

The Utility of Biocide Combinations in Controlling Mixed Microbial Populations in Metalworking Fluids

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Recently it has been shown that appropriate test procedures are good predictors of metalworking fluid bioresistance in which potentially useful biocides may be functional. The variety of microorganisms degrading metalworking fluids suggests that it would be appropriate to utilize more than one chemical agent to institute biological control.

A mixed culture of bacterial species: Pseudomonas aeruginosa, Proteus mirabilis, Pseudomonas fluorescens, Klebsiella pneumoniae, and of fungal species: Cephalosporium and Fusarium species, all isolated from metalworking fluids originally, were evaluated in soluble oil, preformed emulsion, and synthetic metalworking fluid containing either 1,3,5-hexahydrotriazine (2-hydroxyethyl), sodium 2-pyridinethiol-1-oxide, or mixtures of these for two or eight weeks. The following general results were obtained.

- 1. The mixture of the two biocides produced greater than additive antimicrobial effects.*
- 2. A differential effect in the three fluids was noted, suggesting that the amount of dissolved oil reduced activity.*
- 3. There was good correlation between the two-week test for bioresistance and the eight-week test for biocide evaluation.*

INTRODUCTION

The use of mixtures of antimicrobial agents to control either single or mixed microbial populations has been considered acceptable practice in the therapy of infectious diseases for a number of years. There are a number of reasons for using biocidal mixtures. These are:

1. anticipation of microbial resistance
2. extension of antimicrobial spectrum
3. increase in cost effectiveness; and due to reduced levels
4. less solubility problems

5. fewer incompatibilities; and
6. a potential reduction in toxicity.

The current paper deals with a mixture of two biocide types that appear to have excellent practical utility in combination.

The first of these, hexahydro 1,3,5. tris (2-hydroxyethyl)-triazine (HET), has been used to control biological breakdown of water-soluble metalworking fluids with some success for at least 15 years. However, levels and rate of use of biocides in the metalworking industry are difficult to control, i.e., the incorporation of biocides in fluids prior to use can neither take into consideration all possible dilutions of the concentrated fluid nor the length of time the fluid will be used once diluted. With more extensive use of the hexahydrotriazines, it became apparent that abuse as well as misuse (4), (3) could lead to the development of fungal growth which, once established, was difficult to contain. In one of those studies (4), the advantages of adding a known active antifungal agent were explored. Subsequently (1), it was found that sodium 2-pyridinethiol-1-oxide (P) was compatible with the activity of HET and together they could effectively control both bacteria and fungi in oil-in-water metalworking fluid.

This presentation deals with the selection of an optimal ratio and a more rigorous challenge of this combination in the three major metalworking fluid types and in two procedures currently nearing acceptance by two committees of ASTM.

MATERIALS AND METHODS

The first series of tests were done to determine optimal levels of each biocide in the three metalworking fluids.

Procedure One: 48-Hour Screening

Preparation of Bacterial Inoculum. Mixed spoiled metalworking fluid was mixed with an equal amount of sterile tryptic soy digest broth and incubated with shaking at 30°C for 48 hours (6). This constituted the bacterial inoculum.

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The bacterial count was never less than 10^9 organisms per ml.

Preparation of Fungal Inoculum. Isolates of *Fusarium* and *Cephalosporium* species from metalworking fluids were grown in Czapek Dox broth at 30°C with shaking until turbid. The population density was never less than 10^5 colony-forming units (CFU) per ml. Five ml of each of the two inocula were added to 90 ml of metalworking fluid containing various levels of biocide in a 6 oz screw-cap prescription bottle which was capped loosely. Samples were placed on a reciprocal shaker and incubated with shaking at 27°C for 48 hours. At this time, one ml was removed from each sample for bacterial and fungal counts.

Procedure Two: Two-Week Bioresistance Test

After establishing the optimal levels of the two biocides, appropriate amounts were added and with untreated controls the systems were inoculated with the same amount of fungal and bacterial inocula as in Procedure One. The protocol used was described in (6) and is currently under consideration by ASTM/ASLE Committee D2/L1's Task Force "Bioresistance of Aqueous Metalworking Fluids." Samples were removed at indicated times in this protocol for fungal and bacterial evaluations.

