AFRL-RZ-WP-TR-2010-2094

EFFECT OF DIETHYLENE GLYCOL MONOMETHYL ETHER (DiEGME) AND TRIETHYLENE GLYCOL MONOMETHYL ETHER (TriEGME) ON MICROBIAL CONTAMINANTS IN AVIATION FUEL

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MARCH 2010
Interim Report

AIR FORCE RESEARCH LABORATORY
PROPULSION DIRECTORATE
WRIGHT-PATTERSON AIR FORCE BASE, OH 45433-7251
AIR FORCE MATERIEL COMMAND
UNITED STATES AIR FORCE

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
March 2010	Interim	01 August 2007 – 01 August 2009

4. TITLE AND SUBTITLE

EFFECT OF DIETHYLENE GLYCOL MONOMETHYL ETHER (DiEGME) AND TRIETHYLENE GLYCOL MONOMETHYL ETHER (TriEGME) ON MICROBIAL CONTAMINANTS IN AVIATION FUEL

5a. CONTRACT NUMBER
F33615-03-2-2347

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER
62203F

6. AUTHOR(S)

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5d. PROJECT NUMBER
3048

5e. TASK NUMBER
04

5f. WORK UNIT NUMBER
304804AK

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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Air Force Materiel Command, United States Air Force

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Air Force Research Laboratory
Propulsion Directorate
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Air Force Materiel Command
United States Air Force

10. SPONSORING/MONITORING AGENCY ACRONYM(S)
AFRL/RZPF

11. SPONSORING/MONITORING AGENCY REPORT NUMBER(S)
AFRL-RZ-WP-TR-2010-2094

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES

PAO Case Number: 88ABW-2009-3882, Clearance Date: 09 September 2009. Report contains color.

This microbiological study was undertaken as part of a larger Reduction of Total Ownership Cost (RTOC) FSII replacement study. The RTOC efforts were funded by the DoD RTOC office through ASC/EN. The FSII replacement study was conducted jointly by AFRL (RZPF and MLSA), AFPET, ASC/EN, UDRI, UTC, C4e, B-52 SPO, and the KC-135 SPO.

14. ABSTRACT

Diethylene glycol monomethyl ether (DiEGME) is widely used in military aircraft to inhibit both fuel system icing and microbial growth. However, due to the deleterious effects of the additive, the RTOC program was conducted to determine if it is possible to replace DiEGME in military aircraft fuel. The study presented here was performed in support of this program, which was designed to investigate the possible mission impact of replacing DiEGME with TriEGME. The current study was confined to the impact upon the biocidal/biostatic properties of the proposed additive. This study suggests that similar amounts of DiEGME and TriEGME are needed to retain biocidal/biostatic effectiveness, and that the substitution of TriEGME for DiEGME will result in equivalent protection from microbial contaminants.

See Alternate Abstract on reverse →

15. SUBJECT TERMS
bioburden, aviation fuel, bacteria, fungus, microbial contaminants, DiEGME, FSII TriEGME

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT:	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON (Monitor)
a. REPORT	b. ABSTRACT	c. THIS PAGE			Ellen Strobel
Unclassified	Unclassified	Unclassified	SAR	58	19b. TELEPHONE NUMBER *(Include Area Code)* N/A

14. ABSTRACT (alternate)

Triethylene glycol monomethyl ether (TriEGME) is under consideration as a replacement for diethylene glycol monomethyl ether (DiEGME), an additive widely used in military aircraft as a fuel system icing inhibitor (FSII) and as an inhibitor of microbial growth. Currently, DiEGME's high dosage rate results in significant expense for the Air Force and Navy, and DiEGME has also been implicated in a number of aircraft system problems, including fuel tank topcoat peeling in the B-52. As a result, an investigation is underway to determine if it is possible to replace DiEGME with TriEGME. TriEGME is a similar molecule, but its lower vapor pressure would eliminate the topcoat issue, and its higher partition coefficient may allow more of it to enter the water phase at a given temperature, making it more dose effective than DiEGME (that is, given the same concentration of DiEGME and TriEGME, more TriEGME will partition into the water—where it is needed to prevent freezing and/or microbial growth—making it more effective than the same level of DiEGME). This study addresses the microbiological component of the overall investigation; i.e., whether the biocidal/biostatic effectiveness of TriEGME is similar to that of DiEGME. Basic questions addressed in this study include: whether the same microorganisms currently affected by DiEGME are also affected by TriEGME, whether the concentration of TriEGME required to stop their growth is equivalent to that of DiEGME, and whether microorganisms recently gathered from the field may have greater tolerance for TriEGME than lab cultured microorganisms. Methodologies utilized here are primarily based on traditional culture methods. Fuel/water mixtures in French square bottles are used to simulate tank conditions. Microorganisms obtained from the American Type Culture Collection (ATCC) and from the field were introduced into these test setups, where they were challenged by DiEGME and TriEGME concentrations from 0-30% by volume in the water phase. Results suggest that the ability of DiEGME and TriEGME to halt microbial growth is both concentration and microbe dependent. Concentrations greater than 10% DiEGME and 15% TriEGME by volume in the aqueous phase were shown to retain biocidal/biostatic effectiveness in all test cases. However, due to TriEGME's higher partition coefficient, the effective additive concentration in the fuel phase would be similar to that of DiEGME. Due to the persistence of two of the field microbe strains even at 30%, additional tests were conducted at 30-60% in the aqueous phase for both FSIIs. These microbes were still viable even at 60% DiEGME. However, at 40% TriEGME and above they were eventually eradicated. The results suggest that DiEGME and TriEGME at reduced concentrations would still be effective at controlling microbial growth. DiEGME and TriEGME's ability to inhibit biofilm growth is also demonstrated. TriEGME is shown to be a suitable replacement for DiEGME, offering equivalent protection against microbial contaminants in aviation fuel.

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PREFACE

The results presented in this report are part of a larger Reduction of Total Ownership Cost (RTOC) program funded by the RTOC DoD office through ASC/EN. The authors especially thank Ed Wells of ASC/EN for his support of the RTOC efforts. The RTOC study is a FSII replacement study being conducted jointly by AFRL (RZPF and MLSA), AFPET, ASC/EN, UDRI, UTC, Encore Logistics Support Systems, B-52 SPO, and the KC-135 SPO. The authors thank Steven Zabarnick and Matthew DeWitt of the University of Dayton Research Institute and Chuck Delaney of Encore Logistics Support Systems for their technical support and guidance. The authors also thank Charles Bleckmann of the Air Force Institute of Technology (AFIT/ENV) for his technical advice.

1. Summary

The goal of this study was to find whether the replacement of DiEGME with TriEGME in aviation fuel would result in a change in any anti-microbial properties currently attributed to DiEGME. This study evaluated TriEGME's microbial activity at aqueous phase concentrations from 0-30% levels at ambient temperature (~0.0-0.04% in the fuel phase), compared to the ~30-60% levels at ambient temperature (~0.04%-0.10% in the fuel phase) typically expected of a FSII present in aircraft fuel tanks. The lower concentrations of TriEGME were chosen for this study due to the prevailing desire to reduce the current FSII concentration significantly, for the purposes of lower cost, topcoat peeling prevention, and lower toxicity. The lower concentrations of TriEGME were also chosen in order to observe the fall-off of TriEGME's effectiveness level. The current study is similar to one recently conducted by this laboratory on DiEGME at reduced concentrations. Two different groups of microbes were challenged in this study: lab cultured microbes acquired from ATCC, and microorganisms recently collected from aircraft fuel tanks in Roswell, NM and Victorville, CA commercial air bases. These two types of microorganisms were chosen for the current study due to their possible differences in behavior to FSII exposure. The current study suggests a minimum of 10% DiEGME in the aqueous phase at ambient temperature (~0.01% by volume in the fuel phase) or, in the case of the possible replacement FSII, TriEGME, 15% minimum by volume in the aqueous phase at ambient temperature (~0.01-0.02% by volume in the fuel phase) will be necessary to adequately control microbial growth. Additional tests on the field consortia at levels currently expected for FSII in the field of 30-60% by volume in the aqueous phase of DiEGME (~0.05-0.15% by volume in the fuel phase) and TriEGME (~0.04-0.10% by volume in the fuel phase) suggest that two field consortia microbe strains persist at 30-60% in the aqueous phase for the DiEGME, but that TriEGME at 40-60% in the aqueous phase (~0.06-0.10% by volume in the fuel phase) was able to eradicate all field consortia growth. However, eradication did not occur until after 30 days of exposure to TriEGME. These results suggest that both DiEGME and TriEGME are beneficial for controlling microbial growth, and that no negative effects will occur due to the substitution of TriEGME for DiEGME.

2. Introduction

Microbial contamination has been blamed for a multitude of problems in aviation fuel systems, including blockage of fuel filters, surface pitting, degradation of fuel and/or fuel additives, aircraft down time, and aircraft failure (1, 2, 3, 4, 5). Enabling problems associated with microbial growth is the presence of residual water in tanks, which can easily accumulate in the absence of proper fuel maintenance. While fuel provides hydrocarbons, which microbes can utilize as an energy source, water provides nutrients which encourage microbial proliferation. Water can also directly cause clogging in fuel systems if icing occurs (6). As a result of several icing instances, the U. S. Air Force (USAF) added ethylene glycol monomethyl ether (EGME) to the specification for military jet fuel as a precautionary fuel system icing inhibitor. Several microbially related operational delays were also reported prior to the introduction of EGME (2, 3, 4, 7, 8), but it was found that EGME also deterred microbial growth in aviation fuel, an unintended benefit (9, 10). In 1984, the U. S. Navy substituted another FSII to the JP-5 specification, diethylene glycol monomethyl ether (DiEGME). DiEGME also proved to be an effective deterrent to microbial growth in aviation fuel. In the early 1980s, the USAF replaced EGME with DiEGME due to toxicity concerns, whereas the Navy replaced it due to flashpoint concerns surrounding EGME (2, 4, 11, 12). Several studies have explored the effectiveness of DiEGME and other FSII additives in curbing or eliminating microbial growth (13, 14, 15, 16, 17, 18, 19). These studies generally recommended that FSII levels of 15% or greater in the aqueous phase must be maintained for the control and/or elimination of microbial growth.

Years after the introduction of DiEGME into USAF fuel systems, the Air Force has seen a gradual increase in operational problems due to the effects of DiEGME. DiEGME has been implicated in topcoat peeling in the B-52 and in the disarming of filter coalescers (20). As a result, studies have been conducted to determine whether the FSII concentration can be lowered to ameliorate these problems, while at the same time retaining FSII's desirable traits, such as the prevention of ice crystal formation in fuel and control of microbial growth (21). In addition, studies have been conducted to assess whether it is possible to replace DiEGME with another additive with lower volatility, such as TriEGME. Such a replacement would solve the problem of topcoat peeling. In recent years, the USAF has also seen an increase in incidents related to microbial contamination. It has been hypothesized that the number of microbes tolerant of DiEGME has been gradually increasing, resulting in more maintenance issues in fuel systems (22). There is also some contention as to whether DiEGME is still an effective biocide/biostat, and if so, what minimum concentration is required for it to be effective (17, 19, 22, 23). The USAF has similar concerns about TriEGME, the proposed replacement for DiEGME.

The current study was undertaken to consider issues concerning microbial activity of TriEGME at low concentrations, similar to those tested in the reduced FSII (DiEGME) program. The minimum FSII microbial growth data appears in a previous U.S. Air Force technical report (24). No study to date has addressed the biostatic/biocidal activity of DiEGME and TriEGME at low levels on standard lab consortia tested in the past, and also on recently collected field microbes. Due to many variables such as: regional temperature changes, free water differences, humidity, and aircraft tank geometry, it becomes very difficult to duplicate the variety and numbers of microbial contaminants found in the field. Nevertheless, a wing tank sampling study conducted by this lab in 2004-2006, ranging over 93 aircraft, 15 airframes, and 14 airbases made it possible for a fairly representative microbial sampling consortia to be available for the current study (22, 25). Five bacteria and one fungus isolated from Roswell and Victorville air bases were chosen to represent wild consortia for this study, based on their genera's high frequency of occurrence in the overall sampling study across all airbases and airframes, and the fact that viable cultures were obtainable (22).

The methodology of the current study is modeled after a study performed by Phillips in 1964 (26), which used 100 mL French square bottles with 35 mL fuel and 50 mL Bushnell Haas (BH) solution to simulate tank conditions, and traditional plate colony counts to enumerate viable microbes. Additive levels tested in the current study were at or below the lowest levels expected in aircraft fuel systems, which corresponds to 0-30% FSII in the aqueous phase.

Figure 1 expresses the relationship between the amount of TriEGME added to the fuel phase and the volume of TriEGME expected in the aqueous phase (20), and compares the values for TriEGME with the values for the current FSII additive, DiEGME.

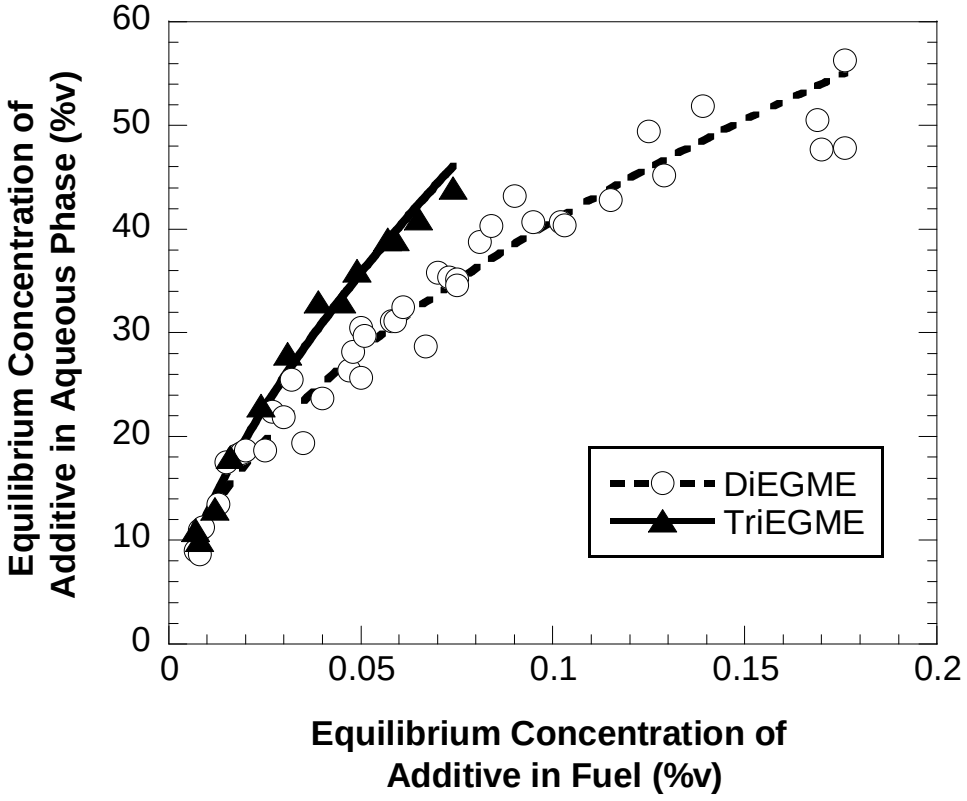

Figure 1. Equilibrium concentration of DiEGME and TriEGME in fuel vs. aqueous phase

Both DiEGME and TriEGME have non-polar and polar features to their molecules, allowing them to partition into either the fuel or aqueous phase. They partition preferentially into the aqueous phase, however, with more DiEGME or TriEGME entering the aqueous phase as the temperature drops, or as the amount added to the fuel increases. DiEGME and TriEGME are similar molecules, but TriEGME has a higher partition coefficient. At equilibrium, the partition coefficient is expressed as:

$$PC = \frac{\text{Volume \% concentration of additive in water}}{\text{Volume \% concentration of additive in fuel}}$$

The higher partition coefficient of TriEGME means that when the same amount of DiEGME or TriEGME is added to fuel, the result will be more TriEGME in the aqueous phase, as Figure 1 suggests. For example, under the same experimental conditions at equilibrium, the partition coefficient for DiEGME has been expressed as 538, compared to 1120 for TriEGME (17). The actual amount of DiEGME or TriEGME in the fuel or aqueous phase, however, is dependent on a variety of factors, such as the amount of water, temperature, and multiple mixing events (changes in a fuel's icing inhibitor content due to the additive's accumulation in the water phase, plus the addition of new additized fuel plus its water content in multiple occurrences). In this study, these variations were minimized by the use of single temperature, static French square liquid setups with water bottoms large enough that it can be assumed that back diffusion (the return of the FSII additive to the fuel phase) does not significantly change the FSII level in the water phase, and that all of the additive remains in the

aqueous phase. All DiEGME and TriEGME used in these experiments was added directly to the aqueous phase at known percentage volumes.

The current study explored TriEGME levels of 0-30% in the aqueous phase, which corresponded to additized fuel levels of 0-0.04% by volume in the fuel, according to the chart above. This chart was obtained at 20° C. The DiEGME data for figure 1 was obtained with 130-560 ppm of water, and the TriEGME data was obtained with 300 ppm of water. Due to the manner in which the information is plotted, however, the water content differences have no impact. The results of several studies (13, 17, 19) suggested that DiEGME and TriEGME levels of 15% by volume and above in the water phase would be adequate for control of microbial growth, which would correspond to a 0.01-0.02% dosage in the fuel phase using the chart above. This dosage level would equal one-seventh or less of the current typical dosage.

3. Methods, Assumptions, and Procedures

3.1 Materials

Clear French square 100 mL bottles from Fisher Scientific were sterilized by autoclave. The test setup for each French square consisted of 35 mL of Jet A aviation fuel POSF 4877 for the fuel phase and 50 mL of aqueous phase made from Bushnell-Haas broth nutrient solution (Sigma-Aldrich, Inc. St. Louis, MO) with DiEGME or TriEGME added as appropriate. Fuel was filtered with a 0.45µm hydrophobic cellulose nitrate filter (Nalge Nunc, Rochester, NY) prior to use in the test setup. The Bushnell-Haas solution was sterilized by autoclave. Microbes were cultured on Luria-Bertani (LB) agar plates. LB broth and Difco granulated agar were obtained from Sigma-Aldrich and Becton-Dickinson (Sparks, MD), respectively. All plating was performed in a laminar flow hood. A Reichert Quebec Darkfield colony counter (Depew, NY) was used to quantify microbial growth.

3.2 ATCC Lab Cultured Microorganisms

Lab culture microorganisms were obtained from ATCC. They included: *Pseudomonas aeruginosa* (ATCC catalog # 33988), *Hormoconis (Cladosporium) resinae* (ATCC # 20495), and *Yarrowia (Candida) tropicalis* (ATCC # 20336). *P. aeruginosa* is a type of bacteria, *C. resinae* is a fungus, and *C. tropicalis* is a yeast. The *P. aeruginosa* originated from a fuel storage tank in Ponca City, OK, and was deposited into the ATCC collection by R. Allred in 1982. The *C. resinae* originated from an aircraft fuel tank and was deposited by J. J. Marshall from the NLABS collection in 1977. The *C. tropicalis* was deposited in 1971. The ATCC microorganisms were chosen based on their prevalence in the fuels literature; for example, they are used in the ASTM method E 1259-01 for evaluating antimicrobials in liquid fuel (27), and they were used by Neihof, Westbrook, and Hill (13, 16, 19).

3.3 Wild Type Microbe Collection

The wing tanks of several civilian aircraft in long term storage were sampled from 2004 to 2005. Military aircraft were also sampled throughout 2005. Preliminary results were compiled in a previously published technical report (22). The Victorville and Roswell microbes were obtained from wing tanks on commercial DC-9 aircraft that had been idle for at least a year. All Victorville and Roswell aircraft fuel tank samples contained fuel with water bottoms. Microorganisms obtained from Roswell and Victorville aircraft fuel tanks in 2004-2005 included: a *Methylobacterium* species, a *Pseudomonas* species, *Bacillus licheniformis*, *Clostridium intestinale*, *Rhodococcus equi*, and *Hormoconis (Cladosporium) resinae*. All are bacteria except for *C. resinae*. These field microorganisms were the most common found overall in the most recent study of microbial contaminants in aircraft fuel, and were chosen to roughly approximate a realistic test set (22). Due to the fact that microbial consortia are typically not dispersed in fuel systems in a homogenous manner, it may not be possible to truly capture a representative picture of microbial growth over an entire fleet of aircraft (27). The extensive sampling efforts undertaken by this lab in 2004-2005 were, however, the best recent attempt at representing the likely widespread classes of consortia encountered in the field today (22). DiEGME levels were not recorded for the commercial aircraft during the 2004-2005 sampling study. The military aircraft in the 2004-2005 study, however, were known to be exposed to DiEGME in many cases, and in many instances data on DiEGME levels was recorded (24). The frequency of different types of microbial contaminants from 2004 to 2005 in both commercial and military aircraft was noted in the 2004-2005 sampling study, with the six most common being chosen to represent the field consortia in the current study.

The microorganisms cultured from the field were identified by 16S ribosomal RNA sequencing as: *Pseudomonas* sp. (obtained from Roswell, NM air base), *Bacillus licheniformis* (Roswell), *Clostridium intestinale* (Roswell), *Rhodococcus equi* (Victorville, CA air base), and *Methylobacterium*

sp. (Victorville). Sequencing was performed by MWG Biotech of High Point, NC. Fuel sampling procedures, DNA extraction, purification, and sequencing procedures are detailed elsewhere (11, 22, 24). Procedures used for bacterial sequence identification are also listed elsewhere (11). *Cladosporium resinae* (Roswell) was identified by light microscopy, performed by Forensic Analytical of Rancho Dominguez, CA.

3.4 Test Procedure

All microorganisms were revived from frozen cultures stored at -80º C. They were incubated in unsealed, autoclaved steel-capped glass test tubes containing 5 mL of LB broth, POSF 4877 fuel + BH broth, and BH broth alone. LB and BH full-strength broth were autoclaved to sterilize, while the fuel was not autoclaved but was filtered before use. After inoculation, when visible inspection showed significant microbial growth (indicated by cloudiness or an increase of solid or fluffy material at test tube bottom) of the LB, the fuel + BH, and/or the BH test tubes, the cultures were deemed viable. Two hundred microliter aliquots of each microorganism grown in BH broth were then pipetted separately in the case of the single organism tests, or they were pipetted and combined to make a mixed culture in the case of the mixed culture tests. One hundred microliters of the single or mixed culture was then used to inoculate each French square bottle at each DiEGME test level. French square bottles were incubated at 28º C. At the time of initial plating, referred to as Day 0, the microbes were exposed to DiEGME for at least 4, but no more than 24 hours. Colony counts were not taken prior to the Day 0 plating. For all test points, the fuel/water French square setups were manually shaken for 30 seconds, the phases were allowed to re-separate, and a 100 µL aliquot was drawn from the aqueous phase. The aliquot was spread on an LB plate. A second aliquot was used to make dilutions as needed with the BH, typically 1:100, 1:1000, and/or 1:10,000. Growth rates were microorganism dependent, with colonies typically appearing 24 to 72 hours after plating.

Following incubation, the colony forming units (CFU) on each plate were enumerated using a counter probe. The countable range for a raw plate is between 30 and 300 CFU (28). In practice, however, colonies were sometimes above or below the countable range, despite the dilutions performed. Due to the dilution method used, the maximum corrected raw colony count in the present experiments was 30,000,000 per mL, values above this were considered to be too numerous to count (TNTC). This corresponded to a raw count above 300 on a plate with a 1/10,000 dilution. Colony counting uncertainty is expected to be plus or minus an order of magnitude. This uncertainty is based on colony counting results obtained from random, multiple platings. Most of the colony counts reported here were the results of single platings. Although there is not always a direct relationship between colony count and level of microbial contamination of aviation fuel—due to the fact that over 90% of microbes in the environment may not be culturable on agar plates (29)—it is safe to assume that relationship in the current study, as all of the microbes utilized have been previously cultured on agar plates. In addition, it is often the case that a large colony count is directly suggestive of a significant potential for microbially-induced problems such as biofilm formation. However, no numerical standards have been universally accepted which define a particular colony count level as problematic (27). This fact is explicitly stated in the standard guide for microbial contamination in fuels and fuel systems, ASTM D 6469-99 (12).

