EVALUATION OF A BIOCIDAL TURBINE-FUEL ADDITIVE

Charles R. Crane, Ph.D.
Donald C. Sanders, M.S.

Approved by

J. Robert Dille, M.D.
Chief, Civil Aeromedical Institute

Released by

P. V. Siegel, M.D.
Federal Air Surgeon

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I. Introduction.

Microbial growth in kerosene-type fuels is often associated with the fouling of fuel screens and capacitance probes, as well as the corrosion of aluminum-alloy fuel tanks (1–13). Since 1956, when fuel system malfunctions in the B–47 aircraft were traced to microbial sludge formation, considerable effort has been expended to develop a fuel additive which would retard the growth of these organisms and, at the same time, be compatible with the fuel system components.

Of particular interest recently has been the potential usefulness of several boron-containing organic compounds which exhibit microbiocidal properties[2,3,10,14,15]. These compounds present a unique solution to the problem of getting the biocidal material into contact with the organisms which proliferate in the water layer, since they could be introduced as a fuel-biocide solution and, due to their water solubility, would tend to saturate any water pockets which exist in the tank. Biobor-JF, developed by the Standard Oil Company of Ohio, is such a product.

An evaluation of the effectiveness of Biobor-JF as a biocidal jet fuel additive was initiated in a two-phase program. One phase was a carefully controlled laboratory study of the biocidal properties of the additive under simulated field conditions. Concurrent tests were conducted by the Aircraft Services Base, Federal Aviation Administration, Oklahoma City, Oklahoma using the fuel-additive mixture in an agency-owned Convair 880 aircraft performing routine flight operations. This report, however, is concerned primarily with the laboratory studies.

The laboratory studies conducted in this evaluation consisted of the following:

a. Comparative evaluation of seven nutrient media to determine the one most suitable for culturing the mixed population of microorganisms found in fuel.

b. Evaluation of a membrane filter procedure for measuring microbial concentration in both fuel and aqueous samples.

c. Viability of microorganisms in turbine fuel.

d. Kinetics of microbial mass transfer in fuel-water systems.

e. Effect of Biobor-JF on microbial growth in fuel-water systems.

f. Effect of Biobor-JF on microbial growth in liquid and solid nutrient media.

II. Experimental.

Fuel. A commercial kerosene fuel, Conoco Jet–50, was used in all laboratory and field studies except as noted in Table IX.

Counting of Microorganisms

1. Fuel Samples: Microorganisms in jet fuel were counted using a modification of the procedure described in the Society for Industrial Microbiology publication, "Proposed Procedures for Microbiological Examination of Fuels"[16]. The measured fuel samples were filtered by suction through membrane filters (Millipore membrane, type HA, 47–mm dia., pore size 0.45μ) supported in sterile, stainless steel filter funnels (Gelman Instrument Co.) of approximately 1 liter capacity. Each filter was washed with approximately 300 ml of sterile 0.1% aqueous Triton X–100[1] to remove the residual kerosene from the membrane, followed by 100 ml rinse with 0.01 M phosphate buffer, pH 6.8. The membrane filter was transferred aseptically from the filter assembly to a petri dish (Lab-Tek Plastics Co., 60 x 20 mm) containing the appropriate growth medium. The filter was placed on the agar, face-up, covered, and incubated.

2. Water Samples: Microorganisms in water samples were serially diluted with 0.01 M phosphate buffer, pH 6.8, and filtered through membrane filters as described above except that they did not receive the detergent wash.

[1] Triton X–100 is an alkyl phenoxy polyethoxy ethanol produced by Rohm and Haas Company, Philadelphia, Pennsylvania.

