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Biocidal Properties of an Anti-Icing Additive

N. I. HENDEY

Admiralty Materials Laboratory
Holton Heath, Poole
Dorset, England

V. J. BAGDON AND G. E. ERNST

U.S. Army Mobility Equipment Research and Development Center
Fort Belvoir, Virginia

D. F. KLEMME AND J. M. LEONARD

Marine Biology and Biochemistry Branch
Ocean Sciences Division

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N.I. Hendey, V.U. Bagdon, G.E Ernst, D.E. Klemme, and J.M. Leonard

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BIOCIDAL PROPERTIES OF AN ANTI-ICING ADDITIVE

INTRODUCTION

A much discussed topic of recent years has been the role of microorganisms, both bacteria and fungi, in the deterioration of hydrocarbon fuels of jet aircraft. The organisms tend to develop as a thick pellicle wherever a water bottom is present to produce a fuel/water interface. Portions of this felt frequently sink to the floor of the tank and become firmly attached to it.

The fungus component of the microflora reproduces by means of spores, and although great care is taken to remove particulate matter from aircraft fuels, most presently used filters fail to prevent the spores from entering aircraft tanks. In such circumstances, the spores have been known to germinate and develop fungal growths which have attached themselves to the interior surfaces of the tanks. The effects of microbiological slimes and mycelia on aircraft function include clogging the various filters, small piping and orifices and shorting the capacitive fuel-quantity probes.

In other situations, most noticeably in steel pipelines, bacteria have been demonstrated to be important agents of metallic corrosion, and it is not unreasonable to suggest that the corrosion of unprotected aluminum alloy in aircraft integral fuel tanks could be aggravated by microorganisms. The possibility of controlling such microbial contaminations in fuel-water systems by adding toxic agents has been examined, e.g., Refs. 1 and 2. The perfect coating of tank interiors is extremely difficult to attain, and small bubbles, pinholes, or hairline cracks in the coating will permit access of the organisms to the metal beneath. Also, instances of fungi penetrating films of protective coatings have been noted (3-5).

Work done in several laboratories indicates that such attack is possible and that fungi might be the causative organisms in the corrosion of aluminum integral fuel tanks (4,6). It has also been suggested that microbially contaminated fuel might develop poor water-separation properties, although researchers at Naval Research Laboratory and U.S. Army Mobility Equipment Research and Development Center have been unable to demonstrate such a phenomenon (7,8).

The causative organisms have been reported from all over the world. They exist in the soil and, since their spores are airborne, they can be found on every exposed surface. Keeping large volumes of kerosene fuels entirely free from fungal contamination, therefore, is practically impossible.

Whatever the real importance of fungi, their physiology demands water for spore germination. So if water can be rigorously excluded from the fuel, the spores are unable to develop. This philosophy has a special significance because there are compelling reasons, not connected with the microorganisms themselves, for keeping jet fuels and fuel systems free from moisture. This, however, is an extremely difficult thing to do, particularly when the route from the refinery to the aircraft includes water-flushed pipelines, underground storage tanks, tankers, or aircraft carriers. The possibility of water entering the fuel either by means of condensed moisture, leakage, or accident is ever present.

The most important reason for keeping water out of aircraft fuels is the risk of ice formation. With aircraft flying at high altitudes, even the water in true solution will separate as a filter-clogging rime when fuel is chilled sufficiently. To overcome this problem, it has been customary to fit preheaters in the fuel supply in aircraft. Another method of combatting the ice menace is to use a freezing-point depressant for the water. The anti-icing

agent which is the subject of this report is dissolved in the fuel at about 0.1% concentration, but its very high solubility in water results in a much higher concentration in the aqueous phase. Thus, water bottoms of storage tanks often become strong solutions of anti-icing additive and concentrations of 10 to 20% are common, one value of 35% having been reported (9). The U.S. Specification MIL-I-27686D, "Inhibitor, Icing Fuel System," was current when the work reported here commenced. This specification requires that the additive shall have the following composition by weight: methyl cellosolve (2-methoxyethanol) 99.6% ± 0.04 and glycerol 0.4% ± 0.04. This is the same formulation of the additive discussed in the Phillips report (10). The recently amended version of the specification, MIL-I-27686E, omits the glycerol with no detriment to the product.