The current study employs a direct comparison between DiEGME and TriEGME to ascertain the relative effectiveness of these two additives in inhibiting microbiological growth. The rest of the test procedure used in this study is based on a Phillips report from 1964 (26). Essentially, the Phillips method requires plating of the liquid test setups approximately every three days during a 46 day test duration. Blank fuel/water mixtures were also maintained throughout the test cycle for each DiEGME and TriEGME concentration level. DiEGME and TriEGME concentration levels tested in this study were: 0, 5, 10, 15, 20, 30, 40, 50, and 60% by volume in the water phase. Blanks (fuel/water mixtures with no inoculants added) at each level were also plated randomly throughout the test period. Blanks did not show any growth throughout the test period.

4. Results and Discussion

4.1 ATCC Microorganism Tests

In these tests, *Pseudomonas aeruginosa*, *Cladosporium resinae*, and *Candida tropicalis* were revived from separate frozen cultures. They were tested singly and collectively for their resistance to DiEGME and TriEGME at low additive concentration levels. These three microorganisms were grown in the test setups, plated on LB plates, and their colonies were counted after 72 hours of incubation for each test point, as *C. resinae* colonies were not clearly visible prior to 72 hours. Three types of information are shown below: 1) Figures 2-9 show French square test setups following the 46 day test duration, which present visual comparisons of the liquid ATCC inoculated samples at different DiEGME and TriEGME concentrations. Test setups which show particulate matter and/or cloudiness in the bottom (aqueous) layer have significant microbial contamination; 2) Figures 10-15 show agar plate growth of the ATCC consortia, Figures 16-33 show plate growth for ATCC *Pseudomonas* alone, ATCC *Cladosporium* alone, and ATCC *Candida* alone, at several different points during the experiment. The agar plates shown were used for visual inspection and/or enumeration of colony growth; 3) Figures 34-41 below summarize ATCC microbial growth for the 46 day test period for the mixed ATCC consortia, as well as the ATCC microorganisms tested singly.

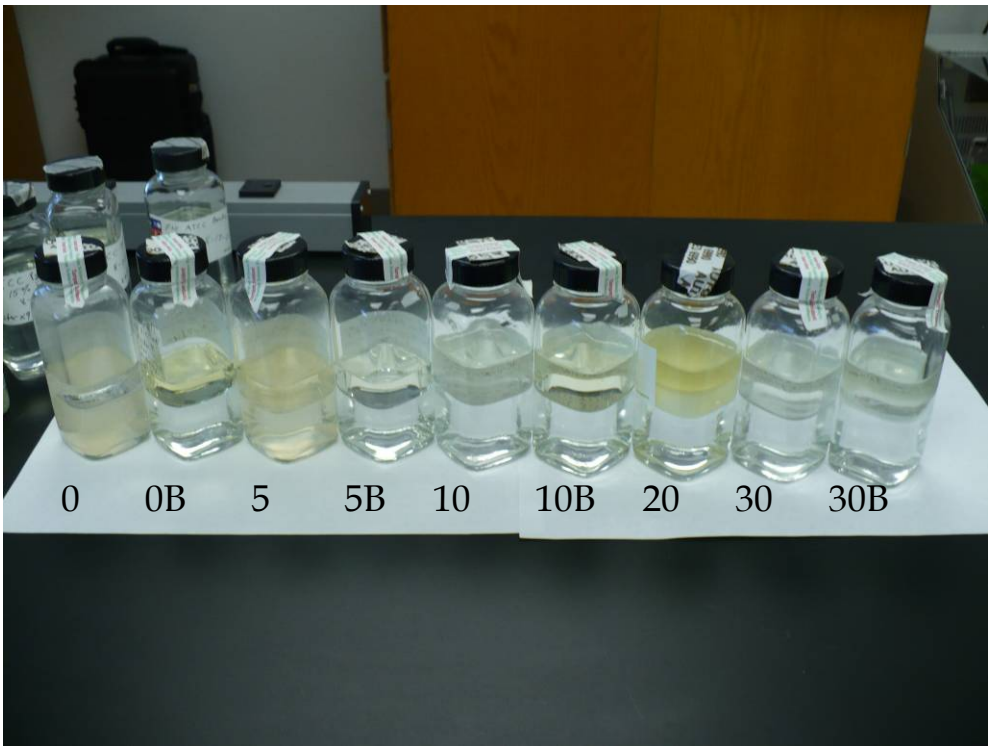

Figure 2. ATCC consortia following 46 day test, side by side with respective blank, except for 20%. DiEGME concentrations are, from left to right: 0, 5, 10, 20, and 30% by volume in aqueous phase

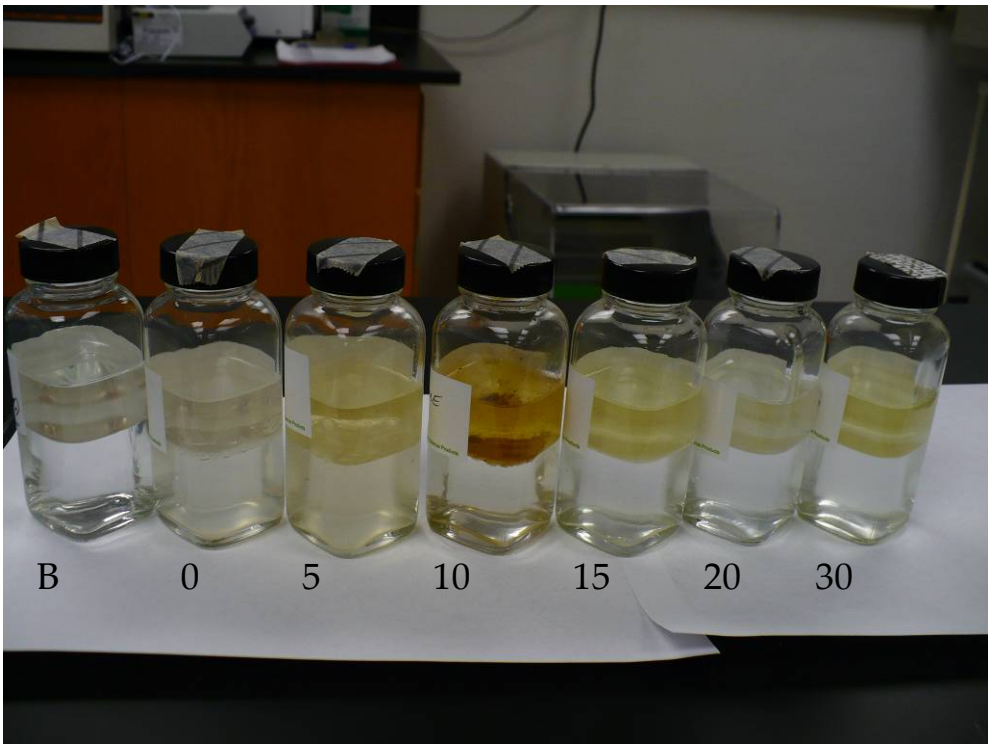

Figure 3. ATCC consortia following 46 day test. A blank (POSF 4877 Jet A fuel/Bushnell-Haas water solution) is shown, followed by TriEGME concentrations of: 0, 5, 10, 15, 20, and 30% by volume in water phase.

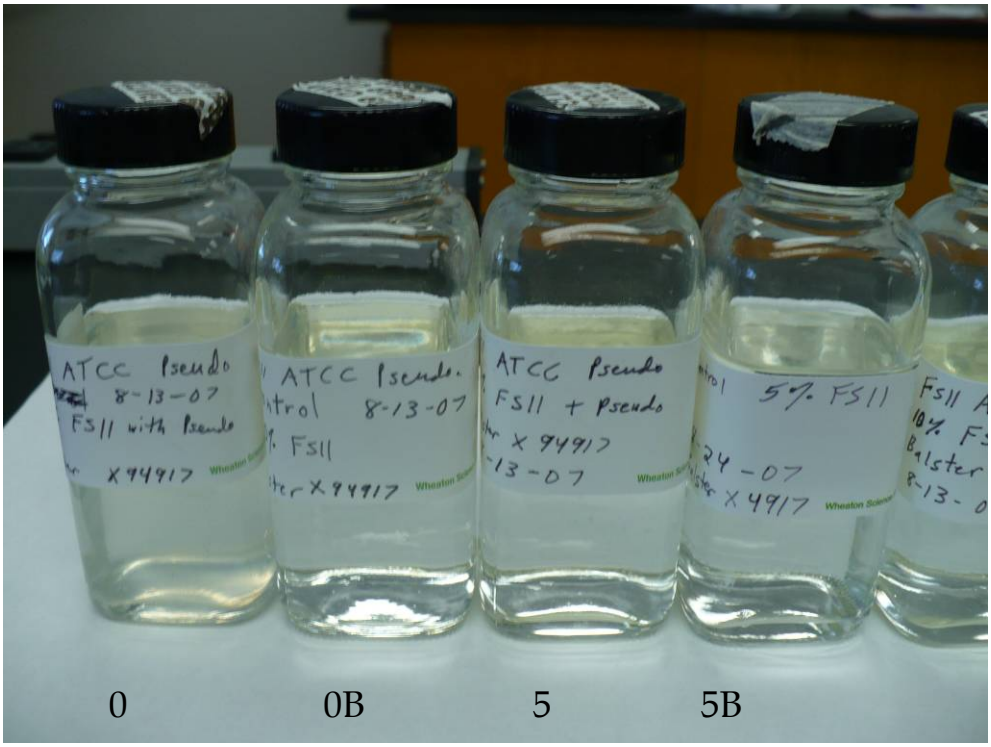

Figure 4. ATCC *Pseudomonas* following 46 day test. DiEGME concentrations are: 0 and 5% by volume in aqueous phase, paired with respective blanks.

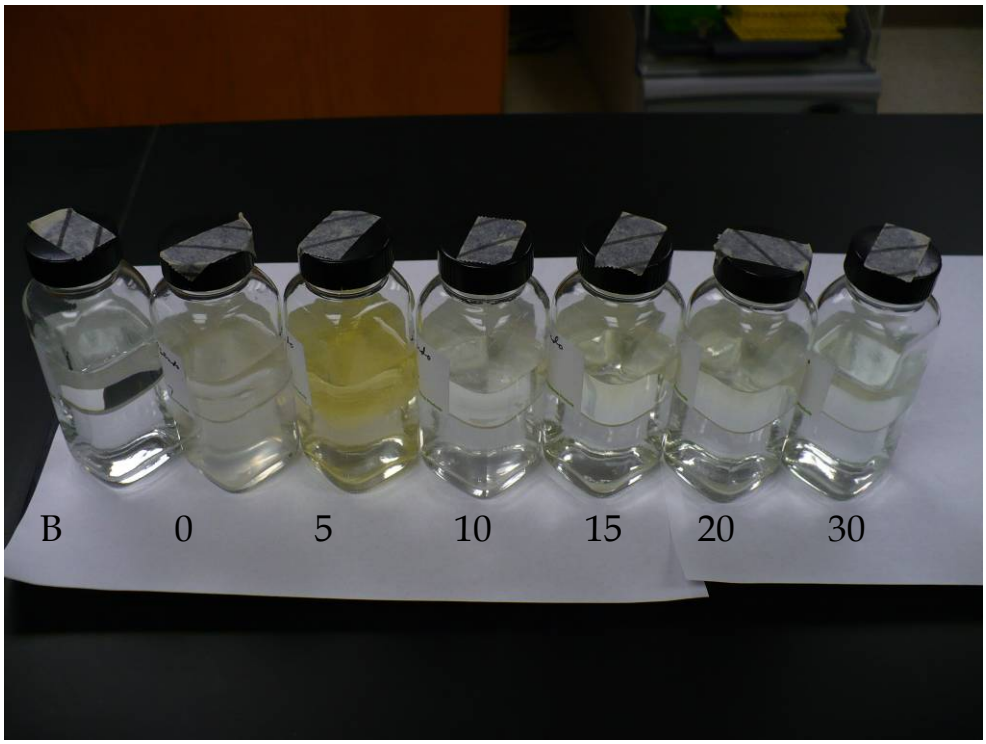

Figure 5. ATCC *Pseudomonas* following 46 day test. A blank is shown followed by TriEGME concentrations of: 0, 5, 10, 15, 20, and 30% by volume in the water phase.

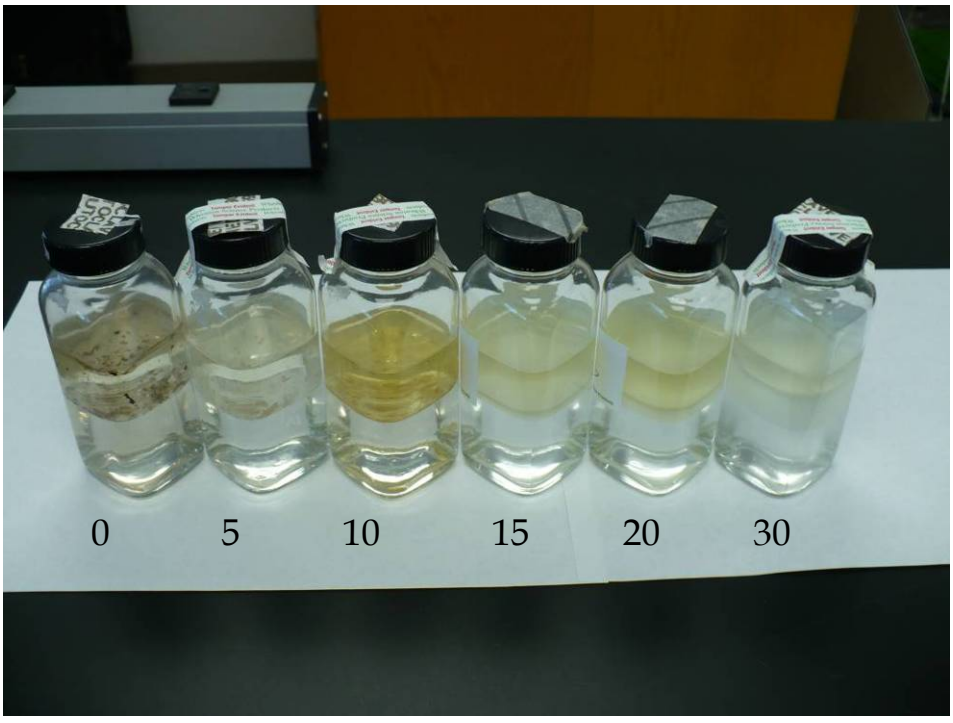

Figure 6. ATCC *Cladosporium* following 46 day test. DiEGME concentrations are: 0, 5, 10, 15, 20, and 30% by volume in aqueous phase.

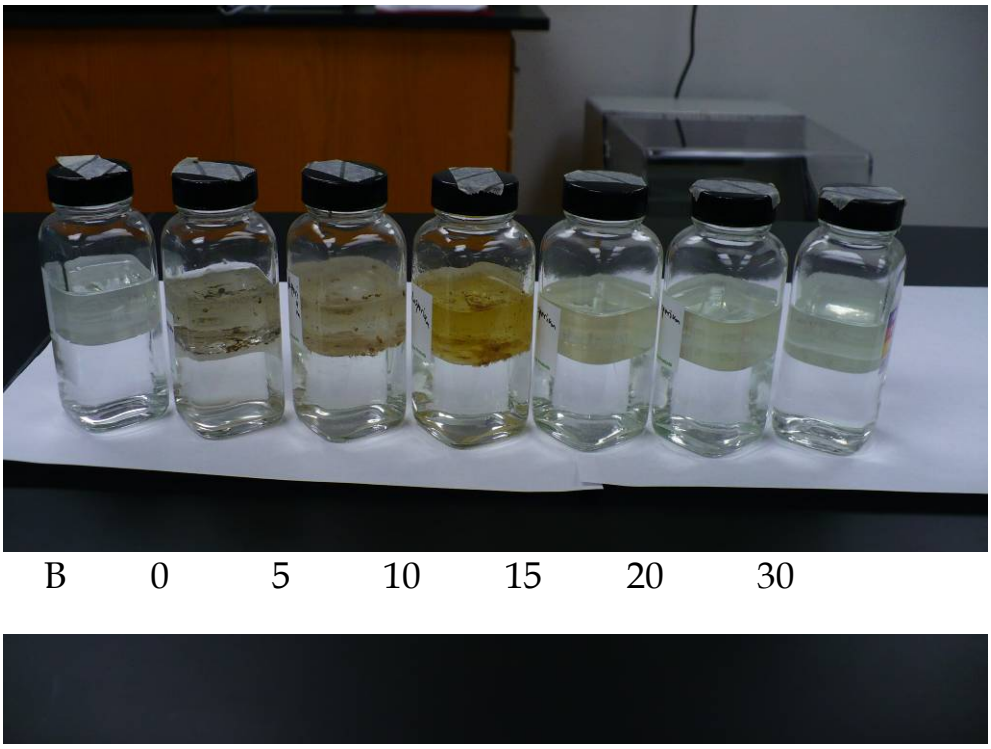

Figure 7. ATCC *Cladosporium* following 46 day test. A blank is shown, followed by TriEGME concentrations of: 0, 5, 10, 15, 20, and 30% by volume in water phase.

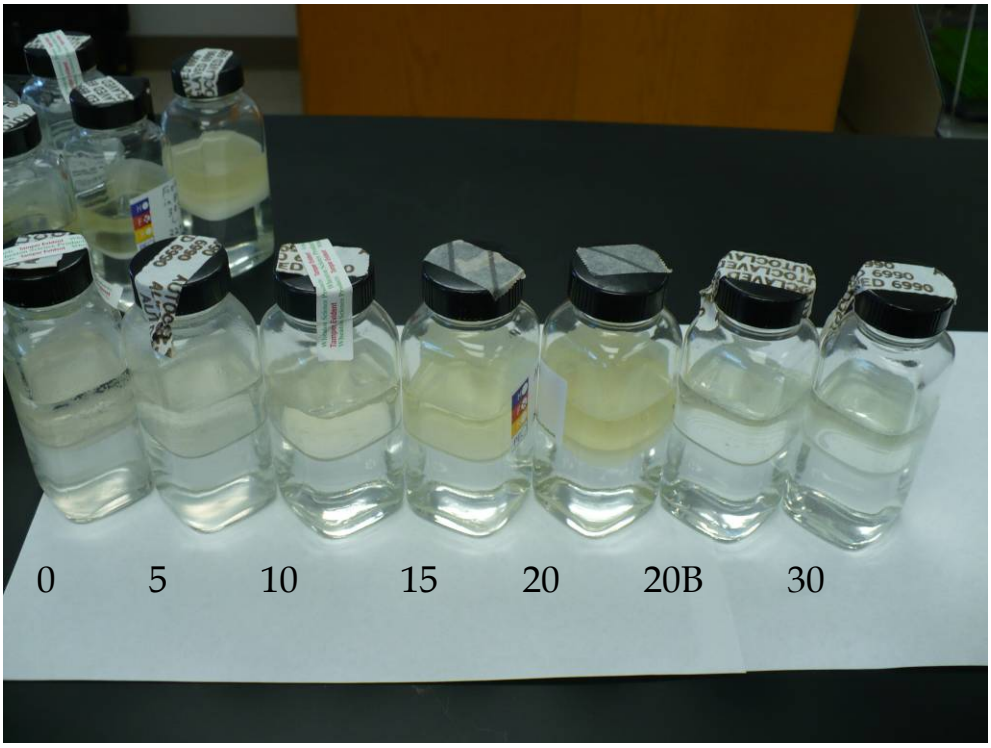

Figure 8. ATCC *Candida* following day 46 of test. DiEGME concentrations are: 0, 5, 10, 15, 20, and 30% by volume in aqueous phase, with 20% next to 20% blank.

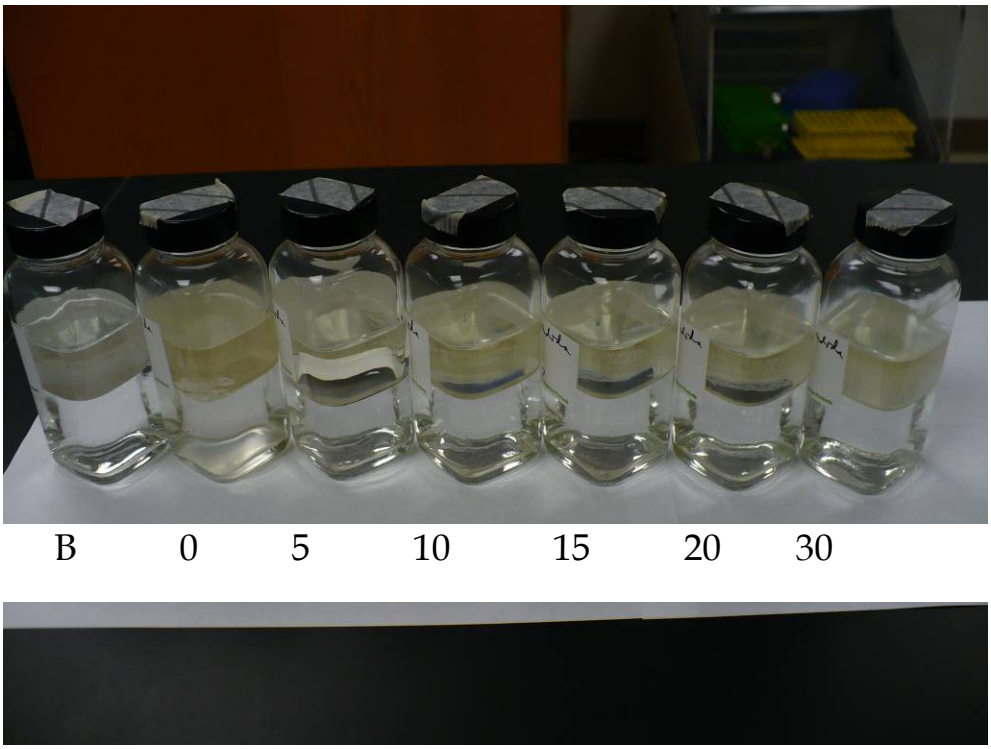

Figure 9. ATCC *Candida* following day 46 of test. A blank is shown, followed by TriEGME concentrations of: 0, 5, 10, 15, 20, and 30% by volume in water phase.

Several observations become apparent from visual inspection of the ATCC consortia test setups, as well as those from the separate ATCC microorganisms. All the DiEGME 0% test setups, shown in Figures 2,4,6, and 8, and all of the 0% TriEGME test setups, shown in Figures 3,5,7, and 9, have cloudiness and/or brown particulates in the water phase. Cloudiness that doesn't dissipate is considered to be a very good indicator of microbial activity, as is the generation of large, brown particulates--hallmarks of biofilm formation. In fuel systems, a biofilm is a microbial growth formation that typically appears as a sheen, pellicule, or mat that forms between the fuel and water layers or on the interior sides of a tank. Biofilms consist of microbes, inert detritus, water, and extracellular polymeric substances (EPS)—also known as the glycocalyx, which is a polysaccharide or peptide slime. The formation of a biofilm in a fuel system can have important consequences. Biofilms protect bacteria, fungus, and/or yeast and encourage their growth, which in turn promotes the deleterious effects of microbial contamination, such as microbially induced corrosion (MIC) and fuel degradation. The presence of biofilms can also lead directly to the plugging of fuel lines and filters (27).

