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CAN NON-CHEMICAL ANTIMICROBIAL DEVICES REPLACE OR AUGMENT FUEL TREATMENT MICROBICIDES?

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KEYWORDS

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ABSTRACT

Despite their history of successful use as fuel system disinfectants and fuel preservatives, antimicrobial pesticide use faces increasing restrictions due to both regulatory control and public concerns. A variety of non-chemical treatments have been used with varying degrees of success to disinfect non-fuel fluids and to at least partially inhibit biofilm development on infrastructure surfaces. Promoters of one technology have claimed successful fuel disinfection and fuel-tank fouling prevention. This paper will review a range of non-chemical treatment technologies and will present the results of preliminary evaluations of several technologies that were tested on Jet A fuels that had been challenged with either *Pseudomonas aeruginosa* or *Hormoconis resinae*. Data are presented on treatment impact on adenosine triphosphate (ATP) concentration, culturability and live/dead direct counts in Jet A-1 and on glass microcosm surfaces.

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INTRODUCTION

Uncontrolled microbial contamination in fuels can cause both fuel and equipment biodeterioration. Common symptoms of fuel biodeterioration include but are not limited to

increased corrosivity, decreased oxidative stability and decreased energy value [1]. Although filter clogging is the most commonly reported fuel system biodeterioration symptom, microbially influenced corrosion (MIC) and biofilm interference with fuel gauge sensors are also common problems [2, 3]. Currently, microbial contamination is controlled by treating fuel systems with additives – microbicides. All microbicides are classified as hazardous chemicals. Consequently, personnel handling these products must receive specialized chemical handling training and wear special, personal protective equipment [4]. Although there are numerous microbicides approved in the U.S. under the Federal Insecticide, Rodenticide and Fungicide Act (FIFRA) [5], only two products have been approved by the aviation industry for use in aircraft [6]. One of these products (Biobor JF®; a 95% active blend of 2,2'-(1-methyltrimethylenedioxy) bis-(40methyl-1,3,2-Dioxaborinanes) + 2,2'-oxybis(4,4,6-trimethyl-1,2,3-Dioxaborinanes)) is known to hydrolyze on contact with water, rendering the microbicide biologically inactive. The other (Kathon® FP1.5; a 1.5% active blend of 5-chloro-2-methyl-4-isothiazolin-3-one + 2-methyl-4-isothiazolin-3-one) is a known skin sensitizer. Given the hazards associated with the handling of microbicidal chemicals, current military regulations prohibit the use of microbicides in U.S. Air Force (USAF) aircraft. Although IATA recommends microbicide use as needed and permits the use of microbicide-treated fuel [6], commercial airlines typically drain treated fuel and replace it with microbicide-free fuel. The IATA-recommended soaking period (12h to 72h, depending on contamination severity and microbicide) is designed to kill-off biofilm microbial communities. During this period, aircraft are grounded. Non-chemical technologies, capable of inhibiting biofilm development and reducing toxic-chemical exposure would bring significant benefits to the aviation and other sectors for which fuel-quality stewardship is important.

This paper reports the results of a preliminary evaluation project in which the performance of four different non-chemical, antimicrobial technologies was tested.

MATERIALS AND METHODS

Test Rigs

Balanced Charge Agglomeration (BCA) and Fuel-Mag Test Stand

Figure 1 shows the fuel treatment test rig. The photo on the left (Figure 1a) shows the front of the rig, and the photo on the right (Figure 1b) shows the back of the rig. The numbered components in Figure 1 are explained in Table 1. The test rig flow-rate was set for 1 gpm. The system was flushed with clean JetA-1 fuel before first use. Samples were collected from the fuel reservoir (5 gal bucket at left in fig 1b), between the pump and treatment (middle gate valve (7) in Figure 1b and post-treatment (6 in Figure 1a and 2b). An array of sample bottles is seen in the Figure 1b foreground.

UV Test Stand

In order to test the effect of UV irradiation on fuel biomass, either a 1mm or 1 cm layer of control or microbially challenged Jet A1 was decanted into a 125 mm dia open Petri dish. A Pyrex glass dish was used for the 23-24 June work. It was replaced with a quartz dish for the 09-

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13 August tests in order to improve UV transparency. Figure 2 illustrates the UV irradiation test set up.

A fuel sample was submitted for analysis to estimate the penetration depth of UV wavelengths between 200 and 400 nm in a typical jet fuel. A stock solution of fuel 4877 was prepared by diluting one gram of the fuel with hexane up to a 100 gram total. The UV-Vis spectrum of the 1% fuel in hexane solution was measured in a quartz cell with a 1 cm path length using a spectrophotometer.

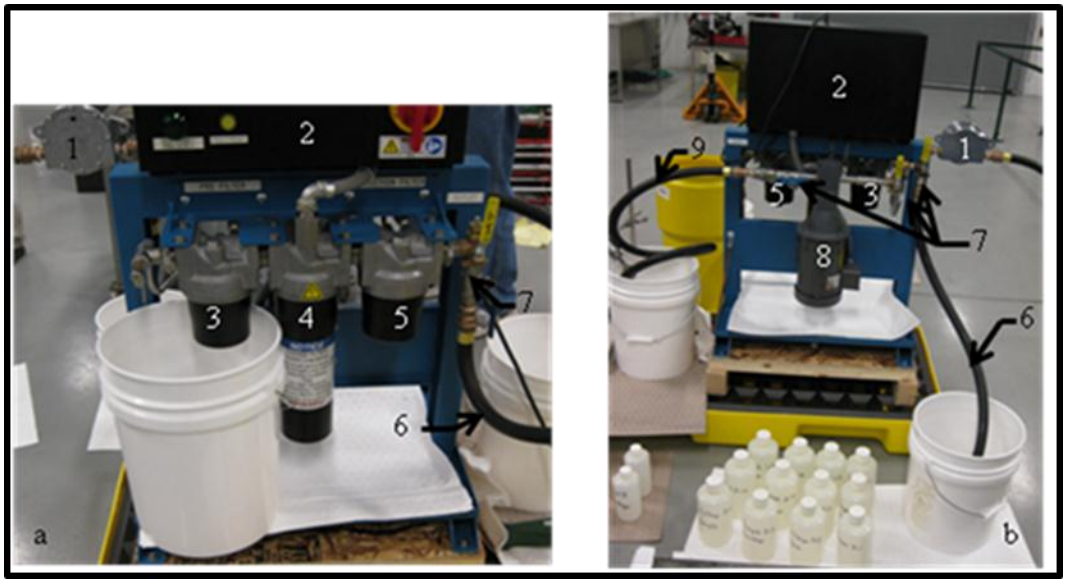

Figure 1: Non-Chemical, Antimicrobial Treatment Test Rig

Table 1. Legend for Figure 1

Item	Description
1	Fuel-Mag Device
2	BCA Control Panel
3	BCA Prefilter Housing (empty)
4	BCA Housing
5	BCA Postfilter Housing (empty)
6	Test Rig Fuel Discharge Line
7	Gate Valves
8	Pump/Pump Motor Housing
9	Test Rig Fuel Suction Line

Electronic Biological Eliminator (EBE) Test Stand

As part of Series 2 testing, challenged fuel was passed through an EBE device (Figure 3) via gravity flow. Before testing, the funnel (Figure 3, top) and quartz chamber (grey cylinder; Figure 3, center) were rinsed with methanol, then unchallenged fuel. Challenged fuel was then

decanted into the funnel, gravity fed through the quartz chamber, and collected into a sample bottle at the chamber's outlet (Figure 3, bottom).

Figure 2: UVm Light Set-up with Jet A-1 in 125 mm dia Quartz Petri Dish

Figure 3: Electronic Biological Eliminator

Fuel

Initially, JRF3 without DiEGME and JRF3 with DiEGME were evaluated to determine the effect of DIEGME on microbial growth. Subsequently, Jet A-1 fuel was used for all non-chemical treatment evaluation testing. All fuel was provided by AFPET Laboratory, WPAFB. The Jet A-1 fuel was from a single tender as characterized in COA 2008LA13647001 of 09 September 2008.

Challenge Microorganisms

The cultures selected for use in this project were one bacterium and one fungus from the list of standard challenge inocula listed in ASTM E 1259 [7]. *Pseudomonas aeruginosa*, ATCC No.

33988 was selected as the bacterial challenge culture and *Hormoconis resinae* (formerly *Cladosporium resinae*), ATCC No. 20495, was selected as the fungal challenge culture. For Series 1 tests, *Acinetobacter* sp. Desig. RAG-1 ATCC No. 31012 (*A.* sp. RAG-1) was substituted for *P. aeruginosa*.

All organisms were grown in Bushnell Haas [8] medium (BHM) to which 5% v/v fuel had been added as the sole carbon source. For preliminary testing, P. aeruginosa and H. resinae were grown in BHM augmented with JRF3 with DiEGME and JRF3 without DiEGME. Cultures were grown in Erlenmeyer flasks at 22°C on a gyrorotary shaker rotating at 180 RPM. The *A.* sp. RAG-1 culture used for the Series 1 tests was in BHM in 250 mL, wide-mouth glass jars that had been inoculated on 5-weeks earlier and had been incubated without agitation at 25°C. Cultures for Series 2 testing were grown in BHM augmented with JRF3 without DiEGME. Growth conditions were the same as for the Series 1 cultures.