The merit of the anti-icing additive (AIA) in serving its primary purpose is outside the scope of this report, but aside from anti-icing considerations, there is evidence that AIA has antimicrobial potency. For some time, the U.S. Air Force has added AIA to JP-4 routinely, and the consensus is that complaints about microbial infestations of aircraft fuel systems have diminished. Whether the improvement in the JP-4 situation is due to the fungicidal activity of the AIA or to the better fuel handling and housekeeping procedures, or to a combination of factors, is open to debate. The authors of this report have learned that the Navy is now giving serious thought to the routine use of the anti-icing additive in its JP-5. The latter fuel is vulnerable to microbes—perhaps even more so than JP-4—so the assays reported herein should be of special interest.

The *in vitro* assays of microbial inhibition of AIA by different laboratories have given results which are in strong disagreement, due in part to essential differences in the methods used. It seemed desirable, therefore, to devise a procedure and to test its reproducibility by running parallel assays on the same material in several independent laboratories. The authors of this report, representing three laboratories, agreed to such a collaborative effort.

PROCEDURE

To minimize variations, one or another of the participants furnished materials to be used by all three laboratories. The items so shared were the fungus culture, the Oxoid filter disks, the AIA mixture, the jet fuel (JP-5), and the small glass incubation jars. The basic procedure, proposed by one of the authors (Hendey), is similar in principle to the phenol coefficient test; viz., the test organisms are exposed to several concentrations of the toxicant for specified periods of time, and the lethal dose-time combinations are determined by plating out the organisms on a nontoxic, favorably nutrient medium. A preliminary trial suggested a few minor alterations of detail in the method as originally proposed.

The test organism was *Cladosporium resinae*, the fungus which is so common in contaminated fuels. The fungus to be used for inoculum was grown to active sporulation (10-21 days) on petri plates of wort agar. Toxicities were determined separately in the single-phase systems i.e., the JP-5 hydrocarbon and the aqueous medium. The JP-5 was filtered through a Millipore filter, pore size 0.45 micron, before distribution to the participating laboratories. The aqueous liquid was Bushnell-Haas medium* diluted to one-tenth strength with distilled water and AIA as required. The AIA was prepared in the laboratory from reagent-grade ingredients.

* Bushnell-Haas Solution, Full Strength

Distilled water	1 liter
NH_4NO_3	1.0 gram
$MgSO_4 \cdot 7H_2O$	0.2 gram
$CaCl_2$	0.02 gram
KH_2PO_4	1.0 gram
K_2HPO_4	1.0 gram
$FeCl_3$	0.01 gram

For growing fungi the pH is adjusted with 1:1 HCl to 6.4 ± 0.2. *C. resinae* grows readily at a pH of about 6.5.

For testing purposes, 40-ml portions of liquid phases containing AIA were placed in 2-ounce, screw-cap, glass ointment jars and sterilized at 15 pounds pressure for 20 minutes. The AIA concentrations are shown in the first column of Tables 1 and 2. Oxoid filter disks were steam sterilized at 10 pounds for 20 minutes and then inoculated by drawing them, with sterile forceps, across a plate of *C. resinae*. A minimum of 12 disks was placed in each jar, with all precautions to avoid introducing extraneous organisms. With the caps screwed on loosely, the jars were placed in an incubator at 30°C ± 1°.

At planned intervals, as shown in the tables, survival of the spores was determined. Three disks were removed from each jar with sterile forceps, drained, and placed on a wort-agar plate. They were incubated at 30°C and examined for growth at the times shown in the tables. In preliminary experiments growth on hydrocarbon-soaked disks was often sparse, even without AIA. The writers attribute this to the disks being rendered non-wettable to water, so that the inoculum starves for lack of the nutrients in the agar medium. To minimize this difficulty, the inoculated side of the disk was pressed (touched off) on the agar surface and the disk was then placed nearby, inoculated side up. Growth was rated on an arbitrary numerical scale*, which is illustrated by Fig. 1. The set of cultures for the photograph did not have the touch-off areas described above. The observations, as functions of AIA concentration, duration of contact with AIA, and incubation time on the agar, are presented in the tables.