For the liquid samples, it was always the case that the unadditized control had significantly more growth, which could be discerned visually as a biofilm at the fuel/water interface and by cloudiness in the aqueous phase. The contrast between the unadditized sample and those containing DiEGME or TriEGME was quite clear. In the 5% DiEGME and TriEGME setups, cloudiness and/or particulates are evident in the ATCC consortia, the *Pseudomonas*, and the *Cladosporium*, shown in Figures 2-5. The *Candida*, however, is clear at 5%. For the 10% TriEGME, only the ATCC consortia and the *Cladosporium* by itself show obvious brown particulates, suggesting that the *Cladosporium* is responsible for the persistence of growth at this TriEGME level. All the other 10% setups are clear. At TriEGME levels of 15% and higher, there is no cloudiness or particulate formation for any of the ATCC microbes tested.

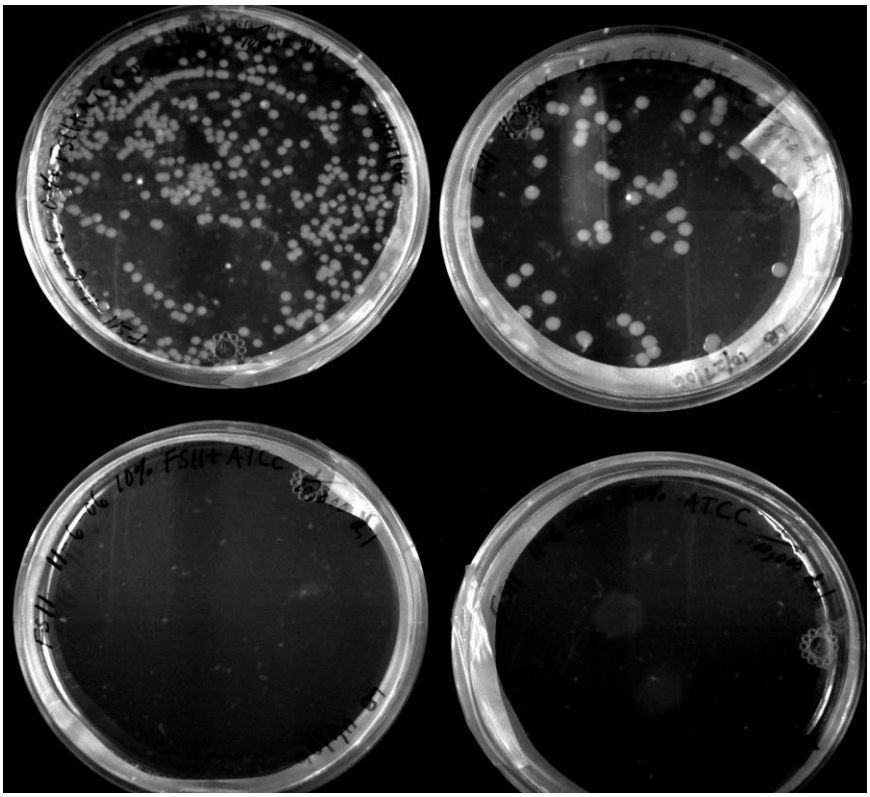

Figure 10. ATCC consortia at day 6 of incubation. DiEGME concentrations, 1/10,000 dilution, are: 0% (upper left), 5% (upper right), 10% (lower left), and 30% (lower right) by volume in water phase. Colonies are only growing at the 0 and 5% levels.

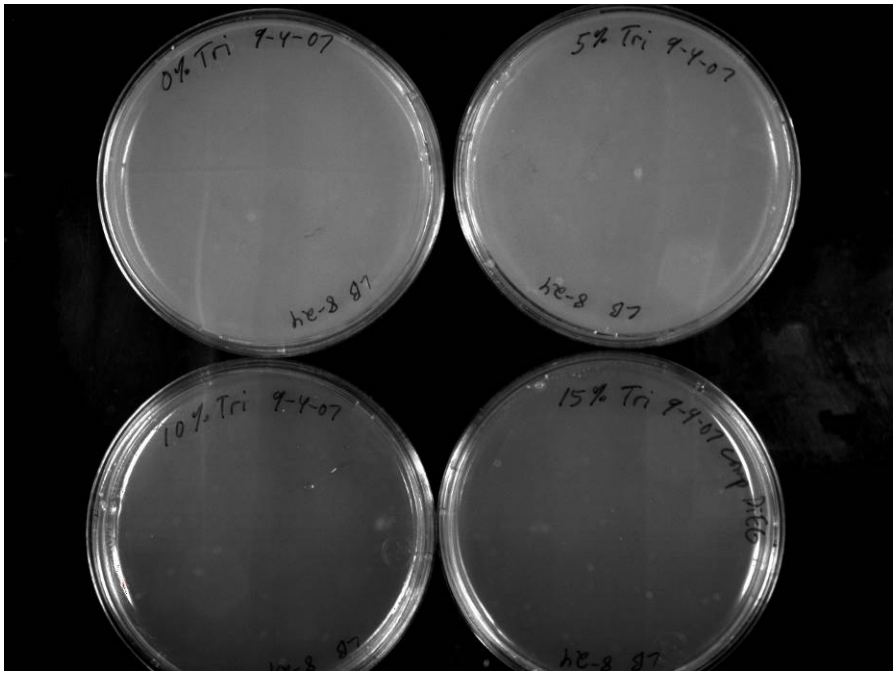

Figure 11. ATCC consortia at day 8 of incubation. TriEGME concentrations are: 0% (upper left), 5% (upper right), 10% (lower left), and 15% (lower right) by volume in water phase. Colonies are swarming the plates at the 0 and 5% levels. The other two plates have no growth.

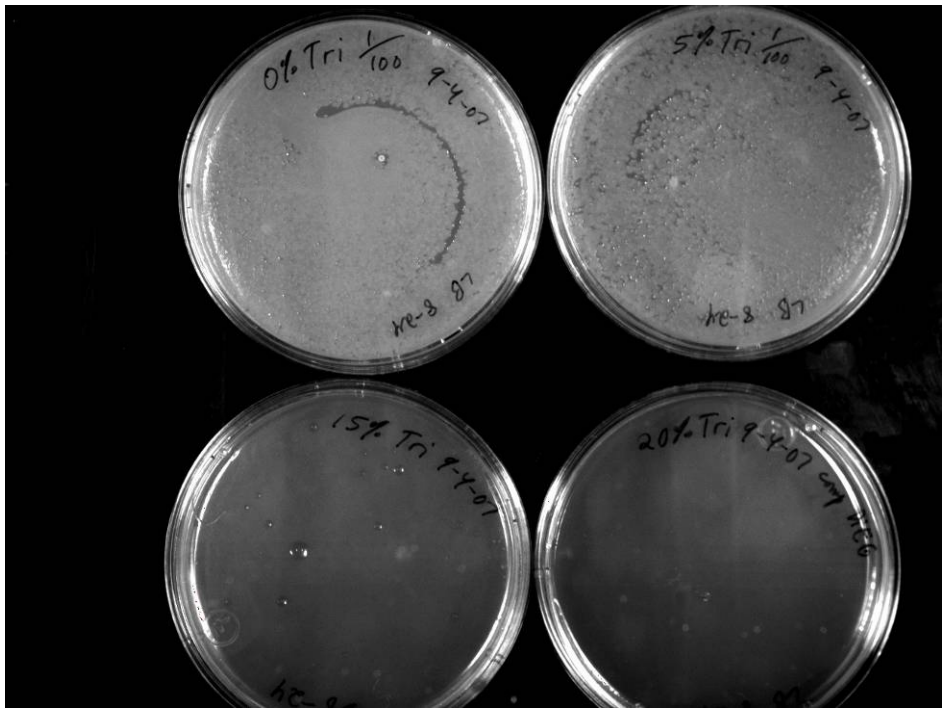

Figure 12. ATCC consortia at day 8 of incubation. TriEGME concentrations, 1/100 dilution, are: 0% (upper left), 5% (upper right), 15% (lower left), and 20% (lower right) by volume in water phase. Colonies are only growing at the 0 and 5% levels.

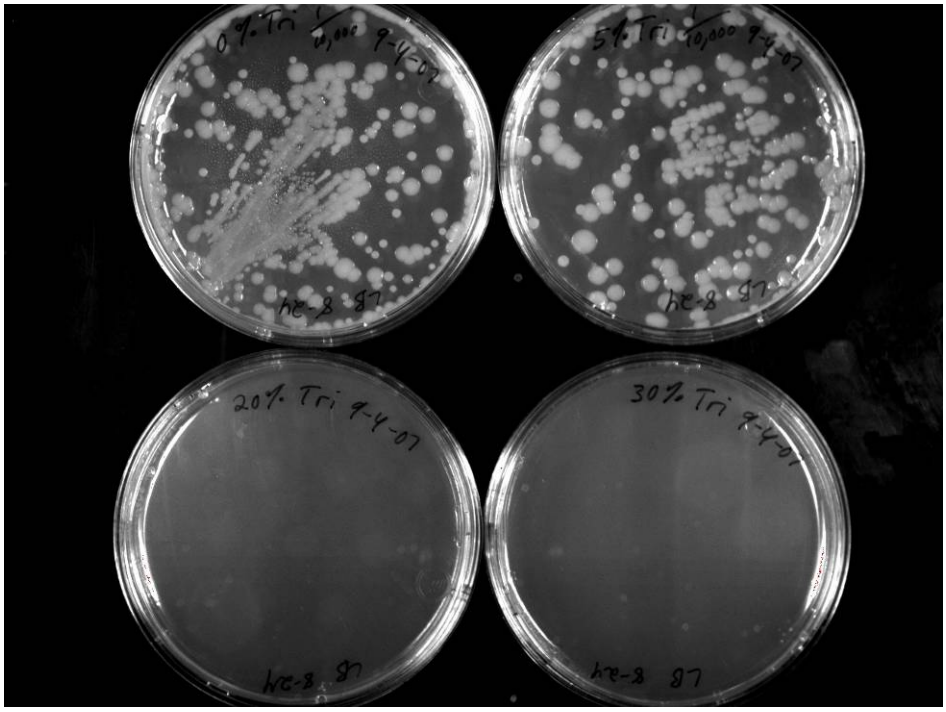

Figure 13. ATCC consortia at day 8 of incubation. TriEGME concentrations, 1/10,000 dilution, are: 0% (upper left), 5% (upper right), and with no dilution, 20% (lower left), and 30% (lower right) by volume in water phase. Colonies are only growing at the 0 and 5% levels.

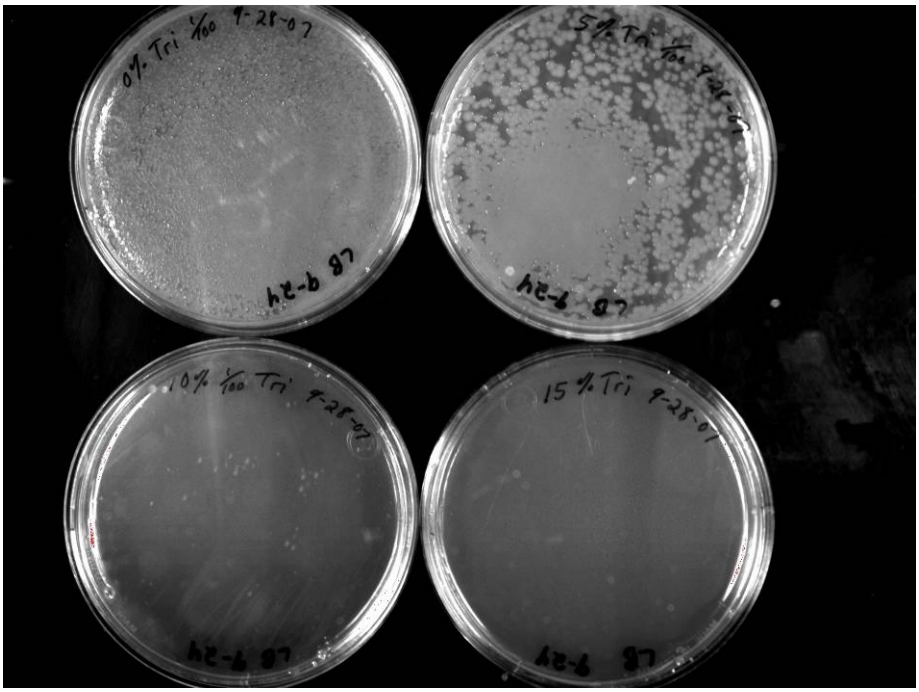

Figure 14. ATCC consortia at day 32 of incubation. TriEGME concentrations, 1/100 dilution, are: 0% (upper left), 5% (upper right), 10% (lower left), and 15% (lower right) by volume in water phase. Bacterial or yeast colonies are growing at the 0 and 5% levels, and fungal colonies from *Cladosporium* are growing at the 10% level.

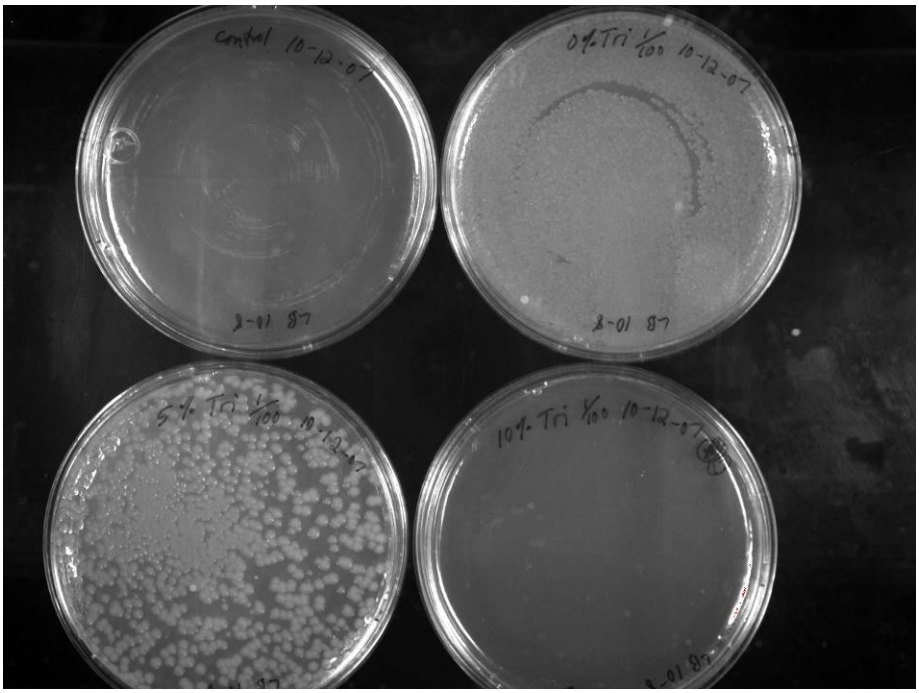

Figure 15. ATCC consortia at day 46 of incubation. The uninoculated control blank (upper left), with no colony growth, is shown with TriEGME concentrations, 1/100 dilution, of: 0% (upper left), 5% (lower left), and 10% (lower right). Bacterial colonies are growing at the 0 and 5% levels, and fungal colonies from *Cladosporium* are growing at the 10% level.

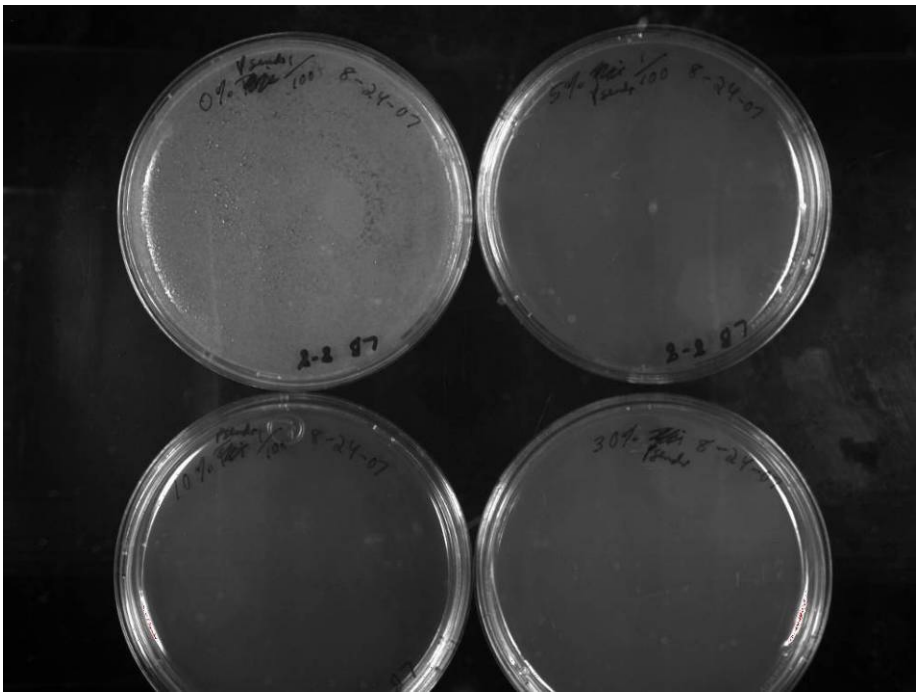

Figure 16. ATCC *Pseudomonas* at day 11. DiEGME concentrations, 1/100 dilution, are: 0,5,10, and 30%. The 0% plate (upper left) is swarming with growth; the other plates have no colonies.

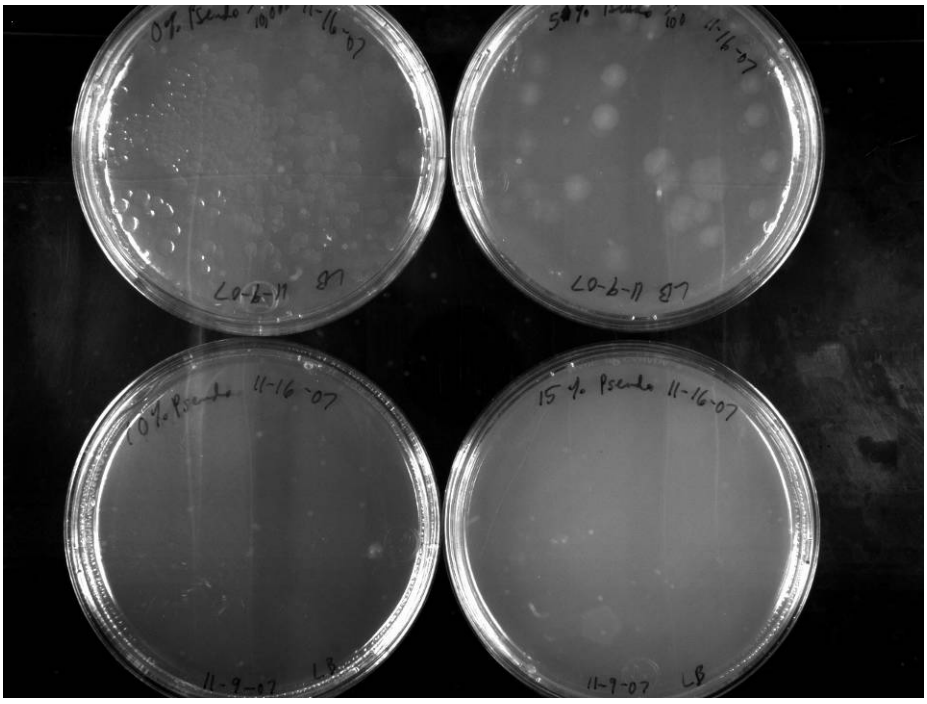

Figure 17. ATCC *Pseudomonas* at day 3. TriEGME concentrations, 1/100 dilution, are: 0,5,10, and 30%. The 0% plate (upper left) is swarming with growth, the 5% plate (upper right) has less growth; the other plates have no colonies.

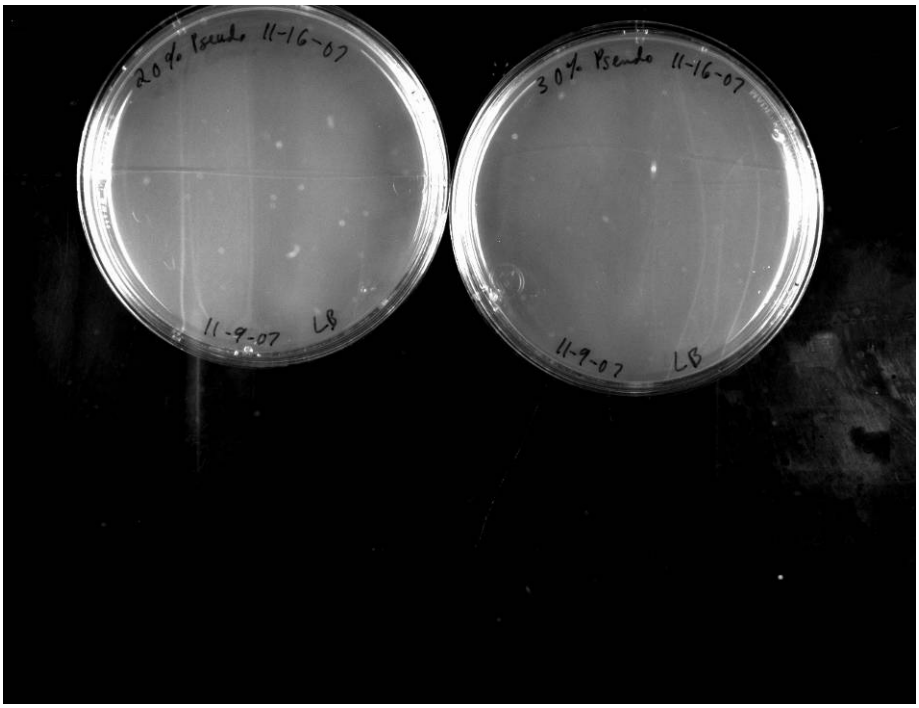

Figure 18. ATCC *Pseudomonas* at day 3. TriEGME concentrations are: 20 and 30%. These plates have no growth.

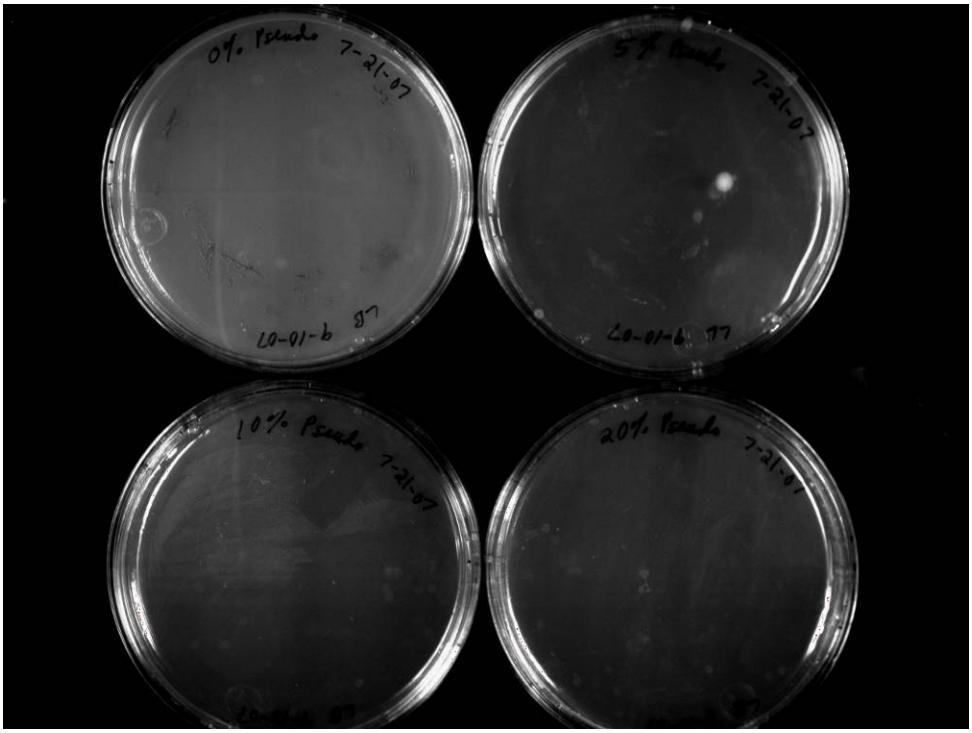

Figure 19. ATCC *Pseudomonas* at day 38. DiEGME concentrations are: 0,5,10, and 20%. The 0% plate (upper left) is swarming with growth; the 5% plate (upper right) has one colony. The 10 and 20% plates (lower left and right) have none.