Procedure Three: Eight-Week Biocide Evaluation Method

Procedure Three was based on a test approved by ASTM Committee E35 entitled, "The Evaluation of Antimicrobial Agents in Aqueous Metalworking Fluids." A selected defined inoculum according to paragraph 5.6.1.2 was opted, as follows:

1. *Pseudomonas aeruginosa* ca 10^9 /ml
2. *Klebsiella pneumoniae* " 10^9 /ml
3. *Pseudomonas fluorescens* " 10^9 /ml
4. *Proteus mirabilis* " 10^9 /ml
5. *Fusarium* sp " 10^5 /ml
6. *Cephalosporium* sp " 10^5 /ml

Bacterial species (1) and (2) above were isolated from soluble oil, and (3) and (4) above from a synthetic fluid, while both fungal isolates, (5) and (6), were from a semisynthetic fluid. Other variations from the published protocol relate to weekly treatments of the test systems which were in response to microbiological conditions. These are specified with the results in the following section. The procedure is a six-week extension of (6).

RESULTS

All of the procedures demonstrated the value of using the two biocides in concert for controlling bacteria and fungi. However, equal effectiveness cannot be claimed in all three metalworking fluids.

In the results from the 48-hour screening test (Table 1), the synergistic action of HET and P against bacteria is evident at the lowest level of the mixture in all fluids. Synergism is defined (2) if log reduction with biocide A and log reduction with biocide B is significantly less than log reduction with biocide A plus B. At higher levels of HET, bacte-

BIOCIDE	LEVEL (ppm)	SOLUBLE OIL		SEMI-SYNTHETIC		SYNTHETIC	
		B	F	B	F	B	F
P ₁	20	2.1	1.2	2.0	0	2.1	3.3
HET ₁	200	1.8	0.6	2.2	0	2.1	0
P ₁ +HET ₁		9.4	1.7	10.0	0.8	10.0	4.8
P ₂	45	1.6	4.3	2.0	3.2	2.0	3.3
HET ₂	450	2.6	1.6	2.5	3.0	5.4	1.6
P ₂ +HET ₂		9.4	4.9	10.0	4.5	10.0	4.8
P ₃	91	1.6	3.9	1.9	3.2	2.0	3.3
HET ₃	910	7.6	3.9	5.5	1.5	5.2	3.3
P ₃ +HET ₃		9.4	4.9	10.0	4.5	10.0	4.8

P —Sodium 2-Pyridinethiol-1-Oxide

HET—Hexahydro 1,3,5 Tris (2-hydroxyethyl)-S-Triazine

B —Bacteria

F —Fungus

BIOCIDE LEVEL (ppm)	MICROBIAL LEVELS AT INDICATED EXPOSURE TIMES (DAYS)						
	2.5	4	6	8	10	14	
CONTROL 0	(B)	10^9	10^9	10^9	10^9	10^9	10^9
	(F)	10^4	10^4	10^4	10^4	10^5	10^5
P ₁ 25	(B)	10^8	10^9	10^9	10^9	10^9	10^9
	(F)	10^3	10^3	10^4	10^3	10^4	10^5
HET ₁ 225	(B)	0	0	10^2	10^6	10^9	10^9
	(F)	10^3	10^4	10^4	10^6	10^5	10^5
P ₁ +HET ₁	(B)	0	0	0	10^6	10^9	10^9
	(F)	10^3	10^3	10^3	10^4	10^5	10^4
P ₂ 51	(B)	10^8	10^9	10^9	10^9	10^9	10^9
	(F)	10^2	10^3	10^3	10^2	10^4	10^4
HET ₂ 509	(B)	0	0	0	0	10	10^9
	(F)	10^3	10^3	10^3	10^3	10^4	10^5
P ₂ +HET ₂	(B)	0	0	0	0	0	10^4
	(F)	0	0	0	40	0	15
P ₃ 76	(B)	10^8	10^9	10^9	10^9	10^9	10^9
	(F)	10^2	0	0	10^3	10^3	10^4
HET ₃ 764	(B)	0	0	0	0	10^3	10^8
	(F)	0	0	18	10^2	10^6	10^5
P ₃ +HET ₃	(B)	0	0	0	0	0	0
	(F)	0	0	0	0	0	50

B = Bacteria/ml

F = Fungi/ml

P = Sodium 2-Pyridinethiol-1-Oxide

HET = Hexahydro 1,3,5 Tris (2-hydroxyethyl)-S-Triazine

rial viability reduction is near maximum thus masking any synergistic effects. Although there was no apparent synergism against the fungi, there was excellent control at the 45 ppm level of P, reinforcing the evidence for the biological compatibility of the two biocides (7), (1). Notice also the

small but perceptible increase in fungal levels at the 450 ppm HET in the synthetic and semisynthetic fluids. This was reported previously (4) and is indicative of the dangers of using less than the recommended and optimal level of HET.

