State University Microbiology Lab, *Pseudomonas fluorescens* and one ATCC acquisition, *Enterobacter aerogenes* ATCC 13048. The other two were isolated from metalworking fluids. Survival was best with the larger inoculum and in the 1:40 fluid, suggesting the need for a fairly large inoculum if the population must adapt to the more toxic environment afforded by the higher (1:20) fluid concentration. The two cutting fluid isolates fared better in these survival studies, one of them being the *Ps. aeruginosa* designated *Pyocyanin+*. Because of the widespread use of *Ps. aeruginosa* 15442 in antimicrobial testing, it was compared to the *pyocyanin+* strain (Table 3).

Although there was considerable death in all systems compared to the previous experiment, it was quite evident that the cutting fluid isolate was much hardier than the ATCC strain. It was decided that future studies involving *Ps. aeruginosa* would only utilize the cutting fluid isolate.

Because of the frequency of isolation of *Klebsiella pneumoniae* from field samples, the same type of survival study was done with that species (Table 4). In this case, it

TABLE 2—DYNAMICS OF SURVIVAL OF MIXED INOCULUM IN SYNTHETIC FLUID				
TIME OF SAMPLING	BACTERIAL COUNTS/ml IN:			
	SMALL INOC.	LARGE INOC.	SMALL INOC.	LARGE INOC.
	2.5 PERCENT FLUID		5 PERCENT FLUID	
0 time	4×10^6	4×10^7	4×10^6	4×10^7
1 hour	1.5×10^7	1.5×10^8	1.3×10^7	2×10^8
3 hours	7×10^6	1.2×10^8	1×10^7	1.2×10^8
8 hours	8×10^6	2×10^8	8×10^6	8×10^7
24 hours	5×10^6	3×10^8	2.5×10^6	4×10^7
48 hours	2.5×10^6	4×10^8	5×10^5	2.5×10^7
SPECIFIC SURVIVAL AT 48 HOURS				
<i>E. aerogenes</i> (LS)	-	+	±	+
<i>P. mirabilis</i> (FI)	+	+	+	+
<i>Ps. aeruginosa</i> (FI)	+	+	+	+
<i>Ps. fluorescens</i> (LS)	-	+	-	±

LS = Laboratory Strain
FI = Field Isolate

TABLE 3—DYNAMICS OF SURVIVAL OF PSEUDOMONAS AERUGINOSA				
TIME SAMPLE TAKEN	PS. AERUGINOSA ATCC 15442		PS. AERUGINOSA CUTTING FLUID PYOCYANIN +	
	2.5 PERCENT FLUID	5 PERCENT FLUID	2.5 PERCENT FLUID	5 PERCENT FLUID
	0 time	4×10^7	4×10^7	6.5×10^7
1 hour	2.3×10^7	1.7×10^7	3×10^7	3×10^7
3 hours	1.6×10^7	7×10^6	4×10^7	3.5×10^7
8 hours	1.5×10^7	5.5×10^5	5×10^7	3×10^7
24 hours	3.5×10^6	<100	7×10^5	8×10^6
48 hours	<100	<100	3×10^4	2×10^4

TABLE 4—DYNAMICS OF SURVIVAL OF MEMBERS OF TRIBE KLEBSIELLA						
TIME AFTER INOCULATION	ENTEROBACTER AEROGENES (13048)		KLEBSIELLA PNEUMONIAE (4352)		KLEBSIELLA PNEUMONIAE (CUTTING FLUID ISOLATE)	
	2.5%	5%	2.5%	5%	2.5%	5%
	0 time	2×10^7	2×10^7	1.6×10^7	1.6×10^7	1.8×10^7
1 hour	1.85×10^7	1.4×10^7	1.9×10^7	1×10^7	1.5×10^7	1.5×10^7
3 hours	1.6×10^7	1.7×10^7	1.3×10^7	1×10^7	9×10^6	1.25×10^7
8 hours	1.6×10^7	2×10^7	1×10^7	1.4×10^7	8×10^5	2.5×10^6
24 hours	1.15×10^7	8×10^5	1.4×10^7	7×10^6	4×10^7	2.3×10^4
48 hours	1.2×10^7	<100	6.5×10^7	<100	3×10^7	3.3×10^4

was joined by two other organisms, *K. pneumoniae* ATCC 4352 and a related species *Enterobacter aerogenes* ATCC 13048, which is also used in many official tests for antimicrobial agents. Again, the cutting fluid isolate had the best survival record.

