

Evaluation of Source of Bacterial Inoculum in Development of a Cutting Fluid Test Procedure

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In an attempt to simplify and standardize the bacterial inoculum for an ASTM/ASLE test procedure, four different sources of inoculum were used. All of these were based on biologically deteriorated fluid originally. Two were undefined mixtures with and without adaptation and two utilized selected isolated strains from three test fluids. All inocula were compared in a synthetic fluid, a soluble oil, and a preformed emulsion. Although the relative rankings of the three fluids based on growth were the same with all inocula, there were significant differences in extent of growth in the same fluid with the different inocula.

INTRODUCTION

For the past several years, the need for a test procedure for evaluating the bioresistance of metalworking fluids has become increasingly important. Although a number of procedures have been used on a rather informal basis (1), (7), (9), no single test has received either official blessing or widespread acceptance. There are a number of reasons for this, including the lack of suitable laboratory facilities and personnel in most locations where metalworking fluids are used, and the inability of those individuals more sophisticated in microbiology to decide upon a uniform procedure.

A set of rules for use must, of necessity, include the following:

(a) For any procedure to be meaningful, it must be accepted by the users of metalworking fluids.

(b) The test must be simple, reliable, and repeatable and must correlate with actual conditions of plant use.

(c) To satisfy (a) above, the procedure ideally should be performable within the currently operating chemistry and/or metallurgy laboratories, without excessive outlay for capital equipment.

(d) The test should be performable by personnel usually functioning in the chemistry and metallurgy laboratory. This means chemical technicians and not microbiologists.

(e) Results should be obtainable in a reasonable period of time without sacrificing the reliability of the test.

For the past three years, a joint committee of ASTM/ASLE has been examining procedures for potential adoption to evaluate the relative bioresistance of metalworking fluids. At the outset, the feasibility of simplifying a procedure and still maintaining a high degree of reliability seemed very probable. The type of fluid and the other reagents available in the chemistry laboratory could all be described in precise language. The sole component that cannot be so described is the microbial inoculum used for evaluating the bioresistance of the fluid. A variety of alternatives has been investigated including single strains of *Pseudomonas* species (8), mixed cultures containing pseudomonads and sulfate reducers (2), and spoiled fluid (5), (7). No one procedure has been evaluated using a variety of different inocula to compare a standard group of metalworking fluids. The purpose of such comparison is two-fold: (1) it would establish whether or not a single known species could be used universally for getting comparative data on fluids, and (2) on a quantitative basis it would determine whether the source of the organisms and/or spoiled fluid gives drastically different results when compared with other inocula.

MATERIALS AND METHODS

The procedure used in this study was a modification of the method of Heinrichs and Rossmore (3) developed to simulate use conditions. One liter samples of test metalworking fluid containing 10 g of cast iron chips and 10 percent v/v of the appropriate bacterial inoculum were treated to a regimen of aeration for four and one-half days followed by a two and one-half day quiescence to simulate a weekend shutdown. At this point, evaporation loss is made up by the addition of deionized water. Aeration was then resumed for an additional four and one-half days. Equal sample portions were removed for viable bacterial levels prior to initiation of aeration, at two days, at four days, after the two and one-half day shutdown, and two days and four days later. Bacterial plate counts were done at the times indicated in soy peptone casein digest broth and incubated at 30 C for 48 hr.

ALTERNATE METHOD FOR ESTIMATING VIABLE BACTERIAL COUNTS

Despite the simplification of this procedure and the inoculum used therein, one drawback might prevent universal acceptance in the average chemistry or metalurgy laboratory; that is the necessity for performing standard microbiological tests with attendant equipment and techniques for maintaining sterility. Several procedures based on dye reduction (4), (6) have been suggested to replace the standard bacterial plate count. A number of products are commercially available utilizing a nutrient medium and the reducible dye, triphenyl tetrazolium chloride, in a "dipstick" configuration. A recent innovation incorporates these ingredients in a filter paper pad. This product was available to use in sufficient quantity to evaluate its utility in comparing bacterial levels in the test fluids. Its use simply involves immersion of the reagent strip and incubating in an enclosed plastic ziplock pouch overnight at room temperature. Although test strips were done alongside every plate count, only the final readings are presented for the purpose of comparing rankings:

Metalworking Fluids—Three fluids were used throughout this study: (a) a soluble oil at a concentration of 4 percent (b) a preformed emulsion at a concentration of 3 percent and (c) a synthetic at a concentration of 3 percent. All fluids were diluted with deionized water.

Description of Inocula—The purpose of this study was to determine if differences in source and preparation of a bacterial inoculum affected the reliability and reproducibility of a biological test procedure for metalworking fluids. In addition, some permutations were examined to evaluate the possibility for a universal inoculum. An overriding consideration in all cases was to keep the procedure simple.