Cell Harvesting

Series 1

Initially, cells were harvested by centrifugation at 5,000 x g for 10 min. They were then resuspended in 5 mL Jet A-1. As presented in the Results section this protocol did not produce a homogeneous cell dispersion. Consequently, for all Series 1 tests, an appropriate volume of *A.* sp. RAG-1 broth was dispersed into each pail of test fuel. The fuel was then mixed aggressively with a high-speed mixer (Figure 4).

Figure 4: Fuel Reservoir with High-speed Mixer

Series 2 – P. aeruginosa

P. aeruginosa cells from ~300 mL of BHM were harvested by centrifugation at 5,000 x g for 10 min at 4° C. Initial centrifugation was done on 15 mL aliquots in 20 centrifuge tubes. After discarding the supernate, ten pellets were resuspended into 7 mL of Jet A-1 and pooled into one centrifuge tube. The remaining ten pellets were resuspended into a second 7 mL portion of Jet A-1 and pooled into a second centrifuge tube. Cells were then centrifuged again for 10 min at 5,000 x g at 4° C. The supernates were discarded and the pellets were each resuspended into 6 mL Jet A. Resuspension was accomplished by first aspirating (drawing and expelling) the pellet

20 times using a 0.5 mL pippeter, and then sonicating the partially resuspended cells for two 10 min cycles at 19 watts, 50 to 60 Hz for 10 min. The two suspensions were then pooled, tested for ATP concentration and distributed as 1.5 mL aliquots into four 15 mL centrifuge tubes. The contents of each tube was used to challenge one 19 L (5 gal) pail of Jet A-1.

Series 2 – H. resinae

Spherical *H. resinae* colonies, growing that the fuel-water interface in BHM with Jet A-1 (Figure 5), were collected aseptically in A-15 mL centrifuge tube (Figure 6) containing 5 mL Jet A-1. The cells were then sonicated for 10 min, transferred to a sterile polyethylene bag, and stomached (ground with fingers against counter top in order to break up cell masses) for 5 min. The cycle of sonication and stomaching was repeated two more times. The cell suspension was stored overnight at 4° C. The mass of *H. resinae* hyphae was transferred to a 500 mL HDPE bottle, from which the top 3 cm had been removed and to which 50 Jet A-1 had been added. This preparation was blended for 2 min at 1,000 rpm with a hand held blender, sonicated for 10 min and blended a second time for 1 min to produce the challenge preparation. The cell suspension was dispensed as 10 mL aliquots into five 15 mL centrifuge tubes, and tested for ATP concentration. The contents of each tube was used to challenge one 19 L (5 gal) pail of Jet A-1.

Figure 5: *H. resinae* growth in Bushnell Haas Medium, augmented with 5% v/v each, Trypticase Soy Broth and Jet A-1 Fuel

Figure 6: H. resinae Colonies Harvested from Bushnell Haas Broth, Fuel Phase

Non-chemical Antimicrobial Technology Testing

System Flushing

The BCA test rig was used to evaluate the BCA and Fuel-Mag technologies. Before use, the test rig was flushed with 19 L (5 gal) Jet A-1. The QGO-M ATP test was used to determine fuel bioburden before and after flushing. After completion of all test runs, the test rig was flushed with lean fuel and drained for storage. However, during storage between Series 1 and Series 2 test runs, the rig became fouled with *A.* sp. Desig. RAG-1. Consequently the rig was disassembled and all parts were washed with methanol. After the rig was reassembled, it was flushed with 3 L methanol and 19 L (5 gal) Jet A-1. The QGO-M ATP test was used to determine fuel bioburden before and after flushing. After completion of all test runs, the test rig was flushed with clean fuel and drained for storage.

Test Runs – Series 1

After being weighed to determine the actual volume of fuel in the pail, a 19L pail of Jet A-1 was dispensed into a 19 L polypropylene reservoir, and 250 mL of fuel was withdrawn as a pre-challenge sample. Mixing was initiated and approximately 100 mL of *A.* sp. RAG-1 broth was dispensed into the fuel. Again, 250 mL was withdrawn as a pre-treatment sample.

The BCA test rig pump, set for 3.8 L/min (1 gpm) was turned on, and a third 250 mL pre-treatment sample was collected from the sample port just upstream of the Fuel-Mag device (center of 3 gate valves (#7) in Figure 1b). The test rig valves were then aligned to direct fuel flow through either the BCA device (Figure 1b, #4) or the Fuel-Mag device (Figure 1, #1). Samples (250 mL) were collected after 60, 90 and 120 sec flow through the designated treatment device, and then the valves were aligned to direct flow through the second device. Again, treated fuel samples were collected after 60, 90 and 120 sec. This provided triplicate samples for each treatment from a single challenged reservoir. The system was flushed with 19 L clean fuel and the test run series was repeated two more times to give three test runs with three replicate samples of treated fuel from each treatment for each run.

The effect of UV irradiation was tested by dispensing sufficient, *A.* sp RAG-1challenge Jet A-1 into a 100 cm dia Petri dish to form either a 1 mm or 1 cm layer of fuel. The fuel was exposed to either UVc for 20 sec or UVm for 30 sec. All of the treated fuel was collected as the sample for biomass testing. Each treatment was run on two portions of fuel.

Test Runs – Series 2

After being weighed to determine the actual volume of fuel in the pail, a 19 L pail of Jet A-1 was dispensed into a 19 L polypropylene reservoir, and 250 mL of fuel was withdrawn as a pre-challenge sample. The fuel was then challenged with the contents of one 15 mL centrifuge tube containing resuspended cells. Challenged fuel was kept homogenized by aggressive mixing (Figure 4). For single treatment runs, the BCA test rig pump, set for 3.8 L/min (1 gpm) was turned on, and a third 250 mL pre-treatment sample was collected from the sample port just upstream of the Fuel-Mag device. The test rig valves were then aligned to direct fuel flow through either the BCA device or the Fuel-Mag device. Samples (250 mL) were collected after 90 sec flow through the designated treatment device, and then the valves were aligned to direct

flow through the second device.

In order to test the effects of multiple treatments, the balance of each 3.8 L portion (after samples had been taken) of treated fuel was retained in a previously unused, methanol disinfected, polypropylene reservoir. Subsamples were collected for UV_m and quartz treatment exposure. The balance of Fuel-Mag treated fuel was then was run through the BCA device and the balance of BCA treated fuel was run through the Fuel-Mag device. Portions of the BCA→Fuel-Mag and Fuel-Mag→BCA treated fuels were then treated by UV_m. As shown in Table 2, 12 of 48 possible treatment combinations were tested.

Table 2. Non-Chemical Treatments Evaluated 09-13 August 2010

Treatment			
BCA	UV_m	BCA→UV_m	Fuel-Mag→UV_m
Fuel-Mag	BCA→Fuel-Mag	Fuel-Mag→BCA	BCA→Fuel-Mag→UV_m
Quartz	BCA→Quartz	Fuel-Mag→Quartz	Fuel-Mag→BCA→UV_m

Biomass Testing

Adenosine Triphosphate (ATP)

ASTM Method D 7687 [9] was used to determine ATP concentrations in Briefly, a 5 mL sample is pressure filtered through a 0.7µm NPS, glass-fiber, in-line filter. The sample is washed and air dried to remove interfering chemicals and extracellular ATP. The microbial cells retained on the filter are then lysed and the ATP extract lysate is captured in a reaction tube. The lysate is then diluted 1 to 10 and 100 µL of diluted ATP extract is mixed with 100 µL Luciferin-Luciferase reagent and placed into a luminometer. Luminescence is recorded as relative light units (RLU). All RLU data are converted to pg ATP/mL by comparing test sample RLU against the RLU obtained from 100 µL of a 1.0 ng of ATP/mL standard.

Live/Dead Direct Counts

Live/Dead® BacLight™ and Live/Dead FungaLight™ test kits were used to quantify biomass in samples. Background luminescence and insufficient cells/microscope field necessitated modification of the manufacturer's protocol. In order to obtain quantifiable cells/field from which to compute cells/mL, 5 mL samples were filtered through a 0.45 µm pore-size, black, polycarbonate filter. The filters were then washed before proceeding with the manufacturer's protocol. In the protocol, live cells fluoresce green and dead/moribund cells fluoresce red. Stained cells on membranes were counted using an Olympus (Tokyo) BX50 microscope equipped with epifluorescence illumination, a DP25 digital camera and cellSens [ver.1.33] imaging software. Raw data were converted to live cells/mL and dead cells/mL, and the live to dead cell ratios were computed. Live/Dead Direct Counts were performed by Situ Biosciences,

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Culture Testing

During the Series 2 testing effort, a sub-set of fuel samples was tested for culturable bacteria and fungi. Initially, culture testing was performed in accordance with ASTM D 6974 [8]. Briefly, 5.0 mL samples were filtered through 0.45 µm NPS, sterile, gridded, cellulose acetate membrane filters, held in in-line filter housings. Each filter was then placed onto either trypticase soy agar (bacterial enumeration) or Sabouraud dextrose agar (fungal enumeration). After just a few filtrations the elastomeric O-ring that provided the seal between the filter and filter-housing swelled. Consequently it no longer provided the necessary seal and fuel leaked from the upstream side of the filter. All subsequent culture tests were performed using the streak-plate method. A 10 µL portion of liquid sample was collected using a standard inoculating loop, and the sample was then streaked across the surface of the culture dish containing the growth medium.