In the next experiment (Table 5), the three fluid isolates with the best survival record and a recent *Ps. fluorescens* fluid isolate was inoculated into 15 different fluids diluted 1:20. In only one fluid, the synthetic labeled #10, did all three bacterial types fail to survive. This fluid was from one

of the companies which did not guarantee freedom from biocides. In two cases, #8 soluble oil and #12 synthetic, there was considerable death with only one of the three species surviving. However, nine of the 15 fluids showed an increase in total count while three others remained the same.

In a longer two-week study (Table 6), three fluids were selected from the 15, representing the three major types currently marketed. All had been used in several previous ASTM collaborative studies for evaluating both bioresis-

TABLE 5—SURVIVAL OF MIXED INOCULUM IN SELECTED METALWORKING FLUIDS				
SPECIFIC SURVIVAL OF:				
FLUID	TOTAL BACTERIAL	PSEUDOMONAS SPECIES	KLEBSIELLA PNEUMONIAE	PROTEUS MIRABILIS
	COUNT AFTER 48 HR INCUBATION			
1. Synthetic	7×10^8	+	+	+
2. Soluble oil	9×10^7	+	+	+
3. Soluble oil	8×10^7	+	+	+
4. Soluble oil	1.4×10^8	+	+	+
5. Soluble oil	1.5×10^8	+	+	+
6. Synthetic	1.1×10^8	+	+	+
7. Soluble oil	1.5×10^7	+	+	+
8. Soluble oil	1.4×10^5	+	-	-
9. Semi-Synthetic	1.7×10^8	+	+	+
10. Synthetic	<100	-	-	-
11. Soluble oil	1.3×10^7	+	+	+
12. Synthetic	2.5×10^4	-	-	+
13. Synthetic	2×10^9	+	+	+
14. Semi-Synthetic	2.3×10^8	+	+	+
15. Semi-Synthetic	4×10^8	+	+	+

TABLE 6—THE EFFECT OF FUNGAL COMPETITION ON BACTERIAL INOCULUM—TWO WEEK STUDY*						
INOCULUM	SYNTHETIC FLUID**		SEMI-SYNTHETIC FLUID**		EMULSION**	
	BACTERIA	FUNGI	BACTERIA	FUNGI	BACTERIA	FUNGI
Bacteria	1.5×10^8	9×10^4	1.9×10^8	0	2.9×10^8	6×10^3
Fungi	2.2×10^8	3.2×10^4	1×10^8	2.6×10^4	4.1×10^8	6×10^4
Bacteria + Fungi	1.3×10^8	1.4×10^4	2.6×10^8	1.5×10^4	3.1×10^8	1.4×10^4

*These tests were conducted in a closed chamber without any attempt to isolate the individual systems so that cross-contamination due to aeration was inevitable. This explains the presence of bacteria in systems only inoculated with fungi and the presence of fungi in systems only inoculated with bacteria. It also demonstrates the capability of growing the two microbial types in test systems without overt interference.

**Zero Time Counts: Bacteria— 7×10^7
Fungi— 1.75×10^4

tance and biocides. The tests were carried out in the one liter systems described earlier. The four bacterial species used (Table 5) were also evaluated in this larger, longer test. In addition, fungi were added as indicated to determine the effect of this microbial type on bacterial survival. Not only did the purposeful presence of fungi have no effect on bacterial levels, but (because of cross contamination) even low accidental levels of each type were able to proliferate in the presence of large levels of the other.