Essentially two broad groups of inocula were used: A, based directly on spoiled fluid, and B, based on pure cultures isolated from fluids originally inoculated with A.

A.1. Adapted spoiled fluid inoculum. Spoiled fluid from one of two designated sources was mixed 50:50 with soy peptone casein digest broth and incubated with agitation at room temperature for 48 hr. This mixture was added to equal parts of the metalworking fluid under test for the purpose of adapting the organisms to this fluid. This latter mixture was also incubated at room temperature for 48 hr and is the definitive inoculum used in this test.

Designated sources of spoiled fluid.

(a) **Old and mixed.** The authors have maintained a 20-liter carboy of spoiled fluid in their laboratory for the past 15 years. This represents samples from all types of fluids and operations from all parts of the country. The level of bacterial count is checked periodically and if below 10^7 /ml, organic nutrients are added and the mixture is aerated for 48 hr. Usually this is sufficient to raise the level above 10^8 /ml.

(b) **Recent and singular.** A 7 percent emulsion from an aluminum machining operation contained $>10^8$ bacteria when received in the authors' laboratory 10 days prior to its use as an inoculum source.

A.2. Spoiled fluid inoculum without adaptation. Spoiled fluid from one of two designated sources was mixed 50:50 with soy peptone casein digest broth and incubated with agitation at room temperature for 48 hr. This incubated mixture is used directly as the inoculum.

B.1. Mixed pure cultures. One dominant bacterial colony type was selected from plate counts done with the A.2.(b) inoculum from each of the three fluids. These three isolates were grown separately with agitation and mixed in equal parts prior to use.

B.2. Pure cultures. The isolates described in B.1. were used alone in each of the three fluids, for a total of nine separate tests.

The levels of viable bacteria reached in all systems can be seen in the zero time counts in Tables 1, 2, 3, and 4.

Results

The results are reported in two tables for each set of data. For each procedure in which a different inoculum is used, the comparative data, i.e., the bacterial count per ml, is presented at each day of sampling. In addition, there are also tables (1A, 2A, 3A, 4A) listing the bacterial count at the end of the test period and the ranking with the lowest number given for best results in each fluid. In case of a tie, the fluids shared the highest ranking. Similarly, rankings with the bacterial test strips are also given.

In the test utilizing the adapted fluid (Table 1) the original source of the inoculum proved to be critical, with the organisms of most recent vintage reaching higher levels after 11 days in two of the fluids. In the absence of adaptation, the most recent inoculum was still more active in two of the fluids (Table 2). This experiment was repeated and the results are presented directly under the first run in Table 2. Notice that in only two cases, the preformed emulsion with the old inoculum and the solu-

TABLE 1—BACTERIAL GROWTH IN THREE METALWORKING FLUIDS:
inoculum adapted in each fluid prior to use

A. Inoculum Source—old and mixed.

FLUID TYPES		TIME AFTER INOCULATION					
		0 DAYS*	2 DAYS	4 DAYS†	7 DAYS*	9 DAYS	11 DAYS
Preformed Emulsion	B/ml:	4.4×10^8	0	0	0	1×10^1	1×10^1
Synthetic	B/ml:	2.9×10^8	0	0	0	1×10^1	0
Soluble Oil	B/ml:	4.1×10^8	4.5×10^7	3.4×10^8	2×10^7	5.5×10^6	4×10^7

B. Inoculum Source—recent and singular.

FLUID TYPES		TIME AFTER INOCULATION					
		0 DAYS*	2 DAYS	4 DAYS†	7 DAYS*	9 DAYS	11 DAYS
Preformed Emulsion	B/ml:	1×10^8	0	0	0	0	0
Synthetic	B/ml:	1.5×10^8	0	8×10^4	1×10^7	1×10^6	5×10^8
Soluble Oil	B/ml:	2.8×10^8	3.5×10^7	4.1×10^8	1×10^8	2×10^7	4×10^8

*Aeration begun.

†Aeration discontinued.

TABLE 2—BACTERIAL GROWTH IN THREE METALWORKING FLUIDS:
inoculum not adapted prior to use

A. Inoculum Source—old and mixed.

FLUID TYPES		TIME AFTER INOCULATION					
		0 DAYS*	2 DAYS	4 DAYS†	7 DAYS*	9 DAYS	11 DAYS
Preformed Emulsion	1. B/ml:	1.2×10^7	1×10^2	0	0	1×10^1	1.8×10^2
	2. B/ml:	1×10^7	1×10^2	0	0	2.2×10^3	1×10^2
Synthetic	1. B/ml:	1.2×10^7	3.5×10^5	1×10^5	1×10^5	0	1×10^2
	2. B/ml:	1×10^7	8.5×10^6	3×10^6	2.2×10^6	5×10^6	6×10^5
Soluble Oil	1. B/ml:	1.2×10^7	8×10^7	9×10^8	8×10^8	5×10^8	2.7×10^9
	2. B/ml:	1×10^7	8×10^6	1.5×10^7	3×10^7	4×10^7	2×10^7