DISCUSSION

A closer simulation of the intended uses of the fuel would have been a test in which the inhibitor was incorporated in the two-phase system of fuel over aqueous solution; but in such a system, the concentration of inhibitor to which the organisms are subjected is affected by several factors. The distribution of inhibitor between two phases is determined by its partition coefficient, and the amount in each phase is a function of both the overall concentration and the fuel-to-water ratio. The partition coefficient, in turn, varies with temperature, as does also the microbial activity of a substance. Bean et al. (11) have shown that the situation is even more complex than these variables would indicate. They found that, for a fixed concentration of phenol in the aqueous phase of a liquid paraffin/water system, the antibacterial activity increased as the oil-to-water ratio was increased, and also that the two-phase system was significantly more antibacterial than an equivalent aqueous solution without the oil. Hence, the importance of the interface between the two phases is indicated. These authors reported adsorption of bacterial cells in multilayers and enhancement of phenol concentration at the interface. Obviously, these many factors would complicate interlaboratory correlation. Hence, at this juncture, the study has been directed toward exploring the inherent toxicities of AIA, rather than determining a dosage range for field use. These results from experiments on one-phase systems should not be translated to two-phase systems.

Water and AIA are miscible in all proportions, so there is no ambiguity in the 0 to 100% concentration range in Table 1. The AIA is soluble in fuel only to about 2.5%, so the percentages above 2.5% in Table 2 are really two-phase systems; disks in these higher percentages were undoubtedly soaked in fuel-saturated AIA.

*Rating scale	Growth coverage of inoculated area
0	none
1	less than 1/4
2	1/4 to 1/2
3	1/2 to 3/4
4	3/4 to complete but not dense
5	complete, dense

Table 1
Inhibition of *Cladosporium resinae* by Anti-Icing Additive in Bushnell-Haas Solution

Percent AIA (v/v)	Lab	Periods of Immersion in AIA															
		2 days				7 days				14 days				28 days			
		Weeks of Incubation of Plates															
		1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
100	A	005*	553	5	5	0	0	0	0	0	0	0	0	0	0	0	0
	B	0	—	—	—	0	0	0	0	0	0	0	0	0	0	0	0
	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
80	A	005	553	5	5	0	0	0	0	0	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0	—	0	0	0	0	0	0	0	0
	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
40	A	022	5	5	5	0	0	0	0	0	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	C	213	5	5	5	0	0	0	0	0	0	0	0	0	0	0	0
20	A	5	5	5	5	5	5	5	5	5	5	5	5	1	5	5	5
	B	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	C	5	5	5	5	5	5	5	5	5	5	5	5	123	5	5	5
10	A	5	5	5	5	5	5	5	5	5	5	5	5	544	5	5	5
	B	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	C	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
5	A	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	B	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	C	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
2.5	A	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	B	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	C	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
0	A	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	B	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	C	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

*Rating: 0 = no growth; 5 = complete overgrowth. (See footnote, page 3). Where the ratings for three replicate disks were the same, only one number is recorded in the table; where different, the three individual ratings are shown.

Table 2
Inhibition of *Cladosporium resinae* by Anti-Icing Additive in JP-5 Fuel

Percent AIA (v/v)	Lab	Periods of Immersion in AIA															
		2 days				7 days				14 days				28 days			
		Weeks of Incubation of Plates															
		1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
80	A	5*	5	554	5	0	0	0	0	0	0	0	0	0	0	0	0
40	A	015	135	5	5	100	500	500	554	0	0	0	0	0	0	0	0
20	A	155	5	5	5	0	0	0	0	0	0	0	0	0	0	0	0
10	A	115	355	5	5	0	0	0	0	0	0	0	0	0	0	0	0
5	A	0	100	300	500	0	0	0	0	0	0	0	0	0	0	0	0
2.5	A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	B	0	0	—	—	0	0	—	—	0	0	—	—	0	—	—	—
	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1.25	A†	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0	—	0	—	—	—	0	0	0	0
	C	0	0	0	0	0	0	0	0	002	004	005	005	0	0	0	0
0.63	A†	443	5	5	5	232	5	5	5	0	455	5	5	100	310	550	551
	B	433	544	544	544	233	4	4	4	233	344	344	544	022	345	355	455
	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.32	A†	5	5	5	5	543	5	5	5	222	5	5	5	0	0	0	0
	B	433	4	4	4	433	433	433	433	554	554	554	554	234	345	345	445
	C	544	5	5	5	0	005	005	005	200	500	500	500	030	050	050	050
0.16	A†	5	5	5	5	5	5	5	5	4	5	5	5	1	4	5	5
	B	443	4	4	4	344	344	344	344	4	5	5	5	344	455	455	5
	C	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
0	A	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	A†	5	5	5	5	5	5	5	5	5	5	5	5	122	5	5	5
	B	543	543	543	543	344	344	344	344	344	344	344	344	344	344	445	455
	C	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