In the two-week bioresistance test (Table 2) done only in the semisynthetic fluid, control of bacteria and fungi by the medium level of the biocide mixture parallels the 48-hour test results. Also, notice the gradual loss of fungal control with the highest dose of HET as well as the eventual loss of bacterial control. It should be pointed out, however, that this level, 764 ppm (active), is only 65 percent of the recommended dose by the manufacturer. Nevertheless, the two-week test reinforces the conclusion of the shorter screening test concerning the efficacy of the mixture.

The final studies in all three metalworking fluids were not only longer but also more complex due in part to the irregular regimen of inoculation and treatment. As men-

tioned previously, this protocol was followed as an attempted simulation of field events; that is, to inoculate when microbial levels dropped and to add biocide when microbial levels increased.

In general, the results (Tables 3, 4, 5) reinforce the previous findings. Two important but related factors bear emphasizing when attempting to construct laboratory models and extrapolate results to field situations. The types of fluid may behave differently with respect to the activity of a biocide, and the presence of oil in the formulations used in this study reduced the effectiveness of the biocidal combination. This conclusion was not evident until four weeks of testing had passed.

Nevertheless, the mixture controlled both fungal and bacterial growth for four weeks in the soluble oil and semisynthetic fluid and for eight weeks in the synthetic fluid even with the addition of added inoculum and without resorting to heroic methods of treatment, that is, with no

TABLE 3—EVALUATION OF PYRIDINE-TRIAZINE BIOCIDAL MIXTURE IN 5% SYNTHETIC METALWORKING FLUID—ASTM E35.15

	BIOCIDAL LEVEL (ppm)	MICROBIAL LEVELS AT INDICATED EXPOSURE TIMES (DAYS)									
			2.5	7	14	20	28	35	42	49	56
CONTROL	0	(B)	10 ⁸	10 ⁸	10 ⁸	10 ⁷	10 ⁸	10 ⁸	10 ⁸	10 ⁸	10 ⁸
		(F)	10 ⁴	10 ⁴	10 ³	10 ³	10 ³	10 ³	10 ³	10 ³	10 ⁴
P ₁	25	(B)	10 ⁷	10 ⁸	10 ⁸	10 ⁷	10 ⁶	10 ⁷	10 ⁶	10 ⁸	10 ⁸
		(F)	10 ⁴	10 ²	0	10 ²	0	0	0	0	10 ⁴
HET ₁	250	(B)	10 ²	0	10 ⁸	10 ⁸	10 ⁸	10 ⁶	10 ⁸	10 ⁷	10 ⁸
		(F)	10 ³	10 ⁴	10 ⁵	10 ⁴	10 ⁴	10 ³	10 ³	10 ³	10 ⁴
P ₁ + HET ₁		(B)	0	0	10 ³	10 ³	10 ²	10 ⁵	10 ⁶	10 ⁷	10 ⁸
		(F)	0	0	0	10	0	0	0	0	10 ³
P ₂	45	(B)	10 ⁷	10 ⁷	10 ⁸	10 ⁷	10 ⁸	10 ⁸	10 ⁸	10 ⁸	10 ⁶
		(F)	10 ⁴	0	10	10 ²	0	0	0	0	10 ⁴
HET ₂	450	(B)	10 ²	0	10 ⁴	0	10 ²	10 ⁵	10 ⁷	10 ⁷	10 ⁶
		(F)	10 ²	10 ³	10 ³	0	10 ⁴	10 ⁴	10 ⁵	10 ⁵	10 ⁵
P ₂ + HET ₂		(B)	0	0	10 ³	0	0	0	0	0	0
		(F)	0	0	0	0	0	0	0	0	20
P ₃	64	(B)	10 ⁷	10 ⁸	10 ⁸	10 ⁶	10 ⁵	10 ⁷	10 ⁷	10 ⁸	10 ⁷
		(F)	10 ⁴	0	0	10 ²	0	0	0	0	10 ³
HET ₃	640	(B)	10 ²	0	10 ³	0	0	10 ⁸	10 ⁸	10 ⁸	10 ⁸
		(F)	0	0	10 ²	0	10 ³	10 ⁵	10 ³	10 ³	10 ⁴
P ₃ + HET ₃		(B)	0	0	0	0	0	0	0	0	0
		(F)	0	0	0	0	0	0	0	0	10

B = Bacteria/ml

F = Fungi/ml

P = Sodium 2-Pyridinethiol-1-Oxide

HET = Hexahydro 1,3,5 Tris (2-hydroxyethyl)-S-Triazine

Week 1—10% biocide (except control), 10% inoculum, 10% fluid makeup

Week 2—nothing

Week 3—10% biocide (except control), 10% fluid makeup

Week 4—10% inoculum, 10% fluid makeup, full dose biocide (HET, P₁, P₂, P₃, HET-P), 10% biocide (HET₂, HET₃, HET₂-P₂, HET₃-P₃)