Both filters and streak plates were incubated at 25°C for up to five days. Each day, the number of colonies was counted. When the number of colonies on a membrane no longer increased, the colony counts were converted to colony forming units (CFU)/mL.

3.7 Microbicide Effect on ATP

An uncharacterized soil bacterium was grown in yeast extract broth, diluted in phosphate buffer to approximately 10 ng ATP/mL and dispensed as 100 mL portions into seven bottles. Each bottle was treated as indicated in Table 3. After 0, 6 and 24h post-treatment, ATP concentration was determined.

Table 3. Test Matrix; Effect of Chemical Microbicides on ATP Concentration of an Uncharacterized, Soil Isolate Bacterium

Bottle No.	Treatment	Dose (mg a.i.[a]/L)
1	Untreated Control	0
2	ADBAC[b]	50
3	ADBAC	100
4	BNPD[c]	93.7
5	BNPD	187.4
6	Hydrogen Peroxide	50
7	Hydrogen Peroxide	100

a – a.i.: active ingredient
b – ADBAC: Alkyl Dimethyl Benzyl Ammonium Chloride
c – BNPD: Bromonitropropanediol

Biofilm Inhibition Testing

A series of four, 250 mL, wide-mouth, glass jars was set up to run a preliminary evaluation of the effect of non-chemical treatments on biofilm formation (Figure 7). Each microcosm contained 100 mL of Jet A-1 over 10 mL of Bushnell-Haas broth. The Jet A-1 samples used are listed in Table 4. Glass microscope slides were rinsed with acetone and deionized water before being placed into microcosm jars. The glass slides were positioned so that at least 1 cm was submerged in the aqueous-phase of the microcosm. After two, four and eight weeks, one glass slide was removed from each microcosm and observed at low and high power magnification. Two 1 cm^2 areas (1 cm2 from portion of slide that had been exposed to the aqueous-phase and 1 cm^2 from the portion exposed to fuel just above the fuel-aqueous-phase interface; Figure 8) of each slide were tested for ATP and AMP.

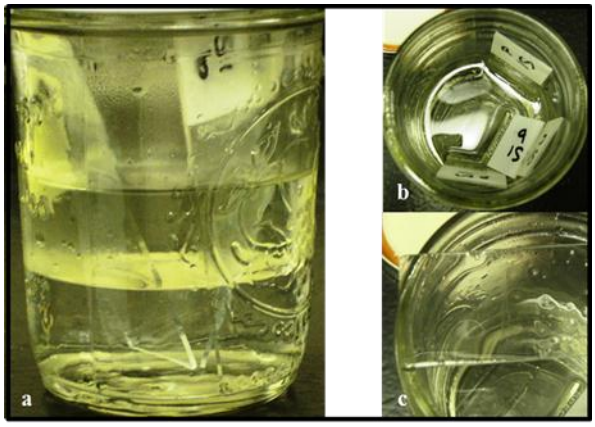

Figure 7: Preliminary Biofilm Evaluation Microcosm – a) *P. aeruginosa* challenged, untreated Jet A fuel over BHM; b) microcosm top view; c) close-up view; glass slide after two-weeks exposure

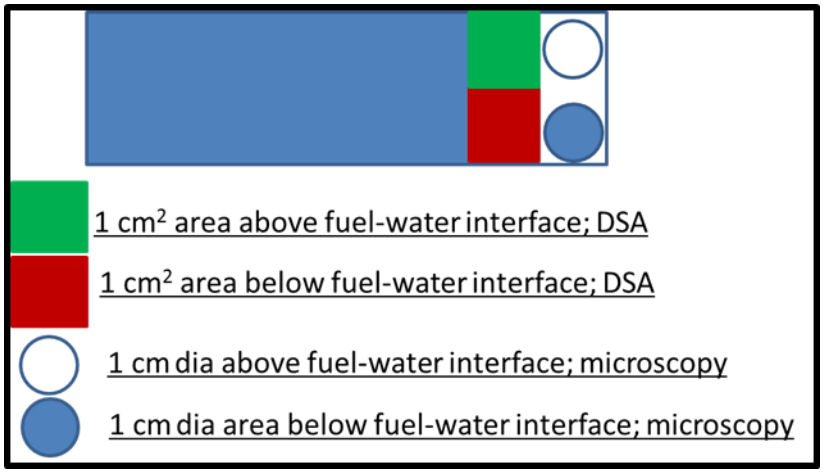

Figure 8: Schematic of Glass Microscope Slide Used for Biofilm Evaluation; Showing Areas Examined Microscopically and Areas Sampled for ATP

Table 4. Biofilm Evaluation Microcosm Fuels

Jar	Treatment	Source [a]
1	*P. aeruginosa* Challenged Control	Composite; 25 mL ea. from samples # 5, 13, 19 & 23
2	BCA	Composite; 50 mL ea. from samples 2 & 4
3	BCA→UV_m	50 mL ea. from samples 64 & 66
4	EBE	50 mL ea. from samples 30 & 32

a – fuels from Series 2 testing, sample numbers refer to Sample I.D. on Fuel Sample inventory sheet [10].

Data Analysis

The effects of each of the BCA and Fuel-Mag treatments and of the 12 different Series 1 treatment combinations were tested for significance using two-way Analysis of Variance (ANOVA) with replication (triplicate samples of three test runs x 2 treatments). The effects of the UV treatments were tested for significance by one-way ANOVA. All ANOVA computations were completed using the Microsoft Excel Analysis Toolpak add-in.

RESULTS

Challenge Culture Biomass
Cultures; Series 1

In preparation for the Series 1 testing effort, *P. aeruginosa* was grown in BHM (85 mL + 5% $^{v}/_{v}$ fuel) augmented with either JRF3 with DiEGME or JRF3 without DiEGME. The ATP data (Table 5) from the two types of broths demonstrated that DiEGME did not affect the growth of *P. aeruginosa* in BHM.

Table 5. *P. aeruginosa* in Bushnell Haas Medium under JRF3 with and without DiEGME; pg ATP/mL

JRF3	Replicate	Log pg ATP/mL
With DiEGME	1	4.39
	2	4.36
Without DiEGME	1	4.34
	2	4.40

Based on these results, and recognition of the possibility that DiEGME might interfere with the treatment effects, it was decided to work with Jet A-1 (DiEGME-free fuel).

After it was determined that there was insufficient *P. aeruginosa* broth with which to challenge the 57 L (15 gal) of Jet A-1to be used in the Series 1 tests, it was decided to determine the biomass of *A.* sp. RAG-1 that was available. The ATP concentration was 4.90±0.002 Log pg

ATP/mL.

Just before being used to test the effects of either the BCA or Fuel-Mag treatment, 19 L portions were doped with approximately 100 mL *A.* sp. RAG-1 broth. Table 6 shows a comparison between the expected (theoretical) and observed ATP concentrations in each of the three 19 L Jet A1 challenged fuel preparations.

Table 6. Recovery of *A.* sp. RAG-1 ATP from Challenged Jet A1 Fuel Reservoirs

Reservoir	Fuel			Inoculum				
	Wt (lb)	Vol (gal)	Vol (L)	Vol (L)	d.f.[a]	Log pg ATP/mL		% Recovery
						Theor.[b]	Obs.	
1	31.0	4.67	17.7	0.10	177	3.28	1.41	1.4
2	31.0	4.67	17.7	0.13	136	3.38	2.05	4.5
3	31.5	4.74	18.0	0.11	164	3.04	2.28	17

a – d.f.: dilution factor
b – Theor.: theoretically expected Log pg ATP/mL, computed from inoculum volume, Log pg ATP/mL inoculum and dilution factor.

Cultures; Series 2
P. aeruginosa

The ATP concentrations in the two broths from which *P. aeruginosa* was harvested were 5.01 and 5.03 Log pg ATP/mL, respectively. After harvesting the ATP concentration in the suspension of cells in Jet A was 5.03 Log pg ATP/mL. This confirmed that the harvesting protocol was effective.

To estimate the effect of resuspending 1.5 mL of *P. aeruginosa* into 17 L fuel, percent recovery was determined. Based on a dilution factor of 1.1×10^3 the challenged fuel should have yielded ~100 pg ATP/mL. The actual pg ATP/mL from six challenge pail samples (Table 7) was 2±0.7 – approximately 2% of the expected yield (where the expected yield was the [ATP] in the *P. aeruginosa* suspension ÷ the dilution factor).

