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**10.4 The role of the inoculum in the microbiological evaluation  
of water-based metalworking fluids  
Die Bedeutung des Keimes zur mikrobiologischen Bewertung  
von wasserhaltigen Kühlschmierstoffen**

The development of a microbial inoculum for use in challenging water-based metalworking fluids can be exactly defined or evolved solely from deteriorated fluids from the field. It can include aerobic bacteria, fungi, and anaerobic sulfate reducers. The selection of the types and sources is dependent upon the origin of the metalworking fluid and the microbial problem that is anticipated or encountered.

Die Abtrennung eines mikrobiellen Keims für die Beanstandung wasserhaltiger Kühlschmierstoffe läßt sich nur aus zerstörten Flüssigkeiten in der Praxis bewerkstelligen. Hierzu können aerobische Bakterien, Pilze und anaerobische Sulfat-reduzierer gehören. Die Auswahl und die Herkunft hängen vom Ursprung des Kühlschmierstoffes ab sowie vom mikrobiologischen Problem, um das es gerade geht.

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## 1. Introduction

### Einführung

The most critical component in the evaluation of the microbiological status of water-based metalworking fluids is the microbial inoculum selected for the test procedure. Metalworking fluids are prone to attack to varying degrees. Their level of resistance is a function of the formulation of the fluid, which can be called an intrinsic property, and also of the external activities impinging on the fluid, which we can refer to as extrinsic factors. Intrinsic factors include those additives that are related to the functionality of the fluid, i. e. their lubricity, corrosion inhibitors, and in some cases specific antimicrobial agents. The extrinsic factors include the size of the system in which the fluid is being used, the nature of the operation, the type of metal in the process, the quality of the water used for dilution, the design of the system, and the personal hygiene and esprit de corps of the work force. How long a fluid resists biodegradation while in place is the net result of all of the factors just mentioned.

## 2. Nature of organisms

### Art der Organismen

In the design of a test method, what should always be uppermost in mind is both the level and biological nature of the contamination impacting on the fluid. Tests for microbial resistance have been devised for two purposes: to determine the relative resistance to attack of fluids as received from the fluid manufacturer, and secondly, to evaluate the relative merits of antimicrobial agents designed for use in metalworking fluids. In addition, it is of importance to separate the aims of the different levels of concern for biocide efficacy.

### 2.1 Inoculum selection

#### Auswahl des mikrobiellen Keimes

The first concern is of the biocide manufacturer who must have broad spectrum approaches. This manufacturer must look for a microbial inoculum that is relatively representative of organisms isolated from the field, and these must be added to fluids equally representative but also those which have a high degree of susceptibility to microbial growth. The needs of the producer of metalworking fluids is slightly more specific. The microbial inoculum selected here should conform to isolations from the fluid associated most frequently with that producer's fluids. Lastly, the actual user of metalworking fluids and of potential biocides must select an inoculum directly from a site in the operation which has developed a microbial problem.

### 2.2 Specific Biodeteriogens

#### Spezifische Bio-Zerstörer

The organisms that have been found to cause problems in most metalworking fluids, whether they be oil-in-water emulsions, true solutions of a variety of chemical wetting agents, dispersed pseudo-solutions, both of which are known as synthetic fluids, or the above with small amounts of oil added to them, belong to three major groups: aerobic bacteria, predominantly members of the genus

*Pseudomonas* (Table 1), although other species of bacteria have also frequently been found; anaerobic bacteria, primarily members of the genus *Desulfovibrio*; and lastly, members of the group referred to as the imperfect fungi, which includes several mold species as well as yeasts.

Table 1: Microorganisms isolated from water-based metalworking fluids  
Tafel 1: Aus Kühlschmierstoffen isolierte Mikroorganismen

Species	Frequency of Occurrence In Contaminated Fluids	Growth and/ or Survival
1. <i>Pseudomonas aeruginosa</i>	Very High	Excellent
2. <i>Pseudomonas fluorescens</i>	Low	Good
3. <i>Pseudomonas cepacia</i>	Low	Good
4. <i>Pseudomonas stutzeri</i>	Low	Good
5. <i>Pseudomonas alcaligenes</i>	Low	Good
6. <i>Pseudomonas pseudomallei</i>	Low	Fair
7. <i>Pseudomonas putida</i>	Low	Good
8. <i>Aeromonas hydrophila</i>	Low	Fair
9. <i>Proteus mirabilis</i>	Moderate	Excellent
10. <i>Proteus vulgaris</i>	Low	Good
11. <i>Proteus rettgeri</i>	Low	Fair
12. <i>Enterobacter cloacae</i>	Moderate	Very Good
13. <i>Enterobacter agglomerans</i>	Low	Good
14. <i>Enterobacter gergoviae</i>	Low	Good
15. <i>Citrobacter freundii</i>	High	Very Good
16. <i>Escherichia coli</i>	Moderate	Good
17. <i>Klebsiella pneumoniae</i> (oxytoca)	High	Very Good
18. <i>Klebsiella ozaenae</i>	Low	Good
19. <i>Serratia liquefaciens</i>	Low	Fair
20. <i>Desulfotribrio</i> sp.	Moderate to High	Fair to Good
21. <i>Salmonella</i> sp.	Rare	Fair
22. <i>Shigella</i> sp.	Rare	None
23. <i>Vibrio</i>	Rare	None
24. <i>Achromobacterium</i> sp.	Low	Poor
25. <i>Bacillus</i> sp.	Low	None
26. <i>Clostridium</i> sp.	None	None
27. <i>Staphylococcus aureus</i>	Rare	None
28. <i>Streptococcus</i> sp.	Rare	None
29. <i>Nocardia</i> sp.	Fair	Fair
30. <i>Candida</i> sp.	Low to Moderate	Good
31. <i>Fusarium</i> sp.	Moderate to High	Good
32. <i>Cephalosporium</i> sp.	Moderate	Good