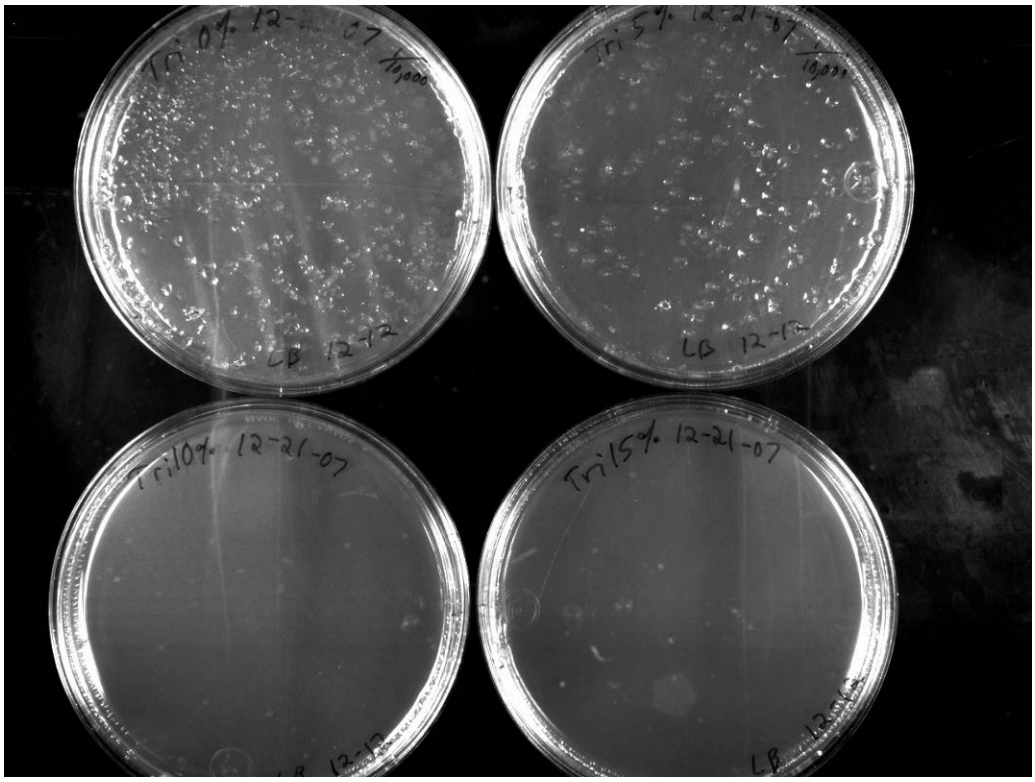

Figure 20. ATCC *Pseudomonas* at day 38. TriEGME concentrations are: 0,5,10, and 15%. The 0% plate (upper left) and 5% plates (upper right) are swarming with growth. The 10 and 15% plates (lower left and right) have none.

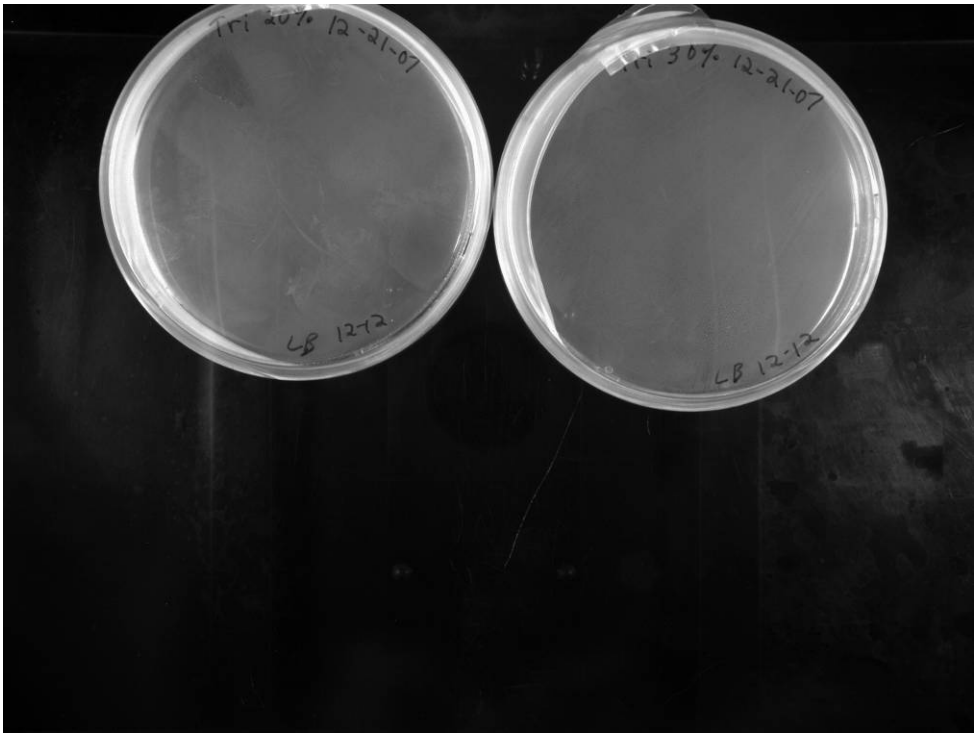

Figure 21. ATCC *Pseudomonas* at day 38. TriEGME concentrations are: 20 and 30%. The plates have no growth.

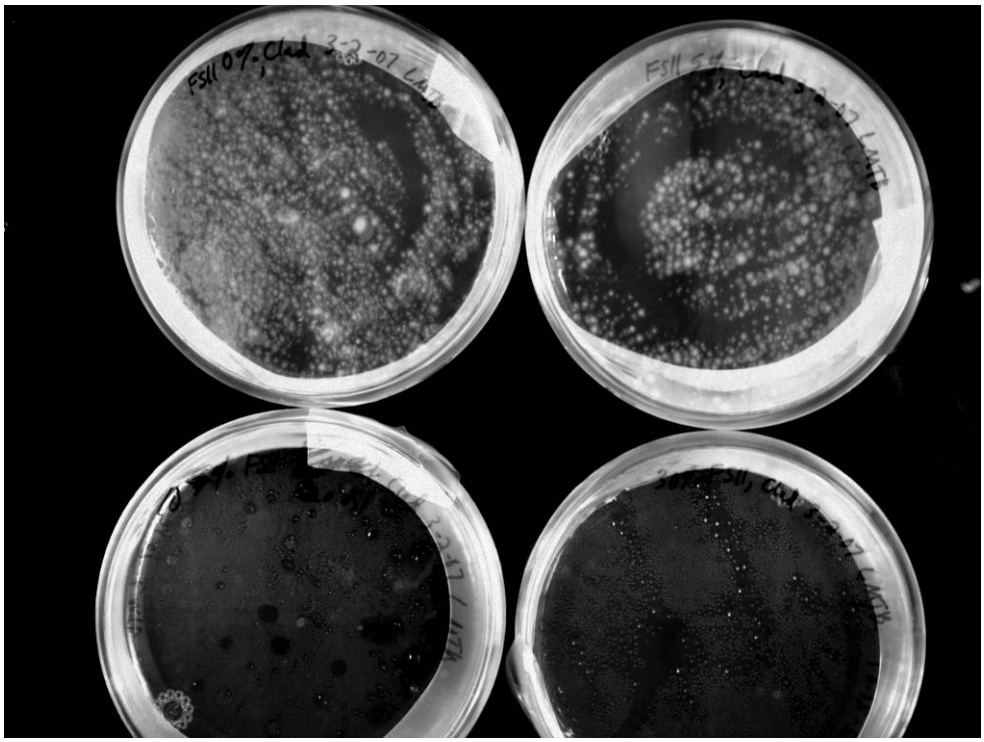

Figure 22. ATCC *Cladosporium* at day 30 of incubation. DiEGME concentrations are: 0 (upper left), 5 (upper right), 20 (lower left) and 30% (lower right). Lower plates have condensation, not colonies.

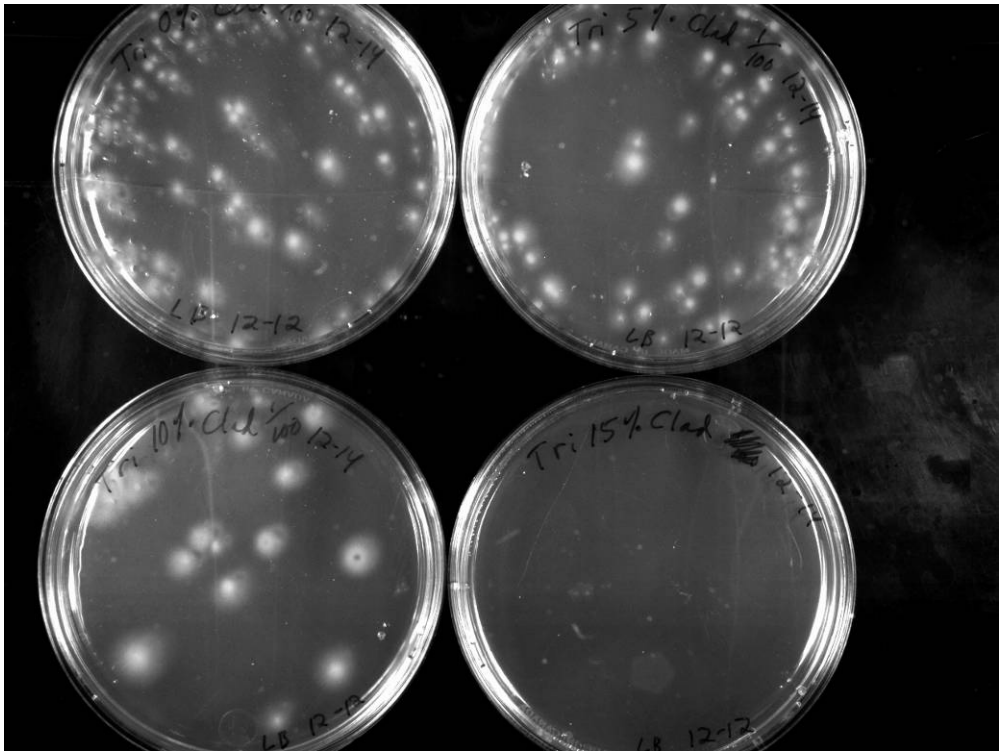

Figure 23. ATCC *Cladosporium* at day 31 of incubation. TriEGME concentrations are: 0 (upper left), 5 (upper right), 10 (lower left) and 15% (lower right).

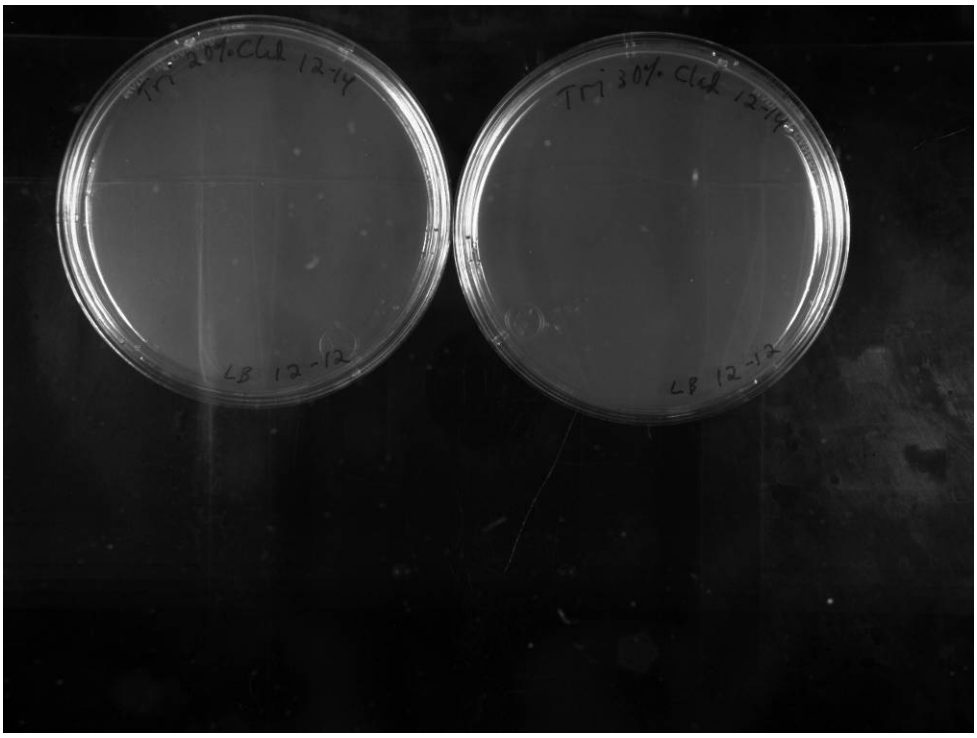

Figure 24. ATCC *Cladosporium* at day 31. TriEGME concentrations are: 20 and 30%. These plates have no colonies.

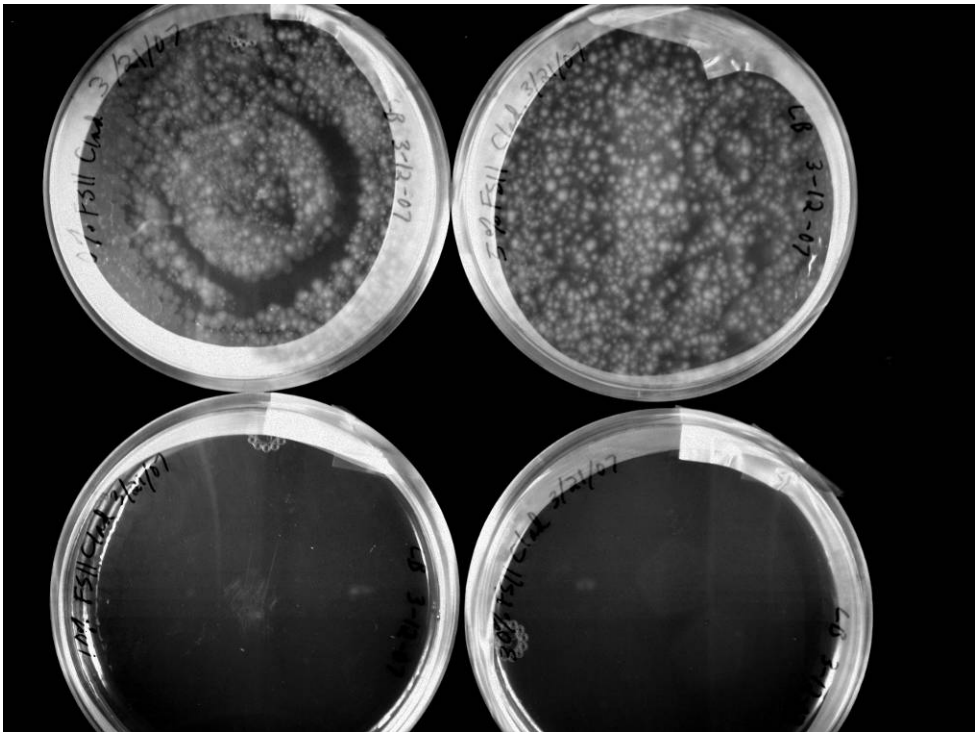

Figure 25. ATCC *Cladosporium* at day 47. DiEGME concentrations are: 0 (upper left), 5 (upper right), 10 (lower left), 30% (lower right). The 10 and 30% plates have no colonies.

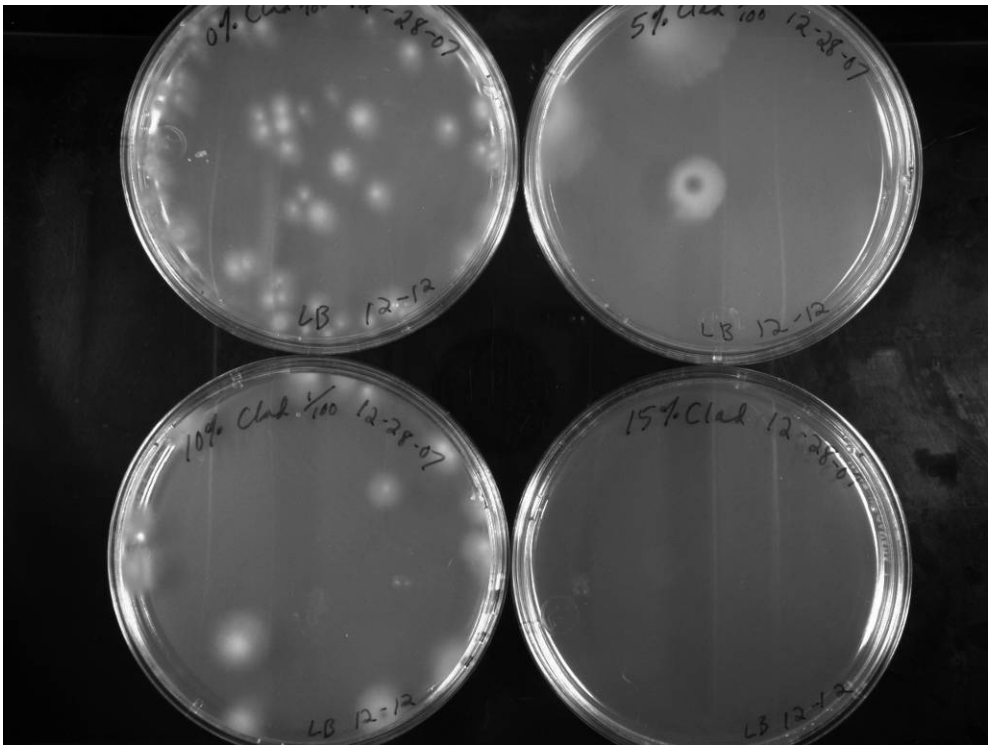

Figure 26. ATCC *Cladosporium* at day 48. TriEGME concentrations are: 0 (upper left), 5 (upper right), 10 (lower left), 15% (lower right). The 15% plate has no colonies.

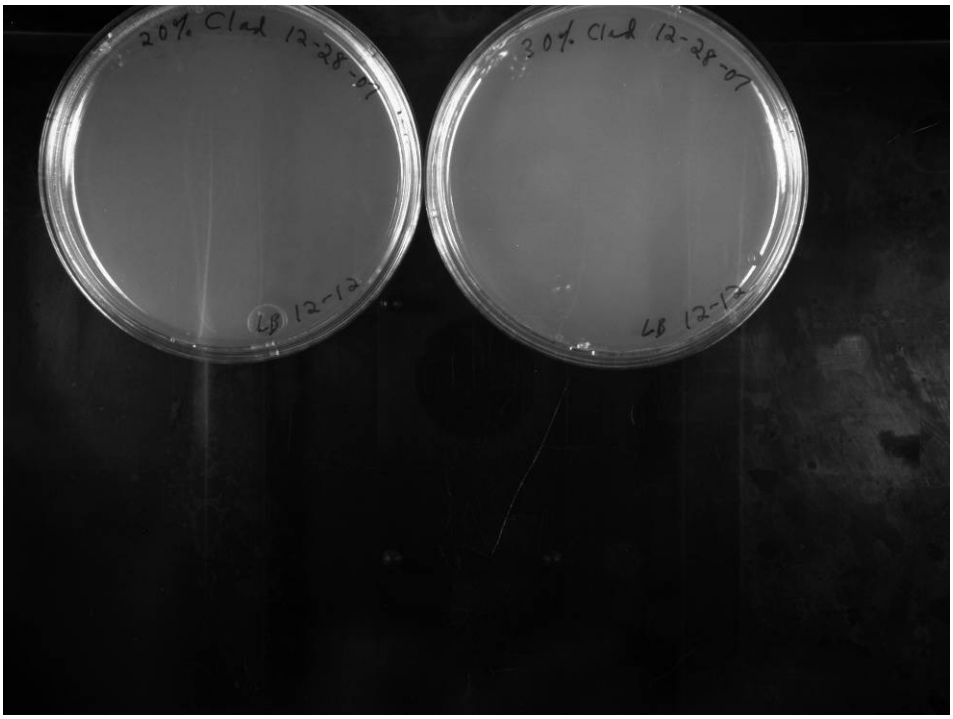

Figure 27. ATCC *Cladosporium* at day 48. TriEGME concentrations are: 20 and 30%. These plates have no colonies.

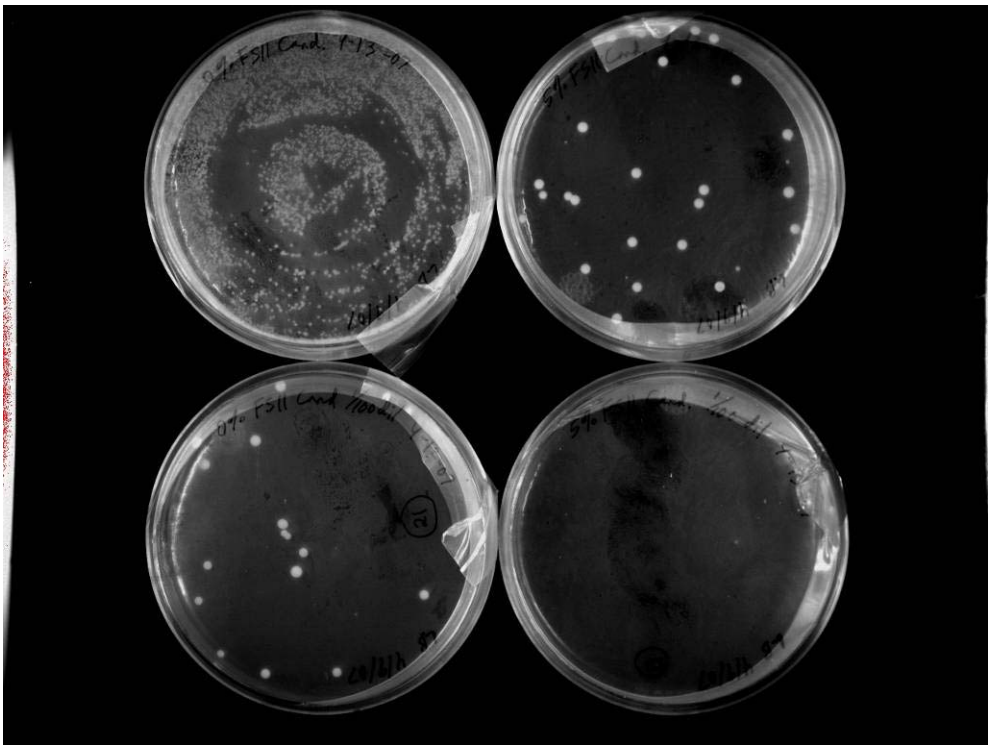

Figure 28. ATCC *Candida* at day 8. DiEGME concentrations are: 0 (no dilution, upper left), 5 (no dilution, upper right), 0 (1/100 dilution, lower left), and 5 (1/100 dilution, lower right). The 5% 1/100 dilution plate has no colonies.