An incidental finding noticed in all the experiments was the relatively poor survival of the *Ps. fluorescens* when compared to *Ps. aeruginosa*. It is suggested that it be omitted from future consideration as part of the defined inoculum.

The results of an eight-week survival study in one-liter systems are shown in Table 7. This study followed the protocol of ASTM Committee E35, "The Evaluation of Antimicrobial Agents in Aqueous Metalworking Fluids," [ASTM Standard E686, *ASTM Standardization News*, March (1979)]. All systems contain fungi purposefully added and, at the end of the study, there was survival of all three bacterial types with some decrease and increase in total bacterial and fungal population.

The three species that were eventually chosen for these studies represent three separate groups of bacteria associated with microbiological problems in metalworking fluid. These include deterioration, odor and slime formation (3), (4), (5), with each species offering a different degree of resistance and survivability.

Neither isolation frequencies (Table 1) nor comparative survival studies (Tables 3, 4) are in agreement with the recommendation of EPA for biocide efficacy evaluation in metalworking fluids (6). In fact, as demonstrated previously (7) *Staphylococcus aureus*, one of those on the EPA list is rarely isolated and survives poorly in metalworking fluid. One unique candidate is missing; sulfate reducers. A case can be made for their inclusion in the inoculum. However, they have specialized growth conditions (anaerobic), grow very slowly and do not compete well in a new environment. Usually, if aerobic organisms are under biocide control, sulfate reducers should also be inhibited. Apparent lack of inhibition of this latter group in the field is most likely due to system design, with contaminated sludge not accessible to circulating biocide. This would not be the case in a laboratory test procedure.

TABLE 7—EFFECT OF FUNGAL COMPETITION ON BACTERIAL INOCULUM EIGHT WEEK STUDY

	SOLUBLE OIL	SEMI-SYNTHETIC	SYNTHETIC
Week One			
Total Bacterial Count/ml	1.8×10^8	1.1×10^9	1.6×10^9
<i>Pseudomonas sp.</i>	+	+	+
<i>Klebsiella pneumoniae</i>	+	+	+
<i>Proteus mirabilis</i>	+	+	+
*Fungi	4×10^4	7×10^4	2.8×10^4
Week Eight			
Total Bacterial Count/ml	9×10^8	1.1×10^8	8×10^7
<i>Pseudomonas sp.</i>	+	+	+
<i>Klebsiella pneumoniae</i>	+	+	+
<i>Proteus mirabilis</i>	+	+	+
*Fungi	4×10^4	8×10^3	5×10^4

*The mixed fungal inoculum contained *Fusarium* and *Cephalosporium* which were both present at the end of 8 weeks.

Conclusions

In conclusion, the results indicate that a defined inoculum can survive and potentially grow in a large number of metalworking fluids and that field isolated strains are better candidates than comparable laboratory strains. However, no claim for a universal inoculum can be made. Even in the 15 fluids, there was evidence of too much "die-off" in three to make such a claim. Perhaps the uses of a defined inoculum should be examined. The biocide manufacturer, the metalworking fluid formulator and the fluid user all have distinct reasons for selecting an appropriate inoculum to challenge the biocide. The biocide manufacturer needs an inoculum for demonstration of efficacy in a reasonable number of different fluids and possibly for registration with the Environmental Protection Agency (8), with assurances from fluid producers that test fluids are biocide-free. The fluid producer must evaluate the defined inoculum in their product line and accommodate their decisions to the results obtained. The ultimate challenge is with the fluid user who has little concern for any successes unless they apply directly to problems in the plant. In this latter case, if microbiological problems exist, it would be more rational to use spoiled fluid directly from the site as the basis for the inoculum.

What then is the advantage of a defined inoculum? It can be stored in a culture repository (e. g. ATCC) so that studies can be repeated in different laboratories; it can be used to compare new generations of biocides; it can be made avail-

able and for legal purposes when claims of efficacy need to be substantiated.

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