### 2.3 Previous studies

#### Frühere Untersuchungen

Although some fluids show a preference to one or the other of these groups of organisms, none escape the predation of all three groups. If the conditions are right, there will be anaerobic sulfate reduction and there will be fungal growth. A number of studies have been previously published [1 – 6] describing methodology and inoculum preparation. In addition, two test methods for the microbiological evaluation of metalworking fluids have been standardized by the American Society for Testing & Materials (ASTM): ASTM E 686 – 80, *Standard*

*Method for Evaluation of Antimicrobial Agents in Aqueous Metal Working Fluids*; and ASTM D-3946-80, *Standard Method for Evaluating the Bioresistance of Water-Soluble Metal-Working Fluids*.

### 3. Development of inoculum Entwicklung des biologischen Keimes

#### 3.1 Species selection Wahl der Spezies

In a survey of 100 samples of metalworking fluid, the dominant microbial species representing different types included *Pseudomonas aeruginosa*, *Proteus mirabilis*, and *Klebsiella pneumoniae* [3]. These organisms are not antagonistic to each other when mixed in equal amounts, and in a susceptible fluid all last for eight weeks under a regimen of five days of aeration and two days of quietude. These three organisms represent a potential for growth in a variety of fluids (*Pseudomonas aeruginosa*), the ability to produce strong fecal odors in the presence of oxygen (*Proteus mirabilis*), and the ability to survive aerosolization, fix nitrogen, and grow anaerobically as well as aerobically (*Klebsiella pneumoniae*), which makes this trio suitable for biocide manufacturers in the evaluation of their products.

#### 3.2 Species survival Überleben der Spezies

In a group of 15 fluids from a variety of manufacturers with no guarantee that all of the fluids were devoid of antimicrobial agents, the three species grew in all but one. The organisms were all grown in a nutrient broth medium to a maximum population (approximately  $10^9$  bacteria/ml) in 48 hr at 25 °C. They were mixed and added at the level of 5 % of the cutting fluid formulation for test purposes. This rather high inoculum made it possible to evaluate biodegradation more rapidly and also to determine the effectiveness of an antimicrobial agent in a highly contaminated system, a situation which is more often than not seen in operational systems where microbes are already causing problems.

#### 3.3 Undefined inoculum Undefinierter biologischer Keim

The second approach to devising an inoculum is perhaps more realistic in terms of the immediate user's concerns. This involves the use of spoiled fluid, i. e. fluid from a particular machine sump which the user considers deteriorated from biological attack. The levels of microbes in these fluids can vary depending upon when the fluids were removed. Frequently, after reaching a maximum population density, there exists a short plateau period where there is a decline in viability. In order to maximize the effectiveness of a microbial inoculum of this sort, the spoiled fluid is mixed with an equal volume of sterile nutrient broth and incubated with shaking for 48 hr. This invariably yields an inoculum size of about  $10^9$  bacteria/ml at the same physiological condition. This is added at 5 % of the fluid formulations in challenge. Thus the dominant aerobic

bacterial species are utilized without necessarily knowing what those species are named.

#### 3.4 Single organisms Einzelorganismus

In a rather extensive study [1] we found that organisms isolated from such a mixed inoculum and grown separately, then used as single species inocula, produced the same relative bioresistance patterns in a selected group of metalworking fluids as did the activated spoiled fluid inoculum. So there is no gain in isolating single species for on-site studies.

#### 3.5 Fungi Pilze

Up to this point only the aerobic bacterial population as an inoculum has been mentioned. It is difficult to rely on spoiled fluid as a source of either sufficient fungi or yeasts or sulfate reducing bacteria to be used as a challenge in a short-term test. If these two latter groups are of concern, and this concern relates to current problems in a manufacturing operation or future concerns as they relate to a claim on an antimicrobial label describing the breadth of activity of the agent, they must be included in the inoculum. The aerobic bacteria grow much more rapidly and in 48 hr with aeration the fungal population would not be sufficiently large to give a consistent challenge to test fluids. Thus, fungi are grown separately (they can be fungal isolates from the same spoiled fluid [1]), with mixed activated inoculum previously described. This too does not necessitate the classification of the species of fungus. Seeing the organism from the fluid is sufficient to characterize it.