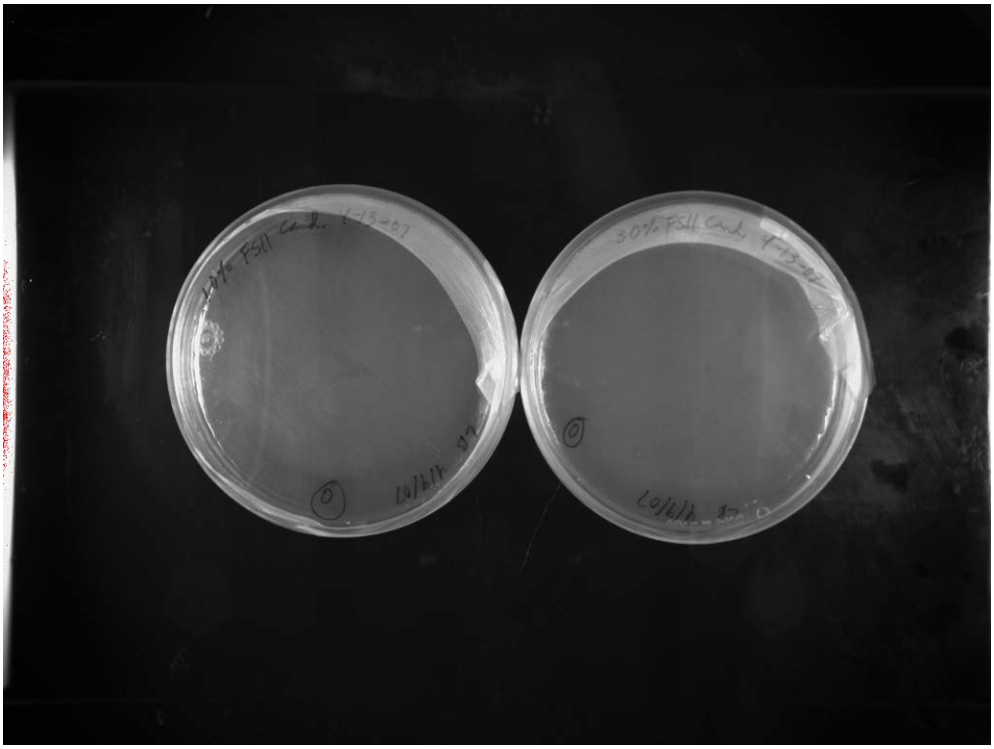

Figure 29. ATCC *Candida* at day 8. DiEGME concentrations are: 10 (left) and 30% (right). Neither plate has colonies.

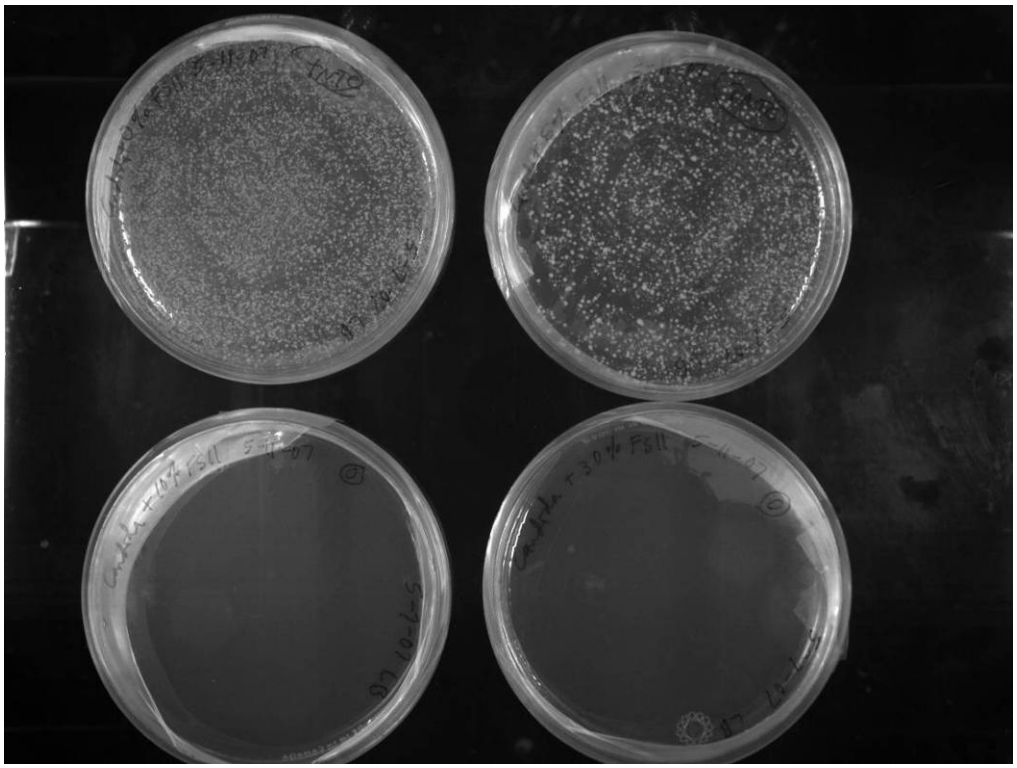

Figure 30. ATCC *Candida* at day 35. DiEGME concentrations are: 0% (upper left), 5% (upper right), 10% (lower left), and 30%. Neither lower plate has colonies.

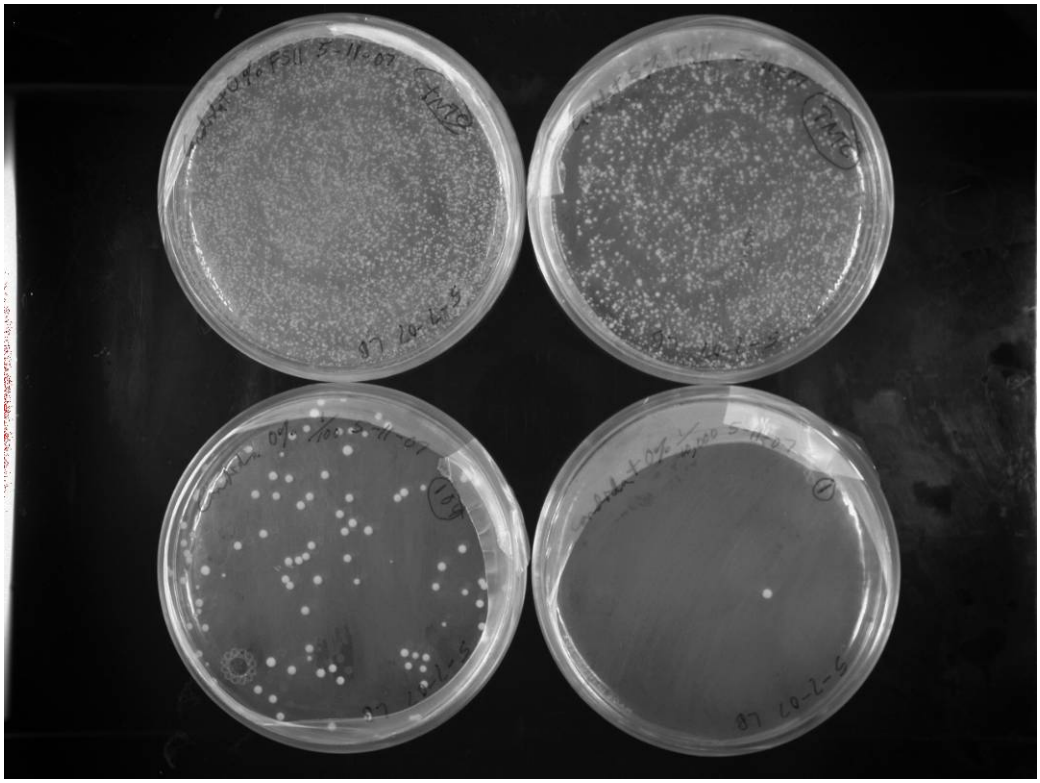

Figure 31. ATCC *Candida* at day 35. DiEGME concentrations are: 0% (upper left), 5% (upper right) at 1/10,000 dilution, 0% at 1/100 dilution (lower left), and 0% at 1/10,000 dilution (lower right). Neither lower plate has colonies.

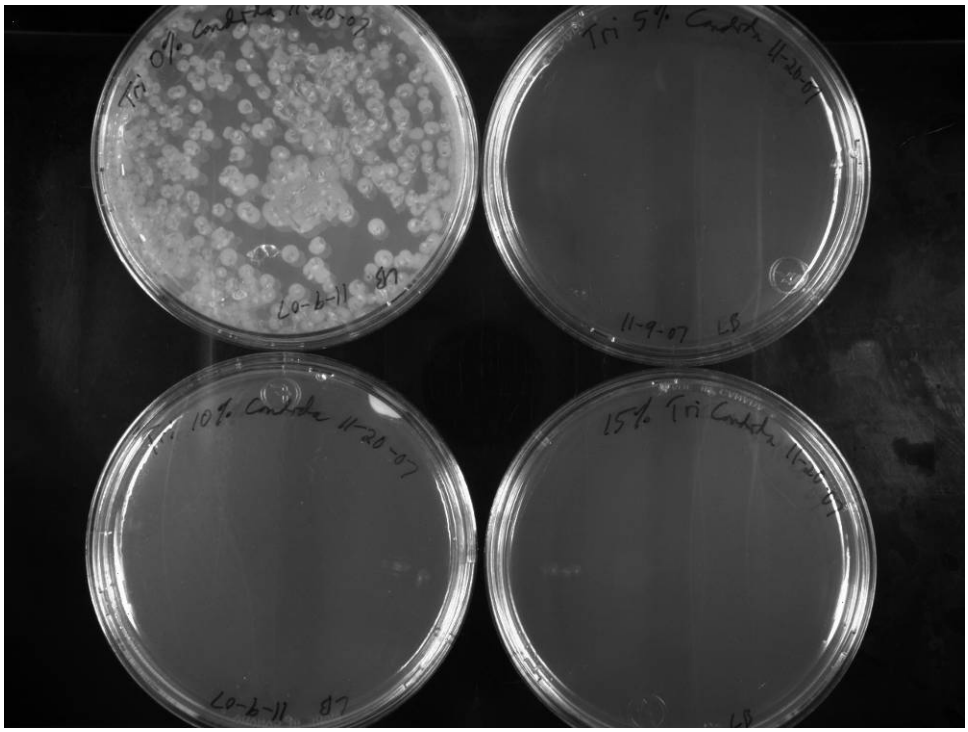

Figure 32. ATCC *Candida* at day 7. TriEGME concentrations are: 0 (no dilution, upper left), 5 (no dilution, upper right), 10 (no dilution, lower left), and 15 (1/100 dilution, lower right). Only the 0% plate has colonies.

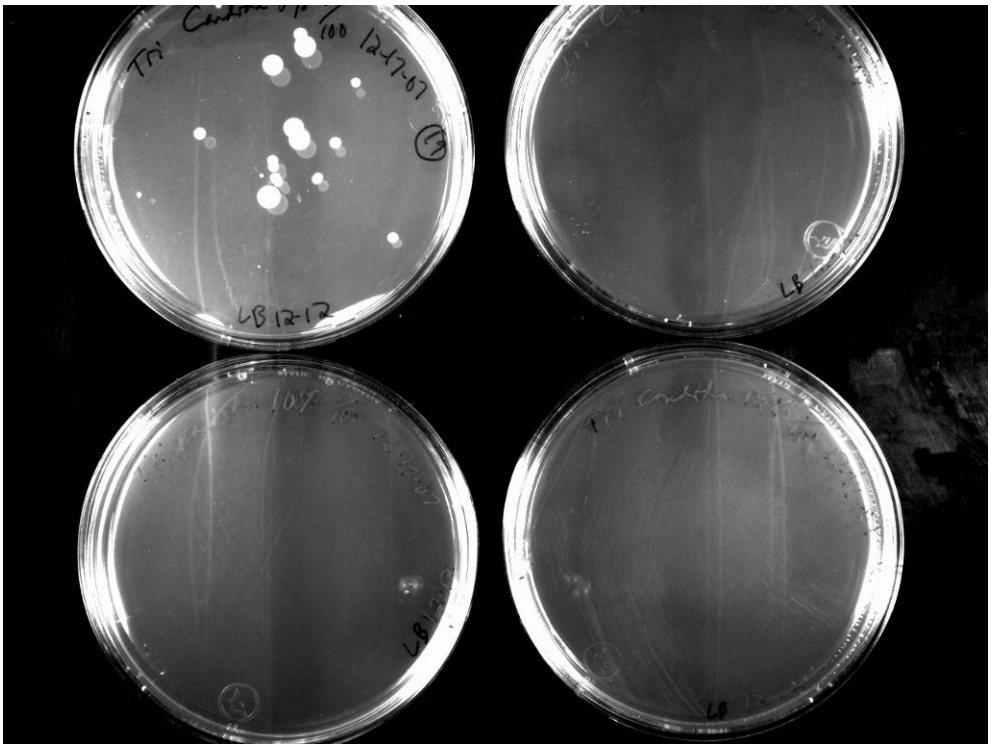

Figure 33. ATCC *Candida* at day 35. TriEGME concentrations are: 0, 5, 10, and 15%. Only the 0% plate has colonies.

Similar observations can be made concerning the colony growth on the LB agar plates over the 46 day test period. The ATCC consortia plates shown in Figures 10-33 clearly indicate that 0 and 5% FSII levels permit microbial growth, although 5% has less growth than 0% FSII. The ATCC single species plates show the same trend, with the exception of *Candida*, which was unable to grow at concentrations of 5% or greater. The FSII levels of 10% and above seem to have the capacity to completely eliminate growth for *Pseudomonas* and *Candida*, but the ATCC consortia and *Cladosporium* alone required TriEGME concentrations of 15% or greater by volume in the aqueous phase to be subdued. Compared to DiEGME, TriEGME seemed to have a greater effect on the *Candida*, and a lesser effect on the *Pseudomonas* and *Cladosporium*. Concentrations of TriEGME at or above 15% seem adequate for control of microbial growth. However, because TriEGME has a greater partition coefficient, the effective dosage rate for TriEGME would be similar to that expected for DiEGME.

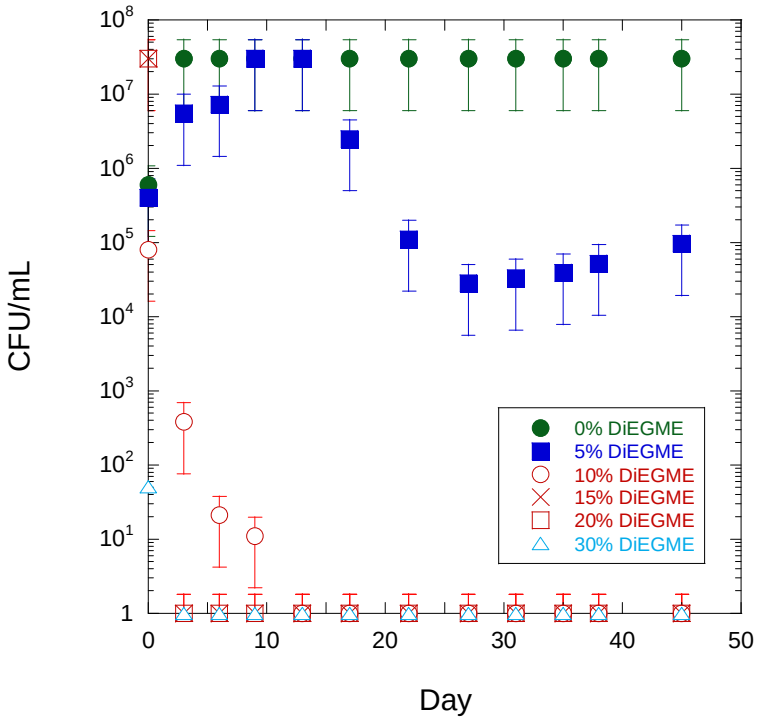

Figure 34. Semi-log plot of ATCC consortia colony forming units (CFU) per mL of liquid sample over the 46 day test period for several DiEGME levels. DiEGME level is indicated as % volume in water phase.

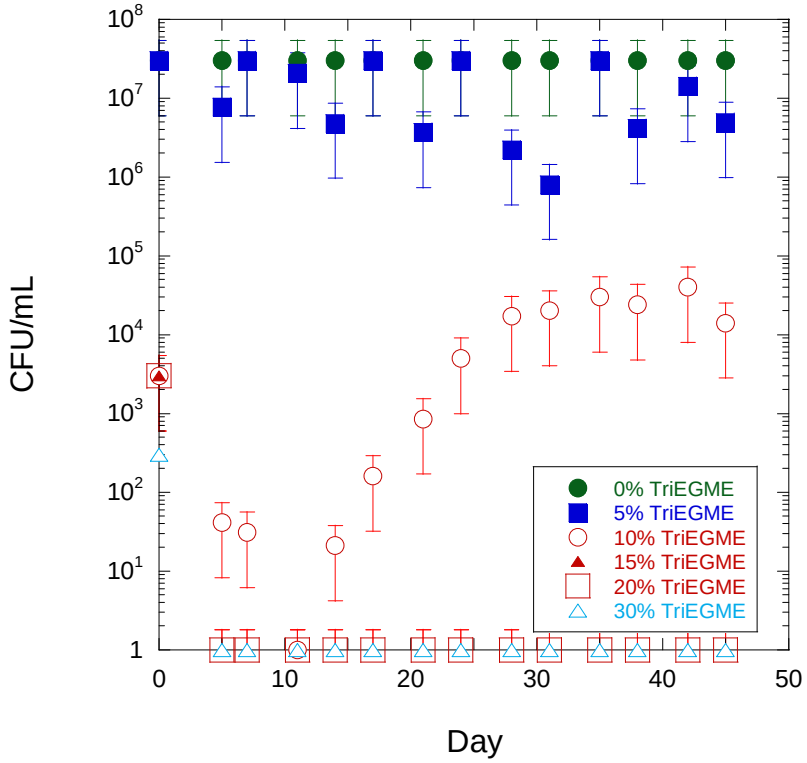

Figure 35. Semi-log plot of ATCC consortia colony forming units (CFU) per mL of liquid sample over the 46 day test period for several TriEGME levels. TriEGME level is indicated as % volume in water phase.

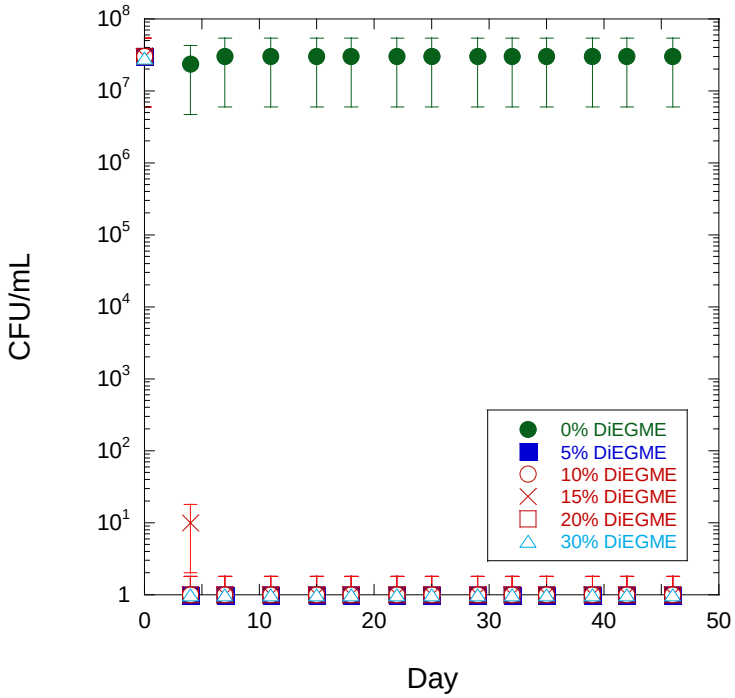

Figure 36. Semi-log plot of ATCC *Pseudomonas* colony counts over a 46 day test period for several DiEGME levels. DiEGME level is indicated as % volume in water phase.

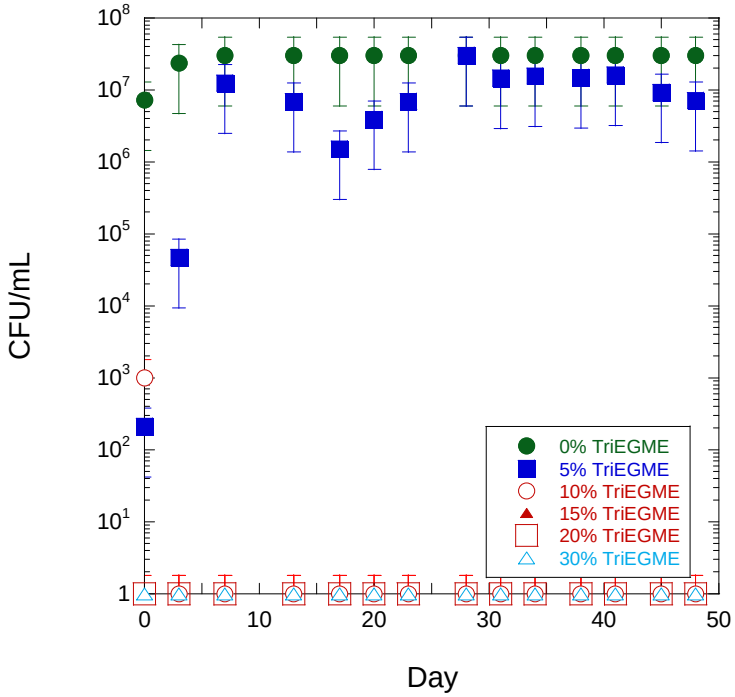

Figure 37. Semi-log plot of ATCC *Pseudomonas* colony counts over a 46 day test period for several TriEGME levels. TriEGME level is indicated as % volume in aqueous phase.

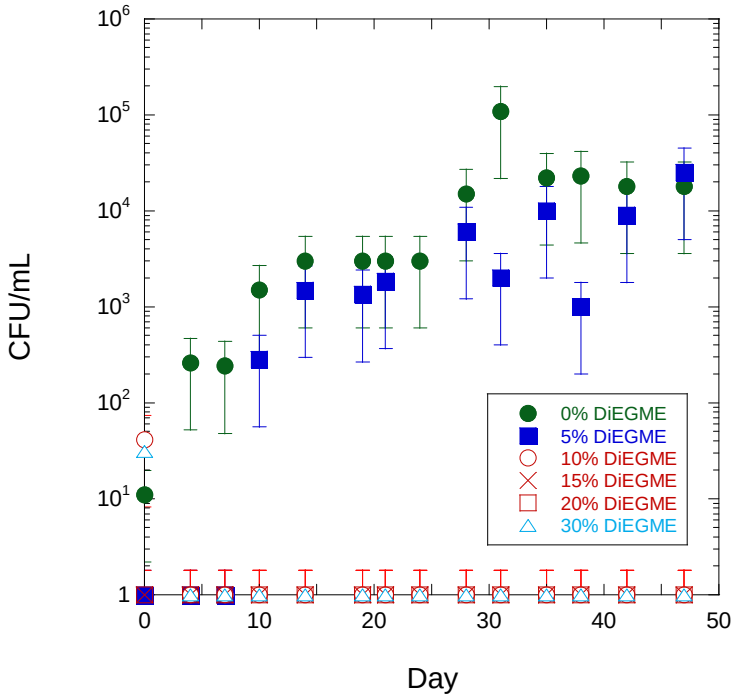

Figure 38. Semi-log plot of ATCC *Cladosporium* colony counts over a 46 day test period for several DiEGME levels. DiEGME level is indicated as % volume in water phase.