Week 5—10% fluid makeup

Week 6—nothing

Week 7—full fungal inoculum

Week 8—nothing

TABLE 4—EVALUATION OF PYRIDINE-TRIAZINE BIOCODE MIXTURE IN 5% SEMI-SYNTHETIC METALWORKING FLUID—ASTM E35.15

	BIOCIDAL LEVEL (ppm)	MICROBIAL LEVELS AT INDICATED EXPOSURE TIMES (DAYS)									
			2.5	7	14	21	28	35	42	49	56
CONTROL		(B)	10 ⁸	10 ⁸	10 ⁹	10 ⁹	10 ⁸	10 ⁸	10 ⁸	10 ⁸	10 ⁸
		(F)	10 ⁵	10 ⁴	10 ⁴	10 ⁴	10 ³	10 ³	10 ⁴	10 ⁴	10 ⁴
P ₁	25	(B)	10 ⁸	10 ⁸	10 ⁸	10 ⁸	10 ⁸	10 ⁸	10 ⁷	10 ⁸	10 ⁸
		(F)	10 ²	10 ²	10 ³	10 ³	10 ²	0	10	10 ⁴	10 ⁴
HET ₁	250	(B)	10 ⁶	10 ⁴	10 ⁶	10 ⁶	10 ⁷	10 ⁴	10 ⁷	10 ⁷	10 ⁸
		(F)	10 ⁴	10 ⁴	10 ⁴	10 ³	10 ³	10 ⁴	10 ³	10 ³	10 ³
P ₁ +HET ₁		(B)	10 ³	10	10 ³	10 ⁸	10 ⁸	10 ⁸	10 ⁸	10 ⁸	10 ⁸
		(F)	0	0	0	0	0	0	0	0	0
P ₂	45	(B)	10 ⁶	10 ⁸	10 ⁸	10 ⁸	10 ⁸	10 ⁸	10 ⁸	10 ⁸	10 ⁸
		(F)	10 ⁴	10 ²	10 ⁴	10 ⁴	10 ³	10	10	10 ⁴	10 ⁴
HET ₂	450	(B)	10 ⁶	10 ⁶	10 ⁶	10 ⁸	10 ⁴	10 ⁶	10 ⁶	10 ⁶	10 ⁸
		(F)	10 ⁴	10 ²	10 ³	0	10	0	10 ³	10 ⁴	10 ⁵
P ₂ +HET ₂		(B)	10 ⁵	10 ⁵	10 ⁵	0	0	0	0	0	0
		(F)	0	0	0	0	0	0	0	0	0
P ₃	64	(B)	10 ⁸	10 ⁸	10 ⁸	10 ⁷	10 ⁸	10 ⁷	10 ⁸	10 ⁷	10 ⁷
		(F)	10 ³	0	10 ⁴	10 ³	10	10 ²	10 ²	10 ³	10 ²
HET ₃	640	(B)	0	0	10 ⁴	10 ⁴	10 ⁸	10 ²	10 ⁶	10 ⁷	10 ⁷
		(F)	10 ⁴	10	10 ²	10 ²	10 ⁴	10	10 ³	10 ⁴	10 ⁴
P ₃ +HET ₃		(B)	0	0	0	0	0	0	0	0	0
		(F)	0	0	0	0	0	0	0	0	0

B = Bacteria/ml

F = Fungi/ml

P = Sodium 2-Pyridinethiol-1-Oxide

HET = Hexahydro 1,3,5 Tris (2-hydroxyethyl)-S-Triazine

Week 1—10% biocide (except control), 10% inoculum, 10% fluid makeup

Week 2—nothing

Week 3—10% biocide (except control), 10% fluid makeup

Week 4—full biocide (except P₃+HET₃ and control), 10% biocide (P₃+HET₃), 10% fluid makeup

Week 5—10% fluid makeup

Week 6—full fungal inoculum

Week 7—nothing

Week 8—nothing

more than the addition of the original dose of biocide at four weeks in some systems (Tables 3, 4, 5).

Especially important were the organoleptic evaluations. In the soluble oil systems, neither the control, HET or P prevented emulsion separation, while the medium and high dose of the mixture did retard this for four weeks. In the other two fluid types, the physical appearances of the microbiologically controlled systems were in sharp contrast to the systems containing either no biocide or unsuccessful levels of HET or P. All of the synthetic fluid systems turned grey-black in eight weeks, except those containing the medium and high doses of the biocide mixture. Observations of the semisynthetic fluids were just as revealing. All samples except those containing the mixture lost their normal, slightly opalescent, translucent appearance, becoming slightly milky with a grey cast. The biocide mixture samples maintained normal appearance for four weeks but by eight weeks looked like the other samples.