#### 3.6 Sulfate reducers Sulfat-Verringerer

If a system has been plagued by anaerobic sulfate reduction, i. e. fluids turn black, excessive hydrogen sulfide odor, pitting and corrosion in isolated parts of the metalworking operation, then it would be necessary to add a source of sulfate reducing bacteria. The best source, again, is from the operation with the problem. The bottom sludge from the sump can be checked directly for sulfate reducing bacteria with any one of a number of test methods recommended by microbiologists [7]. If these tests prove positive and corroborate the organoleptic findings, then the sludge sample is mixed with equal amounts of fungal inoculum and aerobic bacteria inoculum and added to the formulation. This latter addition has a dual purpose. It not only adds another microbial group which may in fact have a different degree of resistance, but also adds another source of chemical contamination capable of interfering with biocide activity. One warning about sulfate reducing bacteria. They are difficult to maintain and grow in the laboratory, and cannot be treated as handily as the aerobic bacterial population and the fungal populations. Thus their use as part of the inoculum must always, for convenience sake, be on an ad-hoc basis from the sites of interest.

### 3.7 Duration of test

#### Versuchsdauer

The selection of an appropriate inoculum is closely related also to the length of time a test method is operational. For a rapid definition of either microbial resistance in a fluid or microbial susceptibility to an antimicrobial agent in the fluid, short-term tests from 48 – 96 hr are valuable. These tests are done in closed containers and rely on the innate activity solely of the added inoculum. The results on reduction or increase in population size are meant to give directions for either field trials or for longer term laboratory tests. For the determination of microbial population successions and the relative survival of the different members of the population as they relate to the change in the physical and chemical characteristics of the fluid, longer term testing is required. ASTM E686–80 takes 60 days for completion. The test method can be carried out with a characterized inoculum, aerated with sterile air, and the results of microbiological activity based solely on those organisms that were added at the beginning of the test method. A more realistic use of this protocol, i. e. one which would be more like an operation in the field, involves aeration with unfiltered air from a typical large compressor and during the 60-day period the fluids would be impacted continually with airborne contaminants from the immediate environment.

This type of study really involves the survival of the fittest and allows all types of organisms to attempt biodegradation of the test fluids or challenge the antimicrobial agent. It is in this type of laboratory test that fungal succession to the aerobic bacterial population was first noted. In this case, the cause was the misuse of a well-known antimicrobial agent, i. e. at less than recommended levels. Coincidentally, regardless of what species are added at the beginning in an inoculum in a long-term test with unfiltered air, we have been able to predict which additional species will be found at the end of the test with a fair degree of accuracy.

### 4. Summary and conclusion

#### Zusammenfassung und Schlussfolgerung

Extensive studies have shown that whether individual dominant species or a mixed population based on physiologically activated metalworking fluid are used, the relative microbiological resistance of the fluids remains the same. Methods have been recommended for treating each individual group separately. Emphasis has been placed on the needs of the specific user of metalworking fluids or antimicrobial agents as a basis for the selection of an appropriate microbial inoculum.

#### Anmerkung

- 1) Fungal isolates are inoculated in suitable liquid growth medium (sabourauds dextrose broth) and incubated with shaking for 3 – 5 days at 25 °C. This growth is added at 5% level to test formulations.

#### References/Literatur

- [1] Rossmore, H. W., Pauli Sceszny, and L. A. Rossmore: Evaluation of Source of Bacterial Inoculum in Development of a Cutting Fluid Test Procedure. *Lubr. Engg.* 33 (7): 372–377, 1977
- [2] Rossmore, H. W., J. F. Steckhaus, L. A. Rossmore, and D. DeFonzo: The Utility of Biocide Combinations in Controlling Mixed Microbial Populations in Metalworking Fluids. *Lubr. Engg.* 35 (10): 559–563, 1978
- [3] Rossmore, H. W. and L. A. Rossmore: The Identification of a Defined Microbial Inoculum for the Evaluation of Biocides in Water-Based Metalworking Fluids. *Lubr. Engg.* 36 (11): 16–20, 1980
- [4] Rossmore, H. W.: Evaluation Techniques for Biodegradation of Water-Miscible Metalworking Fluids. *Biotechn. Research Techniques* 1: 227–242, 1976
- [5] Bennett, E. O.: The Biological Testing of Cutting Fluids. *Lubr. Engg.* 30 (3): 128–135, 1974
- [6] Rossmore, H. W. and B. W. Williams: An Evaluation of a Laboratory and Plant Procedure for Preservation of Cutting Fluids. *Int. Biotechn. Bull.* 7: 55–60, 1971
- [7] Bailey, C. A. and M. E. May: Evaluation of Microbiological Test Kits for Hydrocarbon Fuel Systems. *Applied and Environmental Microbiol.* 37 (5): 871–877, 1979