H. resinae

Similarly, the observed *H. resinae* ATP concentration (Table 7) after dilution into 17 L of Jet A-1 was 11% of that expected based on the dilution factor (50 mL of *H. resinae* suspension into 17 L fuel; d.f. = 340).

Cell Harvesting

The initial plan was to harvest cells from BHM by centrifugation at 5,000 x g for 15 min, dispense supernate and resuspend the cells in a small volume of fuel for further dilution to achieve 2 to 3 Log pg ATP/mL in the challenged test fuel. *P. aeruginosa* grown in JRF3 with DiEGME did not form a pellet. However, P aeruginosa grown in JRF3 without DiEGME did

from a good pellet. Apparently the DiEGME affected cell settling. *A.* sp. RAG-1 also formed a good pellet. However, the cells in the pellet resisted dispersion even after vortexing at maximum speed for 1 min (Figure 9).

After centrifugation for 10 min at 5,000 x g, the ATP concentration in the aqueous-phase supernate over *P. aeruginosa* pellets was 2.5±0.3 x10^4 pg ATP/mL (19% of the pre-centrifugation ATP concentration).

Sonication facilitated the harvesting of *P. aeruginosa*. Figure 10 shows the uniform turbidity of fuel into which *P. aeruginosa* pellets had been redispersed. After all of the pellets were harvested the ATP concentration in the 6 mL of dispersed *P. aeruginosa* was 1.1 x 10^5 pg ATP/mL. Each challenge portion had approximately 0.1 mL of residual *P. aeruginosa* aggregated mass (Figure 11). The ATP in the aggregated mass was not determined.

The *H. resinae* spherical colonies were harvested from the broth's fuel phase (Figure 5) and collected into a 15 mL centrifuge tube (Figure 6). The colonies were broken up by sonicating for 10 min, then stomaching in a plastic bag (Figure 12a). The sonication and stomaching processes were repeated two more times to create a cell dispersion (Figure 12b and 12c). This process created a three-phase product (Figure 12d), which, overnight became two phases (Figure 12e).

Table 7. Effect of Dilution in Jet A on *P. aeruginosa* and *H. resinae* pg ATP/mL

Organism	Pail	[ATP]
P. aeruginosa	Inoculum	107,000
	1	1.9
	2	2.8
	3	2.9
	4	2.7
	1+2 combo	1.1
	3+3 combo	1.0
	AVG	2.1
	SD	0.9
	Computed from inoculum d.f.	97
	% recovery	2%
H. resinae	Inoculum	1,800
	5	0.4
	6	0.8
	7	0.6
	8	0.5
	AVG	0.6
	SD	0.17
	Computed from inoculum d.f.	5.3
	% recovery	11%

Figure 1: *Acinetobacter* sp. RAG-1 After Centrifugation for 15 min at 5,000 x g and Attempted Resuspension in Jet A1 Fuel

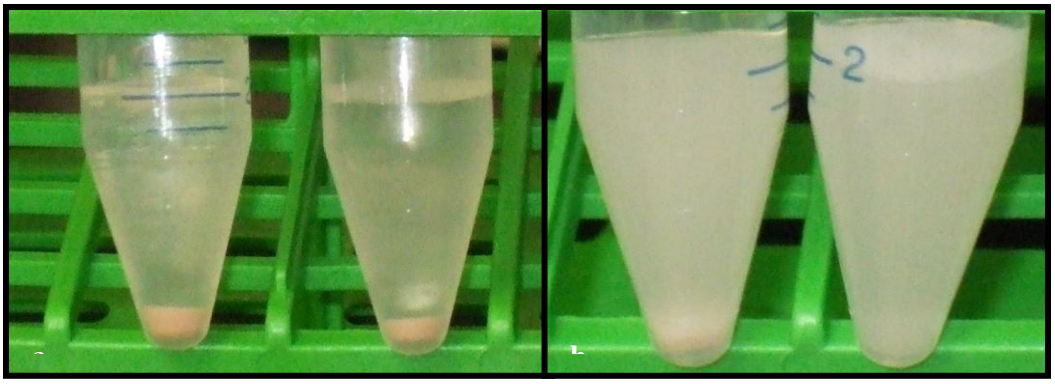

Figure 10: *P. aeruginosa* Centrifuged Pellets Resuspended in Jet A-1

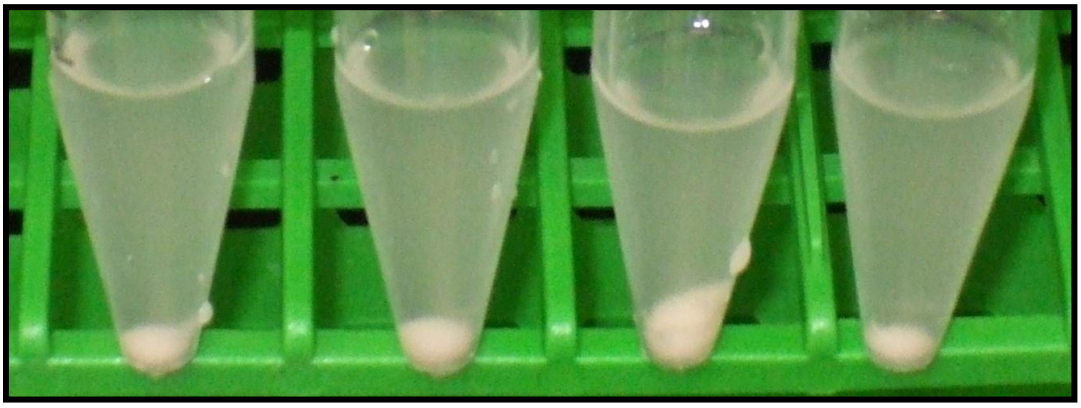

Figure 11: *P. aeruginosa* Challenge Preparations Ready for Dosing Four Pails of Jet A-1; ~17L Fuel per Pail

The concentrations of ATP in the fuel, middle (cell-mass) and bottom (aqueous) phases were:

3.36 Log pg ATP/mL, 6.55 Log pg ATP/mL and 4.79 Log pg ATP/mL, respectively. The mass of *H. resinae* hyphae were transferred to a 500 mL HDPE bottle, from which the top 3 cm had been removed and to which 50 Jet A-1 had been added. This preparation was blended for 2 min at 1,000 rpm with a hand held blender (Figure13a), sonicated for 10 min and blended a second time for 1 min to produce the challenge preparation (Figure 13b). The fuel was dispensed as 10 mL aliquots into five 15 mL centrifuge tubes (Figure 13c). The ATP concentrations in the five challenge portions ranged from 1.2 x 10^3 pg ATP/mL to 2.7 x 10^3 pg ATP/mL (1.8±0.6 x 10^3 pg ATP/mL).

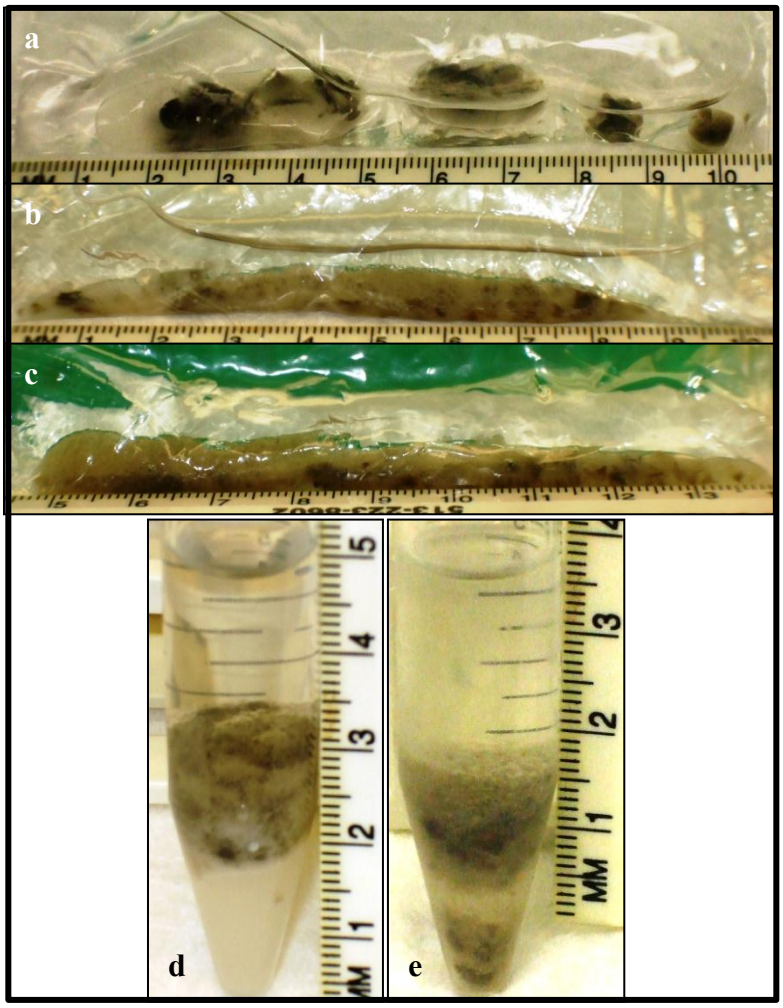

Figure 12: Stages in Preparation of *H. resinae* Dispersion for use as Jet A-1 Challenge

Effects of Non-Chemical Treatments
Series 1 Testing

None of the treatments appeared to affect ATP concentrations significantly. The BCA and Fuel-Mag test results are summarized in Table 8 and the UV irradiation results are presented in Table 9. Table 10 summarizes F-ratios for the three different treatments. The ANOVA results confirm the apparent absence of significant effect suggested by inspection of Tables 8 and 9.