The significance of these findings is twofold:

1. The problem of antifungal deficiency reported for the triazine biocides has been overcome.
2. True synergism between HET and P has been demonstrated against bacteria.

Thus, the mixture is truly complementary, exploiting the excellent antibacterial properties of HET stimulated by P, and keeping any attendant fungal problems under control. The above conclusions are given with this *caveat*. Do not anticipate either synergism or even compatibility between antimicrobial agents. Serendipitous rewards can be a lady or a tiger, and the results of unbiased testing should determine whether biocides can be successfully mixed in metalworking fluids. Certainly, this has been the case with the HET-P mixture.

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TABLE 5—EVALUATION OF PYRIDINE-TRIAZINE BIOCIDES MIXTURE IN 5% SOLUBLE OIL METALWORKING FLUID—ASTM E35.15

BIOCIDE LEVEL (ppm)	MICROBIAL LEVELS AT INDICATED EXPOSURE TIMES (DAYS)									
		2.5	7	14	21	28	35	42	49	56
CONTROL 0	(B)	10 ⁸	10 ⁸	10 ⁹	10 ⁸	10 ⁸	10 ⁹	10 ⁹	10 ⁷	10 ⁸
	(F)	10 ⁵	10 ⁴	10 ³	10 ³	10 ³	10 ³	10 ³	10 ³	10 ⁵
P ₁ 25	(B)	10 ⁷	10 ⁸	10 ⁸	10 ⁸	10 ⁸	10 ⁷	10 ⁸	10 ⁸	10 ⁷
	(F)	10 ⁴	10 ⁴	10 ³	10 ³	10 ³	0	10	0	10 ⁵
HET ₁ 250	(B)	10 ⁷	10 ⁷	10 ⁸	10 ⁸	10 ⁸	10 ⁶	10 ⁷	10 ⁸	10 ⁸
	(F)	0	0	0	0	0	0	0	0	10 ⁴
P ₁ + HET ₁	(B)	10 ³	10 ²	10 ⁶	10 ⁸	10 ⁸	10 ⁷	10 ⁷	10 ⁷	10 ⁷
	(F)	0	0	0	0	0	0	0	0	10 ⁴
P ₂ 45	(B)	10 ⁸	10 ⁸	10 ⁸	10 ⁸	10 ⁸	10 ⁷	10 ⁶	10 ⁸	10 ⁸
	(F)	10 ⁴	10 ³	10 ²	10 ²	10 ²	0	10 ²	10 ²	10 ⁴
HET ₂ 450	(B)	0	0	10 ⁵	10 ⁷	10 ⁶	10 ⁶	10 ⁶	10 ⁶	10 ⁶
	(F)	10 ³	10 ⁴	10 ⁴	10 ³	10 ³	0	0	10 ²	10 ⁵
P ₂ + HET ₂	(B)	0	0	10 ⁵	10 ⁷	10 ⁶	10 ⁶	10 ⁶	10 ⁶	10 ⁶
	(F)	0	0	0	0	0	0	0	0	10 ⁴
P ₃ 64	(B)	10 ⁷	10 ⁸	10 ⁸	10 ⁸	10 ⁸	10 ⁸	10 ⁸	10 ⁸	10 ⁷
	(F)	10 ⁴	10 ³	10 ²	10 ²	10 ²	0	10 ²	10 ²	10 ⁴
HET ₃ 640	(B)	0	0	10 ⁶	0	0	10 ⁶	10 ⁷	10 ⁶	10 ⁸
	(F)	0	10 ⁴	0	0	0	10 ²	10 ⁴	10 ⁴	10 ⁵
P ₃ + HET ₃	(B)	0	0	0	0	10	10 ⁴	10 ⁶	10 ⁶	10 ⁶
	(F)	0	0	0	0	0	0	0	0	10 ⁵

B = Bacteria/ml

F = Fungus/ml

P = Sodium 2-Pyridinethiol-1-Oxide

HET = Hexahydro 1,3,5 Tris (2-hydroxyethyl)-S-Triazine

Week 1—10% inoculum, 10% fluid makeup, 10% biocide (except control)

Week 2—nothing

Week 3—10% fluid makeup, 10% biocide (except control)

Week 4—10% fluid makeup, 10% inoculum, Full biocide dose (except control, HET₁-P₁, HET₃), 10% biocide (HET₁-P₁, HET₃)

Week 5—10% fluid makeup

Week 6—nothing

Week 7—full fungal inoculum

Week 8—nothing

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