B. Inoculum Source—recent and singular.

FLUID TYPES		TIME AFTER INOCULATION					
		0 DAYS*	2 DAYS	4 DAYS†	7 DAYS*	9 DAYS	11 DAYS
Preformed Emulsion	1. B/ml:	1.8×10^7	2×10^6	1×10^7	3.9×10^8	1.6×10^8	1.8×10^6
	2. B/ml:	1.7×10^7	5.6×10^5	4×10^3	9×10^5	4×10^6	5×10^6
Synthetic	1. B/ml:	1.8×10^7	2×10^4	2.5×10^5	6×10^6	2.5×10^6	1.1×10^8
	2. B/ml:	1.7×10^7	7.5×10^5	1.3×10^8	1.2×10^8	3×10^8	1×10^7
Soluble Oil	1. B/ml:	1.8×10^7	3.1×10^8	5.5×10^8	1×10^9	1×10^8	1.5×10^9
	2. B/ml:	1.7×10^7	4.3×10^6	3.4×10^7	1×10^7	8×10^7	7×10^8

*Aeration begun.

†Aeration discontinued.

ble oil with the recent inoculum, was there any reasonable agreement between replicated experiments.

The data in Table 3 reflect in part the capriciousness of biological systems. The organisms used were selected from the 11th day plate counts shown in Table 2 B.1. where the counts were 1.8×10^8 /ml, 1.1×10^8 /ml, and 1.5×10^9 /ml for preformed emulsion, synthetic and soluble oil respectively. These isolates never demonstrated

their previous vigor when used as a mixture of three pure cultures.

Since there was a possibility that the diminished activity seen in Table 3 might be the result of competition, the authors examined the growth of each isolate in each of the three fluids. What is quite evident (Table 4), only in the soluble oil do all three organisms reach the levels seen in Table 2. In addition, the immediate past history of the

TABLE 3—BACTERIAL GROWTH IN THREE METALWORKING FLUIDS:
inoculum consisting of isolates from three fluid types and mixed prior to use

FLUID TYPES		TIME AFTER INOCULATION					
		0 DAYS*	2 DAYS	4 DAYS†	7 DAYS*	9 DAYS	11 DAYS
Preformed Emulsion	B/ml:	1.5×10^7	3×10^2	5×10^2	0	0	1×10^1
Synthetic	B/ml:	1.5×10^7	1×10^3	1.5×10^5	6×10^5	2.5×10^6	9×10^7
Soluble Oil	B/ml:	1.5×10^7	5×10^7	8.5×10^7	2.5×10^7	3.5×10^8	1.5×10^9

*Aeration begun.
†Aeration discontinued.

TABLE 4—BACTERIAL GROWTH IN THREE METALWORKING FLUIDS:
inoculum pure cultures isolated from each of the three fluids

INOCULUM SOURCE	FLUID TYPES	TIME AFTER INOCULATION					
		0 DAYS*	2 DAYS	4 DAYS†	7 DAYS	9 DAYS	11 DAYS
Bacteria per ml							
Preformed Emulsion:	Preformed Emulsion	2.5×10^8	0	1.6×10^2	2×10^1	2.7×10^3	5.7×10^4
	Synthetic	2.5×10^8	4×10^4	1.2×10^4	0	1.2×10^3	1.4×10^5
	Soluble Oil	2.5×10^8	8.5×10^8	7.4×10^9	2.1×10^9	4.3×10^9	4.8×10^9
Synthetic:	Preformed Emulsion	3.4×10^8	5×10^2	3.7×10^6	3×10^7	1×10^6	$< 10^5$
	Synthetic	3.4×10^8	2×10^4	0	0	1×10^1	5.7×10^2
	Soluble Oil	3.4×10^8	6.1×10^8	9.2×10^9	5×10^8	3.8×10^9	3.6×10^9
Soluble Oil:	Preformed Emulsion	3.7×10^8	0	3×10^2	4.2×10^3	4×10^3	6×10^4
	Synthetic	3.7×10^8	1.1×10^5	2×10^4	0	6×10^1	1.9×10^3
	Soluble Oil	3.7×10^8	1.2×10^8	1.5×10^9	1.1×10^9	1.8×10^9	1.8×10^9

*Aeration begun.
†Aeration discontinued.