Figure 2: Shearing *H. resinae* Hyphae with Hand-Held Blender.

Series 1 Samples; Six-Weeks Post-Treatment

Retains of the fuel samples collected on 23 and 24 June 2010 were stored at 4C. On 09 August, ATP and AMP tests were run on retains from the each of the three runs: challenge fuel, Fuel-Mag treated, BCA treated, UVc-irradiated and UVm-irradiated.

The data, presented in Table 11, demonstrate that the *A.* sp. REG-1 populations proliferated in all of the fuel samples. However, ATP concentrations in the fuel samples that had been exposed to either the BCA or Fuel-Mag treatments were 62 to 94% less than ATP concentrations in the untreated challenge fuel samples. In contrast to the BCA and Fuel-Mag treatments, ATP concentrations in the UV irradiated samples were dramatically greater (1 Log pg ATP/mL

greater) than in the control samples.

Table 1. Effect of BCA and Fuel-Mag Exposure on ATP Concentrations in Jet A-1 Fuel

Run	Replicate	pg ATP/mL		
		Pre Treat	BCA	Fuel-Mag
1	1	29	61	101
	2	23	19	154
2	1	111	330	105
	2	113	135	469
3	1	249	131	369
	2	1,057	142	508
AVG		264	136	284
SD		397	107	186

Table 9. Effect of UV_c and UV_m Exposure on ATP Concentration in Jet A-1 Fuel

Rep	Challenge Fuel	pg ATP/mL			
		UV_c 20 sec exposure		UV_m 30 sec exposure	
		1mm	1cm	1mm	1cm
1	72	280	4	48	50
2	56	645	35	42	35
AVG	64	580	20	45	42
SD	11	273	22	4	11

Table 10. ANOVA Summary; Effects of BCA, Fuel-Mag and UV Irradiation on ATP Concentration in Jet A-1 Fuel

Factors	F-ratio $_{observed}$	F-ratio $_{critical\ [P=0.5]}$
BCA x Fuel-Mag x Control	0.13	4.26
UV_c – 1 mm x 1 cm	5.8	18.5
UV_m – 1 mm x 1cm	0.12	18.5
UV_c x UV_m	4.6	7.7

Series 2 Testing

Effects of Single treatments

The effects of the non-chemical treatments were assessed primarily by their impact on ATP concentration ([ATP]). Culturability data were used as a corroborating measurement. Table 12 presents the *P. aeruginosa* data for all treatments. The BCA and EBE treatments were apparently more effective than either the fuel-mag treatment or UV_m irradiation against both [ATP] and CFU/mL. However, since the [ATP] were all near the limit of quantification (LOQ) the differences are not statistically significant. However, the BCA results are consistent with those obtained in the first test series. The earlier series did not include the EBE treatment. Both BCA and Fuel-Mag treatments reduced [ATP] significantly in the June tests. Fuel-Mag did not seem to affect either [ATP] or CFU/mL in this test series.

None of the treatments affected the total cell microscopic direct counts obtained by L/DCD. However for all samples, the average number of cells per microscopic field was <7. Preferably there should be 20 to 80 cells per field in order to obtain statistically valid data. Direct counts were made on membranes through which 50 mL of fuel had been filtered. The percentage of live cells per field was quite variable. Consequently, although both BCA and Fuel-Mag treatments appear to have reduced the percentage of live cells, the differences were not statistically significant ($F_{obs} = 1.21$; $F_{0.05[2,5]} = 5.79$).

The *H. resinae* data are shown in Table 13. As noted above, the [ATP] in the untreated challenge fuel was only 0.6±0.17 pg ATP/mL. This is at the low end of the method's sensitivity limit (0.1 pg ATP/mL). Moreover, the H. resinae culture data were all near or below the lower detection limit (10 CFU/mL) of the method. Consequently, no supportable conclusions can be drawn from the H. resinae test results.

Effects of Multiple, In-Series Treatments

Because each of the candidate treatments is expected to affect the cells differently, it is likely that sequential treatment with multiple types of non-chemical devices will demonstrate synergistic antimicrobial performance. The [ATP] data did not reveal any synergies. However, the culture data suggest that there may be have been synergistic effect when *P. aeruginosa* was exposed to UV_m after having been exposed to BCA, Fuel-Mag or both (Table 12). As noted above, both the [ATP] and CFU/mL data were too close to the LOQ and LDL to permit any assessment of performance against *H. resinae*.

Effects of Antimicrobial Pesticide Treatments

The results reported in Table 8 and 9 suggest that the ATP tests had been run too quickly after the fuel samples had been exposed to the non-chemical treatments. To test this hypothesis, three different types of microbicides were used. ADBAC is a quaternary ammonium compound, known to have strong surfactant properties and consequently to lyse cells rapidly. A rapid, post treatment decrease in ATP would be expected. BNPD is an organic, non-oxidizing agent, known to target the cell's electron transport system. A 6 to 12h delay in observable effects would be expected. Hydrogen Peroxide (H_2O_2) is an oxidizing agent. Catalase enzyme in the cells hydrolyses H_2O_2. Moreover, H_2O_2 has a short half-life in water. At the doses used (Table

3), H_2O_2 would be expected to initially decrease ATP until the population recovers.

Table 2. Effect of BCA, Fuel-Mag and UV Exposure on ATP Concentrations in Jet A-1 Fuel Six-Weeks Post-Treatment

Run	Sample	Log pg ATP/mL	% loss of ATP
1	Pre Treat	4.93	0%
	Fuel-Mag	4.18	82%
	BCA	4.48	65%
2	Pre Treat	5.39	0%
	Fuel-Mag	3.78	98%
	BCA	4.34	91%
3	Pre Treat	5.47	0%
	Fuel-Mag	4.28	94%
	BCA	4.23	94%
1	Pre Treat	6.01	0%
	UV_c 1 mm	6.94	-757%
	UV_c 1 cm	7.53	-3255%
2	Pre Treat	7.51	0%
	UV_m 1 mm	7.97	-190%
	UV_m 1 cm	8.09	-284%

A 6 to 12h delay in observable effects would be expected. Hydrogen Peroxide (H_2O_2) is an oxidizing agent. Catalase enzyme in the cells hydrolyses H_2O_2. Moreover, H_2O_2 has a short half-life in water. At the doses used (Table 5), H_2O_2 would be expected to initially decrease ATP until the population recovers.

The test results, presented in Table 14, confirm the three hypotheses. When treated with ADBAC, the ATP concentration drops in <6h. When treated with BNPD, 24h are needed before the ATP concentration is reduced by >90%. The ATP concentration initially falls by 52 to 70% in the samples treated with 50 and 100 mg a.i. H_2O_2/L respectively, and recovers by T_{24}.

These results confirm that samples should not be tested for ATP until at least six-hours, post-treatment.

UV Light Penetration of Jet A-1 Fuel

Figure 14 shows the light absorbance spectrum for 1% w/w fuel 4877 in hexane (200nm to 400nm). The fuel-hexane solution is opaque to light at wavelengths ≤ 280nm. Hexane absorbance at 280 nm is 0. Consequently, all of the light absorbance is due to the fuel.

Table 3. Effect of Non-Chemical Treatments on *P. aeruginosa*

Treatment	pg ATP /mL	CFU/mL	Cells/ mL x 10^7	% Live Cells
Untreated	3 ±0.5	40±38 [a]	1.5±0.56	40±17
BCA	0.6±0.3	5.5±0.7	1.5±0.64	30±17
Fuel-Mag	2.4±0.9	30±20	2.0±0.92	20±12
UV_m	1.7±0.8	30±45	N.D. [b]	N.D.
EBE	1.0±0.1	<1	N.D.	N.D.
BCA → Fuel-Mag	1.2±0.4	4.0±0.0	N.D.	N.D.
Fuel-Mag → BCA	2.4±0.4	30±47	N.D.	N.D.
BCA → UV_m	1.0±0.2	<1	N.D.	N.D.
BCA → EBE	1.0±0.3	2±2.8	N.D.	N.D.
Fuel-Mag → UV_m	5±4.2	<1	N.D.	N.D.
Fuel-Mag → EBE	1.0±0.2	9±9.4	N.D.	N.D.
BCA → Fuel-Mag → UV_m	1.0±0.9	<1	N.D.	N.D.
Fuel-Mag → BCA → UV_m	3±2.5	<1	N.D.	N.D.

a – The number of colonies on control plates ranged from 2 CFU to too numerous to count (TNTC); The CFU/mL in this table are the averages of the countable plates.
b – N.D.: not determined; not tested
c – Only a single test was run; no replicates

In order to further examine the effect of Fuel 4877 on UV light, test samples were prepared by diluting the 1% stock solution with hexane to fuel concentrations of 1,000, 500 and 250 ppm (wt.) to view the absorbance spectrum below 300 nm. In addition to the test solutions, a hexane blank (0 ppm fuel) was also examined. Figure 15 shows an overlay of the four absorbance curves in the range 200 – 1,100 nm. Figure 16 shows a view of the curve overlays specifically in the 200-300 nm range where the primary fuel absorbances occur. As seen in figure 14, the 1,000 ppm solution provides a 240 – 300 curve that is on-scale to allow penetration depth calculations for the 254 nm wavelength produced by the low pressure (UV_c) mercury lamp and the 200 – 365nm range produced by the medium pressure (UV_m) mercury lamp.