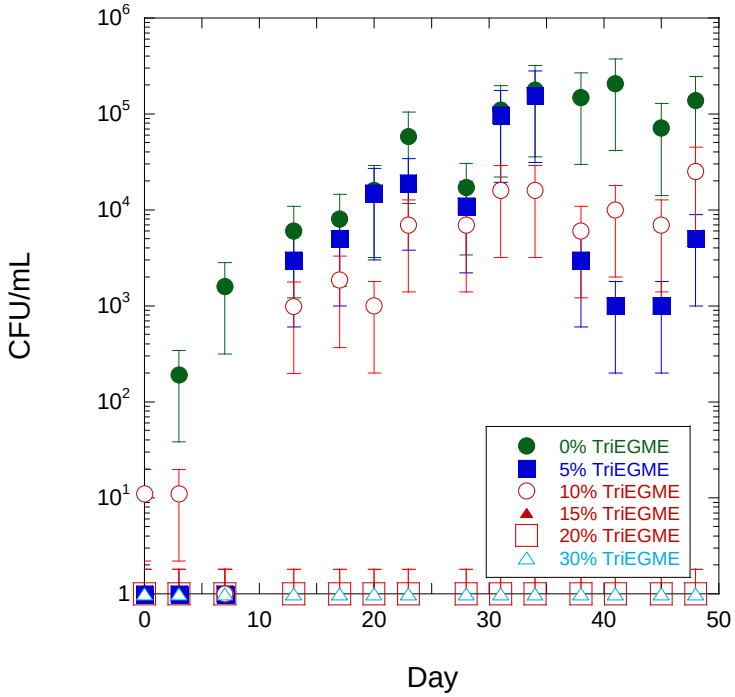

Figure 39. Semi-log plot of ATCC *Cladosporium* colony counts over a 46 day test period for several TriEGME levels. TriEGME level is indicated as % volume in aqueous phase.

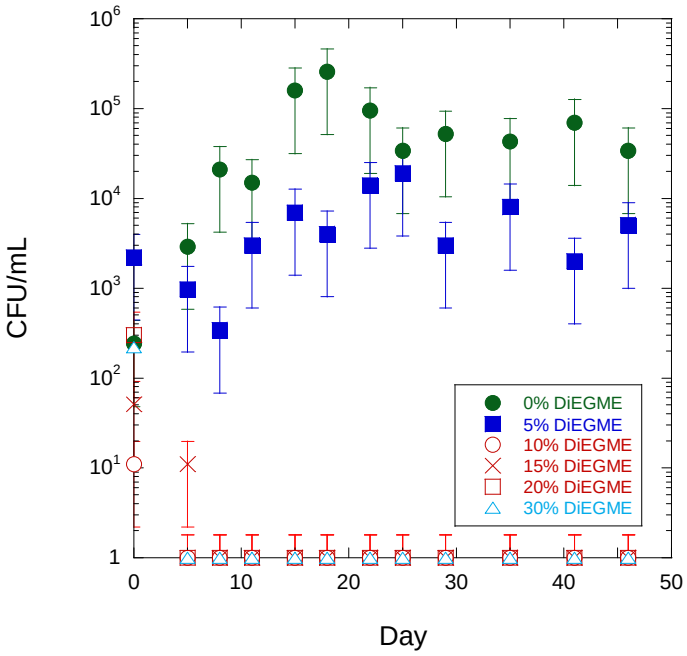

Figure 40. Semi-log plot of ATCC *Candida* colony counts over a 46 day test period for several DiEGME levels. DiEGME level is indicated as % volume in water phase.

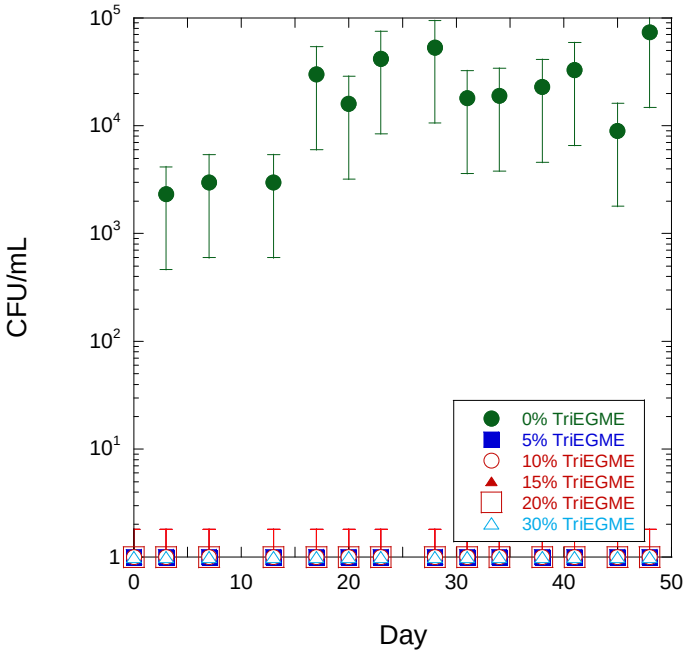

Figure 41. Semi-log plot of ATCC *Candida* colony counts over a 46 day test period for several TriEGME levels. TriEGME level is indicated as % volume in aqueous phase.

It is clear from the Figures 34-41 shown that *Pseudomonas* produces the greatest number of colonies compared to the other ATCC microorganisms, but it also experiences the greatest inhibition due to DiEGME, with only 5% DiEGME completely eliminating its population after 4 days. Although the *Candida* and *Cladosporium* produce fewer colonies, their growth continues unabated at the 5% DiEGME level, though it is also halted at 10% DiEGME in nine days or fewer. The ATCC consortia test with DiEGME containing all three microorganisms shows similar results, though the growth patterns of the consortia are somewhat different, most likely due to interaction among the three types of microorganisms and their metabolites. The microorganisms at all levels of DiEGME treatment still showed growth after four hours exposure to the icing inhibitor. However, as early as Day 1, there were significant declines in colony count for all DiEGME levels above 5% by volume in the water phase. Results suggest that a DiEGME level of 10% by volume in the water phase is adequate for elimination of microbial growth for these microorganisms. This level is slightly lower than that suggested by previous studies.

With regards to the TriEGME tests, it is clear from the plots shown that neither 0 nor 5% TriEGME reduces ATCC consortia or single microorganism colony counts to a significant degree, with the exception of *Candida*, whose colonies are significantly reduced with only 5% TriEGME. Ten percent TriEGME and above reduced colony count significantly, with the exceptions of the ATCC consortia and *Cladosporium* alone, due to the persistence of *Cladosporium*, even at 10% levels of TriEGME. These results suggest that 15% TriEGME and above in the aqueous phase is adequate for suppression or elimination of microbial growth. Using the Figure 1 partitioning data, this would correspond to a TriEGME in fuel concentration of ~0.015 volume % at 23° C.

4.2 Field Microorganisms Consortia Test

Six microorganisms were obtained from the field, cultured, isolated, then frozen at -80ºC. These microorganisms included: *Pseudomonas* sp.*, Rhodococcus equi, Bacillus licheniformis, Clostridium intestinale, Methylobacterium* sp., and *Cladosporium resinae*. They were revived from the frozen state separately by incubation at 28ºC on an LB agar plate, then utilized in the same procedure listed above. The field microorganisms were tested as a consortia only. The same DiEGME and TriEGME levels were used as previously mentioned. Figures 42-44 below shows the French square test setups following the 46 day test. Figures 45-50 below show agar plate growth at several different points during the experiment. Figures 51 and 52 show colony counts at all DiEGME and TriEGME levels over the 46 day test period.

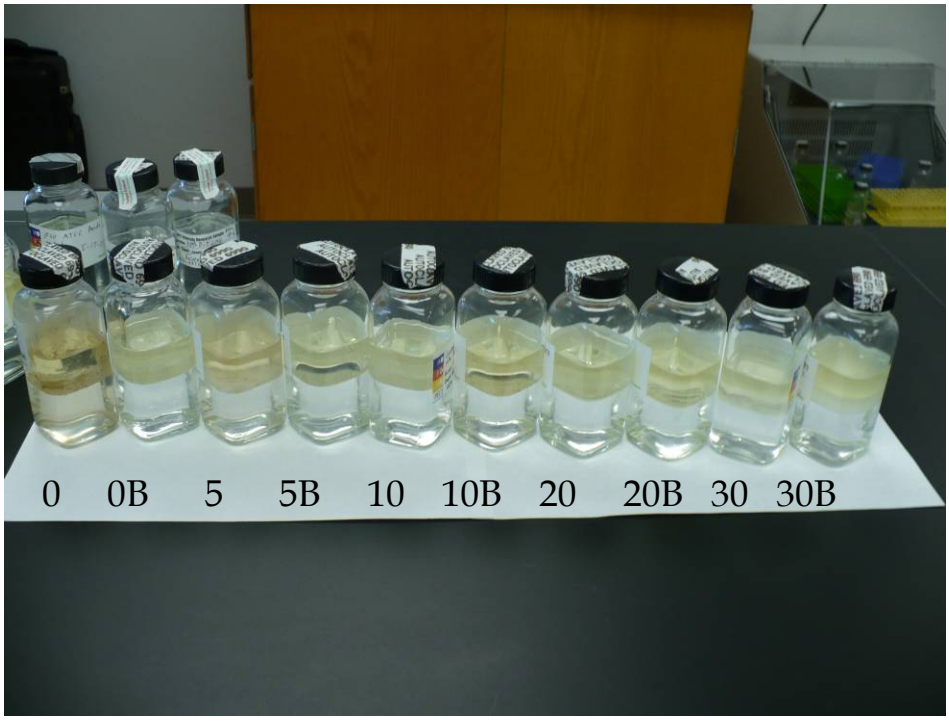

Figure 42. Field consortia following day 46 of test. DiEGME concentrations are: 0, 5, 10, 20, and 30% by volume in water phase, with each next to its respective blank.

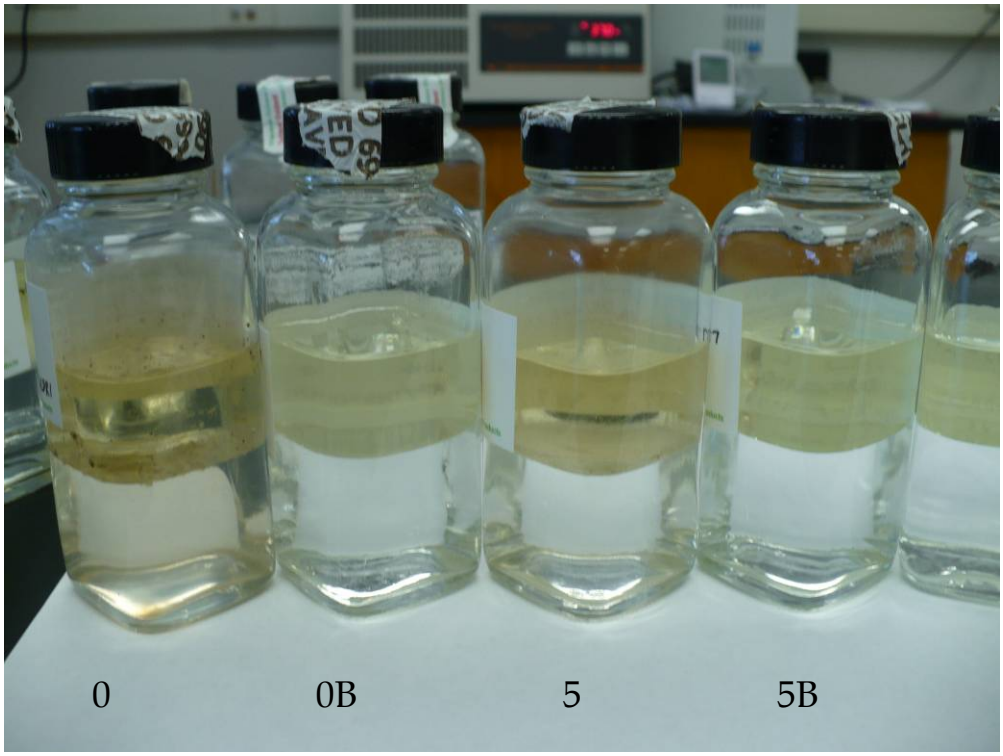

Figure 43. Field consortia following day 46 of test. DiEGME concentrations are: 0 and 5% by volume in water phase, with each next to its respective blank.

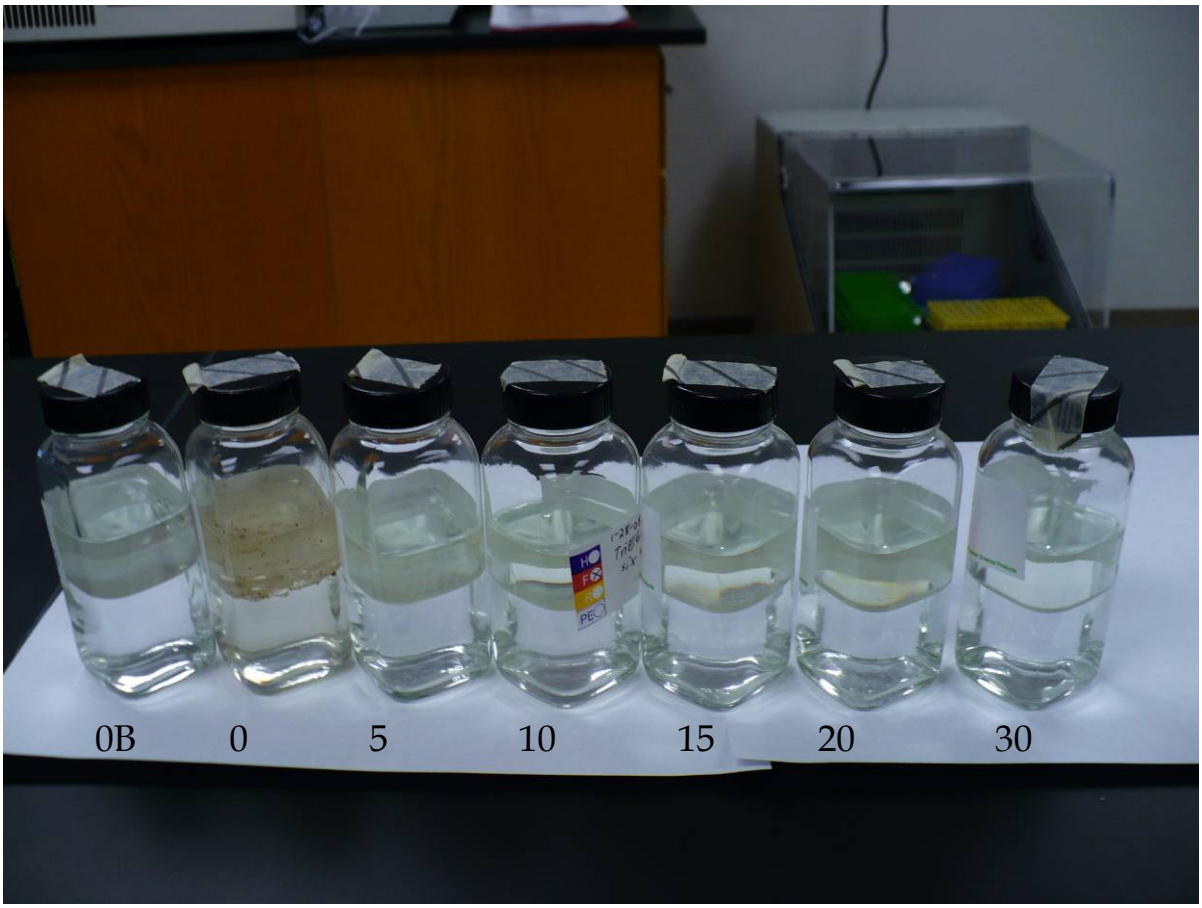

Figure 44. Six field consortia following day 46 of test. The blank fuel/water setup is on the left, followed byTriEGME concentrations of: 0, 5, 10, 15, 20, and 30% by volume in the aqueous phase.

Figures 42-44 show that the field consortia test setups are visually similar to the ATCC consortia setups. Like the ATCC setups, the field consortia setups show some cloudiness in the water layer, as well as particulates and cloudiness in the fuel layer for the 0% TriEGME levels. However, no visual differences were apparent for the 5% level and above test setups.

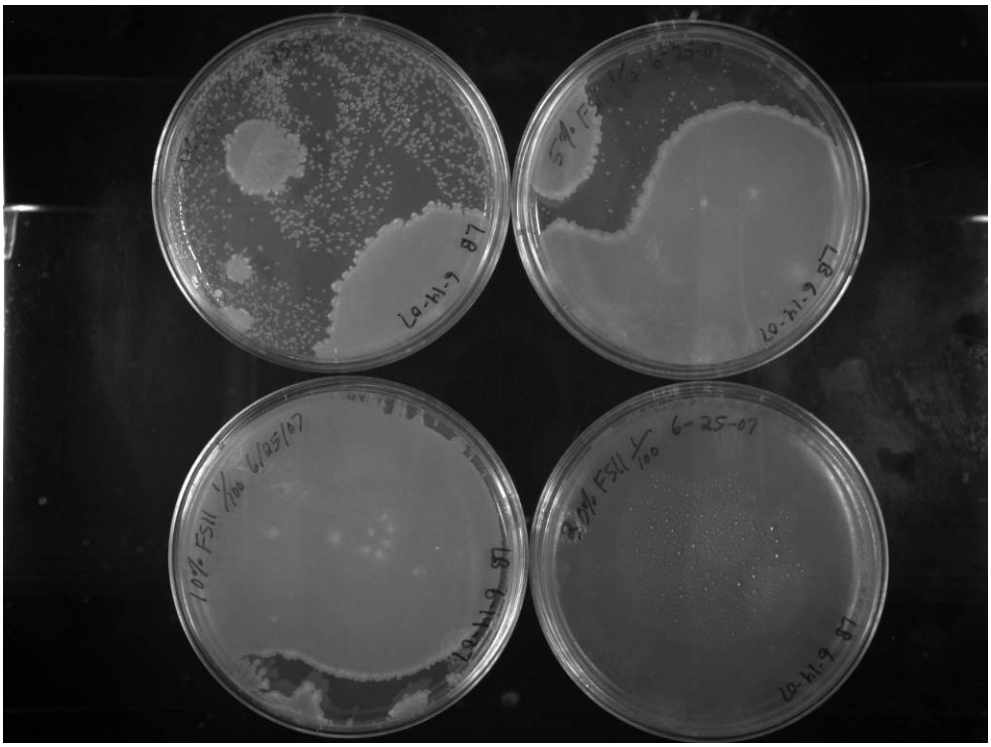

Figure 45. Six field consortia at day 11. DiEGME concentrations are: 0 (upper left), 5 (upper right),10 (lower left), 20% DiEGME (lower right), 1:100 dilution. All plates are swarming with growth.

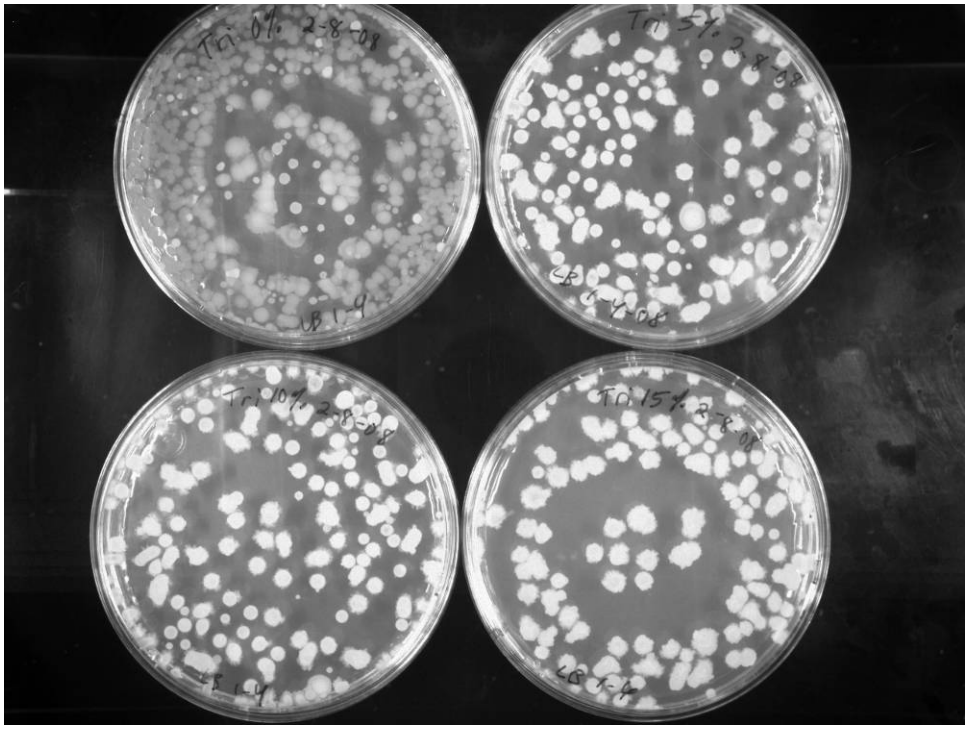

Figure 46. Six field consortia at day 11. TriEGME concentrations are: 0 (upper left), 5 (upper right), 10 (lower left), and 15% (lower right). All plates show growth.

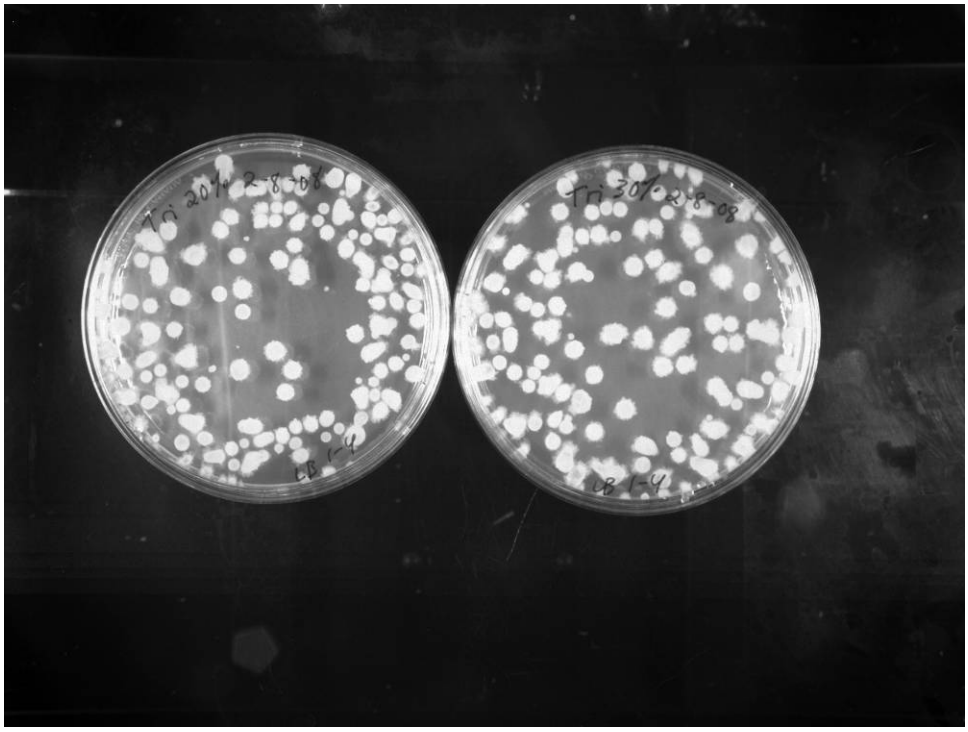

Figure 47. Six field consortia at day 11. TriEGME concentrations are: 20% (left) and 30% (right). Growth shown on both plates.