TABLE 1A—BACTERIAL GROWTH IN THREE METALWORKING FLUIDS:
inoculum adapted in each fluid prior to use; rankings after 11th day

FLUID TYPES	BACTERIAL PLATE COUNT (PER ML)	RANKINGS*	TEST STRIP COUNT (PER ML)	RANKINGS
A. Inoculum Source—old and mixed.				
Preformed Emulsion	0	1	0	1
Synthetic	0	1	0	1
Soluble Oil	4×10^7	3	10^8 or more	3
B. Inoculum Source—recent and singular.				
Preformed Emulsion	0	1	0	1
Synthetic	5×10^8	3	10^7 - 10^8	2
Soluble Oil	4×10^8	2	10^8 or more	3

*Lowest number means lowest count.

TABLE 2A—BACTERIAL GROWTH IN THREE METALWORKING FLUIDS:
inoculum not adapted prior to use; rankings after 11th day

FLUID TYPES	BACTERIAL PLATE COUNT		TEST STRIP COUNT		
	(PER ML)	RANKINGS*	(PER ML)	RANKINGS	
A. Inoculum Source— old and mixed.					
Preformed Emulsion	1.	1×10^2	1	0	1
	2.	1.8×10^2	2	0	1
Synthetic	1.	6×10^5	2	10^5	2
	2.	1×10^2	1	0	1
Soluble Oil	1.	2×10^7	3	10^8 or more	3
	2.	2.7×10^9	3	10^8 or more	3
B. Inoculum Source— recent and singular.					
Preformed Emulsion	1.	5×10^6	1	10^7	2
	2.	1.8×10^8	2	10^8 or more	2
Synthetic	1.	1×10^7	2	10^5	1
	2.	1.1×10^8	1	10^7	1
Soluble Oil	1.	7×10^8	3	10^8 or more	3
	2.	1.5×10^9	3	10^8 or more	2

*Lowest number means lowest count.

TABLE 3A—BACTERIAL GROWTH IN THREE METALWORKING FLUIDS:
inoculum consisting of isolates from three fluid types and mixed prior to use; rankings after 11th day

FLUID TYPES	BACTERIAL PLATE COUNT		TEST STRIP COUNT		
	(PER ML)	RANKINGS*	(PER ML)	RANKINGS	
Preformed Emulsion	0	1	0	1	
Synthetic	9×10^5	2	10^5	2	
Soluble Oil	1.5×10^8	3	10^8 or more	3	

*Lowest number means lowest count.

TABLE 4A—BACTERIAL GROWTH IN THREE METALWORKING FLUIDS:
inoculum pure cultures isolated from each of the three fluids; rankings after 11th day

INOCULUM SOURCE	FLUID TYPES	BACTERIAL PLATE COUNT		TEST STRIP COUNT	
		(PER ML)	RANKINGS*	(PER ML)	RANKINGS
Preformed Emulsion:	Preformed Emulsion	5.7×10^4	1	10^6	2
	Synthetic	1.4×10^5	2	0	1
	Soluble Oil	4.8×10^9	3	10^8 or more	3
Synthetic:	Preformed Emulsion	$< 10^5$	2	10^4	1
	Synthetic	5.7×10^2	1	10^6	2
	Soluble Oil	3.6×10^9	3	10^8 or more	3
Soluble Oil:	Preformed Emulsion	6×10^4	2	10^5	1
	Synthetic	1.9×10^3	1	10^5	1
	Soluble Oil	1.8×10^9	3	10^8 or more	3

*Lowest number means lowest count.

organisms seems to have no effect on subsequent performance; for example, isolates from preformed emulsion should grow better in that fluid. In no case was this true.

The major focus of this test procedure was to evaluate comparative bioresistance of metalworking fluids. Although the various inocula give different absolute final counts in most of the tests, the relative results were essentially the same. That is (Tables 1A, 2A, 3A, 4A), there were no significant differences among the inocula with respect to the final rankings. In addition, the bacterial test strips rankings were equally repeatable.

These findings indicate that the original source of an inoculum is a critical factor in determining the levels of growth in several metalworking fluids.

This fact has practical value in the actual use of the test in the field. With confidence that relative rankings of bioresistance are independent of the source of the inoculum, the individual responsible for evaluating fluids for eventual purchase could use spoiled fluid from his own plant to perform the test. The significance of the quantitative differences among inocula would then be minimized since the real concern in the final analysis should be how well fluids survive in that particular operation. Each plant should set its own test performance standards for the acceptance or rejection of metalworking fluids. Bioresistance is only one of a battery of characteristics demanded of a metalworking fluid which must be evaluated on the basis of total performance. It is worth noting that the lack of sterility in the test system has made it impossible to know by counts alone if the organisms in the inoculum were also the dominant organisms at the end of the test. This is particularly true with the pure cultures, and although they do not seem to offer any advantage in evaluating bioresistance, the results with

three fluids strongly suggest they would be useful in screening microbiocides in that environment. Further study in that area is continuing to determine not only the types and numbers of organisms optimal for an inoculum but also to qualify their ability to grow in a larger and more representative group of fluids.

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