Based on the spectra in Figure 16, it was calculated that a penetration depth of 254 nm in Fuel 4877 would be approximately10 μm (penetration depth increases as aromatics decrease; > 1cm for hexane with 0% aromatics).

Effect of Treatment on Biofilm Development

The primary treatment objective is substantial delay of biofilm development and a measurable adverse effect on the tenacity of the biofilm. As a first step, fuels exposed to BCA, BCA→UV_m or EBE treatment were placed over BHM (Table 4).

The ATP-biomass of a mature biofilm community is expected to range from 104 to107 pg

ATP/mL. After two week's exposure, [ATP] ranged from 180 to 810 pg ATP/cm2 on the surface of glass slides that had been immersed in the aqueous-phase (BHM) biofilm and ranged

Table 43. Effect of Non-Chemical Treatments on *H. resinae*

Treatment	pg ATP/ mL	CFU/mL	Cells/mL	% Live Cells
Untreated	0.6±0.17	1±2.0	<5.94E+05	N.D.
BCA	0.4±0.09	<1	5.94E+05	100
Fuel-Mag	0.5±0.45	2±2.1	N.D.	N.D.
UV_m	0.6±0.3	<1	N.D.	N.D.
Quartz	7 [a]	<1	N.D.	N.D.
BCA ® Fuel-Mag	0.2±0.02	1±1.0	N.D.	N.D.
Fuel-Mag ® BCA	0.2±0.02	<1	N.D.	N.D.
BCA ® UV_m	0.4±0.21	<1	N.D.	N.D.
Fuel-Mag ® UV_m	0.5±0.5	<1	N.D.	N.D.
BCA ® Fuel-Mag ® UV_m	±	±	N.D.	N.D.
Fuel-Mag ® BCA ® UV_m	±	±	N.D.	N.D.

a – Only a single test was run; no replicates

from 50 to 480 pg ATP/cm2 on the surface of glass slides that had been exposed to the fuel-phase. However, after 30 day exposure, significant differences were observed. The results in Figures 19 and 20 demonstrated that exposure to the treatments inhibited the abilities of the surviving microbes to form biofilms. After 45days immersion, the ATP biomass on the area of the coupon that was exposed to fuel was 4.39, 1.53, 3.11 and 1.18 Log pg ATP/cm^2 for coupons in the control, BCA-treated, BCA$\rightarrow UV_m$ treated (fuel disinfected by BCA then exposed to UV_m) and EBE-treated fuels respectively (Figure 17). ATP biomass on the areas of coupons that were exposed to the aqueous-phase was 4.40, 1.08, 1.18 and 1.30, respectively (Figure 18). The BCA and EBE treatments inhibited biofilm formation by > 99%.

DISCUSSION

Experimental Design

The four primary parameters that influenced the experimental design of this project included:

- Selection of challenge microbes
- Test rig design; including treatment technology selection
- Parameter selection
- Replication

Each of these parameters affected the outcome of the research effort and warrants consideration in this section.

Selection of Challenge Microbes

As noted previously, extenuating circumstances dictated the use of *A.* sp. RAD-1 for Series 1.

Neither *P. aeruginosa* nor *H. resinae* was available in quantities sufficient to achieve ≥ 100 pg ATP/mL in the volume of challenged Jet A-1 fuel needed for the test series. Had *A.* sp. RAD-1 post-suspension recoveries been ≥ 10% of the theoretical pg ATP/mL expected after dilution, the challenge fuel [ATP] would have been ≥ 100 pg ATP/mL. However, for reasons not yet fully understood, recoveries averaged 7.7±8.2%. Consequently, data variability eclipsed any variability due to treatment effects.

Table 5. Effect of Three Microbicidal Chemicals on Sample ATP Concentrations for First 24h Post-Treatment

Treatment	Dose (mg a.i./L)	Time (h) Post-Treatment	ATP	
			Log pg/mL	% T_0 Ctrl
Control	0	0	4.00	-
		6	3.97	92
		24	3.88	75
ADBAC [a]	50	0	3.52	33
		6	2.72	5
		24	2.67	5
	100	0	3.08	12
		6	2.72	5
		24	2.74	5
BNPD [b]	93.7	0	4.04	100
		6	3.30	20
		24	2.52	3
	187.4	0	4.04	100
		6	3.15	14
		24	2.30	2
H_2O_2 [c]	50	0	3.93	84
		6	3.68	48
		24	3.90	80
	100	0	8,300	82
		6	3,000	30
		24	8,000	70

a – ADBAC – Alkyl Dimethyl Benzyl Ammonium Chloride
b – BNPD – Bromonitropropanediol
c – H_2O_2 – hydrogen peroxide

However, the selection of *A.* sp. RAD-1 was fortuitous in several respects. In contrast to the *P. aeruginosa* and *H. resinae* cultures – which had been maintained in the ATCC culture collection for decades since they were originally isolated from fuel systems – the *A.* sp. RAD-1 culture had only been maintained as a type culture for several years. It is well known that type-culture strains are less robust than wild type microbes, recently isolated from the environment. Additionally, the proliferation and voluminous polymer production in the stored test rig

illustrated a central thesis of this project. It is insufficient to disinfect fuels. Surviving microbes will subsequently colonize surfaces and develop into biofilm communities. Moreover, during six-weeks of storage, *A.* sp. RAD-1 in untreated control and UV_m-treated retain samples had proliferated to biomass concentrations of 4.90 to 8.09 Log pg ATP/mL (a two to six Log increase in [ATP]). In contrast, the ATP biomass increase in BCA and Fuel-Mag tested Jet A-1 retains was only one to three Log pg ATP/mL. The latter two treatments had unequivocally inhibited *A.* sp. RAD-1 proliferation in the fuel retain samples.

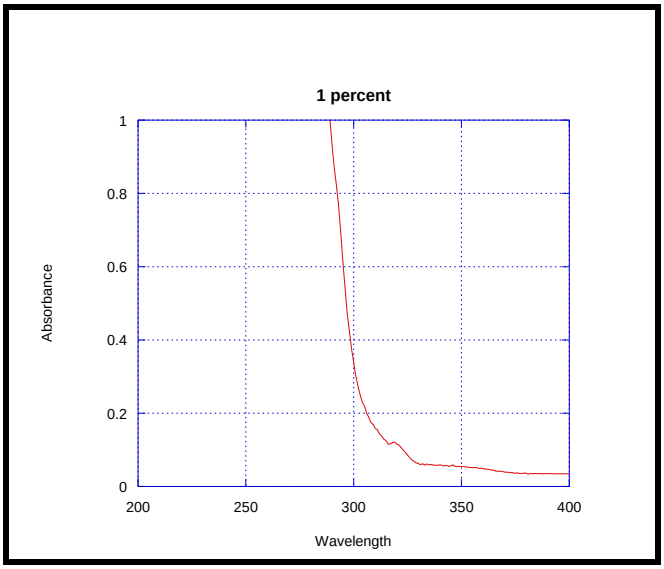

Figure 3: Absorbance Curve of 1% Fuel 4877 Solution in Hexane

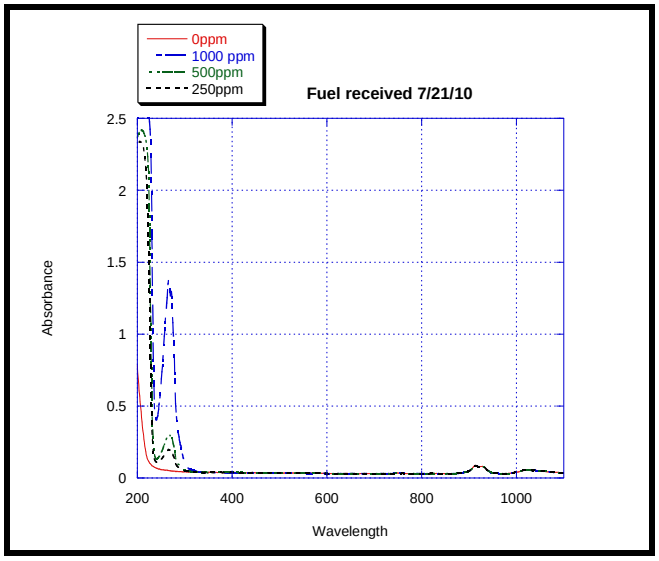

Figure 4: 200nm to 1,100nm Absorbance Curves of Various Fuel Concentrations

The two intended challenge cultures – *P. aeruginosa* and *H. resinae* were selected because they are among the test microbes listed in ASTM E 1259 [7]. This represents a compromise in that they no longer behave like wild type strains of the same organisms. Moreover, biodeterioration is

more typically mediated by microbial consortia [11]. Passman [12, 13, 14] has found that natural, mixed, uncharacterized microbial populations – either from contaminated fuel systems or commercial preparations – are more reliable fuel inocula than are pure cultures. Our experience during Phase I research as consistent with Passman's previous observations. The vulnerability of the test cultures to inhibition by exposure to Jet A-1 fuel created greater challenges than those that would otherwise have need posed by having used a challenge mixture. Future testing will use only mix-culture challenge populations.