Media Selection. Media tested for use with the membrane filter counting method included Nutrient Agar (Fisher Scientific Co.), Standard Plate Count Medium (Baltimore Biological Laboratory), Double Strength Nutrient Agar, Tryptone-Glucose Extract Agar (Difco Manual #9, p. 57), Nutrient Broth enriched with 0.1% yeast extract and 0.1% glucose, Sabouraud Dextrose Agar (Fisher Scientific Co.) and Czapek Solution Agar (Difco Manual #9, p. 245). Replicate samples were withdrawn from a constantly-stirred suspension of mixed bacteria and fungi which had been isolated from fuel. Each sample was filtered and the filters incubated at 37°C on poured plates of the above test media. Counts of bacterial colonies present after 24, 48, and 72 hours of incubation on the first five media failed to show any significant differences among the various media in terms of the number of colonies or rate of growth. Of the last two media, which are designed primarily for the cultivation of fungi, Sabouraud Dextrose Agar supported fungal growth considerably better than the Czapek Solution Agar. Nutrient Agar and Sabouraud Dextrose Agar were therefore selected for the cultivation of bacteria and fungi respectively.

Accuracy of Membrane Filter Method. To establish the degree of precision to be expected from the membrane filter method of counting, the following experiments were conducted.

1. Aqueous Suspensions: Separate suspensions of bacteria and fungi were prepared in phosphate buffer (0.01 M, pH 6.8) by the direct addition of a diluted broth culture of the organism. Replicate samples were prepared by transferring 1.0 ml samples from each constantly-stirred microbial suspension to the sterile funnel-filter assembly which contained 100 ml of phosphate buffer. The addition of the 1.0 ml sample to the buffer provided a more uniform distribution of the organisms over the filter surface. After application of suction, an additional 100 ml of sterile buffer was used to wash down the sides of the funnel.

2. Fuel Suspensions: Separate suspensions of bacteria and fungi in fuel were prepared by shaking fuel with a broth culture of each organism, allowing the broth to settle, then carefully decanting the fuel. Samples of 100 and 500 ml volumes were removed from the constantly-stirred fuel suspension and transferred to the filter-funnel assembly, then washed with detergent and buffer as previously described.

The washed filters from both aqueous and fuel suspensions were then transferred to petri dishes containing the appropriate medium and incubated at 34°C. Colony counts were made using a standard Quebec counter after 24 hours of incubation for the bacterial plates and 48 hours for the fungal plates. The results are shown in Table I; the coefficient of variation ranged from 5% to 12%.

Detergent Effect. Since the jet fuel must be removed from the membrane filter prior to incubation to allow the nutrient to diffuse to the entrapped cells, a detergent rinse is necessary. To determine the effect of this detergent on the growth of the bacteria, aliquots from suspensions of known species (both gram-positive and gram-negative) were transferred into approximately 10 ml of sterile, aqueous 0.1% Triton X–100 contained in a sterile filter-funnel unit. After removal of the detergent solution by suction, the filter was washed with 0, 25, 100, or 500 ml of sterile phosphate buffer (0.01M, pH 6.8); the 500 ml rinse was added in five 100-ml portions to assure complete removal of the detergent. Colony counts were made after 26 hours of incubation on nutrient agar at 34°C.

Settling Rate. In order to estimate the settling rate of bacteria placed in suspension by tank turbulence, a laboratory tank was prepared using a 2-liter, stoppered, graduated glass cylinder. Each such tank was inoculated with 100 ml of Bushnell-Haas salts solution[17] containing 2 ml of a suspension of 2 bacterial and 2 fungal types previously isolated from fuel. 1900 ml of fuel was layered over the 100 ml of salts solution. After 8 days' incubation at room temperature, the cotton plug was replaced by a glass stopper and the fuel-water mixture was shaken vigorously. At various time intervals after shaking, samples of 25 ml each were aseptically withdrawn from exactly 4 inches below the fuel surface, plated, and counted.

Distribution of Organisms in Fuel-Water System. To establish the distribution of bacteria in fuel tanks under static conditions, 3 "tanks" were prepared as described above and incubated for 8–12 days at room temperature. Using a long, sterile, tubular probe, fuel samples were removed at various distances above the fuel-water interface and from the water layer itself;

these samples were then filtered, plated, incubated, and counted.

Long-Term Growth of Microorganisms in Fuel Phase. To determine whether microorganisms can live and multiply in jet fuel without the presence of a discrete water phase, several gallons of untreated jet fuel were chilled in a cold room (3°C) for 3 days and filtered (Whatman #1 filter paper) into a dry container. This system contained such organisms as were indigenous to the fuel and only that amount of water which could be maintained in solution at 3°C; no separate, discrete water phase was present when the system was brought to room temperature. Samples were removed for counting immediately after filtration and at intervals up to 63 days. Incubation temperature was 23–25°C for this 63–day period.