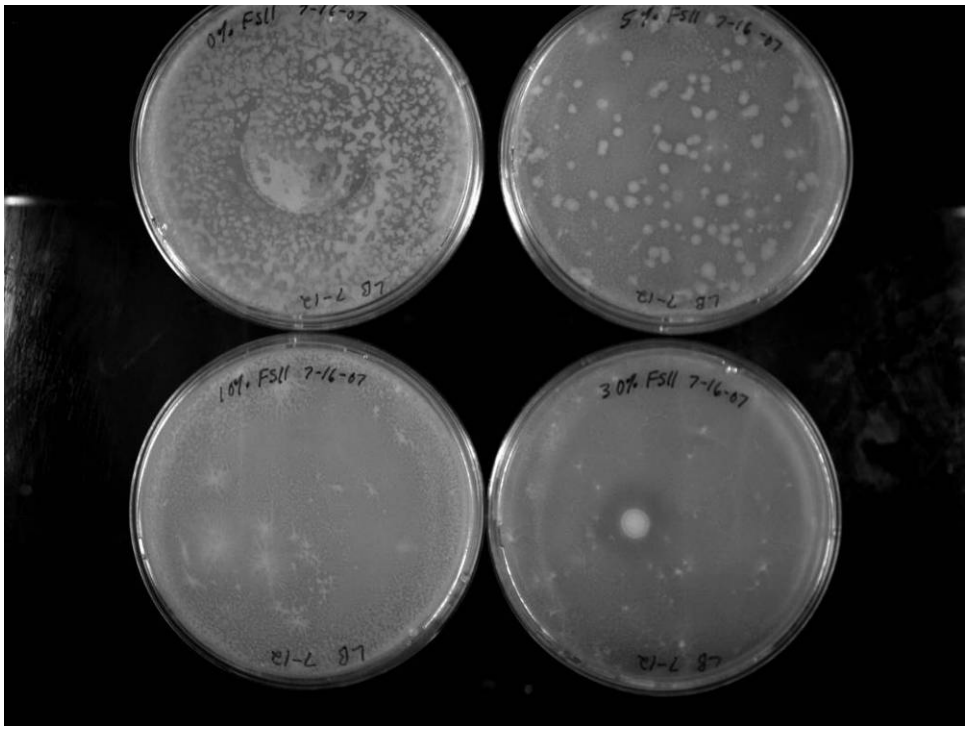

Figure 48. Six field consortia at day 32. DiEGME concentrations are: 0 (upper left), 5 (upper right),10 (lower left), and 30% DiEGME (lower right).

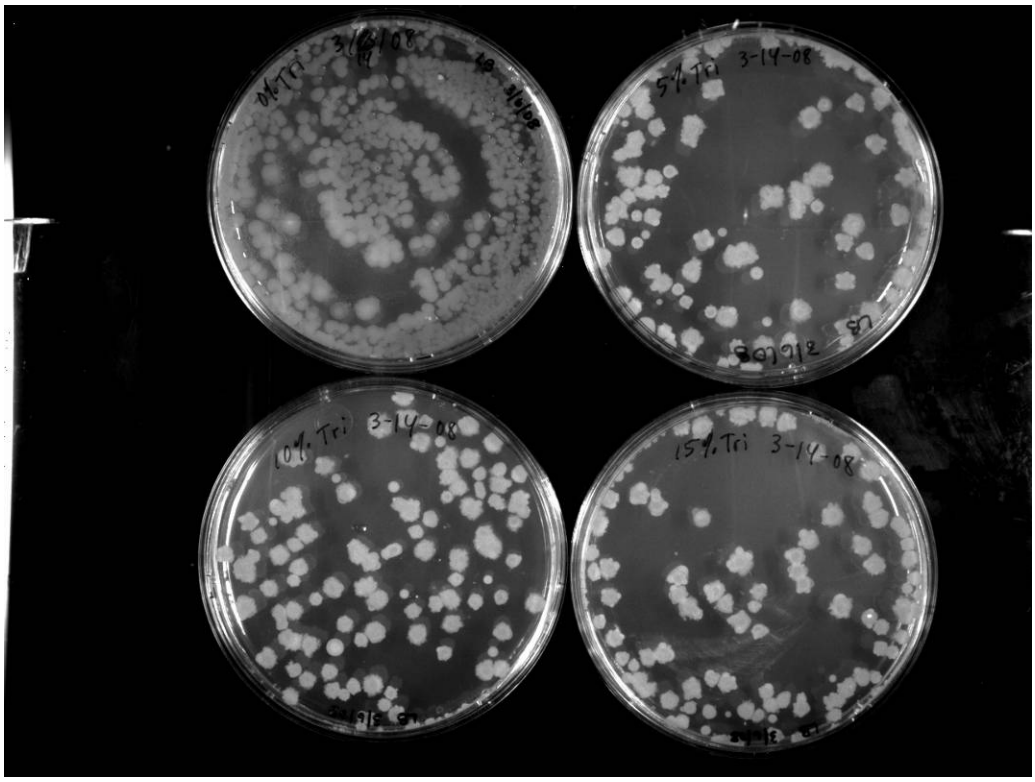

Figure 49. Six field consortia at day 46. TriEGME concentrations are: 0 (upper left), 5 (upper right),10 (lower left), and 15% DiEGME (lower right). Growth on all plates.

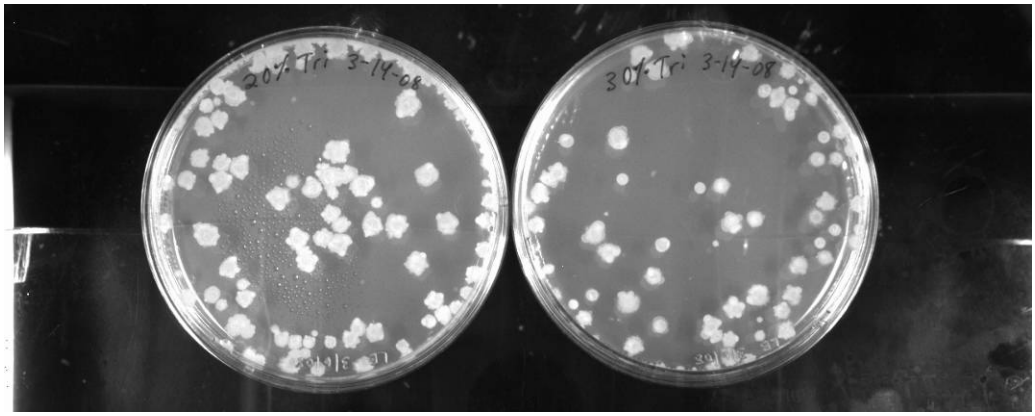

Figure 50. Six field consortia at day 46. TriEGME concentrations are: 20% (left) and 30% (right). Growth shown on both plates.

Figures 45-50 show agar plates at several points during the 46 day test period. Differences can be seen between the ATCC microorganisms' colony growth and that of the field consortia. It is apparent that, although the 5% and higher FSII levels are reducing levels of microbial growth, they are not halting the growth completely for the field microorganisms. However, the addition of higher levels of TriEGME, greater than 5%, did not result in further antimicrobial benefits. Once the "biostatic" concentration for the field microorganisms was reached, higher additive concentrations did not significantly reduce microbial growth.

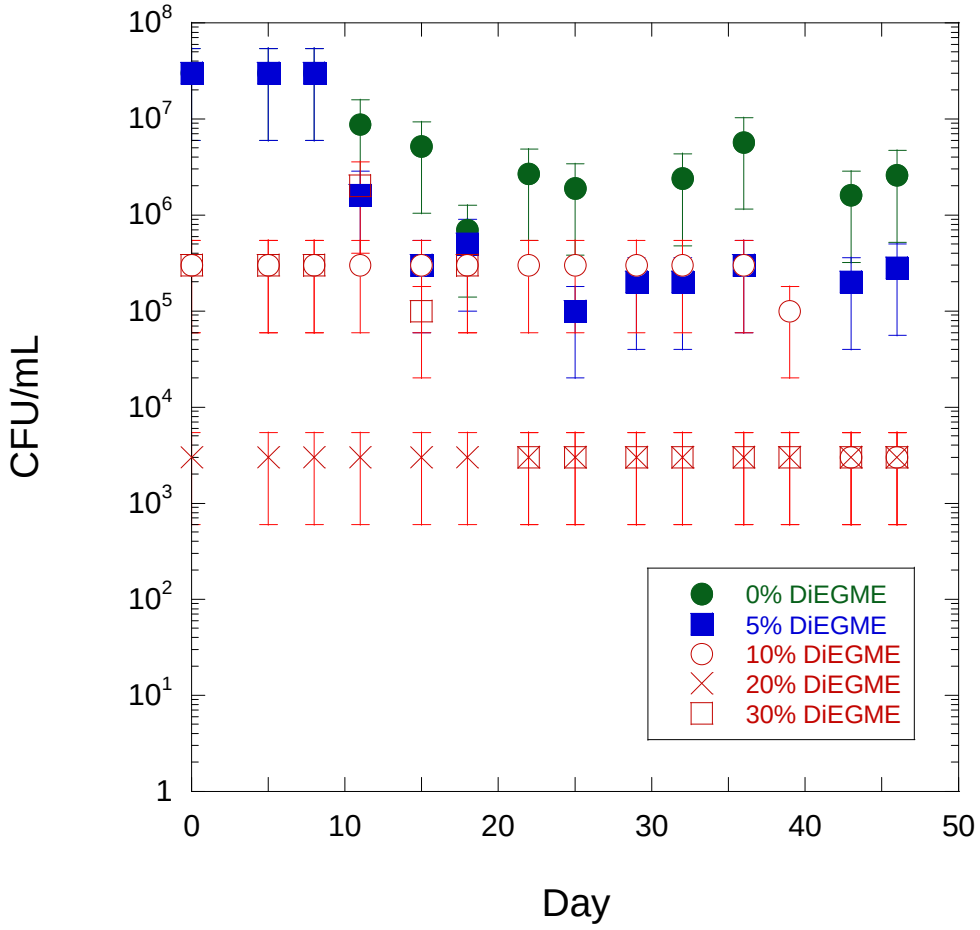

Figure 51. Semi-log plot of six field consortia colony counts over a 46 day test period for several DiEGME levels. DiEGME level is indicated as % volume in water phase.

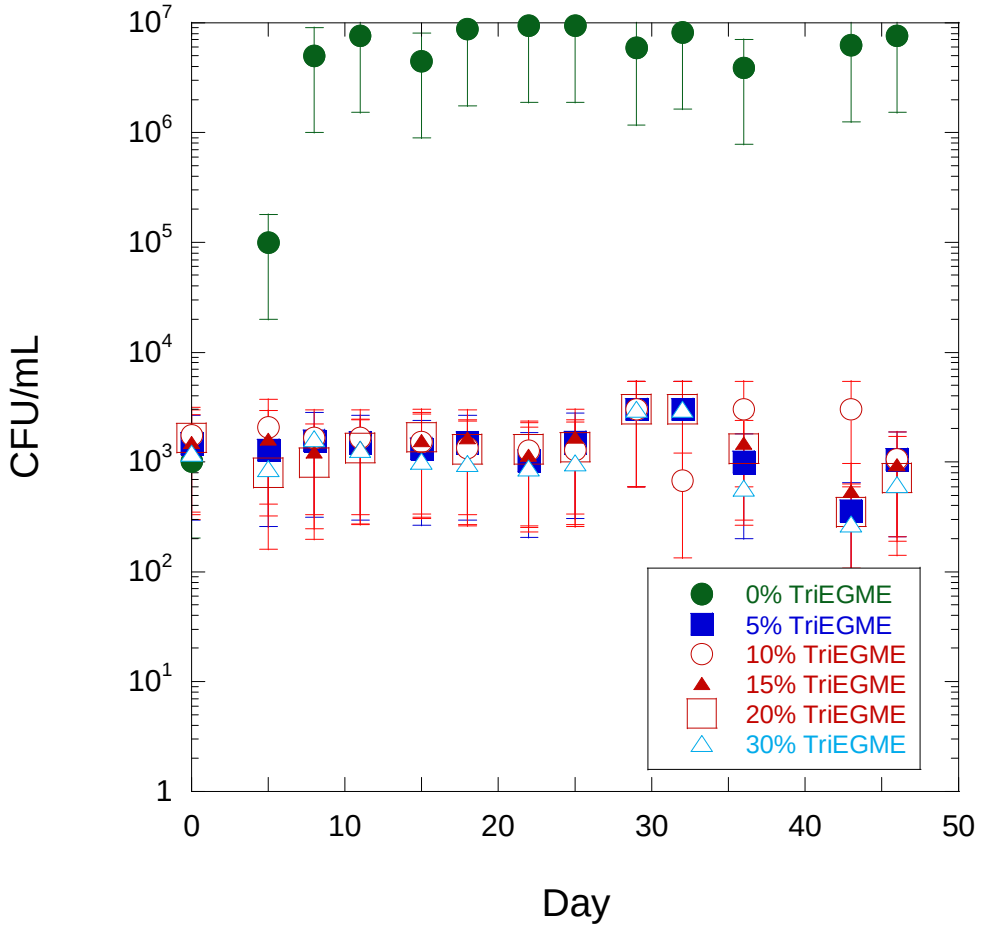

Figure 52. Semi-log plot of six field consortia colony counts over a 46 day test period for several TriEGME levels. TriEGME level is indicated as % volume in water phase.

Figures 51 and 52 show that, unlike the ATCC consortia, the mixed field consortia grew throughout the test period at each tested DiEGME and TriEGME concentration. However, the presence of DiEGME or TriEGME reduced the amount of growth at each test period. It seems possible that differences in response to FSII are the result of genetic mutations within the field microbes. These mutations can occur gradually, due to low exposure to a similar substance in the environment over time, or it can occur quickly, after a single exposure to the additive. Another possibility is that resistance was transferred to the field microbes tested here by other microbes in the environment that became resistant following their own exposure to a similar substance, via plasmid swapping. Still another possibility is hypermutation, wherein microorganisms under stress (due to a toxic agent or environmental change) mutate at increased rates, leading to a higher probability of survival by at least some mutated microorganisms (30). Also, it is possible that some of the microbes are naturally resistant, requiring no exposure to the additive at all.

Until the field consortia are tested singly, or colony morphology is studied further, it will not be clear which members have developed tolerance or if, perhaps, all of them have. Although it is clear that the low levels of DiEGME and TriEGME in this study did not kill the six field microorganisms, the difference between the 0% DiEGME and TriEGME samples and the other low level samples was dramatic, indicating the continuing positive effect of a biocidal/biostatic fuel additive. Generally, it can be

said that, regardless of whether the microorganisms are lab cultured or from the field, DiEGME and TriEGME still have a beneficial biocidal/biostatic effect. The current study suggests that a DiEGME level of 10% in the aqueous phase (~0.01-0.02% by volume added to the fuel), or a TriEGME level of 15% by volume in the aqueous phase at minimum (~0.01-0.02% by volume added to the fuel) is required to control microbial growth and prevention of harmful biofilms.

4.3 Additional Field Microorganisms Consortia Test at Higher DiEGME/TriEGME Concentrations

Because two of the six field microbes persisted in the original microbiological study using 0-30% DiEGME and TriEGME by volume in aqueous phase, an additional study was conducted to determine whether DiEGME and TriEGME were more effective on these microbes at typical DiEGME concentrations seen in the field, which can be ~30-60% in the aqueous phase. A similar study was also conducted for corresponding levels of TriEGME. Morphological features suggested that the two surviving microbes were *Bacillus* and *Clostridium*. The additional study was conducted with the same methodology utilized in section 3.4, except the concentrations tested were: 30, 40, 50, and 60% DiEGME or TriEGME by volume in the aqueous phase, which corresponds to ~0.05-0.15% in the fuel phase for DiEGME, and ~0.04-0.1% for TriEGME. Figures 53-56 show liquid test setups following the 46 day test. Figures 57-62 show colony plating results at several points during the test. Figures 63 and 64 illustrate colony counts throughout the 46 day test period.

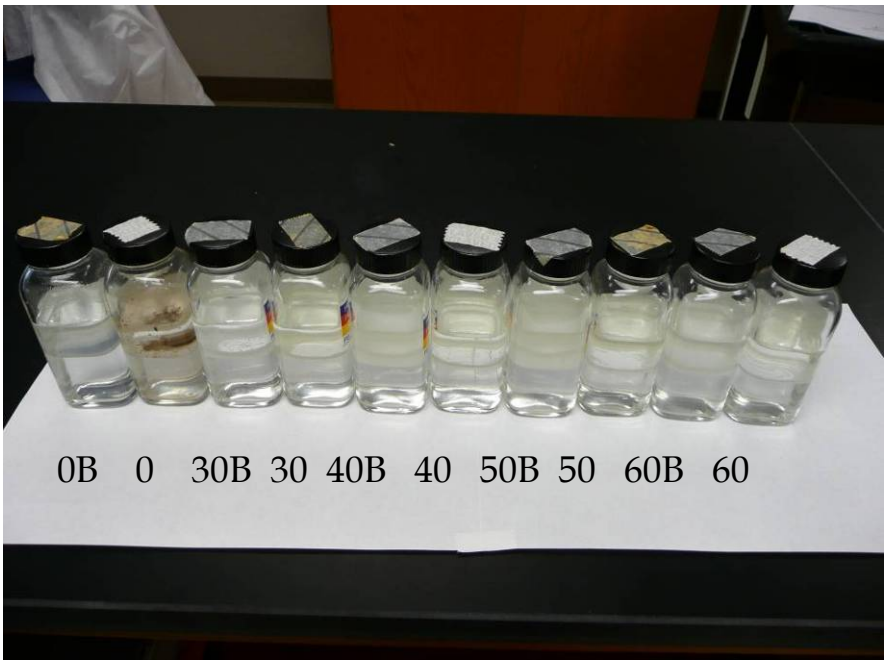

Figure 53. Additional field consortia liquid setups after 46 days. Concentrations are 0, 30, 40, 50, and 60% DiEGME in the aqueous phase. Each is shown with its respective blank.

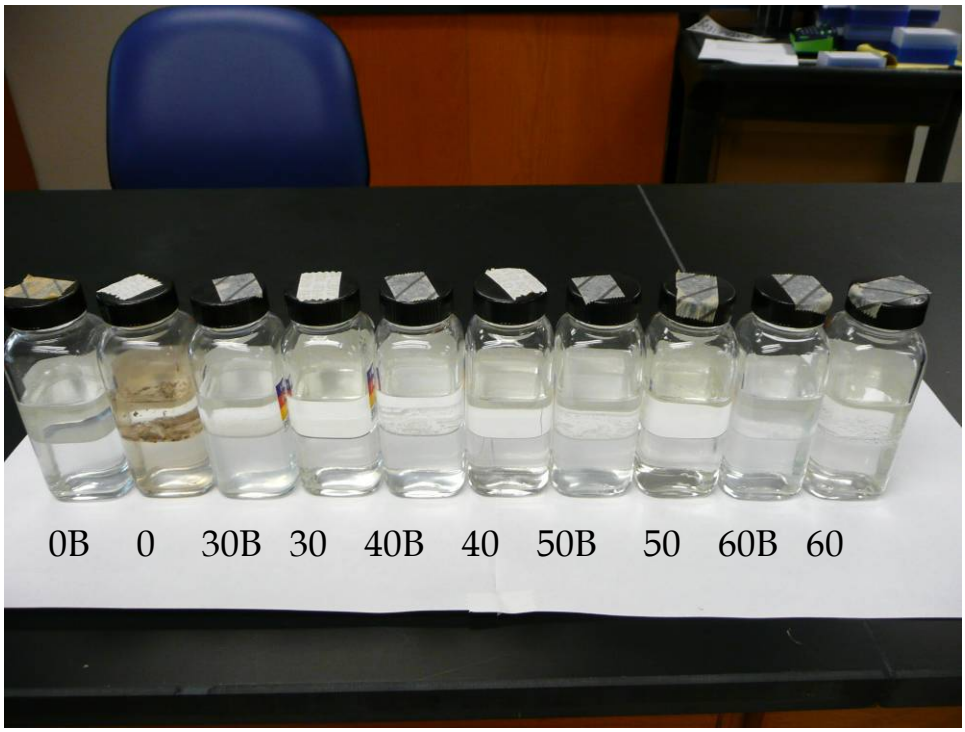

Figure 54. Additional field consortia liquid setups after 46 days. Concentrations are 0, 30, 40, 50, and 60% TriEGME in the aqueous phase. Each is shown with its respective blank.

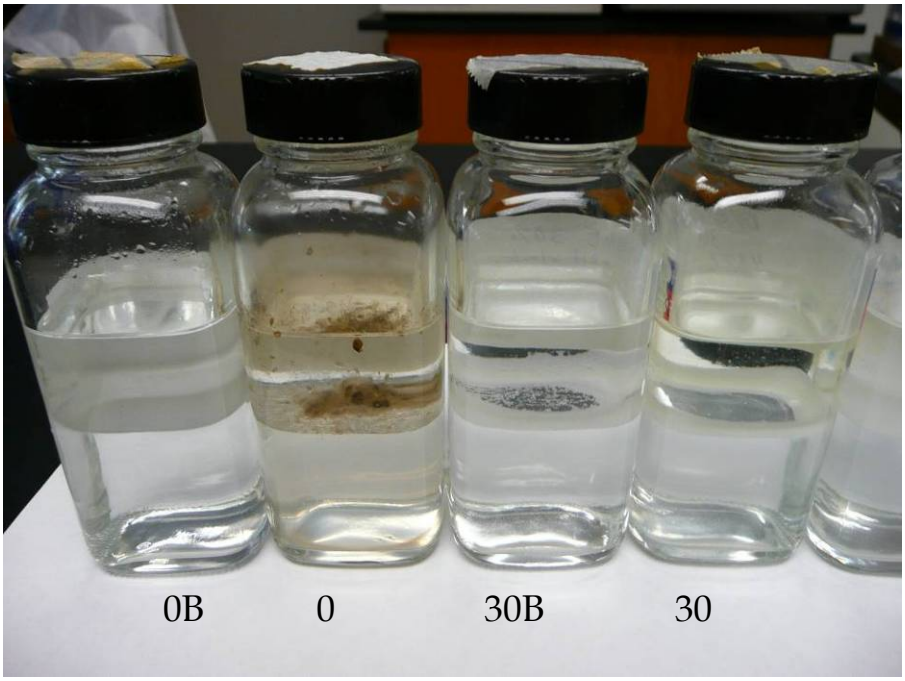

Figure 55. Additional field consortia on day 46. Closeup of 0 and 30% DiEGME with their blanks.

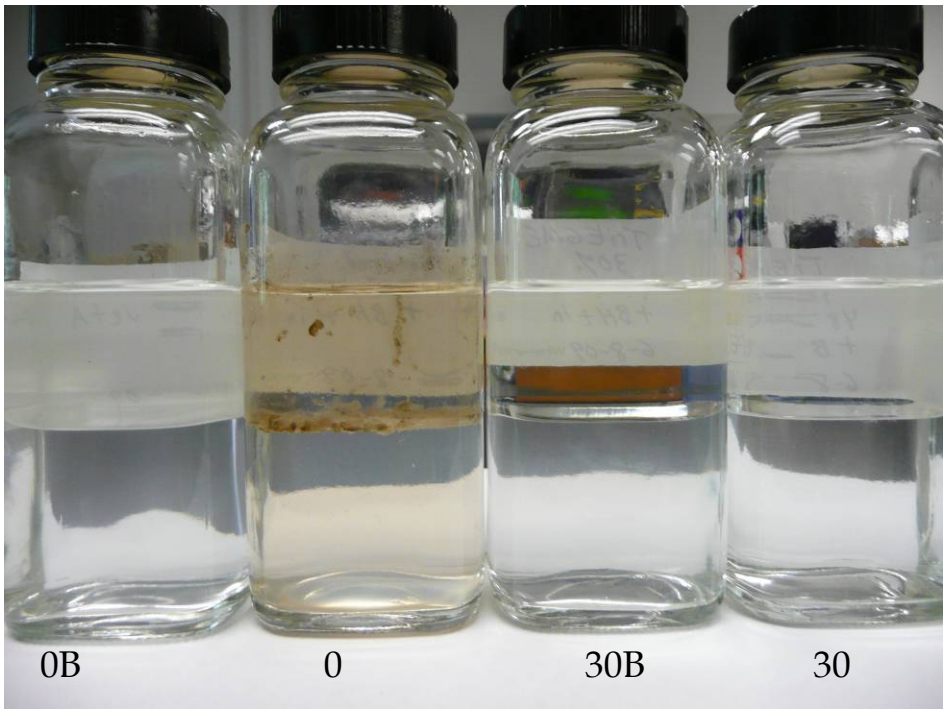

Figure 56. Closeup of additional field consortia liquid setups after 46 days. Concentrations are 0 and 30% TriEGME in the aqueous phase. Each is shown with its respective blank.