*Rating: 0 = no growth; 5 = complete overgrowth. (See footnote, page 3). Where the ratings for three replicate disks were the same, only one number is recorded in the table; where different, the three individual ratings are shown.

†These determinations were made at a separate time by Laboratory A.

The authors believe that the interlaboratory agreement of results was good. An obvious consensus noted in the aqueous system was that, for a week of contact with AIA, a concentration in the 20 to 40% range is lethal. For 2 days of contact, Laboratories A and C agree that 40% AIA is insufficient for kill; Laboratories B and C agree that 80% AIA is sufficient. It seems that 2 days is probably a critical time; had the contact time been either slightly longer or shorter, the results probably would have been more concordant. In the fuel, on the other hand, all three laboratories agree that 2.5% AIA is lethal, even for 2 days of contact. The positive growth which Laboratory A observed at high concentrations is hard to explain, but the observation is consistent with what the same laboratory saw at high concentrations of AIA in the aqueous medium. Laboratories A and B differ with C as to whether 0.63% provides a lethal dose, but one must note that this debatable concentration is six times that prescribed by the specification.

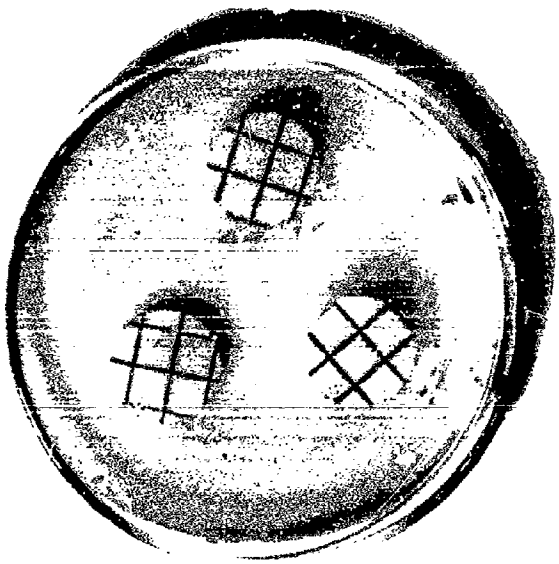
The terms "lethality" and "kill" must be understood in the context of the test. To illustrate, a "0" reading means that not one spore had the vigor to propagate when introduced to a nontoxic, nutritionally favorable environment. If the residual AIA had been washed off the spores, or if a more nearly optimum growth medium had been used, or if better contact between spores and agar medium were achieved, perhaps many "dead" spores would have germinated and grown.

But there is a point of considerable practical importance in the difference between "kill" and "inhibition." A dose lower (much lower, perhaps) than the killing dose should suffice to arrest growth without necessarily killing the spores. The Phillips report (10) stated (Table III, page 15) that 15% anti-icing additive suffices for kill; this is in a jet fuel/Bushnell-Haas system. Careful study of the report, however, suggests that lethality is not necessarily indicated—the action may be simply inhibitive. As already noted, in the present study the lethal concentration was in the 20 to 40% range in a one-phase aqueous system. Advocates of simple inhibition can argue that inhibition is all that is required—that a fuel storage system can tolerate the dirt load represented by an ordinary infection with spores, if the spores do not proliferate. But the advocates of "kill" can argue that the transit of fuel over several successive water bottoms can lower the AIA concentration even below the inhibitive level. In such a situation, a previous contact with a lethal concentration of AIA would have served a useful purpose by reducing the amount of viable inoculum which comes into the nontoxic environment.