Inhibition of Microbial Growth in Fuel-Water Systems by Biobor-JF. To study the rate and extent of microbial inhibition by Biobor-JF in a fuel-water system, four laboratory "tanks" were prepared using 2-liter graduated cylinders stoppered with cotton. 1950 ml of untreated commercial jet fuel were placed in each cylinder. Two bacterial and 2 fungal isolates were grown in separate broth cultures, and 10 ml of each culture were withdrawn, mixed together, and washed twice with Bushnell-Haas salts solution followed by centrifugation. The final mixed suspension was added to 1 liter of the sterile salts solution and a 50 ml aliquot of this suspension was added to each tank. Tanks 1 and 2 were designated as controls; tanks 3 and 4 were treated by adding Biobor-JF to the fuel layer to a concentration of 270 ppm by weight (equivalent to 20 ppm elemental boron). After initial mixing, samples were removed for counting at intervals up to 33 days. Two hours after a uniform agitation of 4 inversions (to simulate normal refueling turbulence), samples were taken with a sterile probe from the aqueous layer and from the fuel layer (4 inches below the fuel surface).

Inhibition of Microbial Growth on Solid Nutrient Media by Biobor-JF. Eight bacterial and 5 fungal isolates, selected from a total of 19 apparently discrete types isolated from fuel (see Appendix A)[1], were grown in separate broth cultures; these were uniformly inoculated onto the surfaces of individual petri dishes of solid media using a sterile glass rod spreader. Bacteria and fungi were grown on nutrient agar and Sabouraud Dextrose Agar respectively, using a single isolate per dish. Whatman #2 filter paper was cut into 6-mm discs using a paper punch; the sterilized discs were dipped into Biobor-JF and applied to the surface of the inoculated plates. Plates were inspected for a zone of inhibition after a 48-hour incubation at 34°C.

In a second experiment, Biobor-JF was added directly to nutrient agar and Sabouraud Dextrose Agar to a final concentration of 400 ppm; it was added after steam sterilization but before the agar had solidified. Petri dishes were poured with the treated agars and inoculated as previously described.

A third experiment was designed to determine whether the microorganisms could utilize Biobor-JF as a sole source of carbon. After sterilization of a solid medium prepared by adding 15 g/liter of agar to Bushnell-Haas salts solution, Biobor-JF was added in concentrations of 0, 4,000, and 10,000 ppm. Four different bacterial isolates were inoculated onto each of the 3 media.

Inhibition of Microbial Growth in Liquid Nutrient Media by Biobor-JF. Flasks containing 5 ml of sterile nutrient broth (beef extract 0.3%, peptone 0.5%), in which 0, 200, 500, 1,000, 5,000, or 10,000 ppm of Biobor-JF was incorporated, were separately inoculated with 1-drop suspensions of broth cultures of individual bacterial isolates. These flasks were cotton-stoppered and incubated with constant shaking at room temperature (26°C) for 22 hours. Optical density of the bacterial suspension was determined at 600 mμ using a Coleman Junior spectrophotometer with nutrient broth as the blank.

For fungal inhibition studies, separate flasks containing 75 ml of sterile Sabouraud Dextrose broth in which 0, 200, 500, 1,000, 5,000, or 10,000 ppm of Biobor-JF was incorporated (after sterilization) were inoculated with 1-drop suspensions of broth cultures of single fungal isolates from jet fuel. The flasks were incubated at room temperature (26°C) for 5 days with intermittent shaking. The medium was removed by filtering the entire flask contents through a membrane filter (Millipore, Type RA, pore dia. 1.20μ, filter diameter 47 mm); the mycelium was then washed with 20 ml of 10% formalin. The pre-weighed

[1] Although the taxonomic characterization of the fuel isolates was beyond the scope of this study, the more obvious physical and cultural characteristics noted during the tests are shown in Appendix A.

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