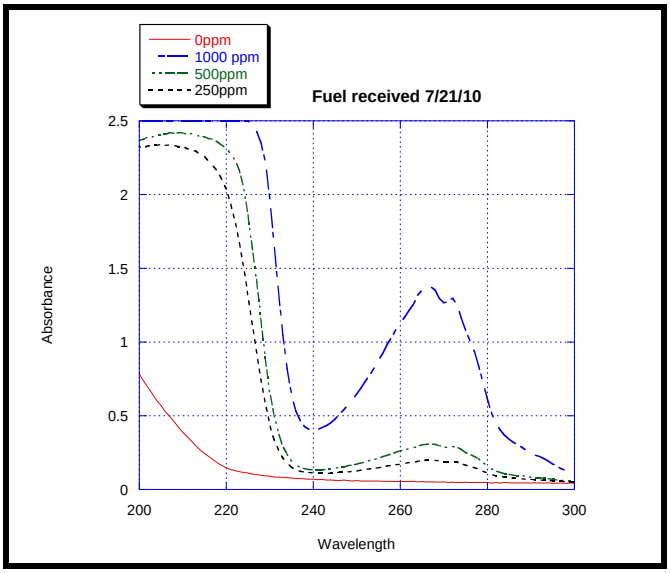

Figure 16: 200nm to 300nm Absorbance of Various Fuel Concentrations

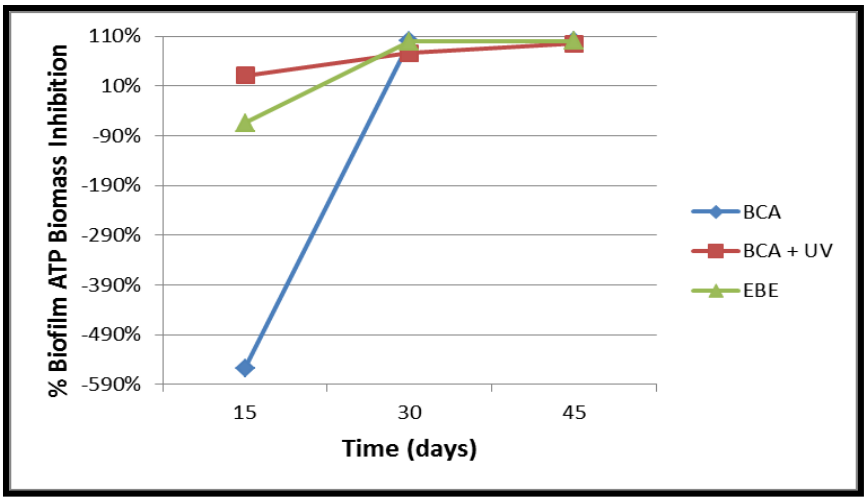

Figure 5: Inhibition of Biofilm ATP Biomass after Single-Pass Non-Chemical Treatment; Coupon-Fuel Interface

The challenge population preparation process also presented several unforeseen challenges. Concentrating culture biomass by centrifugation was simple enough. However, resuspending either bacteria or fungal cell pellets required considerable effort. The repeated cycles of sonication and high shear used to disperse cells into Jet A-1 fuel may have contributed to the low

ATP recoveries (< 2% of recovery expected, based on [ATP] in inoculum x dilution factor) in the challenge fuels. For future testing, the challenge population will be cultivated in larger vessels, in order to minimize the requirement for either cell concentration and resuspension or subsequent dilution into the challenge fuel.

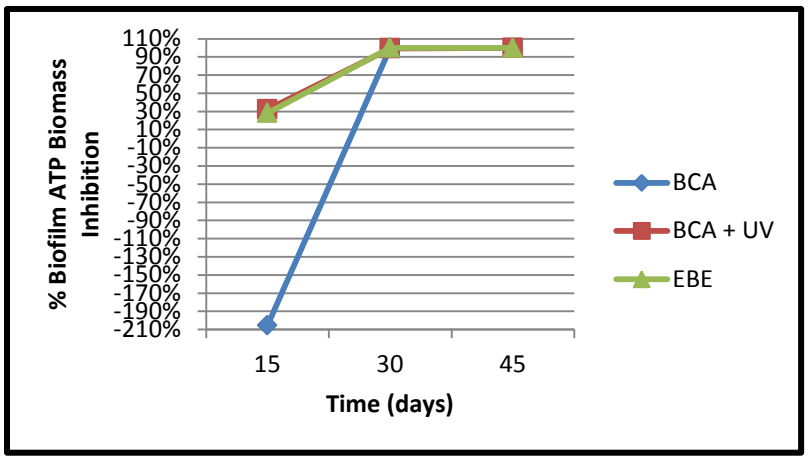

Figure 6: Inhibition of Biofilm ATP Biomass after Single-Pass Non-Chemical Treatment; Coupon-Aqueous-Phase Interface

Test Rig Design

The test rig worked well for most intended purposes. One limitation was that not all of the candidate technologies were in configurations that could be set up in the rig. Consequently, UV irradiation testing was completed on static samples in shallow petri dishes, and EBE testing was completed using gravity feed though a funnel. The UV data showed no discernable difference between UV_c and UV_m irradiation. The requirement for the fuel film to be ≤ 10 μm thick suggests that it is unlikely that it will be cost effective to fabricate a UV system that will effectively disinfect fuels at the volume and flow rates (>300 gpm) expected in aviation fueling systems.

The rapid proliferation of *A.* sp. RAG-1 in the system demonstrated that a rigorous chemical disinfection process was needed in order to prevent such proliferation between test runs. The use of a dense population of *Acinetobacter* probably represented a worse-case scenario, in-terms of post-treatment population recovery. Bacteria in the genus *Acinetobacter* are known to produce prodigious volumes of extracellular polymer. During the Series 1 testing *A.* sp. RAD-1 was used as a pure culture. As depicted in Figure 9, the cells were only partially disaggregated when the pellet was resuspended in Jet A-1. Cells within large (>100 μm dia clusters of cells embedded in biopolymer) were likely to have been protected from the full effects of the treatments. Although, as noted above, regrowth in BCA or Fuel-Mag treated fuel retain samples was substantially less than in untreated retains, regrowth did occur. This phenomenon will be investigated directly in the recirculating biofilm reactors planned for future studies.

For future work, the test rig's piping and valve system must allow for greater flexibility in directing flow through one or more treatment devices, creating single-pass or multi-pass

treatment exposures and for selecting whether to circulate treated fuel through the biofilm reactor.

Parameter Selection

The selection of which parameters to test can have a substantial impact on the apparent effect of treatment exposure. Passman [13] has demonstrated that culturability tends to provide optimistic indications of treatment efficacy. In tanks of ULSD fuel the Log CFU/mL dropped from 6 to <2 (<100 CFU/mL; the test method's LDL) within 24h after treatment with a microbicide. In contrast, [ATP] decreased by 1 to 1.5 Log pg ATP/mL after 72h. In other studies (Table 14 and unpublished) [ATP] decreases over the course of approximately 24h, then approaches a lower asymptote above which [ATP] remains for at least 7-days. This phenomenon has been observed whether the ATP test method includes aqueous extraction [15] or filtration and lysis [9], and is not yet understood.

ATP

The USAF Phase I solicitation letter specified the use of ATP as the primary test parameter to be used for measuring the antimicrobial effect of non-chemical treatment technologies. Passman [12] has discussed the advantages of ATP over culture methods. Briefly, ATP is present in all metabolically active cells. Therefore, it is a direct measurement of the active microbial population in the sample at the time of testing. For routing condition monitoring, the ability to obtain test results within a few minutes after collecting a sample is a significant advantage. During the course of this project, the research team determined that when testing for treatment efficacy, it is appropriate to wait for 24 to 48h after treatment before testing for ATP. Unless immediate cell lysis occurs, the cellular [ATP] will be consumed as long as metabolism continues. Cell death (in contrast to inability to elaborate into colonies on growth media) can take hours or days, depending on the antimicrobial treatment's mechanism of action. The ASTM D 7463 [15] method detects total ATP (cell-associated) and extracellular (dissolved ATP from lysed cells, ATP bound to cell fragments). The protocol used in this study [9] measures only cell-associated ATP. In this protocol extracellular ATP either passes through the filter (dissolved ATP) or is washed away (cell fragment bound ATP) before cells are lysed to extract cellular ATP. Consequently the protocol is considered to be a more accurate measure of metabolically active biomass.