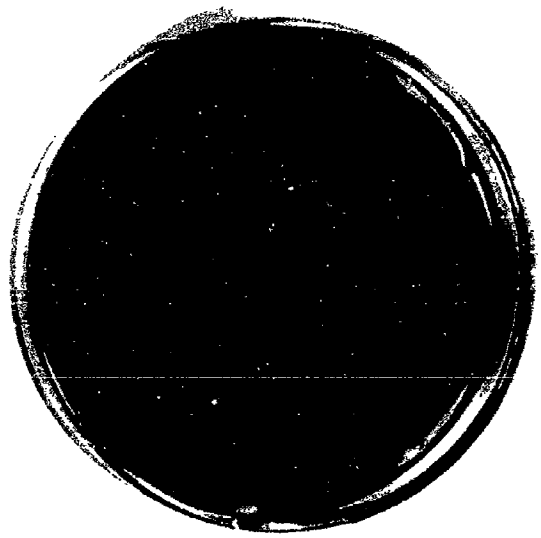
Perhaps the alternative is to maintain the inhibitive concentration throughout the fuel's transit, by adding AIA as needed, rather than dosing the fuel at the refinery so as to meet all possible contingencies of aqueous extraction. In any event, it is clear that an estimate of inhibitive concentration is essential to establishing the minimum concentrations that must be maintained for effectiveness. If the value is reliably determined, for the practical situation it must simply be maintained at every new contact, in the final service tank especially. This point is now being explored.

The investigator in Ref. 9 has analyzed many water bottoms submitted by the U.S. Air Force and has stated that the AIA concentration in most is in the 10 to 25% range. He notes that the samples generally are clean, even though the AIA concentrations may be below the "lethal" level. The observation would be a strong endorsement for AIA, but some of the improvement must be ascribed to the better attention to water separation, to filtration, and to general cleanliness.

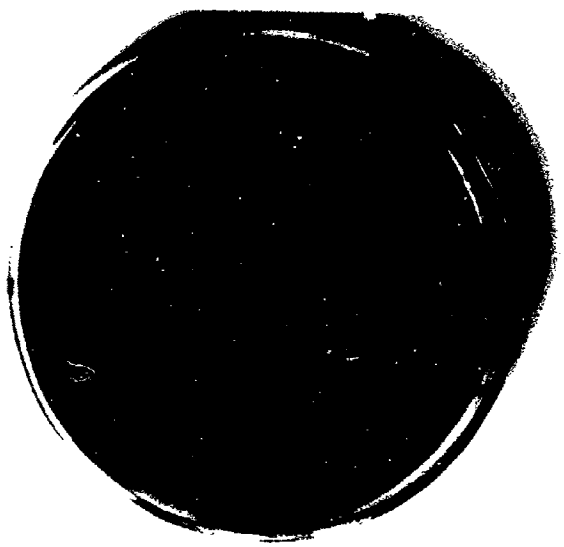
The experiments described here have been limited to one organism, the fungus, *Cladosporium resinae*. This is by far the most frequently encountered fungus contaminant of fuel, but to ignore bacteria would be a serious omission. If circumstances permit, the merit of AIA against at least one of the bacterial contaminants, possibly *Pseudomonas*, may be explored.



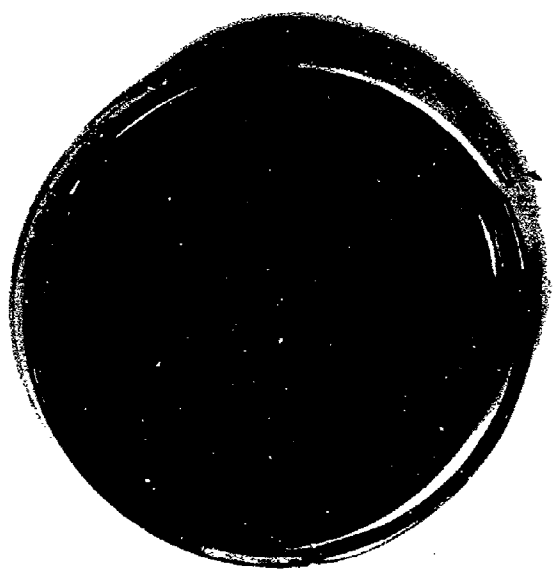
(a) Growth rate = 0



(b) Upper-left-disk growth rate = 4; lower left and right disks = 3



(c) Left and upper right disks = 3; lower right disk = 2



(d) Growth rate = 5

Fig. 1 - Culture plates showing typical growth ratings. A rating of 1 is not illustrated (Photograph by U.S. Army MERDC).

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