Figures 53-56 indicate that substantial growth is only present in the 0% DiEGME or TriEGME liquid setup. A brown biofilm in the hydrocarbon phase is readily apparent, as is significant cloudiness in the aqueous phase. Both suggest significant microbial contamination. The corresponding blank has no growth nor obvious cloudiness. The 30-60% DiEGME and TriEGME liquid setups appear very similar to their blanks, although the blanks appear to be slightly less cloudy in the aqueous phase. At these concentrations of DiEGME and TriEGME, emulsions are persistent and occur for both inoculated and blank liquid setups.

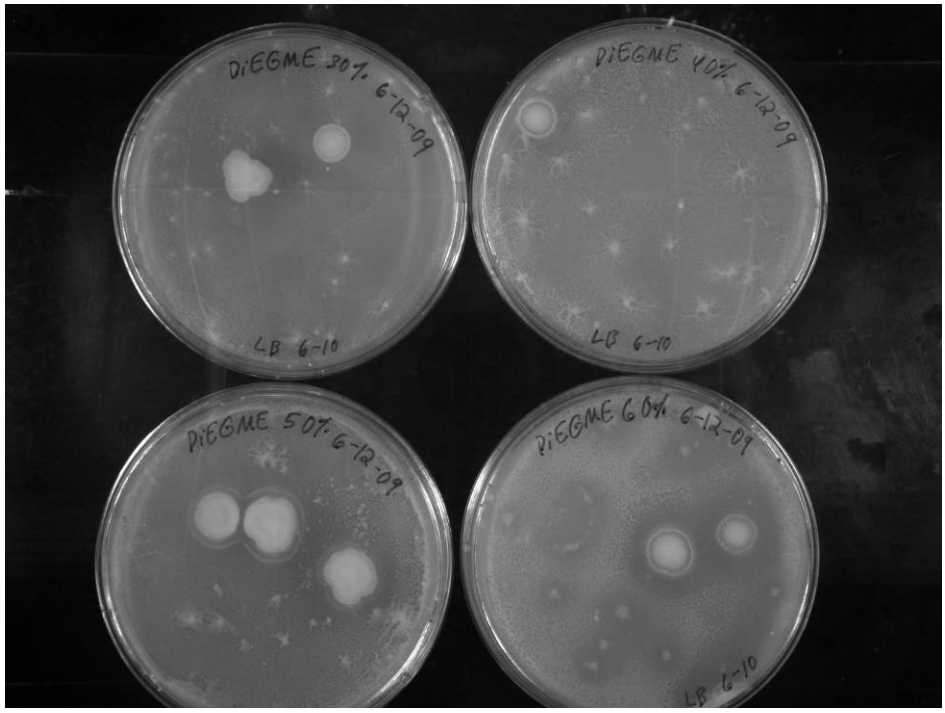

Figure 57. Additional field consortia study on day 4. DiEGME concentrations of 30, 40, 50, and 60% in the aqueous phase are shown.

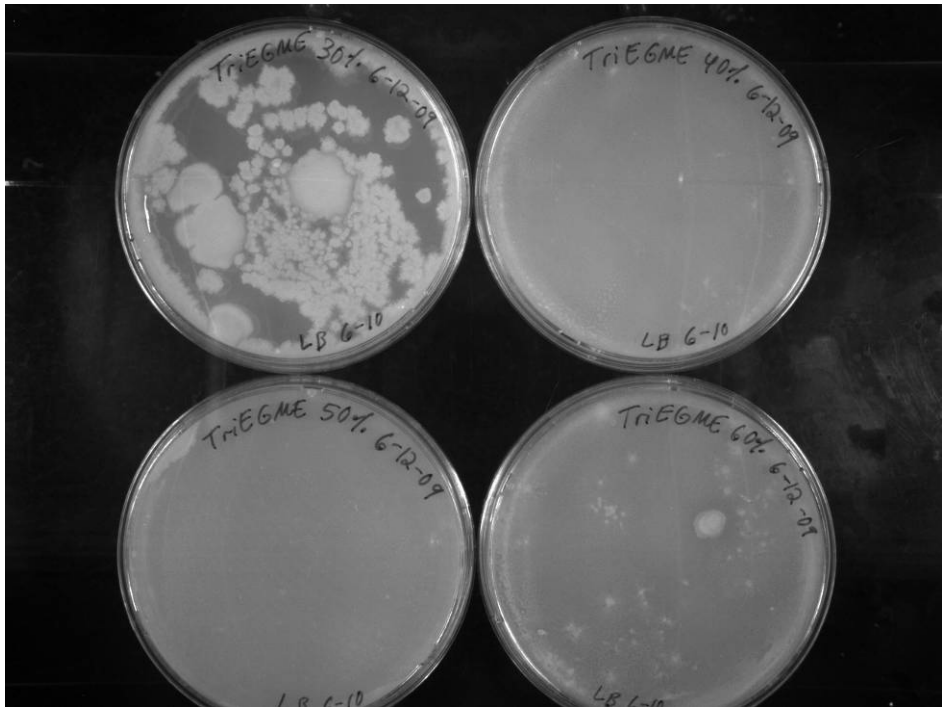

Figure 58. Additional field consortia study on day 4. TriEGME concentrations of 30, 40, 50, and 60% in the aqueous phase are shown.

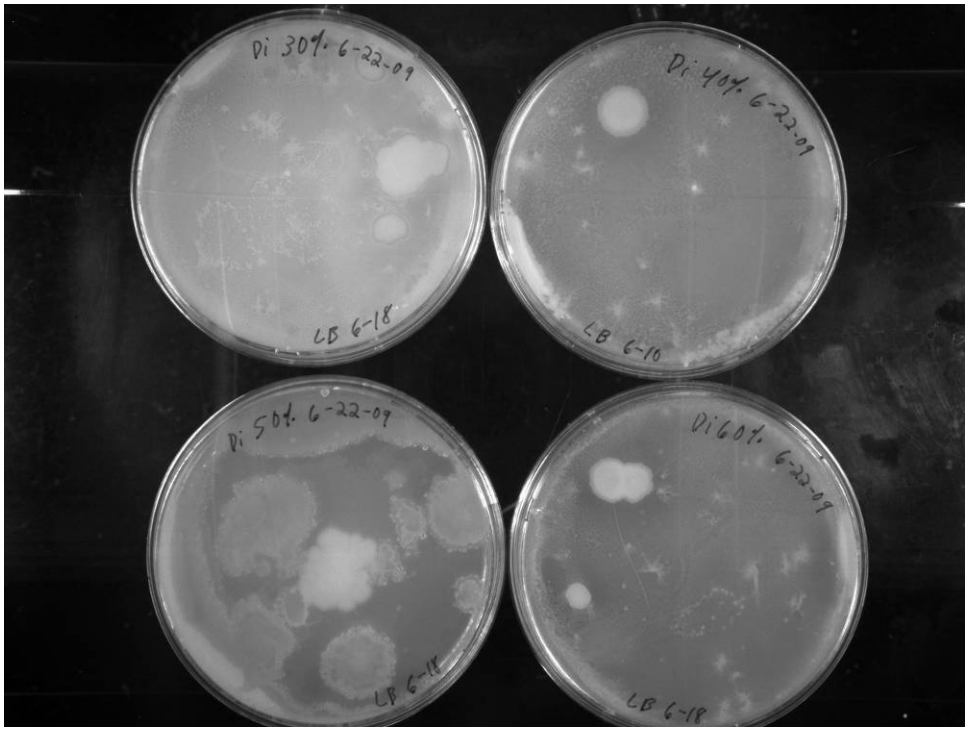

Figure 59. Additional field consortia study on day 14. DiEGME concentrations of 30, 40, 50, and 60% in the aqueous phase are shown.

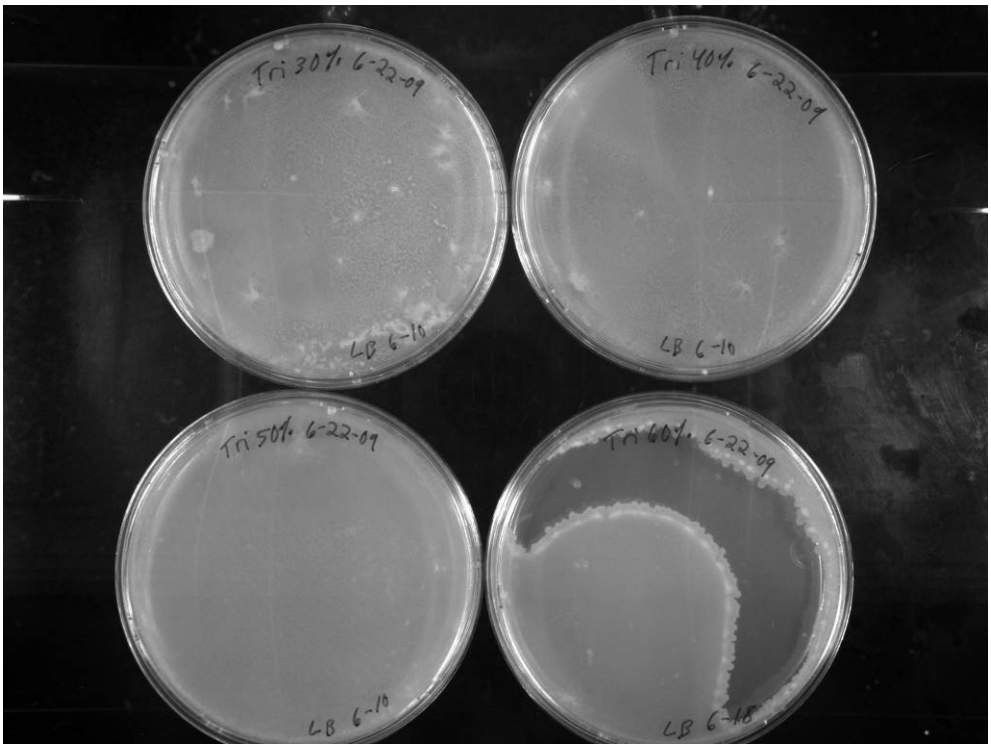

Figure 60. Additional field consortia study on day 14. TriEGME concentrations of 30, 40, 50, and 60% in the aqueous phase are shown.

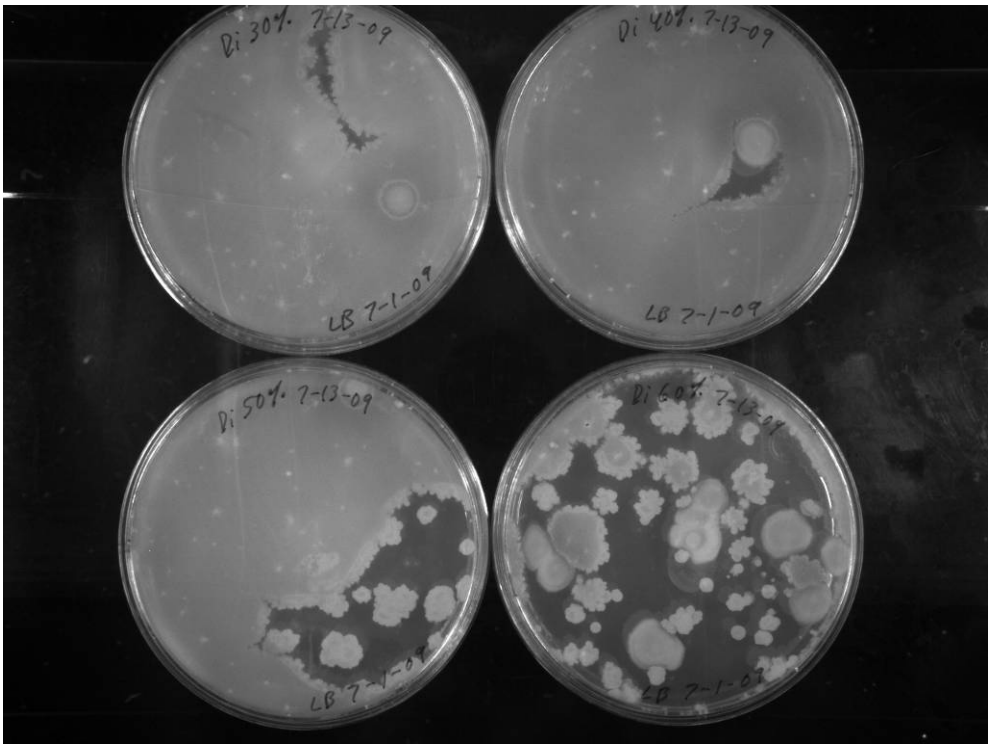

Figure 61. Additional field consortia study on day 35. DiEGME concentrations of 30, 40, 50, and 60% in the aqueous phase are shown.

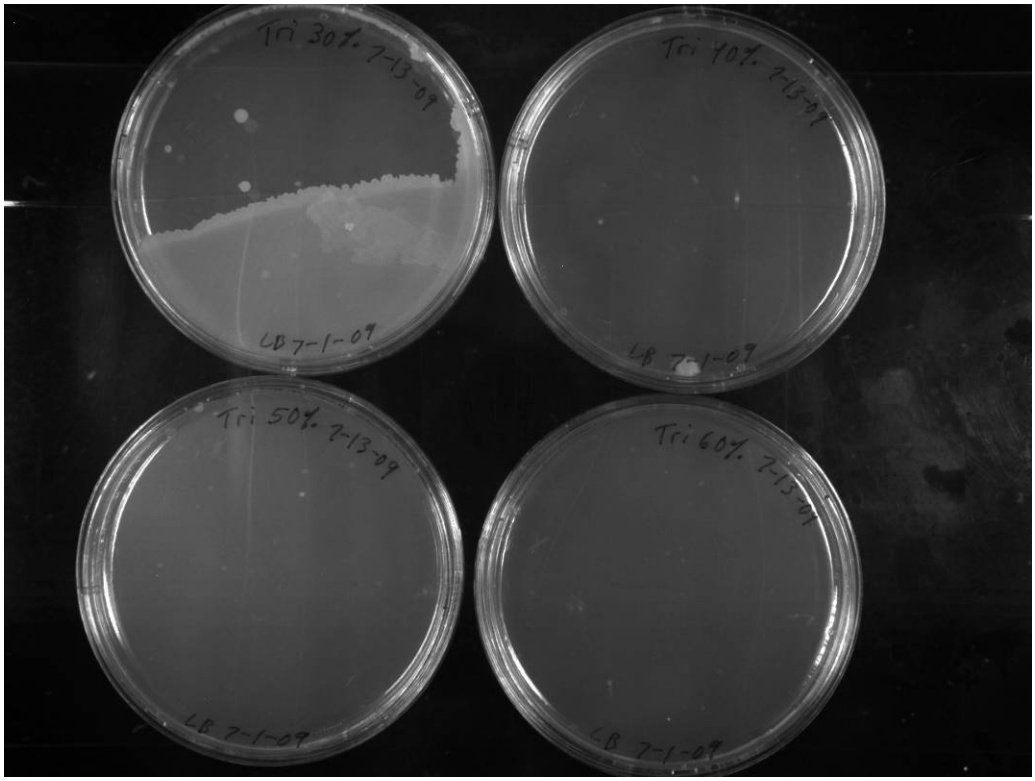

Figure 62. Additional field consortia study on day 35. TriEGME concentrations of 30, 40, 50, and 60% in the aqueous phase are shown.

The colony plate results shown in Figures 57, 59, and 61indicate healthy microbial growth at all test points shown for DiEGME, although it does appear that the amount of colonies is somewhat decreased in the second half of the test duration. Morphological evaluation of these colonies suggests that the *Bacillus* and *Clostridium* obtained from the field are able to survive in the presence of DiEGME, even at concentrations of 30-60% DiEGME in the aqueous phase.

For TriEGME, shown in Figures 58, 60, and 62, clearly at 40% and above growth is eventually eradicated. The 30% growth is lower than reported in the previous TriEGME chart, but still present.

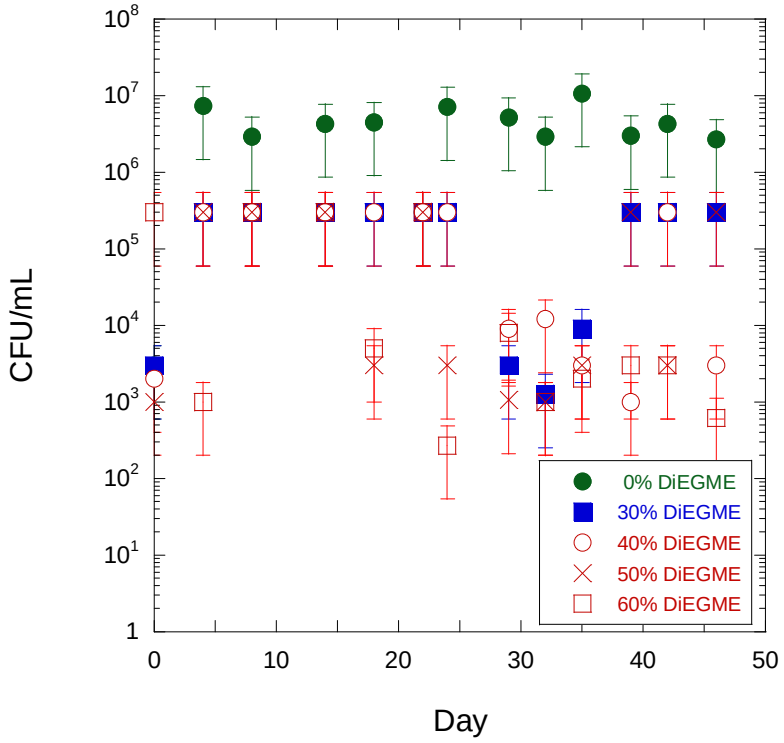

Figure 63. Semi-log plot of additional field consortia test colony counts over a 46 day test period for several DiEGME levels. DiEGME level is indicated as % volume in water phase.

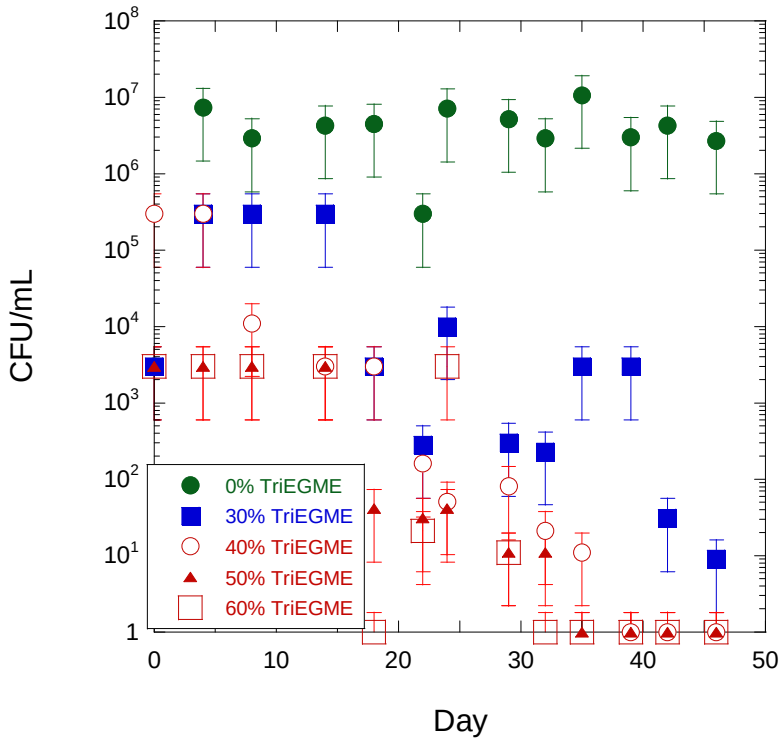

Figure 64. Semi-log plot of additional field consortia test colony counts over a 46 day test period for several TriEGME levels. TriEGME level is indicated as % volume in water phase.

Figure 63 illustrates that, over the 46 day test period, the presence of DiEGME at 30-60% decreases the amount of field consortia colonies present, but does not eliminate growth completely. Furthermore, decreases in numbers are not necessarily obtained with an increase in DiEGME concentration. Rather, the threshold seen for the original study—DiEGME present at 10% or greater in the aqueous phase—seems to provide approximately the same protection as the higher DiEGME concentrations currently present in the field (24). As Figure 64 suggests, over the 46 day test period for TriEGME the results are different at higher concentrations than they are for DiEGME. At levels of 40% and above of TriEGME, even *Bacillus* and *Clostridium* growth was eventually eliminated. This is an interesting result, as TriEGME is generally thought to be less toxic than DiEGME (31). It is possible that, since the growth at 30% was lower than before, that this result reflects experimental variation in colony growth measurements, and should be performed again to determine if the elimination of the field microbes at higher concentrations is repeatable. On the other hand, it is possible that the two microbes' resistance of DiEGME over the entire test period is a reflection of their natural or acquired resistance, one that they do not have to quite the same extent for TriEGME, though they might develop it over time.

5. Conclusions

The current study provides valuable information regarding potential effects of adding DiEGME or TriEGME to fuel systems, and it also provides a better understanding of the current role of DiEGME and TriEGME with respect to microbial contamination. This study of the biocidal/biostatic effects of TriEGME at reduced levels, similar to the levels targeted for the reduced FSII (DiEGME) program, was conducted in support of an RTOC program aimed at replacing DiEGME with TriEGME. TriEGME has preferential vapor properties that would prevent topcoat peeling problems in the B-52 and also has a partition coefficient which would make possible similar additive dosage rates compared to DiEGME. This study explored the biological impact of replacing DiEGME with TriEGME at the reduced levels expected to be implemented for DiEGME, i.e. from ~30-60% by volume in the aqueous phase to 0-30%. In this study, where field microorganisms were included, in addition to lab cultured ATCC microbes, it was found that TriEGME levels of 15% by volume in the water phase or greater were sufficient to eliminate microbial growth of all three lab cultured, ATCC microorganisms tested and were sufficient to significantly limit the growth of the field microorganisms. The results of this study suggest that DiEGME levels of 10% and above and TriEGME levels of 15% and above in the aqueous phase at ambient temperature (~0.01-0.02% in the fuel phase) are beneficial for controlling microbial growth in aircraft fuel systems. Additional tests at higher DiEGME levels suggest that even 30%-60% DiEGME in the aqueous phase did not completely eliminate field consortia growth. The field *Bacillus* and *Clostridium* strains tested were shown to be viable, even at these high concentrations. However, TriEGME levels of 40% and above were sufficient to eliminate all field microbes tested here, but only after 30-40 days of exposure. Examination of post-test liquid setups suggests that the presence of DiEGME or TriEGME can dramatically curtail active microbial growth and/or biofilm formation in fuel/water liquid samples. Overall, it appears that DiEGME or TriEGME would still act beneficially, even at reduced levels, to control microbial growth in current fuel systems. It is expected that a reduction in dosage to the minimum levels indicated would result in the same performance as is currently seen at the higher additive levels used in the field today.

6. References

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LIST OF ACRONYMS, ABBREVIATIONS, AND SYMBOLS

ACRONYM	*DESCRIPTION*
AFPET	Air Force Petroleum Office
AFRL	Air Force Research Laboratories
ASC	Aeronautical Systems Command
ATCC	American Type Culture Collection
CFU	Colony Forming Units
DiEGME	Diethylene Glycol Monomethyl Ether
DoD	Department of Defense
EPS	Extracellular Polymeric Substances
FSII	Fuel System Icing Inhibitor
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC	High Performance Liquid Chromatography
LB	Luria-Bertani
MIC	Microbially Induced Corrosion
RTOC	Reduction of Total Ownership Cost
SPO	Systems Program Office
TNTC	Too Numerous To Count
TriEGME	Triethylene Glycol Monomethyl Ether
UDRI	University of Dayton Research Institute
USAF	United States Air Force
USN	United States Navy
UTC	Universal Technology Corporation