Without introducing a pre-incubation step, designed to induce dormant cells and spores to become metabolically active, no ATP test effectively detects the presence of dormant cells (also called *persistor cells*), bacterial endospores or fungal spores. None of these cells are metabolically active. Consequently, the [ATP] cell is << 1 fg/cell. The greatest challenge with having used the ATP test in this study was that its lower detection limit with 50 mL sample portions is ~20 metabolically active cells/mL (~20,000 cells/L). As will be discussed in the next section, this limitation is largely offset by the low percentage recover of viable cells by culture methods.

Culture

The limitations of culture methods are well documented [16 to 21]. Typically <0.1% of the cells in a

sample will form colonies under the specific growth conditions under which they are cultivated, within the time allocated. Specific growth conditions include the chemical composition and water-activity of the growth medium, chemical composition of the atmosphere (concentrations of oxygen, nitrogen, carbon dioxide and other gases), temperature, pH, atmospheric pressure and incubation period. Viable cells that do not form colonies are classified as *viable but not culturable - VBNC*. The designation VBNC is an umbrella term that includes cells that are injured, but cannot proliferate on the growth medium, under the specified growth conditions, cells with long generation times, and taxa that will not grow under the test conditions. Standard test protocols such as ASTM D 6974 [8] specify both growth conditions and days of incubation before finalizing the colony count. Given the small size of bacteria (0.5 to 1 μm long), it take ~10^9 cells to form a colony that's sufficiently large to be visible to the naked eye. That's 30 generations. For bacterial species with short generation times (~30) visible colonies can form with 15h. A colony of a species with a 12h generation time needs 15 days to form a visible colony. Since most tests are terminated after 72h, this species would be classified as VBNC, unless the incubation period was extended to >15 days. Various types of non-lethal cell injuries impair a cell's ability to proliferate into a colony on solid growth media [16, 17, 18]. This is the major reason for the discrepancy between antimicrobial treatment effects on culturability and the effect on [ATP].

When ASTM D 6974 [8] was published, in-line filter housings that could be used for capturing planktonic microbes from fuel samples were readily available. Currently, these housings are not being produced. We attempted to use a similar in-line filter housing, but leakage through the elastomeric seal resulted in considerable sample loss. The alternative protocol that we used captured too small a sample volume (10 μL) to detect <1 CFU/mL (≈1,000 cells/mL). Moreover, the protocol was too labor intensive to be used practically for the large numbers of samples generated during each test series.

Live/Dead Direct Count

Theoretically, the L/DDC method was designed to quantify the relative numbers of live (green fluorescing) and dead (red-orange fluorescing) cells in a sample. Background fluorescence and low cell population densities rendered this method inapplicable. The UDRI facility did not have the imaging software needed to provide quantitative data. At Situ Biosciences, samples were pre-concentrated by filtration through black, polycarbonate filters. Still, for most samples, there was <1 cell/microscope field. Consequently, quantification of live and dead cell numbers in either control or treated fuel samples was impossible. The L/DDC method did provide data for biofilm development, however, depth of field issues made it impossible to obtain usable images. The biofilm ATP data sufficed for the biofilm inhibition tests.

Antimicrobial Treatment Performance

Acute Effects of Non-Chemical Treatment

Samples collected immediately before and after fuels were exposed to the candidate treatments were tested for acute effects; impact on [ATP], CFU/mL and L/DDC. As noted in the *Results* section, test results for all three of these parameters were at or below the method's LDL, LOQ or both. Consequently, few conclusions can be drawn from these data. There did not appear to be

any significant immediate effect on [ATP] during Series 1 testing. Series 2 results suggested that BCA exposure reduced both [ATP] and viable counts, but did not affect either total cells/mL or the live to dead cell ratio significantly (Table 12). These results must be verified using higher challenge population densities in the fuel to be treated. Future test plans provide for verification testing.

Immediate kill/knock-down of the contaminant population is helpful, but not sufficient to prevent biofilm development on system surfaces downstream of the treatment. Normally, when microbicides are used to disinfect fuels and fuel systems, a residual concentration remains in the treated tank to inhibit regrowth [4]. However, aircraft fuel tanks are routinely drained after microbicide treatment [6]. Although the chemical agent is left in contact with fuel-tank surfaces for 24h, after the fuel is drained, little residual microbicide remains to prevent regrowth on tank surfaces. Passman (unpublished) has observed that after tank cleaning and disinfection, it takes three to six months for a biofilm community to become reestablished. These observations are based on numerous client-confidential biodeterioration risk surveys during which one or more fuel storage tanks received a high biodeterioration risk score (a proprietary rating based on numerous variables, including climate, engineering, maintenance, gross observations, and fuel and fuel-associated water physical, chemical and microbiological data) and were subsequently cleaned and treated with a microbicide. The duration of this reestablishment period depends on a variety of factors including fuel quality, fuel turnover rate, housekeeping practices, residual microbicide concentrations and environmental conditions.

Prolonged Effects of Non-Chemical Treatment

The aircraft fuel-tank disinfection procedure is similar to the non-chemical treatment strategy. Once the fuel has been treated, there is no residual microbicidal effect. Consequently, the prolonged effects of treatment are critical to the cost-effective use of non-chemical technologies. During this investigation, we had a serendipitous opportunity to evaluate the impact of treatment exposure, six weeks post-exposure. Two of the three technologies that had been evaluated during Series 1 testing – BCA and Fuel-Mag – significantly inhibited *A.* sp. RAD-1 proliferation in Jet A-1 retain samples. These very preliminary results suggest that exposure had a prolonged effect on the exposed cells and their progeny. This phenomenon will be tested more thoroughly as part of future research.

More significantly, the Series 2 populations that had been treated by either the BCA or EBE devises inhibited biofilm development almost completely (Figures 17 and 18). The impact on biofilm formation is the most critical indication of treatment efficacy. Planktonic microbes suspended in fuel do little damage. Their population density is unlikely to be sufficient to plug filters or to affect fuel nebulization/combustion in the engine. However, biofilm communities can attack system surfaces; resulting in MIC [3, 23]. Biofilm accumulations on sensor surfaces cause inaccurate gauge readings [2]. Any treatment that does not inhibit biofilm formation is unlikely to be cost effective. Consequently, the preliminary biofilm inhibition results obtained during the Phase I research were very encouraging.

CONCLUSIONS

At this point in the research effort, it appears that both BCA and Fuel-Mag treatments inhibit proliferation and cause physiological stress. The next phase of testing is designed to first confirm the effects of the individual treatments and then assess the combined effects of exposing the fuel to two and three treatments in series.

No disinfection technology, short of sterilization, achieves 100% kill. Typically, 99.9% kill/inhibition is considered to be effective control. However, kill or inhibition of culturability of 99.9% of the planktonic microbes in fuel being delivered to an aircraft does not imply inhibition of subsequent colonization of aircraft fuel tank surfaces by microbes that survive the treatment. Consequently, the Series 1 and 2 tests were meant to provide an indication of the likelihood of a non-chemical treatment's efficacy in inhibiting biofilm formation.

We have demonstrated that the non-chemical treatment technologies are effective in reducing axenic culture biomass in Jet A. Data from both test series confirmed BCA performance. Data for the Fuel-Mal were more equivocal. A new technology – EBE – was tested as part of the Series 2 effort. It also demonstrated antimicrobial performance. Although there are insufficient data to draw statistically supportable conclusions, the preliminary work completed to date supports our theory that used singly or in combination the technologies tested during this research effort show promise.

Unintentionally, we validated the hypothesis that microbes that survive single-point, non-chemical treatment are likely to colonize downstream surfaces and proliferate into biofilm communities. The *Acinetobacter* sp. RAG-1 culture used for the Series 1 testing proliferated in the test rig during six-week's storage. We will also have preliminary data on the impact of single exposure treatment to inhibit the ability of *P. aeruginosa* to form biofilm communities on immersed glass slides.

The work completed to date provides a strong foundation for continued research. The candidate technologies are well suited for recirculating systems characteristic of airfield fuel systems. Equipment designed to handle the 300 to 1,200 gpm flow-rates used to pumps Jet A to fueling hydrants is well suited for pump-house installation. To be commercially viable, however, the treatment system must inhibit biofilm formation. Consequently, the next phase of research will focus on testing biofilm inhibition. Additionally, the axenic, ATCC cultures used during Phase I will be replaced with either an indigenous or commercially available mixed population of organisms adapted to growth in fuel systems. Once the appropriate treatment system has been properly vetted at 1 GPM operation, we will fabricate and test an appropriately scaled-up unit to demonstrate that the technology will be scalable for use in a variety of fuel handling systems, including airfields, power generation systems and marine vessels.

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